PRODUCTION OF
POLY(ADENOSINE DIPHOSPHATE-RIBOSE) POLYMERASE-1

Development of an efficient production and purification protocol

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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This thesis is dedicated to

my parents, Ian and Dorothy and my sister and brother, Melanie and Luke.

Declaration

I hereby declare that all work described in this thesis was performed while I was enrolled as a student for the degree of Doctor of Philosophy in the Unit for Food Safety, Authenticity and Quality at Victoria University of Technology. The work described in this thesis has not been submitted, in whole or in part, for any other degree or diploma in any University.

To the best of my knowledge, no material contained in this thesis has previously been published by any other person, except where due reference is made in the text. Any published or unpublished work performed by any other person in this thesis has been duly acknowledged.

Matthew Knight

August 2003

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I am extremely thankful for the careful guidance and supervision of my supervisor, Dr. Paul Chambers. In the difficult times, without your support, I would never have survived. You will always be a great mate, friend and mentor.

Finally I would like to thank my family and friends for their continuing support and encouragement throughout my PhD candidature. Mum and Dad you were always there. Luke (and Nicole) and Melanie, it's finally over.

Abstract

PARP-1 is a nuclear enzyme involved in a range of activities associated with DNA metabolism, playing a key role in maintaining the integrity of DNA and chromatin structure. As such, this enzyme is likely to provide a useful target when using a rationale drug design approach to develop pharmaceutical reagents including cancer therapeutics. A major obstacle to this work however is that our knowledge of the relationship between structure and function of PARP-1 is rather limited.

Structure – function studies of enzymes typically require the application of techniques for producing reasonable quantities of high quality protein. To this end, this thesis describes the development of a rapid and relatively simple approach to producing and purifying PARP-1. Unlike traditional PARP-1 purification protocols, the method described here requires only one chromatography step thus minimising losses of the enzyme. The method also avoids the use of a competitive inhibitor-based affinity chromatography step, which is common to several other protocols within the literature. The method developed here was published in Knight, M.I. and Chambers, P.J. (2001) Production, extraction and purification of human poly(ADP-ribose) polymerase-1 (PARP-1) with high specific activity. *Prot. Exp. Purif.*, **23**, 453-458.

In carrying out this research, it was discovered that quantifying levels of purified PARP-1 protein is problematic. A range of methods for determining protein concentration was trialled, including the two most commonly used methods: the Lowry and the Bradford protein assays, neither of which were adequate. This thesis describes how, by using a fluorescence-based assay and amino acid analysis, I overcame the problem. This work was published in Knight, M.I. and Chambers, P.J. (2003) Problems associated with determining protein concentration: A comparison of techniques for protein estimations. *Mol. Biotech.*, **23**, 19-28.

With an accurate measure of PARP-1 protein concentration in place, enzymatic analysis of the expressed and purified PARP-1 product, made using the methods developed for this thesis, was performed. This work showed that the purified PARP-1 is a high-quality native enzyme with high specific activity and K_m and V_{max} values

similar to what is reported by other workers in the field. The yield was also more than adequate for future structure – function studies.

Publications arising from work in this thesis

Journal Publications (attached as appendices to this thesis)

- Knight, M.I. and Chambers, P.J. (2001) Production, extraction and purification of human poly(ADP-ribose) polymerase-1 (PARP-1) with high specific activity. *Prot. Exp. Purif.*, **23**, 453-458.

- Knight, M.I. and Chambers, P.J. (2003) Problems associated with determining protein concentration: A comparison of techniques for protein estimations. *Mol. Biotech.*, **23**, 19-28.

Conference Publications

- Knight, M.I. and Chambers, P. (1998) Structure-function studies of Poly(ADP-ribose) Polymerase (PARP). Australian Society of Biochemistry and Molecular Biology, Adelaide, Australia.

- Knight, M.I. and Chambers, P. (1999) Structure-function studies of Poly(ADP-ribose) Polymerase (PARP). Australian Society of Biochemistry and Molecular Biology, Gold Coast, Australia.

Abbreviations

3-AB	3-aminobenzamide
AD	Automodification domain of PARP-1
ADP	Adenosine-diphosphate
ADPRTs	ADP-ribosyl transferases
Amp	Ampicillin
ATP	Adenosine triphosphate
BER	Base excision repair
Ci	Curie
cDNA	complimentary DNA
CD	Catalytic domain of PARP-1
CHO	Chinese hamster ovarian
DBD	DNA binding domain of PARP-1
ddH_2O	double-distilled water
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EDTA-$2H_2O$	hydrated ethylenediamine tetra-acetate
g	Gram
g	centrifugal field being applied radially outwards required for centrifugation
Gent	Gentamycin
GF/C	Glass fibre filter paper (Class C – suitable for scintillation counting)
HCl	Hydrochloric acid
HT-PARP-1	Hexahisitidine-tagged human PARP-1
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kb	kilobases
kDa	kiloDalton
K_m	Michaelis – Menton constant
L	Litre
LB	Luria Bertani Agar
LiOAc	Lithium Acetate

M	Molar
mM	Millimolar
mL	millilitre
mg	milligram
min	minutes
MMS	methylmethanesulfonate
MVP	Major vault protein
N	Normality
NaCl	Sodium Chloride
NAD^+	Nicotinamide adenine dinucleotide
$NaHSO_3$	Sodium dithionite
NaN_3	Sodium azide
NaOH	Sodium hydroxide
nm	Nanometers
^oC	Degrees Celcius
OD	Optical density at given wavelength or absorbance
PAGE	Polyacrylamide gel electrophoresis
PARG	Poly(ADP-ribose) glycohydrolase
PARP-1	Poly(ADP-ribose) polymerase-1
pmol	picomole (10^{-12} moles)
PMS	protamine sulphate
PMSF	phenylmethyl sulfonyl fluoride
PPO	2-,5-diphenyl-oxazole
RNA	ribonucleic acid
r.p.m. (rpm)	revolutions per minute
SCE	Sister chromatid exchange
SDS	Sodium dodecyl sulphate
SFM	Serum free media
sPARP	short PARP
TAE	Buffer containing Tris HCl, Acetic acid and EDTA
TANK-1, TANK-2	Tankyrase-1, Tankyrase-2
TCA	Trichloracetic acid
TEMED	N,N,N',N'-tetramethylethylethylene-diamine
TEP-1	Telomerase-associated protein

Tet	Tetracyclin
TRF-1, TRF-2	Telomeric repeat binding factor-1,-2
Tris HCl	Tris(hydroxymethyl)methylamine
μCi	microCurie
μl	microlitre
μg	microgram
UV	ultraviolet light
V_{max}	maximum velocity (maximum rate of enzyme reaction)
V-PARP	Vault - PARP
w/v	weight (grams)/volume (litres)
YEPD	Yeast extract, Peptone and Dextrose
YNB	Yeast nitrogen base
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase
Zn^{2+}	Zinc dication

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1 Literature Review

1.1 Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme involved in DNA repair and, putatively, several other nuclear activities. PARP-1's role in the cell is to synthesize polymers of ADP-ribose attached to itself (automodification) or other target proteins (heteromodification). The following literature review will cover what we know of ADP-ribosylation, and the structure, activity and functions of PARP-1.

1.2 ADP-Ribosylation

ADP-ribosylation reactions are catalysed by a group of enzymes known as ADP-ribosyl transferases (ADPRTs). ADPRTs catalyse the transfer of the ADP-ribose moiety of NAD^+ to proteins. NAD^+ comprises of an ADP-ribosyl moiety that is covalently attached to nicotinamide through a β-N-glycosidic linkage (See Figure 1.1). This bond is a so-called high-energy linkage and supplies the driving force for ADP-ribosylation reactions.

ADPRTs have been isolated from the nuclei and cytoplasm of many organisms (Shall 1989). ADPRT enzymes isolated from the cytoplasm are only able to catalyse the formation of mono-ADP-ribosyl protein products, commonly referred to as mono(ADP-ribosylation), whereas ADPRT enzymes isolated from the nucleus synthesize homopolymers of ADP-ribose on suitable acceptor proteins and these homopolymers may be linear or branched. This is commonly referred to as poly(ADP-ribosylation).

1.2.1 Mono(ADP-Ribosylation) occurs in prokaryotes and eukaryotes

Mono-ADPRTs catalyse the transfer of one ADP-ribose unit from NAD^+ to a specific amino acid residue on suitable acceptor proteins (See Figure 1.2); these enzymes have been identified in prokaryotes, eukaryotes and viruses.

The first reported mono(ADP-ribosylation) reaction was that catalysed by the bacterial toxin, diphtheria. Many bacterial toxins catalyse mono(ADP-ribosylation) of eukaryotic regulatory proteins such as GTP-binding proteins and non-muscle actin,

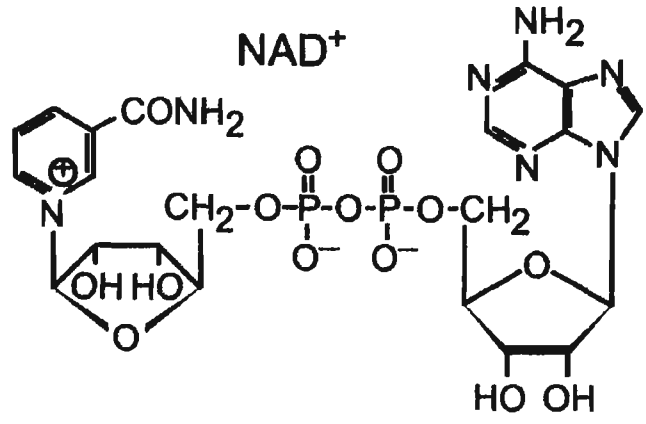


Figure 1.1: Structure of NAD^+ (From Smith 2001).

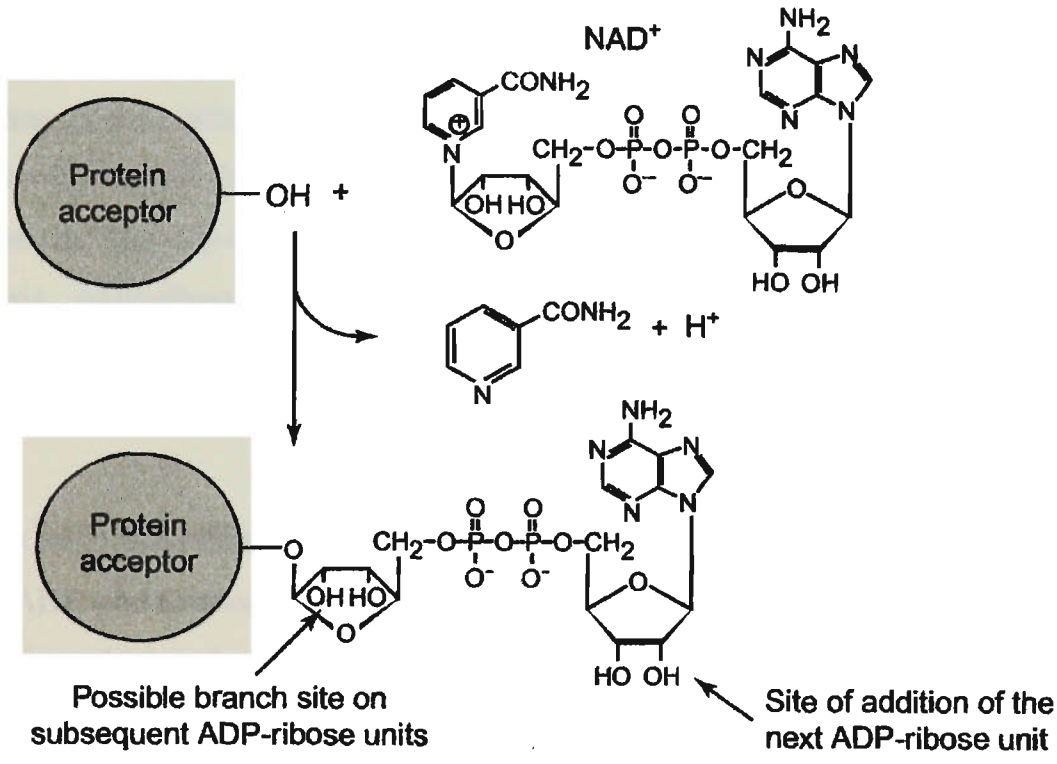


Figure 1.2: Mono-ADPRT activity in prokaryotes and eukaryotes.

Mono(ADP-ribosylation) is the covalent attachment of the ADP-ribose moiety from NAD^+ to an acceptor amino acid (mainly glutamate) on the protein acceptor. This diagram also shows the sites of attachment of subsequent ADP-ribose units for poly(ADP-ribosylation) (From: Smith 2001).

and this is the underlying cause of pathogenesis for what is a rather heterogeneous family of bacterial protein toxins (Aktories 1994).

Other examples of bacterial mono-ADPRTs include the diphtheria toxin (Collier 1975), pertussis toxin (Antonie and Locht 1994), cholera toxin (Moss and Vaughan 1977), exotoxin A from *Pseudomonas aeruginosa* (Li *et al.* 1995) and *Escherichia coli* heat labile enterotoxin (Passador and Iglewski 1994). Each of these toxins contains a domain that catalyses ADP-ribosylation of an acceptor residue within a specific protein target. Toxins with ADPRT activity are defined by the amino acid residue in the target protein that accepts the ADP-ribose moiety. Arginine, asparagine, cysteine, and diphthamide (a post-translationally modified histidine) residues have been identified as amino acid acceptors of the ADP-ribose moiety.

For example, the diphtheria toxin of *Corynebacterium diphtheriae* enters the cytoplasm of the eukaryotic target cell and ADP-ribosylates the side chain of a diphthamide residue found in elongation factor-2 (EF-2) (Pappenheimer 1977); EF-2 catalyses the last step of the elongation cycle and translocation during protein synthesis. ADP-ribosylation of EF-2 terminates protein synthesis, killing the cell (Collier 1975). Exotoxin A from *Pseudomonas aeruginosa* also ADP-ribosylates the diphthamide residue of EF-2 with the same deleterious affects (Li *et al.* 1995).

Cholera toxin and related enterotoxins target the adenylate cyclase system. Mono(ADP-ribosylation) enhances cyclase activity resulting in increased cAMP production which induces a variety of physiological changes depending upon the type of cell infected (Carroll and Collier 1984). The pertussis toxin also ADP-ribosylates a component of adenylate cyclase; it modifies a specific cysteine residue that inactivates the inhibitory component of the adenylate cyclase system (Takada *et al.* 1994). In the photosynthetic bacteria, *Rhodospirillium rubrum*, mono(ADP-ribosylation) controls the reversible regulation of dinitrogenase reductase, a key enzyme in nitrogen fixation (Ludden 1994).

The latter observation led Tanaka *et al.* (1994) to propose that some ADP-ribosylation events may be reversible post-translational modifications that play a role in regulating the function of substrate proteins.

At least six eukaryotic mono-ADPRTs have been identified (Shall 1989). Multiple forms of arginine and cysteine-specific ADPRTs have been purified from avian erythrocytes and polymorphonuclear leukocytes, and mammalian skeletal muscle (Zolkiewska *et al.* 1994). A group of hydrolases remove ADP-ribose group, regenerating free protein (see Figure 1.3). This is evidence for ADP-ribosylation cycles and it is likely that these cycles are involved in regulating protein activities (Takada *et al.* 1994).

1.2.2 Poly(ADP-Ribosylation)

Poly(ADP-ribosylation) is a type of covalent modification of proteins in which ADP-ribose moieties are added sequentially to an initial protein-bound ADP-ribose to form a linear or branched polymer (de Murcia *et al.* 1995); the first ADP-ribose unit is attached to a glutamate residue in the target protein. Poly(ADP-ribosylation) was first demonstrated by Nishizuka *et al.*(1968) as an activity associated with a chromatin-bound enzyme, poly(ADP-ribose) polymerase (PARP). PARP (later called PARP-1 as other members of the PARP family were discovered) was found to transfer the ADP-ribose moiety of NAD^+ to histones and other nuclear proteins forming linear and branched ADP-ribose polymers (Hayaishi and Ueda 1977; Hayaishi and Ueda 1982; Ueda and Hayaishi 1985).

1.2.2.1 Structural Features of Poly(ADP-ribose)

Poly(ADP-ribose) is a homopolymer of adenosine diphosphate ribose units (see Figure 1.4). It is the third most common naturally occurring species of nucleic acid after DNA and RNA. It is a polyanion that has the ability to neutralize the positive charge of many basic proteins, such as histones. Minaga and Kun (1983) suggest a helicoidal conformation for the long ADP-ribose chains and immunological studies suggest a similarity between the secondary and tertiary structure of poly(ADP-ribose) and single-stranded DNA (Sibley *et al.* 1986). The length of ADP-ribose polymers is variable, ranging from 2 to more than 200 ADP-ribosyl residues and the rate of synthesis and degradation varies (Althaus and Richter 1987).

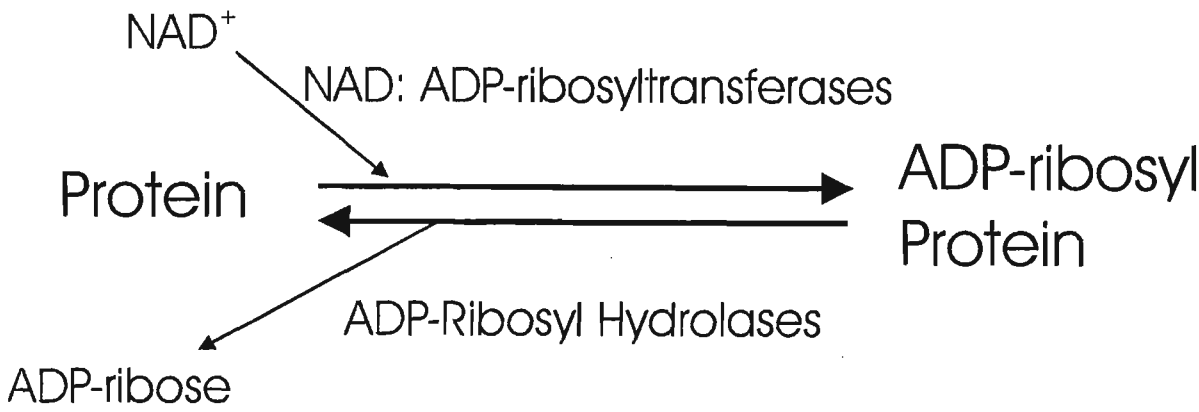


Figure 1.3: Reversible mono-ADP-ribosylation of proteins in eukaryotic cells (Adapted from Takada *et al.* 1994).

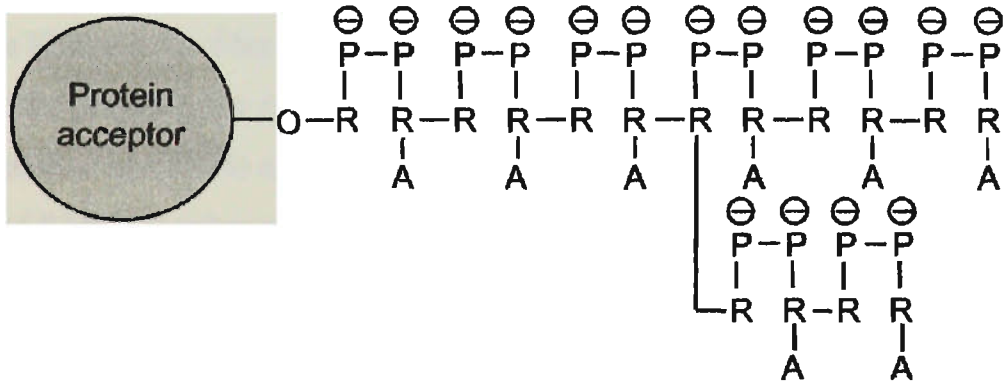


Figure 1.4: Structure of poly(ADP-ribose).
This diagram also shows the overall charge distribution of the poly(ADP-ribose) chain. R – ribose, P – phosphate and A – adenine (From: Smith 2001).

1.2.2.2 Enzymatic Synthesis of Poly(ADP-ribose)

PARP-1 builds polymers of ADP-ribose by catalysing three separate but related reactions that are dependent on DNA for activity:

i. Activation: the glutamate-specific binding of one ADP-ribose moiety from NAD^+ to a protein acceptor to form mono(ADP-ribosyl) protein (Althaus and Richter 1987);
ii. Elongation: the synthesis of a linear poly(ADP-ribose) chain (see Figure 1.5), the subunits of which are glycosidically linked by an α (1"→2') ribosyl-ribose bond to the adenine ribose of the preceding ADP-ribose unit (Althaus and Richter 1987); and
iii. Branching: the transfer of ADP-ribose moieties from NAD^+ to a protein bound poly(ADP-ribose) via a 1'"→2" glycoside branch (Althaus and Richter 1987) (see Figure 1.5).

1.2.2.3 Enzymatic Degradation of Poly(ADP-ribose)

Three enzymes degrade poly(ADP-ribose). The main catabolic route within the cell is hydrolysis of the poly(ADP-ribose) polymers by poly(ADP-ribose) glycohydrolase (PARG). PARG splits ribose-ribose linkages in the polymer to release ADP-ribose units (see Figure 1.5). Many factors modulate the activity of PARG including the length of the polymer, the nature of the acceptor protein and the phase of the cell cycle. Satoh *et al.* (1994) and Alvarez-Gonzalez and Althaus (1989) reported that the activity of PARG is substantially increased when the concentration of ADP-ribose polymers is greater than 5 μM *in vivo*. This explains why, in cells where ADP-ribose polymer synthesis is stimulated, these polymers have a very short half-life and in cells where ADP-ribose synthesis is not stimulated, the ADP-ribose polymers have a very long half-life (Althaus and Richter 1987). The function(s) of ADP-ribose polymers will be covered in Section 1.3 and 1.5.

The second enzyme that degrades poly(ADP-ribose) polymers is poly(ADP-ribose) phosphodiesterase. Poly(ADP-ribose) phosphodiesterase degrades the poly(ADP-ribose) polymer by cleaving the pyrophosphate bonds (see Figure 1.5) to produce 5'-AMP, ribose-5-phosphate and *iso* ADP-ribose. Poly(ADP-ribose) phosphodiesterase

has been isolated from both liver and thymus nuclei (Shall 1984) and therefore poly(ADP-ribose) phosphodiesterase probably plays a lesser role than PARG in poly(ADP-ribose) catabolism (Boulikas 1992a).

The third enzyme able to degrade poly(ADP-ribose) is ADP-ribosyl protein lyase, which cleaves the remaining ADP-ribose residue on the acceptor protein following PARG action (see Figure 1.5). The overall chemistry of poly(ADP-ribosylation) and the enzymes involved in poly(ADP-ribose) metabolism is shown in Figure 1.5.

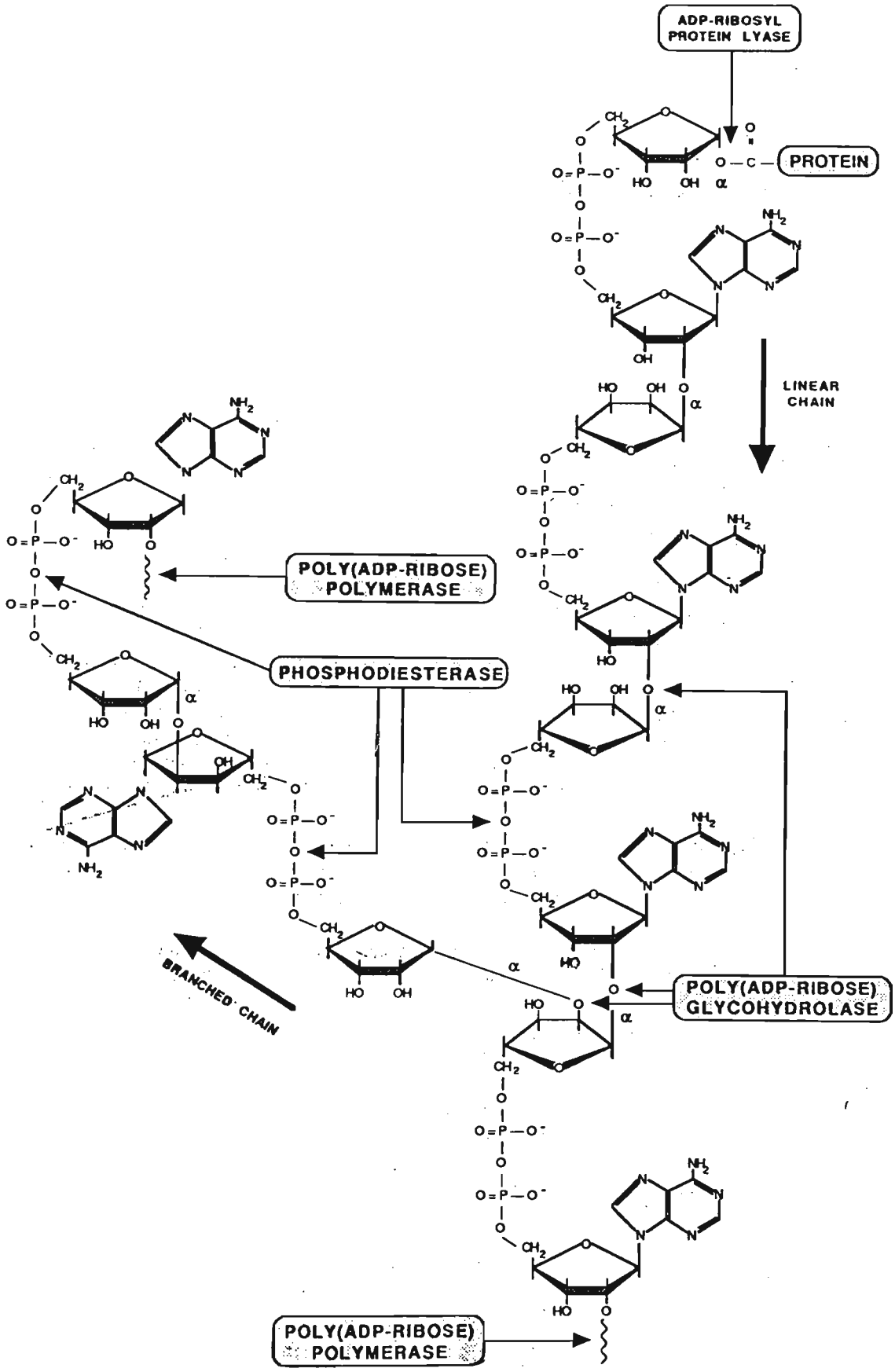
1.3 The Structure of PARP-1

Human PARP-1 mRNA has an open reading frame of 3042 nucleotides and encodes a protein of 113,135 Da made up of 1014 amino acids (Alkhatib *et al.* 1987). PARP-1 has three distinct functional domains, which were initially identified following partial proteolysis with papain and/or α-chymotrypsin (Kameshita *et al.* 1986). These domains include: a 46 kDa DNA-binding domain located at the amino-terminus, a 22 kDa central domain containing an automodification region and a 54 kDa fragment containing the catalytic region. These major domains are divided into subdomains, as described in the following and illustrated in Figure 1.6.

The primary structure of PARP-1 is highly conserved at the amino acid level. For example, human PARP-1 (Uchida *et al.* 1987) has 98%, 97% and 92% homology with bovine (Saito *et al.* 1990), rat (Beneke *et al.* 1997) and mouse PARP-1 (Huppi *et al.* 1989), respectively. The overall conservation is 62% across a wide range of vertebrate species (de Murcia and Menissier de Murcia 1994). The most highly conserved residues are clustered into blocks that correspond to regions within PARP-1 functional domains. Schematic representation of the three functional domains of human PARP-1 is shown in Figure 1.6.

Figure 1.5: Enzymes involved in PARP-1 metabolism (From: Boulikas (1992b)).

PARP-1 transfers the ADP-ribose moiety from NAD^+ to proteins. To produce linear chains, PARP-1 catalyses the formation of ribose α (1"→2') ribose linkages leading to the synthesis of a linear poly(ADP-ribose) polymer. To produce branched chains, PARP-1 also catalyses the formation ribose α(1'"→2") ribose linkages leading to the synthesis of a branched poly(ADP-ribose) polymer. Poly(ADP-ribose) polymers are rapidly degraded by poly(ADP-ribose) glycohydrolase. The last ADP-ribose residue is removed from the protein by ADP-ribosyl protein lyase. The pyrophosphate bond in mammalian cells can also by degraded by phosphodiesterase.


ADP-RIBOSYL PROTEIN LYASE
PROTEIN
LINEAR CHAIN
POLY(ADP-RIBOSE) POLYMERASE
PHOSPHODIESTERASE
BRANCHED CHAIN
POLY(ADP-RIBOSE) GLYCOHYDROLASE
POLY(ADP-RIBOSE) POLYMERASE

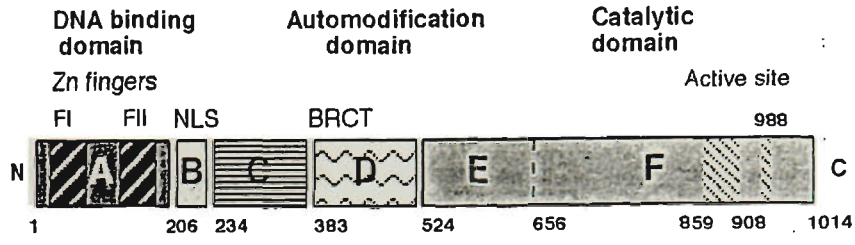


Figure 1.6: Schematic representation of the three functional domains of human PARP-1.

N and C represent the amino and carboxyl terminal of the human PARP-1 protein respectively. The DNA binding domain consists of subdomains A, B and C. Subdomain A contains FI and FII which represents zinc finger I and II respectively. Subdomain B contains the NLS which represents the nuclear localisation signal. The automodification domain consists of subdomain D. BRCT represents the breast cancer susceptibility protein motif. The catalytic domain consists of subdomains E and F. All numerals represent the number of amino acids relative to the amino terminal of PARP-1 (From de Murcia and Menissier-de Murcia 1994).

1.3.1 The DNA-binding Domain of PARP-1 has three subdomains: A, B and C

The N terminal, DNA-binding domain (DBD) extends from amino acid residues 1 to 373. This domain is divided into three subdomains: A, B and C. Subdomain A has two zinc finger motifs and two helix-turn-helix motifs. The two zinc finger motifs are located between a duplicated sequence of amino acid residues at positions 2-97 and 106-207, in which 35 residues are highly conserved in each (See Figure 1.7). These highly conserved regions each contain a zinc-finger motif of the form C-Xaa_2-C-$Xaa_{28,30}$-H-Xaa_2-C that is found in several DNA binding proteins (Alvarez-Gonzalez *et al.* 1999). These features, known as zinc-finger motif's I and II (FI and FII respectively), bind specifically to single and double or single strand breaks, respectively (Molinete *et al.* 1993). Zahradka and Ebisuzaki (1984) determined that each molecule of PARP-1 contains two zinc ions, one bound to each zinc finger in Cys-Cys-His-Cys motifs (See Figure 1.8). Disruption of this metal binding ability dramatically reduces the affinity of PARP-1 for DNA (Molinete *et al.* 1993).

The zinc finger motifs in PARP-1 are structurally and functionally unusual. Zinc fingers FI and FII are comprised of 28 and 30 residues respectively whereas most other zinc fingers contain only 12 – 13 residues. Also the zinc finger motifs of PARP-1 recognize altered structures in DNA rather than specific nucleotide sequences (Gradwohl *et al.* 1990). However PARP-1 zinc fingers are not unique. The duplicated amino acid sequence of zinc finger FI between residues 2 and 97 corresponds exactly to the N-terminal zinc finger motif in DNA ligase III (Lindahl *et al.* 1995).

Two features of interest in subdomain B of the DBD include a nuclear localizing signal (NLS) and a caspase recognition sequence. The NLS has the consensus sequence 2K/R-X10-12-3K/R and constitutes a PARP-1 nuclear homing signal. The caspase recognition sequence constitutes a cleavage site (D214, in the sequence $_{211}$DEVD$_{214}$) for several apoptotic caspases (de Murcia and Shall 2000) making PARP-1 a target for degradation during apoptosis.

Important structural features of subdomain C of the DBD were identified when a 36 kDa fragment containing the subdomain C of the DBD and the entire automodification domain of PARP-1 (amino acid residues 233 – 525) was obtained from partial proteolysis of PARP-1. Buki *et al.*. (1995) found that this 36 kDa fragment of PARP-1 bound strongly to DNA via the helix-turn-helix motif and that elimination of a single amino acid at the N-terminus of this fragment completely abolished the capacity to bind DNA. Chou-Fasman analysis of bovine and human PARP-1 predicted the existence of two helix-turn-helix motifs in this fragment at residues 200 – 220 and 280 – 285 (Uchida *et al.* 1987). Helix-turn-helix motifs have been shown to mediate strong DNA – protein interactions. Further evidence to support the role of PARP-1 helix-turn-helix motifs in DNA binding was provided when Buki's group found that the 36 kDa plasmin fragment of PARP-1 acted exactly like the full-length enzyme in DNA footprinting experiments demonstrating the importance of the helix-turn-helix motifs in DNA binding (Buki *et al.* 1995).

1.3.2 The Automodification Domain of PARP-1

The automodification domain of PARP-1 is the region to which poly(ADP-ribose) polymers are attached. It is in the central region of the enzyme covering amino acid residues 374 to 525 in human PARP-1 and contains 15 highly conserved glutamate residues, which are potential sites of auto-poly(ADP-ribosylation) (Uchida *et al.* 1993).

Many researchers have shown the automodification domain to contain several potential protein-protein binding motifs putatively involved in both homo- and heteromodification reactions (Buki *et al.* 1995). As homodimerisation is believed to be crucial for PARP-1 activity, homodimer intermolecular interactions have been implicated in regulating interactions between PARP-1 and protein acceptor molecules during automodification and heteromodification reactions (Buki *et al.* 1995). Kameshita *et. al.* (1986) showed that this domain is not capable of accepting ADP-ribose polymers when cleaved from the active site or zinc finger domains.

Figure 1.7: A comparison of the primary structure of the zinc finger motifs from human, mouse, bovine, chicken, *Xenopus* and *Drosophila* PARP-1.

The boxes indicate the consensus cysteine (C) and histidine (H) residues in the zinc fingers and (") indicates that the amino acid sequence is homologous with the human sequence whereas (-) indicates a gap. Amongst species, the conserved basic amino acid residues and conserved amino acid residues between the two zinc fingers are indicated by (*) and (+) respectively (From Uchida *et al.*, 1994).

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                            +   *         + *   *                +    +
Human         1 MAESSDKLYRVEYAKSGRASCKKCSESIPKDSLRMAIMVQSPMFDGKVPHWYHFSCFWKVGHSIRH  66
Mouse         1 """A"ER"""""Q""""""""""""""""""""""""""""""""""""""""""""""""Q""""  66
Bovine        1 """"""""""""""""""""""""K"""""""I"""F""E"""""""I"""""L"""""""F""W"  66
Chicken       1 """TG""P""A"""""""""""""G"""A"""""L"L""""""""""""""H"Y"""""RARIVS"  66
Xenopus       1 """A""""""A"""""""""""""GDN"A"E"""L""""""""""""""""H"Y"""""RARVLSQ  66
Drosophila    1 .....MDIELP"LA"""RT"""T""G"KST"S""T""I"V""""AFH"A"""N"F"KT""F"NQRPSSV  64

                          +   *           *   *                   +    +
Human       108 KT_LGDFAAEYAKSNRSTCKGCMEKIEKGQVRLSKKMVDPEKPQL_GMIDRWYHPGCFVKNREE 169
Mouse       108 ""_""""""""""""""M""""L"""""""M""""""""""""""_"""""""""T""""K"D" 169
Bovine      111 ""_"I""G"G""""""""""S"""""D"""""""""V"Y"D""""_""V"C""""K"""QK""" 172
Chicken     108 "S_"T"""""""""""""""""EQ""""""I"I"""""H""""""_""""N""""D"""SR"A" 169
Xenopus     107 M"_"N""""""""""""A""""EQ""""""I"I"""S""V"R"""_"""""""""D"""SS""" 168
Drosophila  105 NLA"K""GI"""""S""""R""EQ""N"DL"""R"TVY"T"VGMKY"GQPL"H"LE""AQL"S" 168
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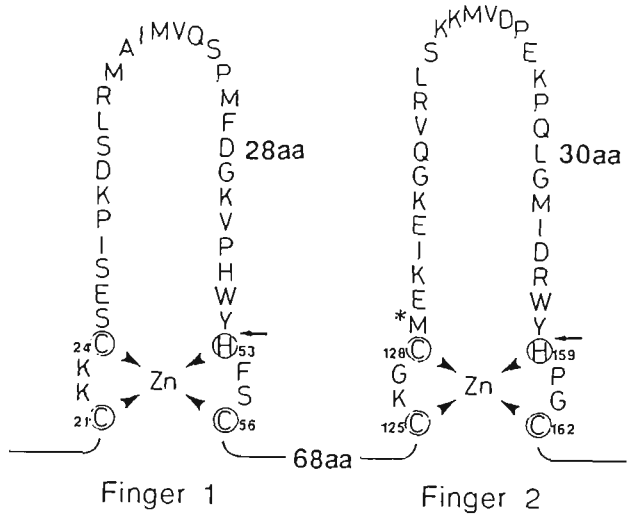


Figure 1.8: Amino acid sequence of the zinc finger motifs of PARP-1 showing the zinc (Zn^{2+}) coordinating cysteines and histidines residues which are circled.

The arrow indicates a Kpn I cleavage site in the DNA. The (*) represents the first internal methionine (Met 129) in the PARP-1 sequence (From Ikejima *et al.* (1990)).

Interestingly PARP-1 from *Drosophila melanogaster* contains a leucine-zipper motif between amino acid residues 388 – 409. Leucine zippers have been implicated in many protein-protein interactions and have been found in many eukaryotic transcription factors and various oncoproteins such as Fos, Jun, CREB, C/EBP and GCN4 (Landschulz *et al.* 1988; O'Shea *et al.* 1991). A possible function for the leucine zipper in *Drosophila* PARP-1 may be to regulate homo- and/or hetero-dimerisation of PARP-1. Uchida *et al.* (1993) proposed that this leucine zipper motif is involved in "protein-interactions" (See Figure 1.9), that stimulate catalytic activity of the enzyme (Alvarez-Gonzalez *et al.* 1994). However, in vertebrates the leucine zipper in PARP-1 is poorly conserved.

While there may not be a leucine zipper in vertebrate PARP-1, there is a BRCT (breast cancer susceptibility protein, *BRCA*1, C-terminus) motif. The BRCT motif has been identified in many DNA repair and cell cycle checkpoint proteins (Bork *et al.* 1997; Callebaut and Mornon 1997). In PARP-1, it is located in the automodification domain between amino acids 372-476, and is weakly conserved across species (de Murcia and Shall 2000). There is increasing experimental evidence to suggest that the BRCT motifs provide modules for strong and specific protein-protein interactions between the BRCT-motif-containing proteins (D'Amours *et al.* 1999). The BRCT motif in PARP-1 appears to be fully functional regulating specific interactions between X-ray cross-complementing protein 1 (XRCC1) (Masson *et al.* 1998), human ubiquitin-conjugating enzyme Ubc9 (hUbc9) (Masson *et al.* 1997) and histones (Buki *et al.* 1995). This will be covered in more detail in Section 1.5.1.

1.3.3 The Catalytic Domain of PARP-1 has two subdomains: E and F

The catalytic domain of PARP-1 is by far the most conserved region of the enzyme. It includes amino acid residues 654 to 1014 and contains a block of 50 amino acids that are strictly conserved between vertebrates (100 %) and highly conserved (92 %) among species as diverse as mouse and *Xenopus* (See Figure 1.6). This region is considered to be the "PARP signature" (de Murcia and Menissier de Murcia 1994).

The catalytic domain is composed of two subdomains: E and F. Subdomain E is located between amino acid residues 662 – 784 and is purely α-helical, with no

structural similarity to any other protein domain found in the Swiss Protein Data Bank (http://kr.expasy.org). To date, the functional significance of subdomain E remains unknown. The second domain, subdomain F is located between amino acids residues 785 – 1012 and contains the substrate (NAD^+) binding site. This site has a chain fold similar to the NAD^+ binding domains of bacterial ADPRTs and consists of a five-stranded antiparallel sheet and a four-stranded mixed β-sheet. Surrounding this core are eight α-helices and three- and two-stranded β-sheets.

1.3.4 Physicochemical Properties of PARP-1

The molecular weight of PARP-1 has been reported to range from 50 to 130 kDa depending on the preparation (See Table 1.1). However researchers found that the low molecular weight variants (50kDa from rat liver (Okayama *et al.* 1977) and 63.5kDa from pig thymus (Tsopanakis *et al.* 1978)) were due to protease activity in the preparation. A detailed description of the intricacies of PARP-1 purification is given in Chapter 4 of this thesis.

One of the first detailed studies investigating the physicochemical properties of PARP-1 was performed by Petzold *et al.* (1981) using PARP-1 purified from lamb thymus. These researchers reported that PARP-1 is a single polypeptide chain with a molecular weight of 116kDa and an isoelectric point of 9.6 and a pH optimum of 8.6 to 8.8 (Petzold *et al.* 1981). The characteristics of this preparation are similar to those of PARP-1 purified from other tissues and from a range of different organisms (Berger and Petzold 1985).

1.3.5 Substrate Specificity, K_m and V_{max} of PARP-1

Studies investigating substrate specificity conclude that β-NAD^+ is the natural substrate of PARP-1 (Hilz *et al.* 1974). Most preparations of PARP-1 have a K_m value for NAD^+ of around 50 μM with a range of 20 to 80 μM and a V_{max} ranging from 570 to 5,800 nmol/min/mg (See Table 1.1). These values are however dependent on the nature and concentration of DNA, histones and protein acceptors in the assay (Shall 1984).

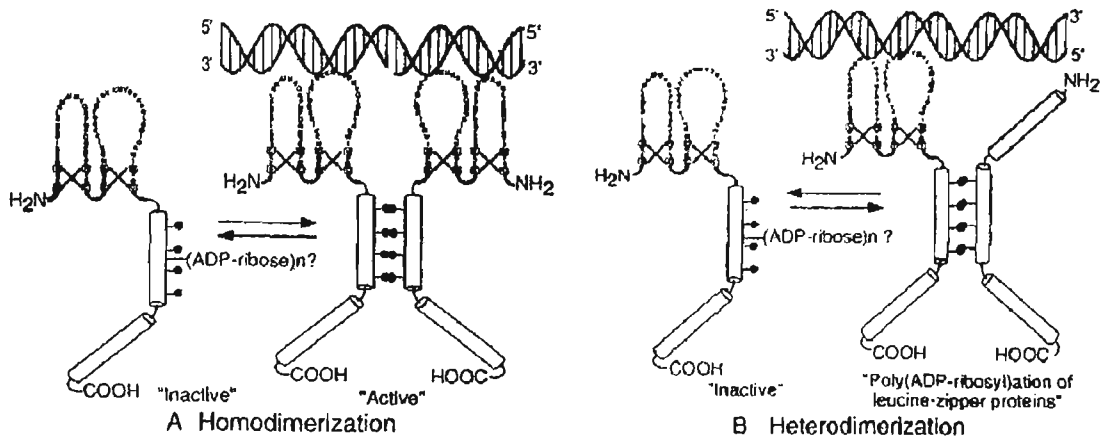


Figure 1.9: Proposed homo- and hetero- dimerisation models of PARP-1 via the leucine-zipper of *Drosophila.*

(A) Homodimerisation : Monomeric PARP-1 becomes enzymatically active following interaction with another PARP-1 monomer via the leucine zippers motifs. **(B)** Heterodimerisation : Monomeric PARP-1 only becomes enzymatically active upon association with other proteins via their leucine zipper motifs. This may include transcription factors such as oncoproteins; Fos, Jun, CREB, C/EBP and GCN4 (From: Uchida *et al.* (1993)).

Table 1.1: Physicochemical and enzymatic properties of PARP-1 from a range of sources (Adapted from: Althaus and Richter 1987).

Source of Enzyme	Molecular Weight[a]	V_{max}[b] (nmol/min/mg)	References
Human tonsils	116,000	862	Carter and Berger (1982).
Pig thymus	63,500	1,900	Tsopanakis *et al.* (1978).
Rat liver	50,000	1,560	Okayama *et al.* (1977).
Human placenta	115,000	1,560	Burstcher *et al.* (1986).
Calf thymus	130,000	570	Mandel *et al.* (1977).
Lamb thymus	135,000	2,540	Pertzold *et al.* (1981).
Bovine thymus	130,000	910	Yoshihara *et al.* (1978).
Calf thymus	114,000	3,730	Zahradka and Ebisuzaki (1984).
Calf thymus	120,000	1,430-2,200	Ito *et al.* (1979).
Mouse testicles	116,000	1,370	Agemori *et al.* (1982).
Sheep testis	120,000	1,265	Zhang and Qui (1986).
Ehrlich ascites tumor cells	130,000	5,800	Kristensen and Holtlund (1978).
HeLa cells	112,000	1,470	Jump and Smulson (1980).
Human placenta	120,000	-[c]	Shizuta *et al.* (1985).
Human PARP-1	116,000	1010	Giner et al. (1994)
Human PARP-1	116,000	480	Knight and Chambers (2001)

[a] Determined by SDS-polyacrylamide electrophoresis.
[b] Reaction conditions varied.
[c] Not specified in this report.

1.3.6 Optimum pH and Temperature

While there have been numerous reports on pH optima for PARP-1 (e.g. See Section 1.3.4) most workers in the field take it to be between 8 and 8.5 and the temperature optimum for mammalian sources of PARP-1 is 25°C (Shall 1984).

1.3.7 Co-factors for PARP-1 Enzyme Activity

There are two co-factor requirements for PARP-1 activity: DNA and Mg^{2+}. As reported by many investigators, PARP-1 activity is dependent on DNA breaks or polynucleotide termini but is not dependent on specific DNA sequences. The earliest observations supporting PARP-1's dependence on DNA for activity was reported by Chambon and co-workers in 1966 (Chambon *et al.* 1966). These workers found that poly(ADP-ribose) synthesis decreased after exhaustive DNaseI treatment in hen liver. Similar observations were made by Nishizuka *et al.* (1967) using mammalian nuclei and by Nishizuka *et al.* (1969) and Hayaishi and Ueda (1974) using rat liver chromatin. Therefore, DNA is an absolute requirement for full PARP-1 activity, however to be most effective, the DNA must contain single or double strand breaks, the most effective break being double strand breaks with blunt ends (Benjamin and Gill 1980).

Another co-factor which stimulates PARP-1 enzyme activity is Mg^{2+} which is thought to favour DNA-PARP-1 interactions by neutralising the negative charge of poly(ADP-ribose) and/or DNA (Benjamin and Gill 1980).

Many researchers believed that because Zn^{2+} ions are essential for the co-ordination of the two zinc fingers in the DNA-binding domain and that disruption of this co-ordination dramatically reduced the affinity of PARP-1 for DNA (Molinete *et al.* 1993) that Zn^{2+} may also be an essential co-factor for PARP-1 activity. However, Ito *et al.* (1979) found that the addition of endogenous Zn^{2+} ions inhibited PARP-1 activity dramatically. From these observations, these researchers proposed that when PARP-1 is purified there is enough Zn^{2+} ions present for PARP-1 enzyme activity and thus, there was no need for the addition of endogenous Zn^{2+} ions to the PARP-1 reaction mixture (Ito *et al.* 1979).

Histones, whilst not a co-factor for PARP-1 activity, can also stimulate activity of the enzyme (Althaus and Richter 1987). Cox and Lehman (1981) and Berger and Pertzold (1985) believe that this is because histones play a role in the formation and/or stabilisation of the DNA-PARP-1 complex. Histones also act as potential targets for poly(ADP-ribosylation) within the cell. Thus, histones are able to accept ADP-ribose monomers which then act as targets for poly(ADP-ribosylation). This was first demonstrated *in vitro* by Hayaishi *et al.* (1977). Kawaichi and colleagues (1980) subsequently found that the concentration of histones, relative to DNA is crucial for preferential initiation of poly(ADP-ribosylation) of histones over PARP-1.

1.4 PARP-1 is a member of a family of poly(ADP-ribosylation) proteins

The first evidence that there are other poly(ADP-ribosyl)ating enzymes came when mouse embryonic fibroblasts derived from PARP-1 knockout mice appeared to have PARP-1 activity (Shieh *et al.* 1998). Soon after this, several new PARPs were identified including PARP-2 (Ame *et al.* 1999), PARP-3 (Johansson 1999; Smith 2001), vault-PARP (V-PARP) (Smith 2001), tankyrase 1 and 2 (TANK-1 and TANK-2) (Smith *et al.* 1998; Smith 2001) and s-PARP (Smith 2001). The following section describes the features and functions (where known) of each of these new members of the PARP family.

1.4.1 PARP-2

PARP-2 has the strongest resemblance to PARP-1 (60% identity in the "PARP signature" domain) and thus is believed to be functionally similar to PARP-1. PARP-2 is located in the nucleus and binds to and is activated by DNAse-treated DNA (Ame *et al.* 1999). However the DBD of PARP-2 is completely different from PARP-1 perhaps indicating that PARP-2 has a different affinity for the DNA strand breaks to PARP-1. PARP-2 is likely to contribute to the residual poly(ADP-ribose) activity observed by Shieh *et al.*(1998) in PARP-$1^{-/-}$ cells after treating them with DNA-damaging agents. Thus, many researchers believe that PARP-2's role within the cell is DNA repair (Smith 2001; Shall 2002).

1.4.2 PARP-3

The smallest protein in the PARP family consists of a unique N-terminal of 39 amino acids followed by the PARP homology domain (the region identified as the PARP

homology domain is the catalytic domain of PARP-1 between amino acids 524 – 1014) (Johansson 1999). Very little is known about PARP-3 but future studies will reveal whether it has a nuclear localization domain like PARP-1 and PARP-2 and whether it has the capacity to bind DNA or the ability to be activated by damaged DNA. These properties may be encoded by the unique N-terminal domain or the PARP homology region (Smith 2001; Shall 2002).

1.4.3 Vault – PARP (V-PARP or PARP-4)

Vault-PARP (V-PARP) is part of the vault complex, which is a large ribonucleoprotein assembly found in many eukaryotes and primarily located in the cytoplasm with a small number localised at the cytoplasmic face of the nuclear pore complex in isolated nuclei. The vault complex has a unique barrel structure and whilst its function is unknown several lines of research point to a role in cellular transport (Smith 2001). This complex consists of two other highly conserved proteins, major vault protein (MVP) and telomerase-associated protein (TEP-1), as well as untranslated vault RNA (vRNA) (Smith 2001). V-PARP contains a domain that binds to and modifies MVP. TEP-1 is also a component of the human telomeric complex. The function of V-PARP is not known. However V-PARP association with TEP-1 and localisation at mitotic spindles are consistent with functions which include cell death-signaling pathways. The discovery of V-PARP suggests that poly(ADP-ribosylation) may be associated with activities other than repair of genotoxic damage within the cell (primarily located in the nucleus).

1.4.4 Tankyrase-1 (PARP-5) and Tankyrase-2

Tankyrase-1 (TANK-1) has been identified as an essential component of the human telomeric complex (Smith *et al.* 1998) which is associated with telomeres. Telomeres are essential for chromosome maintenance and stability and are maintained by telomerase, a specialised reverse transcriptase (Nugent and Lundblad 1998). Telomeric repeat binding factor-1 (TRF1) and telomeric repeat binding factor-2 (TRF2) are specific DNA binding proteins that regulate telomere structure and function (Chong *et al.* 1995). TANK-1 was initially identified through its ability to poly(ADP-ribosyl)ate itself and TRF1 *in vitro* (van Steensel and de Lange 1997). TRF1 was unable to bind telomeric DNA once it had been poly(ADP-ribosyl)ated. Smith *et al.*(1998) proposed that when TRF1 is poly(ADP-ribosyl)ated it is released

from telomeres, inducing a change in telomere structure and allowing access to telomerase. This is similar to the way PARP-1 is thought to be released from DNA once it becomes poly(ADP-ribosyl)ated (see Section 1.5.1). Further experimental evidence to support this model was obtained when TANK-1 was overexpressed in human cells. Overexpression of tankyrase-1 in human cells affected the association of TRF1 with telomeres, promoting telomere elongation (Smith and de Lange 2000). The catalytic domain of TANK-1 is 29% homologous to the catalytic domain of PARP-1 although its overall primary structure is vastly different to PARP-1. Recent studies by Chi and Lodish (2000) revealed a homologue to TANK-1, tankyrase 2 (TANK-2). However, not much is known about this new member of the PARP family.

1.4.5 sPARP

sPARP has a calculated molecular mass of 55.3 kDa and is identical in deduced amino acid sequence to the catalytic domain of PARP-1 (Sallmann *et al.* 2000). It is also located very close to the PARP-1 gene on chromosome 1 at position H5-H6. sPARP was initially identified in PARP-$1^{-/-}$ cells and is believed to contribute to the residual PARP-1 activity observed in these cells. However, further studies have identified that it is also produced in $PARP^{+/+}$ cells. Unlike PARP-1 and PARP-2, sPARP activity is not stimulated by DNA damage. sPARP is found in the nucleus of cells indicating that the PARP homology domain alone (the region identified as the PARP homology domain is the catalytic domain of PARP-1 between amino acids 524 – 1014) is sufficient for nuclear localisation (Smith 2001).

Schematic representation of the five members of the PARP family (and sPARP) is shown in Figure 1.10 along with a summary of the PARP family of proteins is shown in Table 1.2. It should be noted that two more members of the PARP family, PARP-6 and PARP-7 have been identified (Shall 2002) but these are yet to be characterised. Prof. G. Poirier reported at the 13th International Symposium on ADP-ribosylation reactions in 2001 that there may be as many as 16 sequences related to PARP-1 in the human genome.

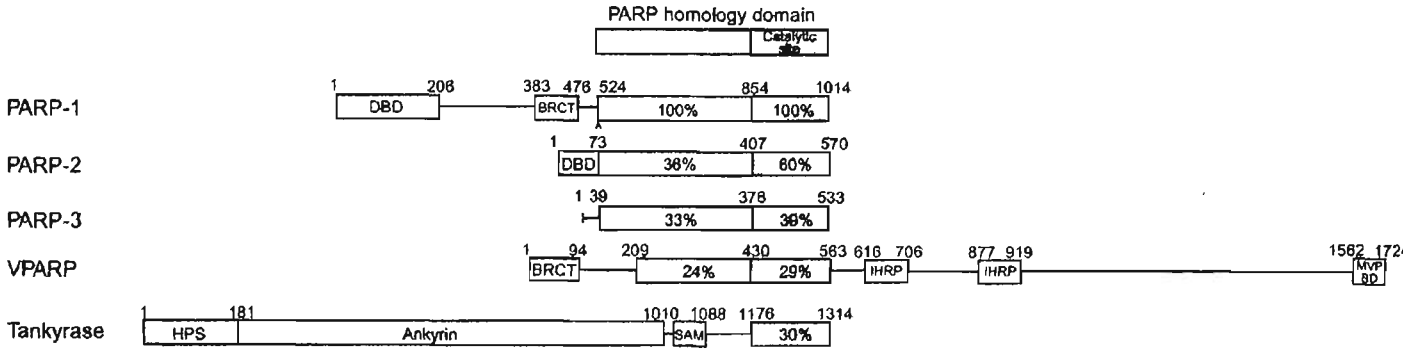


Figure 1.10: Schematic representation of the primary structures of the five members of PARP family.

The percentages indicate the homology with PARP-1 and (^) indicates the start of sPARP (from Smith (2001)).

Table 1.2: A summary of properties of proteins in the PARP family (from de Murcia and Shall (2000)).

Protein	Size, kDa	Activated by Damaged DNA	Catalyses Automodification	Substrates	Cellular Localisation	Reference
PARP-1	113	Yes	Yes	Histones, Other DNA binding proteins	Nuclear	Althaus and Richter (1987);de Murcia and Menissier-de Murcia (1994) and de Murcia *et al.* (1994)
PARP-2	62	Yes	Yes	Unknown	Nuclear	Ame *et al.* (1999)
PARP-3	60	Not known	Not known	Unknown	Centrosome	Johansson (1999)
V-PARP (PARP-4)	193	No	Yes	MVP	Cytoplasmic, nuclear	Johansson (1999) and Smith (2001)
TANK-1 (PARP-5)	142	No	Yes	Telomere specific protein TRF1	Chromosome telomere, peri-nuclear	Smith *et al.* (1998); Smith (2000) and Chi and Lodish (2000)
sPARP	56	No	-	PARP-1, Other DNA binding proteins	Nuclear	Sallmann *et al.* (2000)

1.5 Regulation of PARP-1 Activity and its Putative Functions

PARP-1 activity is probably regulated in the cell at several levels. There is some evidence that suggests the transcription of the PARP-1 gene is negatively regulated by the enzyme itself (de Murcia *et al.* 1995). However, many researchers believe that PARP-1 activity in the cell is mainly regulated by modulation of enzyme activity by DNA strand breaks (de Murcia *et al.* 1995). There is a plethora of experimental evidence to support this (see reviews by Shall (1994) and Lindahl (1995)), the most compelling of which is that catalytic activity of PARP-1 has been shown to increase 500 times over its basal activity when it is stimulated by DNA with strand breaks (Simonin *et al.* 1993b).

This led many researchers to postulate that PARP-1 activity and poly(ADP-ribosylation) may be involved in a number of cellular processes in which DNA breaks feature, including replication, DNA repair, the modulation of chromatin structure, recombination and apoptosis, as well as genomic stability (Review: Shall (1994); Lindahl (1995)). The following section looks at the experimental evidence that implicates PARP-1 activity in these cellular processes in detail.

1.5.1 PARP-1 and DNA Repair

Cells are constantly exposed to a range of DNA damaging agents from endogenous and environmental sources. The result of this exposure is the formation of modified bases, abasic sites or single strand breaks in DNA. One of the multi-enzymatic repair pathways in cells that removes these potentially mutagenic and/or cytotoxic lesions and protects the integrity of the genome is base excision repair (BER).

Experimental work of Roitt (1956) showed that cells treated with DNA damaging agents have depleted levels of NAD^+ suggesting possible involvement of this co-enzyme in DNA repair. However, it was not until nearly twenty years later that the true implications of these observations were realised. Whish *et al.*(1975), Smulson *et al.* (1975) and Cleaver *et al.*(1983) each reported that the decrease in the level of cellular NAD^+ coincided with the utilisation of NAD^+ as a substrate for poly(ADP-ribose) synthesis following DNA damage. These findings were supported by results from many laboratories around the world.

Whilst the synthesis of poly(ADP-ribose) polymers was clearly associated with DNA damage, there was still no direct evidence to associate PARP-1 activity with DNA repair, in particular BER. The first experimental evidence that suggested a direct role for PARP-1 in BER was reported by Creissen and Shall (1982). These workers found that an increase in alkylation damage increased DNA ligase activity, particularly that of the repair enzyme DNA ligase II. The increase in the activity of DNA ligase II was totally inhibited by poly(ADP-ribose) polymer synthesis.

In support of the findings of Creissen and Shall (1982) and Yoshihara *et al.*(1985) found that elongation of DNA replication intermediates in a cell free system was inhibited by poly(ADP-ribosylation) of DNA polymerases by PARP-1, and the presence of any DNA ligase *in vitro* only led to the inhibition of DNA replication. This led to the proposal that PARP-1 may have a direct functional role in DNA repair by regulating the activities of several repair enzymes (Yoshihara *et al.* 1985).

In light of these observations, Satoh and Lindahl (1992) developed a human cell-free system that enabled them to explore the role of poly(ADP-ribose) synthesis in DNA repair. They found that unmodified PARP-1 bound tightly to DNA strand breaks probably blocking access of other proteins to the site of damage. Also, in the presence of various PARP-1 inhibitors, such as 3-aminobenzamide, this situation persisted (Satoh and Lindahl 1992). In the absence of inhibitors, PARP-1 became automodified with the addition of ADP-ribose polymers and this led to the liberation of the protein from the DNA due to electrostatic repulsion between the newly synthesised ADP-ribose polymers and DNA (both poly(ADP-ribose) polymers and DNA are highly negatively charged due to the presence of numerous phosphate groups). Thus, the rapid synthesis of ADP-ribose polymers has been described as an intrinsic release mechanism for removal of PARP-1 from DNA, thereby making the strand break available for DNA repair.

These observations enabled Satoh and Lindahl (1992) to propose the following model. PARP-1 has the ability to bind DNA strand breaks within the cell. Once bound PARP-1 is activated, leading to automodification (i.e. poly(ADP-ribosylation) of the enzyme). This automodification decreases the affinity of PARP-1 for DNA and thus

the enzyme is released. Poly(ADP-ribose) polymers were then degraded by PARG, regenerating PARP-1s ability to bind to DNA (See Figure 1.5 and Figure 1.11). This gives rise to shuttling in which the polymerase attaches to damaged DNA, is subsequently removed following automodification then re-attaches following degradation of poly(ADP-ribose) polymers. The authors of this model propose that binding of PARP-1 protects damaged DNA while the repair machinery is assembled. Once assembled, PARP-1 must re-expose the site of damage to repair emzymes. In this context PARP-1 acts as a temporal protector of damaged DNA, the timing of its release being determined by the rate of synthesis of poly(ADP-ribose). Schematic representation of this model of PARP-1 action in DNA BER is shown in Figure 1.11.

Subsequent studies by Masson *et al.* (1998) and Dantzer *et al.*(1998) shed more light on the role of PARP-1 in DNA repair. Masson *et al.* (1998) and Dantzer *et al.*(1998) showed that PARP-1 interacts with X-ray cross complementing-1 (XRCC-1) protein and DNA polymerase β through a specific interaction with the BRCT motif in the automodification domain (see section 1.3.2). XRCC-1 is known to bind DNA ligase III and DNA polymerase β and plays an important role in the single nucleotide patch repair pathway within the cell (Vodenicharov *et al.* 2000). This suggested to many researchers that PARP-1 is an integral part of a putative DNA repair complex that recruits XRCC-1 and other DNA repair proteins to facilitate the BER pathway within the cell, or PARP-1 may be a factor that directly influences the activity of other DNA repair enzymes through poly(ADP-ribosylation) (Vodenicharov *et al.* 2000). However, the actual relationship between PARP-1, poly(ADP-ribosylation) and DNA repair is still unknown.

1.5.2 PARP-1, Chromatin Structure and Histone Shuttling

Many nuclear processes such as transcription, replication, DNA repair and recombination require chromatin structure to be relaxed; condensed chromatin is generally viewed as being inaccessible to DNA machinery. One mechanism that allows chromatin structure to be relaxed is achieved by poly(ADP-ribosylation) of histones (Niedergang *et al.* 1979; Poirier *et al.* 1982; Boulikas 1990). While the role of this modification is not known, it is likely that individual nucleosomes become relaxed allowing various proteins (e.g. DNaseI, microsomal nuclease, DNA helicase

A and DNA topisomerase I) access to bind to DNA (Althaus 1992; Realini and Althaus 1992; Thommes and Hubscher 1992).

Althaus and Richter (1987) proposed histones go through cycles of binding to and release from DNA. This shuttling is triggered by the binding of PARP-1 to DNA strand breaks. Once bound, PARP-1 specifically targets histone tails, adding multiple ADP-ribose polymers, leading to electrostatic repulsion between histones and DNA which displaces the histones from DNA. This in turn allows other proteins to access DNA. PARG subsequently digests the ADP-ribose polymer on the histone, thereby releasing the histone for reassociation with the DNA.

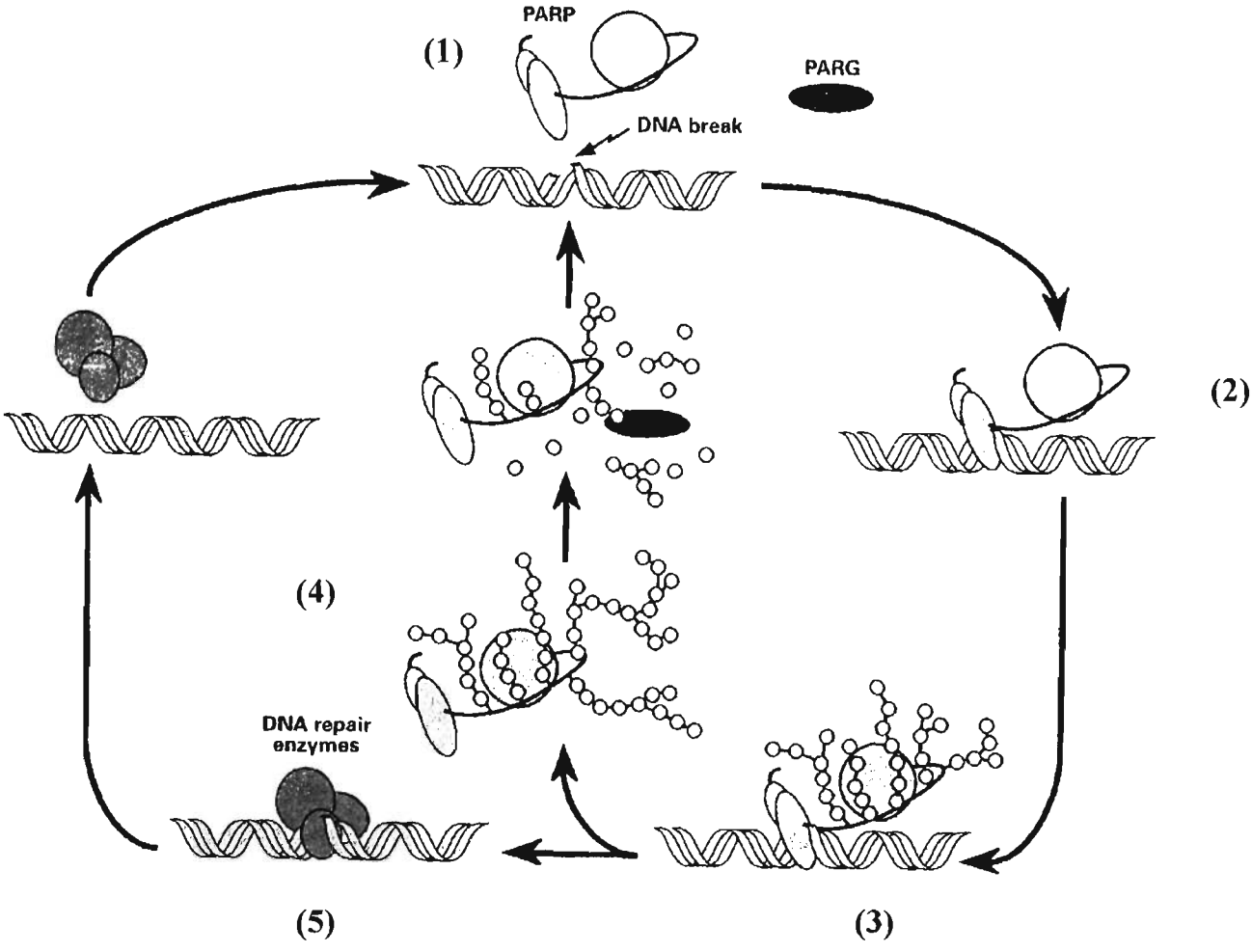


Figure 1.11: Schematic representation of the model for PARP-1 interactions with DNA.

After the introduction of DNA strand breaks PARP-1, DNA excision enzymes and DNA polymerases compete for the binding to the damaged site (1); attachment of PARP-1 interferes with the access to the strand break for DNA repair enzymes (2); poly(ADP-ribose) synthesis is activated by the binding of PARP-1 to damaged DNA (3); the automodified PARP-1 has reduced affinity for DNA and is released, allowing access to the lesion by DNA-repair enzymes (4); excision-repair seals the DNA strand break (5). PARP-1 is regenerated following degradation of ADP-ribose polymers by PARG (from D'Amours *et al.*(1999)).

1.5.3 PARP-1's Role in Sister Chromatid Exchange

Sister chromatid exchange (SCE) involves double-strand break recombination between homologous DNA duplexes, although the exact mechanism for this remains unclear. However, SCE almost certainly involves recombination events for which breaking and rejoining of DNA is essential.

The role of PARP-1 in SCE has been investigated by several laboratories. It was reasoned that inhibiting PARP-1 activity should increase the frequency of SCE (Utakoji *et al.* 1979) and this was found to be the case in studies utilising potent PARP-1 inhibitors such as 3-aminobenzamide (3-AB). The inhibition of PARP-1 activity by 3-AB was very effective at inducing SCE (Utakoji *et al.* 1979). Further studies by Natarajan *et al.* (1981) found that SCE in chinese hamster ovarian (CHO) cells increased in a dose dependent manner when PARP-1 inhibitors such as 5-bromodeoxyuridine were present. Cleaver *et al.* (1982) also observed an increase in SCE in CHO cells treated with methylmethanesulfonate (MMS), a potent PARP-1 inhibitor. Random mutagenesis was used to generate rodent cell lines deficient in PARP-1 activity and these cell lines also showed an increased frequency of spontaneous SCE (Chatterjee *et al.* 1989). Subsequent studies by Meyer *et al.* (2000) showed that PARP-1 overexpression was extremely effective at suppressing the induction of SCE by the alkylating agent methyl-N'-nitro-N-nitros-guanidine (MNNG). On the basis of these findings, Meyer and his collaborators proposed that the abrogation of PARP-1 activity leads to the upregulation of DNA alkylation-induced SCE.

Thus, PARP-1 has emerged as a regulator of alkylation-induced SCE that is strictly negatively controlled by the level of enzyme activity (Burkle 2001). This suggests that PARP-1 activity is not only crucial for BER in the cell but it may also play a crucial role in maintaining chromosomal integrity and genomic stability.

1.5.4 PARP-1's Role in Maintaining Genomic Stability

Initial studies to investigate PARP-1's role in maintaining genomic stability involved the generation of stable HeLa cell lines depleted of PARP-1 (Ding *et al.* 1992). This was achieved by the induction of PARP-1-antisense RNA expression. HeLa cell lines

depleted of PARP-1 showed alterations in cell morphology, chromatin architecture and DNA repair suggesting that PARP-1 activity plays a role in the maintenance of genomic stability (Ding *et al.* 1992).

To further define and clarify PARP-1's role in maintaining genomic stability, PARP-1 "knock-out" mice were generated. Wang *et al.* (1997) found that PARP-$1^{-/-}$ splenocytes exhibited a high frequency of SCE, suggesting that PARP-1 plays an important role in DNA recombination and stabilisation of chromatin structure. To substantiate PARP-1's role in genomic stability, Wang *et al.* (1997) enumerated micronuclei; micronuclei are often used as a biomarker for genomic stability. Wang *et al.* (1997) found that cells deficient of PARP-1, treated with γ-irradiation or mitomycin C (MMC) had a significantly higher number of micronuclei when compared to wild-type and heterozygous cells. This is further evidence that cells deficient of PARP-1 or poly(ADP-ribosylation) have compromised genomic stability following experimental stress. From this work, Wang *et al.* (1997) argued that PARP-1 has the ability to suppress recombination at DNA ends.

To investigate the possibility that PARP-1 may be involved in telomere maintainence in the cell, d'Adda di Fagagna *et al.* (1999) studied the length of the TTAGGG telomeric repeat in PARP-$1^{+/+}$, PARP-$1^{+/-}$ and PARP-$1^{-/-}$ mice using quantitative fluorescence *in situ* hybridisation. These workers found that PARP-1-deficient mice exhibit telomere shortening. Telomere shortening was observed in cells from different genetic backgrounds and tissues, both from embyros and adult mice. However, *in vitro* telomerase activity was not altered in PARP-$1^{-/-}$ mouse fibroblasts (d'Adda di Fagagna *et al.* 1999). Further studies by the same authors using cytogenetic analysis revealed severe chromosomal instability evident in PARP-$1^{-/-}$ mouse fibroblasts due to the increased frequencies of chromosome infusions and aneuploidy (d'Adda di Fagagna *et al.* 1999). On the basis of these findings it was proposed that PARP-1 plays a crucial role in the regulation of telomere length, providing further insights into its function in maintaining genomic integrity.

1.5.5 PARP-1 and Apoptosis

Apoptosis or programmed cell death (PCD) is a biological process that plays an important role in early development, cell homeostasis and disease in metazoans (for reviews see Kerr (1999) and Wyllie *et al.* (1980)). Apoptosis can be induced in cells by a variety of stimuli such as excessive genotoxic damage, withdrawal of growth factors or activation of specific receptors (CD95 or TNF receptors). Apoptosis is characterised by a distinct set of structural changes such as membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and finally fragmentation into membrane bound apoptotic bodies.

Numerous studies have demonstrated that PARP-1 is rapidly and specifically cleaved during apoptosis (Lazebnik *et al.* 1994; Nicholson *et al.* 1995; Tewari *et al.* 1995). PARP-1 is cleaved by caspase 3 (also known as CPP32 or Yama or apopain), a mammalian interleukin-converting enzyme (ICE)-related protease involved in apoptosis (Nicholson et al. 1995; Tewari et al. 1995). Duriez *et al.* (1997) showed that inhibiting PARP-1 cleavage by Bcl-2 over-expression or inhibiting caspase activity also blocked apoptosis. On the basis of these findings, Duriez *et al.* (1997) proposed that PARP-1 cleavage was essential for the efficient completion of apoptosis. However, it should be noted that it is possible that Bcl-2 over-expression or the inhibition of caspase activity may also severely impact on several other aspects of apoptosis - not just PARP-1 cleavage, blocking the apoptotic pathway. Thus, further work was required to determine the role of PARP-1 cleavage in apoptosis.

Oliver *et al.*(1998) conducted experiments in which a non-cleavable variant of PARP-1 (D214A-PARP-1) was expressed in PARP-1 deficient cells. Cells expressing the non-cleavable variant exposed to anti-CD95 antibody showed a significant delay in cell death, whilst morphological analysis revealed cell shrinkage and nuclear condensation. Thus, it appears that PARP-1 cleavage during apoptosis facilitates cellular disassembly and completion of cell death. Oliver *et al.* (1998) proposed that PARP-1 cleavage by caspases promotes apoptosis by disabling a key aspect of the cellular genomic surveillance network, preventing unnecessary DNA repair that would delay chromatin breakdown. A key component of the apoptotic process is the specific degradation of chromatin and DNA fragmentation, resulting in numerous single-stranded nicks in the linker regions of chromatin (Peitsch *et al.* 1993) that,

presumably, PARP-1 would interact with (Le Cam *et al.* 1994). However, inactivation of PARP-1 by apoptotic cleavage would prevent this and thus improve access of endonucleases to chromatin leading to nuclear disintegration (Oliver *et al.* 1998).

1.5.6 PARP-1 and Transcription

Numerous studies have identified the role of PARP-1 and poly(ADP-ribosylation) in the regulation of transcriptional activity within the cell (Mullins *et al.* 1977; Hough and Smulson 1984). Many researchers believe that the remodeling of the DNA architecture induced by the negatively charged poly(ADP-ribose) polymer helps maintain the chromatin loops in transcriptionally active structures (D'Amours *et al.* 1999). For example, Hough and Smulson (1984) showed that PARP-1 is localised in regions of actively transcribed chromatin and they believe that its presence perhaps facilitated the detection of transcription initiation sequences. Recently, PARP-1's association with transcription initiation sequences was shown to promote the activity of various transcription factors including activator protein 2 (AP-2) (Kannan *et al.* 1999), p53 (Wang *et al.* 1998) and nuclear factor κB (NF-κB) (Hassa and Hottinger 1999). This suggests that PARP-1 is able to promote transcription by enhancing protein complex formation. Therefore PARP-1 can either up- or down- regulate gene expression. For example, upon recognition of DNA damage in the cell, PARP-1 initiates the DNA repair process and other vital processes such as transcription or replication are postponed until the DNA repair process have been completed (Ziegler and Oei 2001). Ziegler and Oei (2001) proposed that the immediate goal of poly(ADP-ribosylation) following the detection of DNA damge is to avoid the transcription (or replication) of damaged genes by preventing new transcription from being initiated and speeding up ongoing transcription. Once the damaged DNA has been repaired, transcription or replication proceeds. However, the exact nature of these interactions between PARP-1, poly(ADP-ribosylation) and transcription remains unknown.

Further evidence supporting PARP-1's involvement in gene transcription was reported by Soldatenkov *et al.* (2002). Soldatenkov *et al.*(2002) found that PARP-1 was able to bind to DNA secondary structures (hairpins) in heteroduplex DNA in a

DNA end-independent fashion. The ability of PARP-1 to bind DNA hairpins was inhibited by the automodification of PARP-1 in the presence of NAD^+. Using atomic force microscopy, chromatin cross-linking and immunoprecipitation, Soldatenkov *et al.* (2002) showed that PARP-1 bound to the PARP-1 promoter *in vitro* and *in vivo*. This suggests that the PARP-1 protein may be a potent regulator of transcription, including regulation of its own promoter.

1.6 Scope and Aim of this Thesis

Whilst a considerable amount is known about PARP-1's structure and the list of its putative role(s) increases, there is still a great deal of work to be done to define the precise functions of this enzyme and its mode(s) of action in the cell. Studies on the relationship between the structure and function of this somewhat enigmatic protein will go a long way to resolving this. A major aim of the laboratory at Victoria University is to produce wild-type and site-directed mutant versions of human PARP-1 in quantities and of a quality that will enable approaches such as X-ray crystallography to be applied to structure-function studies. This necessitates the development of techniques for the production and purification of the enzyme with high specific activity.

Several laboratories have had some success in producing purified PARP-1 from a range of tissues (Mandel et al. 1977; Okayama et al. 1977; Yoshihara et al. 1978; Petzold et al. 1981; Carter and Berger 1982; Burtscher et al. 1986) and/or recombinant expression systems (Simonin *et al.* 1990; Giner *et al.* 1992; Kaiser *et al.* 1992; Avila *et al.* 1994; Jung *et al.* 1994; Trucco *et al.* 1996; Rolli *et al.* 1997; Perrin *et al.* 2000) and this work will be reviewed in the introduction to Chapters 3 and 4. In brief, extracting PARP-1 from tissues is problematic because large amounts of starting material are required for producing yields of PARP-1 suitable for X-ray crystallography. Producing PARP-1 from recombinant sources has been met with varying degrees of success. Using bacterial and yeast expression systems for example has often led to the production of truncated protein products or the precipitation of protein as insoluble inclusion bodies, yielding relatively low quantities of poor quality PARP-1 protein. To date, the most efficient and successful recombinant expression system for PARP-1 protein has been the insect cell-based baculovirus expression system. Only one paper in the literature reports baculovirus expression of full-length

human PARP-1 (Giner *et al.* 1992) which produced a relatively high quality protein product. Thus research in the field of PARP-1 structure-function analysis awaits the development of a method for producing high levels of good quality enzyme. This thesis describes such a development.

This thesis describes:

i. the development of an efficient expression system to produce large amounts of recombinant human PARP-1, and

ii. the development of a purification procedure that is simple, rapid and efficient.

While carrying out this research it was found that determining protein concentration for purified preparations of PARP-1 can be problematic. This thesis describes how this problem can be dealt with, making recommendations to other workers in the field.

2 Materials and Methods

2.1 Materials

2.1.1 General Chemicals and Materials

All general and common chemicals were analytical grade and obtained from the Sigma Chemical Company, the British Drug House (BDH), or the Ajax Chemical Company.

2.1.2 Chromatographic Materials

The column and packing reservoir used throughout the purification of PARP were obtained from Amersham Biotech Ltd., Uppsala, Sweden. The DNA-Cellulose (Native DNA) resin was also obtained from Amersham Biotech Ltd., Uppsala, Sweden. The column was packed according to the manufacturer's instructions.

2.1.3 Materials Required for the Detection of PARP Activity

Histone H1 (H 5880) and 2-,5-diphenyl-oxazole (PPO) were obtained from the Sigma Chemical Company.

2.1.4 General Equipment used for Experimental Procedures

The spectrophotometer used for all experimental analysis was the LKB Ultraspec Plus, 4054 UV/Visible spectrophotometer. The scintillation counter used for all experimental analysis was the Wallac 1410 Liquid Scintillation Counter from Amersham Biotech. The peristaltic pump used for column chromatography was the Pharmacia, LKB Pump P1 and the fraction collector was the Pharmacia, LKB Bromma 7000 Ultrorac Fraction Collector. Centrifugation was carried performed in a Beckman J2-HS Refrigerated Centrifuge or Beckman Optima L-70 Ultracentrifuge or a microfuge. Protein preparations were separated using a mini protean II dual slab cell from Bio-Rad laboratories Hercules, California, USA. The stained protein gels were photographed with a Fujifilm LAS1000 charged-coupled device (CCD) and analysed using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998).

2.1.5 Strains

All the *Escherichia coli*, *Saccharomyces cerevisiae* and *Spodoptera frugiara* strains used in this thesis are listed in Table 2.1.

2.1.6 Vectors

All the cloning and protein expression vectors used in this thesis are listed in Table 2.2.

2.1.7 Media Requirements and Antibiotic Preparation

2.1.7.1 Media for Growth and Maintenance of Escherichia coli

All *Escherichia coli* strains were maintained in Luria Bertani (LB) medium: 10 g tryptone, 5 g yeast extract and 5 g NaCl per litre (15 g agar per litre was added for solid medium). The pH of the medium was brought to 7.5 and autoclaved 121°C for 15 minutes. If an antibiotic was required in the medium, the medium was allowed to cool to 55°C before the antibiotic was added.

2.1.7.2 Media for Growth and Maintenance of Yeast Cultures

Yeast cultures were grown in rich media containing glucose (YEPD) or defined media: YNB glucose, YNB galactose, YNB raffinose or YNB glycerol and glucose. The components of all these media are shown in Table 2.3. For solid media, agar was added to a final concentration of 1.5 % (w/v). The media was autoclaved at 121°C for 15 minutes and allowed to cool to 55°C before the addition of any supplements (if required). Yeast stock cultures were maintained at -80°C in 2x YEPD plus 15 % (v/v) glycerol.

2.1.7.3 Media for the Growth and Maintenance of Insect Cell Cultures

Growth and maintenance of insect cells was carried out in *Sf*-900 II SFM liquid medium supplied by Invitrogen Inc. (Catalogue number: 10902-013).

Table 2.1: Bacteria, yeast and insect cell lines.

Organism	Strain	Genotype	Source
Escherichia coli	TG2	*supEhsdΔ5thiΔ(lac-proAB) Δ(sr1-recA)306::Tn10(tet)^r*	VUT Culture Collection
	BL21 (DE3)	*hsdSgal(λcIts857ind1Sam7nin5lacUV5-T7 gene1)*	VUT Culture Collection
	HB101	*supE44hsdS20($r_B^-m_B^-$)recA13ara-14 proA2lacY1galK2rpsL20xyl-5mtl-1*	VUT Culture Collection
	DH10Bac		Invitrogen Inc., Carlsbad, California, USA
Saccharomyces cerevisiae	PMY 1.1	*αleu2ura3his4*	VUT Culture Collection
	BJ2168	*αleu2trp1ura3 phb1-1122pep4-3 prc1-407gal2*	VUT Culture Collection
	SUB61	*αleu2ura3his3lys2trp1*	VUT Culture Collection
Spodoptera frugiara	*Sf-9*		Invitrogen Inc., Carlsbad, California, USA Dr. Michelle, St. Vincent's Medical Research Institute, Fitzroy, Victoria, Australia
	Sf-21		Invitrogen Inc., Carlsbad, California, USA Dr. White, Animal Health Laboratories, CSIRO, Geelong, Victoria, Australia.

Table 2.2: Vectors.

Plasmid	Type	Host	Size (kb)	Selection Marker	Source
pPARP	Cloning	*E. coli*	5.8	Amp*	Prof. G. de Murcia, Institut de Moleculaire et Cellulaire, Centre National Recherche, Strasbourg, France.
pGEX-5X-3	Expression	*E. coli*	3.9	Amp*	Amersham Biotech Ltd., Uppsala, Sweden.
pYES2	Yeast Expression	*E. coli*	5.9	Amp*	Invitrogen Inc., Carlsbad, California, USA.
pFastBac1	Baculovirus Expression	*E. coli*	4.8	Amp*	Invitrogen Inc., Carlsbad, California, USA.
pFastBacHTb	Baculovirus Expression	*E. coli*	4.9	Amp*	Invitrogen Inc., Carlsbad, California, USA.
pFastBac1-CAT	Baculovirus Expression	*E. coli*	5.6	Amp*	Invitrogen Inc., Carlsbad California, USA.

*Amp is an abbreviation for ampicillin.

2.1.7.4 *Preparation of Antibiotic Stock Solutions*

Antibiotic stock solutions were prepared by dissolving the antibiotic in sterile distilled water, ethanol or dimethylformamide and then filter sterilizing the dissolved solution through a 0.22 μm filter. The stock concentration of each antibiotic is listed in Table 2.4. The solutions were dispensed into 1 ml aliquots and stored in light proof containers at –20°C until use.

2.1.8 Standard Stock Solutions

Acrylamide Stock Solution: 29.1 g Acrylamide (National Diagnostics) and 0.9 g NN'-Methylbis-acrylamide (BDH) were dissolved in 60 ml distilled water. Once dissolved, the acrylamide stock solution was made up to 100 ml with distilled water and stored in an amber coloured bottle at 4°C, for up to 2 weeks (LKBPharmacia 1986).

10 % (w/v) Ammonium Persulphate Solution (APS): 0.5 g Ammonium persulphate was dissolved in 4 ml of distilled water. Once dissolved, the APS solution was made up to 5 ml with distilled water (LKBPharmacia 1986).

Bradford Protein Reagent: 100 mg of Coomassie Brilliant Blue G250 was dissolved in 50 ml of 95% (v/v) ethanol. The solution was then mixed with 100 ml of 85 % (w/v) orthophosphoric acid and made up to a final volume of 1000 ml with double-distilled water (Bradford 1976).

Coomassie Staining Solution: 1.25 g Coomassie Brilliant Blue (CBB) G250 was dissolved in 230 ml methanol and 230 ml distilled water. This solution was stirred for an hour and then 40 ml glacial acetic acid was added. After the addition of the glacial acetic acid, if any particles appeared, the solution was filtered through Whatman 3M filter paper (Whatman International Ltd., Maidstone, England) (LKBPharmacia 1986).

Developing Solution for Silver Staining SDS-PAGE Gels: 5.0 g Sodium carbonate and 20 μl of formaldehyde (added immediately before use), made up to a final volume of 200 ml with distilled water (LKBPharmacia 1986).

Table 2.3: Composition of media required for the growth and maintenance of Yeast cultures.

Type of Media	**Media Composition** (all percentage values expressed as (w/v)
YEPD	1 % yeast extract, 2 % bactopeptone and 2 % glucose
YNB glucose	0.67 % yeast nitrogen base without amino acids, 0.5 % casamino acid and 2 % glucose.
YNB galactose	0.67 % yeast nitrogen base without amino acids, 0.5 % casamino acid and 2 % galactose.
YNB raffinose	0.67 % yeast nitrogen base without amino acids, 0.5 % casamino acid and 4 % raffinose.
YNB glycerol and glucose	0.67 % yeast nitrogen base without amino acids, 0.5 % casamino acid 5 % glycerol and 0.1 % glucose.

The following supplements were added as required:
L-leucine (30 μg/l), L-lysine (30 μg/l), L-tryptophan (20 μg/l), L-histidine (20 μg/l) and uracil (20 μg/l).

Table 2.4: Preparation of Antibiotic and Indicator Stock Solutions.

Antibiotic	**Stock Concentration**	**Working Concentration**	**Reference**
Ampicillin (Amp)	50 mg/ml	100 μg/ml	Sambrook *et al.* 1989.
Kanamycin (Kan)	12.5 mg/ml	50 μg/ml	Sambrook *et al.* 1989.
Tetracyclin* (Tet)	10 mg/ml	10 μg/ml	Sambrook *et al.* 1989.
Gentamycin (Gent)	6.7 mg/ml	7 μg/ml	Sambrook *et al.* 1989.
IPTG	200 mg/ml	upto 40 μg/ml	Sambrook *et al.* 1989.
X-gal**	50 mg/ml	100 μg/ml	Sambrook *et al.* 1989.

* dissolved in ethanol.
**dissolved in dimethylformamide.

10X DNA loading buffer: consisted of 0.25 % Bromophenol blue 0.25 % Xylene cyanol FF and 40 % (w/v) sucrose dissolved in sterile double-distilled water (Sambrook *et al.* 1989).

Fixing Solution for Coomassie and Silver Stained SDS-PAGE Gels: 80 ml ethanol and 20 ml glacial acetic acid was made up to a final volume of 200 ml with distilled water (LKBPharmacia 1986).

Incubation Solution for Silver Staining SDS-PAGE Gels: 60 ml Ethanol, 13.6 g Sodium acetate.$3H_2O$, 0.4 g Sodium thiosulphate and 1.04 ml Glutardaldehyde (added immediately before use), made up to a final volume of 200 ml with distilled water (LKBPharmacia 1986).

Lowry Protein Assay Solution A: consisted of 1 ml 5 % (w/v) $CuSO_4$ and 9 ml 1 % (w/v) potassium sodium tartate was added to 100 ml 10 % (w/v) Na_2CO_3 in 0.5 M NaOH (Lowry *et al.* 1951).

Lowry Protein Assay Solution B: consisted of 1 ml Folin-Ciocalteu's reagent and 10 ml double-distilled water (Lowry *et al.* 1951).

1X NanoOrange® protein quantitation diluent: was prepared by diluting NanoOrange® Protein diluent (Component B) 10 fold in double-distilled water (MolecularProbes 2001).

1X NanoOrange® working solution: was prepared by diluting NanoOrange® protein quantitation reagent (Component A) 500 fold in 1X NanoOrange® protein quantitation diluent. It was essential to protect the 1X NanoOrange® working solution from light and the solution was used within a few hours after preparation (MolecularProbes 2001).

10X Phosphate Buffered Saline (PBS): was prepared by dissolving 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4.7H_2O$ and 2 g KH_2PO_4 in 1000 ml of double distilled water.

The solution was then autoclaved at 121°C for 15 minutes and stored at room temperature until use (Sambrook *et al.* 1989).

1X PBS: was prepared by diluting 100 ml of 10X PBS in 900 ml of sterile double distilled water. The solution was filter sterilized and ready for use (Sambrook *et al.* 1989).

*Protein Extraction Buffer:*consisted of 500 mM Tris HCl, 50 mM $MgCl_2$, 2 mM EDTA, 2 % SDS, 2 % β-mercaptoethanol, 0.5 μg/ml RNase (optional), 1 ml 100X protease inhibitors (TPCK, 0.2 mg/ml pepstatin, 100 mM PMSF).

Scintillation Cocktail: consisted of 0.5 % (w/v) 2-,5-diphenyl-oxazole (PPO) dissolved in 100 % toluene (Tsopanakis *et al.* 1978).

10 % (w/v) SDS Solution: consisted of 10.0 g SDS dissolved in 85 ml of distilled water. Once dissolved, the SDS solution was made up to 100 ml with distilled water (Sambrook *et al.* 1989).

10X SDS-PAGE Electrode Buffer: consisted of 144.2 g Glycine, 30.3 g Tris base and 10.0 g SDS dissolved in 800 ml distilled water. Once dissolved, the 10X Electrode buffer was made up to 1 L with distilled water and stored at room temperature for up to 2 months (LKBPharmacia 1986).

1X SDS-PAGE Electrode Buffer: consisted of 40 ml of 10X Electrode buffer diluted to a final volume of 400 ml with distilled water (360 ml) (LKBPharmacia 1986).

2X SDS-PAGE Loading Buffer: consisted of 2.5 ml 1.25M Tris-HCl pH 6.8, 1 g SDS, 2.5 ml 2-Mercaptoethanol, 5.8 ml Glycerol (87 %), 5 mg Bromophenol blue and 35 ml distilled water. This solution was stirred until all of the SDS and Bromophenol blue had dissolved. Once dissolved, the 2X SDS-PAGE loading buffer was brought to a total volume of 50 ml with distilled water and stored at -20°C for up to 2 months (LKBPharmacia 1986).

Silver Solution for Silver Staining SDS-PAGE Gels: 0.2 g Silver nitrate and 40 µl of formaldehyde (added immediately before use), made up to a final volume of 200 ml with distilled water (LKBPharmacia 1986).

Stop Solution for Silver Staining SDS-PAGE Gels: 2.92 g of EDTA-$Na_2.2H_2O$, made up to a final volume of 200 ml with distilled water (LKBPharmacia 1986).

Solution I for Bacmid DNA preparation: consisted of 15 mM Tris HCl (pH 8.0), 10 mM EDTA and 100 µg/ml RNase A (Gibco-BRL-LifeTechnologies 1990).

Solution II for Bacmid DNA preparation: consisted of 0.2 N NaOH and 1 % SDS. This solution must always be prepared fresh (Gibco-BRL-LifeTechnologies 1990).

50X TAE Buffer System for Agarose Gel Electrophoresis: 50X TAE Buffer was prepared by dissolving 242.2 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) in a total volume of 1000 ml of double-distilled water. The solution was autoclaved at 121°C for 15 minutes. The working concentration was 1X (Sambrook *et al.* 1989).

1.875 M Tris-HCl Buffer, pH 8.8: 28.4 g Tris was dissolved in 80 ml of distilled water. The pH of the solution was then carefully adjusted to 8.8 with 5 N HCl. Once adjusted to pH 8.8, the 1.875 M Tris-HCl buffer, pH 8.8 was brought to a final volume of 125 ml with distilled water. This solution was then stored at 4°C and is stable for a period of 4 weeks (LKBPharmacia 1986).

1.25 M Tris-HCl Buffer, pH 6.8: 37.8 g Tris was dissolved in 150 ml of distilled water. The pH of the solution was carefully adjusted to 6.8 with 5 N HCl. Once adjusted, the 1.25 M Tris-HCl buffer, pH 6.8 was made up to 250 ml with distilled water and stored at 4°C for up to 4 weeks (LKBPharmacia 1986).

2.2 Methods

2.2.1 Recombinant DNA Techniques

2.2.1.1 Preparation and Transformation of Competent Cells

Competent *E. coli* cells were prepared by $CaCl_2$ treatment as described in Sambrook *et al.* (1989). 1 ml of an overnight culture of *E. coli* was inoculated into 100 ml of LB broth and grown at 37°C, 200 rpm until the OD at 600 nm was between 0.3-0.4. This took approximately between 2 and 3 hours and ensured that the cells were in mid-exponential growth phase and thus had the ideal cell density of approximately $5x10^7$ cells/ml to maximise transformation efficiency. The cells were then harvested by centrifugation at 4000 *g* for 5 minutes at 4°C and resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$ containing 15 % glycerol. This procedure was repeated. After 20 minutes incubation on ice, the cells were then collected by centrifugation at 4000 *g* for 5 minutes at 4°C and resuspended in 0.5 ml of ice-cold 0.1 M $CaCl_2$ containing 15 % glycerol. The cells were immediately dispensed into 50 µl aliquots and store at -80°C until required for transformation.

Transformation was performed as described in Sambrook *et al.* (1989). Frozen competent cells (50 µl aliquots) were thawed on ice and incubated with the DNA (100 – 500 ng) on ice for 20 minutes. The cells were then heat shocked for 2 minutes at 42°C and transferred immediately to a waterbath at 37°C with the addition of 0.5 ml of prewarmed LB broth. After incubation at 37°C for one hour, cells were plated on selective growth media (preferably 150 µl per plate) and incubated overnight at 37°C. All transformation procedures were performed with a no DNA control.

2.2.1.2 Small Scale Isolation of Plasmid DNA

Small scale plasmid DNA isolation was performed as described by Du (1997) (http://genome.wustl.edu/tools/finishing/Plasmid_mini_prep_II.pdf) with some minor alterations. 1.5 ml of an overnight culture was harvested by centrifugation at 14,000 *g* in a benchtop microfuge for 2 minutes. The pellet was resuspended in 150 µl ET buffer (25 mM Tris-HCl, pH8.0, 10 mM EDTA) containing RNase A (add 10 µl of 10 mg/ml RNase A/DNase-free per ml of ET buffer). The sample was then vortexed vigorously for 3 minutes following which 150 µl of freshly prepared lysis solution (1

ml of 10 M NaOH, 5 ml of 10% SDS, 44 ml of double-distilled H_2O) was added. The sample was then mixed by gently inverting a few times and left at room temperature for 5 minutes. 150 µl of 2.55 M KOAc, pH 4.8 (10 ml 3 M KOAc, pH 4.8 and 1.7 ml double-distilled H_2O) was added to the preparation, inverted twice to mix and incubated on ice for 5 minutes. The preparation was then centrifuge in a benchtop microfuge at 14,000 *g* for 5 minutes to pellet chromosomal DNA and cell debris. The supernatant (approximately 400 µl) was removed to a clean tube and an equal volume of phenol:chloroform (1:1) added. The preparation was then vortexed for 10 seconds and centrifuged for 5 minutes at 14,000 *g* in a benchtop microfuge to separate the phases. The aqueous phase was then transferred to a clean tube containing 1 ml of absolute ethanol. The sample was placed on ice for 10 minutes and then centrifuged in a benchtop microfuge at 14,000 *g* for 10 minutes to pellet plasmid DNA. The supernatant was discarded and the DNA pellet washed with 150 µl of 70% ethanol. The DNA pellet was dried briefly under reduced pressure and resuspended in 40 µl of sterile ddH_2O. The plasmid preparation (3 µl) was then analysed using agarose gel electrophoresis (Section 2.2.1.8).

2.2.1.3 Rapid Small Scale Isolation of Plasmid DNA

Rapid small scale plasmid DNA isolation was performed as described by Sambrook *et al.*(1989). 1.5 ml of an overnight culture grown at 37°C was harvested by centrifugation in a benchtop microfuge at 14,000 *g* for 2 minutes and resuspended in 350 µl of STET buffer (0.1 M NaCl, 10mM TrisHCl (pH 8.0), 1 mM EDTA (pH 8.0) and 5% Triton X-100). 25 µl of freshly prepared lysozyme solution (10 mg/ml in 10 mM TrisHCl, pH 8.0) was added and the mixture vortexed for 3 seconds. After vortexing briefly, the mixture was placed in a boiling waterbath for 40 seconds and then centrifuged at room temperature at 14,000 *g* for 10 minutes. Cellular debris was removed using a sterile toothpick and then 40 µl 2.5 M sodium acetate (pH 5.2) and 420 µl isopropanol added to the supernatant. The sample was mixed by vortexing and stored at room temperature for 5 minutes. Plasmid DNA was recovered by centrifugation at 14,000 *g* for 5 minutes at 4°C. The supernatant was removed via gentle aspiration and the DNA pellet washed with 150 µl of 70% ice-cold ethanol. The sample was again centrifuged at 14000 *g* for 2 minutes and the DNA dried briefly under reduced pressure. The DNA was then resuspended in 40 µl of sterile ddH_2O

and 3 µl of the plasmid preparation was then analysed using agarose gel electrophoresis (Section 2.2.1.8).

2.2.1.4 *Large Scale Purification of Plasmid DNA*

Large scale production of recombinant plasmid DNA was isolated by the boiling method described in Sambrook *et al.*(1989). The recombinant plasmid DNA was then separated from the chromosomal *E. coli* DNA using Ethidium Bromide-Caesium Chloride (EtBr-CsCl) gradient centrifugation. The purified plasmid DNA was removed from the quick-seal tube using a syringe; followed by Et-Br removal using isoamyl alcohol (this was repeated several times) and CsCl removal by precipitating the DNA with a final volume of ethanol (~70 % ethanol).

2.2.1.5 *Spectrophotometric Method for determining DNA Quality and Quantity*

DNA concentration was determined using the method described by Sambrook *et. al.* (1989). DNA samples or oligonucleotides (10 µl) were added to sterile distilled water (990 µl). The optical density (OD) of the diluted DNA samples were measured at 260 and 280 nm using an Ultraspec III UV/Vis spectrophotometer (Amersham Biotech). Sterile distilled water was used as a blank. The reading at 260 nm allowed for calculation of nucleic acid concentration in the sample: an OD of one corresponded to 50 µg/ml double stranded DNA or 33 µg/ml single stranded DNA. Pure preparations of DNA had an OD260/OD280 value of 1.8. If there was contamination with protein or phenol, the OD260/OD280 value obtained was less 1.8. If this occurred the DNA sample was again precipitated with 70 % ethanol and the procedure repeated.

2.2.1.6 *Agarose Gel Method for determining DNA Quantity*

The agarose gel method for determining DNA concentration was performed as described by Sambrook *et al.*(1989). A 1.0 % agarose solution containing ethidium bromide (2 µl) was prepared in 1 x TAE buffer (50 ml). DNA samples were prepared in sterile distilled water (5 µl) containing loading buffer (2 µl) and DNA (3 µl). DNA samples (10 µl) were loaded into the gel and run for one hour at 80 V with 1X TAE as the running buffer. DNA concentration standards (varying from 1-20 µg/ml) were run parallel with the DNA samples. The quantity of DNA was estimated after

photography of the gel comparing the intensity of sample DNA bands with the DNA standards.

2.2.1.7 *Restriction Enzyme Digestion of Plasmid DNA*

As in the manufacturer's (New England Biolabs) instruction: 1 μg of DNA was digested with 10 U of enzyme, restriction enzyme buffer and BSA (if appropriate). Sterile water was added to the restriction digests to achieve a final volume of 10 μl or 30 μl.

2.2.1.8 *Agarose Gel Electrophoresis of Plasmid DNA, Plasmid DNA Restriction Fragments and PCR Products*

To determine the size of plasmid DNA, restriction fragments and PCR products, samples were electrophoresed in 1% agarose gels. All gels were prepared using 1X TAE and 1/500 volume of ethidium bromide (10 mg/ml) (Sambrook *et al.*, 1989). The samples were mixed with 10X DNA loading buffer. The gels were electrophoresed in 1X TAE buffer at typically 80-100 V for 1-2 hours. Appropriate marker DNA, e.g., Lambda DNA – *Eco*RI/*Hind*III (0.564 kb-21.22 kb) or PCR marker (0.1 kb-2 kb) were used for estimating the size of the DNA bands as per Sambrook *et. al.* (1989). The gels were viewed on a UV Transilluminator and photographed using a MP4 Land Camera (Polaroid) and 667 positive film (Polaroid) using a F5.6 aperture and 1/8 second exposure time.

2.2.1.9 *Purification of DNA Fragments from Agarose Gel using Progen BANDPURE™*

The purification of DNA fragments from agarose gels using BANDPURE™ from Progen was performed as described by the supplier's protocol. The approximate DNA concentration of the purified product was determined on agarose gel, using the appropriate intensity of one band of the marker DNA for comparison. The products purified by this method were ready for cloning into various vectors as described in Section 2.2.1.10.

2.2.1.10 *Preparation of Products for Ligation and Ligation Reactions*

Before ligation, 5 µg of vector DNA was digested with the appropriate restriction enzyme (10-20 U) and dephosphorylated using 0.1 U calf intestinal alkaline phosphatase (CIP) according to the manufacturer's instruction (Boehringer Mannheim). The reaction volume varied from 10 µl to 30 µl. To ensure complete digestion, an aliquot of the restriction enzyme digest was separated using agarose gel electrophoresis and visualised on a UV transilluminator. After complete digestion and dephosphorylation was achieved, the reaction was heat inactivated at 85°C for 15 minutes. Vector DNA was then purified for ligation by phenol-chloroform extraction followed by ethanol precipitation or by BANDPURE™ extraction. Insert DNA was prepared by digestion and agarose gel electrophoresis followed by BANDPURE™ extraction. The following ligation ratios of insert – vector DNA were set up 1:1, 3:1, 1:3 and 5:1 using the following formulae:

For 1:1 ratio of insert – vector: $A = (C*D)/B$

For 3:1 ratio of insert – vector: $A = [(C*D)/B]*3$

For 1:3 ratio of insert – vector: $A = [(C*D)/B]/3$

For 5:1 ratio of insert – vector: $A = [(C*D)/B]*5$

Where A = Amount of Insert required for ligation procedure (ng)

B = Size of insert to be ligated (bp)

C = Amount of vector used in the ligation procedure (ng) (always 10 ng)

D = Size of vector in the ligation procedure (bp) (Sambrook *et al.* 1989).

2.2.1.11 *Synthesis and Purification of Oligonucleotides*

Oligonucleotides were either synthesised commercially by Pacific Oligos or in house, using a DNA synthesier 391 (Applied Biosystems Inc.). The oligonucleotides that were synthesised in house required the oligonucleotide to be cleaved from column, deprotected, dehydrated and precipitated. This was performed as per the instruction manual provided by the supplier (Applied Biosystems Inc.).

Cleavage of the oligonucleotide from the Synthesis Column:

1. 5-7 ml concentrated ammonia solution (30-35 %) was dispensed into a clean glass McCartney bottle. The tubes were tightly capped.
2. The tip of a 1 ml syringe was inserted into one end of the column. The syringe fitted snugly with the plunger into the column.
3. 0.75 ml concentrated ammonia was drawn into another 1 ml syringe. Any air bubbles at the tip were removed.
4. The second syringe was inserted into the other end of the column. The syringe-column-syringe unit was held horizontally.
5. The plunger of the syringe containing ammonia was pushed gently and the ammonia was allowed to pass through the column onto the other side of the syringe. Pushing the ammonia back and forth between the syringes 4-5 times.
6. The syringe-column-syringe unit was laid down on a flat surface and incubated for 15 minutes at room temperature, making sure that there was ammonia solution inside the column.
7. Step 5 was repeated.
8. The ammonia solution was drawn into one of the syringes, which was then removed from the column. The ammonia was expelled into a pre-cleaned 4 ml vial and the vial was tapped tightly with a teflon-lined screw cap.
9. Steps 2-8 were repeated four times. The ammonia solution containing the oligonucleotide eluted at each stage were pooled together (total volume, 3 ml) into a vial, capped tightly and stored at –20°C or deprotected.

2.2.1.12 *Deprotection of Oligonucleotides*

The tightly closed vial containing the oligonucleotide was placed at 55°C overnight or for 8-12 hours. The oligonucleotide was ready for deprotection.

Dehydration of Oligonucleotides:

The deprotected oligonucleotide was incubated in a waterbath at 40°C for 16 hours with the vial being capped loosely so that the ammonia evapourated, then frozen at – 70°C before dehydration. The frozen oligonucleotide was dehydrated overnight under vacuum in a freeze dryer.

Precipitation of Oligonucleotides:

The freeze-dried oligonucleotide was dissolved in 600 µl distilled water and 100 µl 3 M sodium acetate, pH 5.2; these volumes were calculated from the expected 20 optical density unit (ODU) of oligonucleotide (30 µl distilled water and 5 µl 3 M sodium acetate per 1 ODU of oligonucleotide product, according to the Manufacturer's instructions). 2 ml of ethanol were added to the oligonucleotide solution, which was vortexed briefly. The mixture was stored at –20°C for 30 minutes then centrifuged at 12,000 *g* in a microfuge for 5 minutes. The supernatant was removed and the oligonucleotide pellet was washed using 70 % ethanol. Finally, the oligonucleotide pellet was dried under vacuum and dissolved in 300 µl of distilled water. The oligonucleotide concentration was determined as described in Section 2.2.1.5.

2.2.1.13 Polymerase Chain Reaction

The polymerase chain reaction was performed according to the manufacturer's (Perkin Elmer Inc.) instructions. The standard polymerase chain reaction was performed in a total volume of 50 µl. The reaction contained 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of the dNTPs (dATP, dTTP, dGTP and dCTP), 2.5 units Ampli Taq DNA polymerase, 1 µM of each of the primers and 100 ng of template DNA. The PCR cycle consisted of an initial denaturation step at 94°C for 5 minutes, followed by 25 cycles of 94°C for 1 minute (denaturation), 54°C for 1 minute (annealing) and 72°C for 1.5 minutes (extension time). At the end of the cycling, a final extension time of 5 minutes was used. The PCR product was visualised by agarose gel electrophoresis (Section 2.2.1.8) and purified for further analysis as described in Section 2.2.1.14.

2.2.1.14 *Purification of PCR products for DNA Sequencing*

PCR products were purified from contaminants, primer-dimers and amplification primers by Wizard Preparations (Promega Inc., USA). Mini columns were prepared by removing the plunger from a 3 ml disposable syringe (Talus Manufacturing, Sydney) and attaching a mini column to the syringe outlet. 0.1 ml DNA purification resin was added to 40 µl PCR product and vortexed for 20 seconds. The resin/DNA was mixed together by vortexing or inversion for 20 seconds. This was repeated a repeated a further two times. The resin/DNA mixture was pipetted into the syringe barrel and carefully pushed into the mini column with the plunger. Isopropanol (80%, 2 ml) was used to wash the column. The column was removed from the syringe, placed in a 1.5 ml Eppendorf tube and centrifuged for 20 seconds at 12,000 *g* to dry the resin. The column was then transferred to a new Eppendorf tube and 30 µl of sterile water was applied to the column. After two minutes, the column was centrifuged (2 minutes at 12,000 *g*) to elute the bound DNA fragment. The purified DNA was stored at –20°C until use.

2.2.1.15 *Automated DNA Sequencing*

All automated DNA sequencing was performed by the DNA Sequencing Service at the Department of Microbiology, Monash University, Clayton Campus, Melbourne, Australia on an ABI 373A Sequencer (Applied Biosystems Inc., USA). DNA sequencing reactions were prepared at Victoria University using one of the following DNA sequencing kits; the ABI™ Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Standard FS) or the ABI™ Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. The composition of the reaction mixes are given in Table 2.5. Cycle sequencing reactions were performed for 25 cycles, each cycle consisting of step 1 (96°C, 30 seconds), step 2 (50°C, 15 seconds) and step 3 (60°C, 4 minutes) on the Peltcer Thermal Cycler (PTC-200, MJ-Research) and the product was further purified according to the manufacturer's instruction.

2.2.1.16 *Analysis of DNA sequence results and Alignment of amino acid sequence for the PARP-1*

DNA sequence alignments, comparisons and translation and alignment of amino acid sequences of PARP-1 were carried out using the BioEdit sequence alignment program, version 5.0.6 (Hall 1999).

Table 2.5: Composition of Sequencing Reactions.

Composition	Standard FS Kit	BigDye Kit
DNA Sample	30-90 ng PCR product or 300-500 ng plasmid DNA	30-90 ng PCR product or 300-500 ng plasmid DNA
Primer	1 µl (3-5 pmol)	1 µl (3-5 pmol)
*Premixed Reagents	8 µl	6 µl
Total Volume	20 µl	16 µl

*Premixed reagents were provided by Applied Biosystems.

2.2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Proteins

SDS-PAGE gels were prepared for electrophoresis according to LKB Pharmacia (1986). Electrophoresis was conducted in 1X SDS-PAGE electrode buffer (Section 2.1.8) at 20 mA in a Mini-Protean® electrophoresis tank (Bio-Rad Laboratories, Hercules, California, USA) for a specified time. The gels were 0.75mm thick and polymerised by the addition of 0.01% (v/v) of TEMED. Preparations of PARP-1 were treated with denaturing and reducing agents and so were suspended in an equal volume of 2X SDS-PAGE loading buffer (Section 2.1.8) and denatured by boiling at 100°C for 3 minutes prior to loading unless otherwise stated. Samples containing approximately 1-2 μg of protein were applied to wells of the gels using a 100 μl syringe.

2.2.2.1 Preparation of SDS-PAGE High Molecular Weight Markers

The samples were run against marker proteins for molecular weight determination. These marker proteins were obtained from Sigma Chemical Company, St. Louis, USA. The molecular weights of the marker proteins were myosin (205,000 Da), β-galactosidase (116,000 Da), phosphorylase B (97,400 Da), bovine serum albumin (66,000 Da), egg albumin (45,000 Da) and carbonic anhydrase (29,000 Da).

The SDS-PAGE High Range Molecular Weight Standards were prepared according to the specification table in the Bio-Rad Instruction Manual, Bio-Rad Laboratories, Hercules, California, USA. The standards contained approximately 400 μg of each protein listed and was placed in a 50 % glycerol, 300 mM NaCl, 3 mM NaN_3 and 100 mM dithiothreitol solution. The standards were then diluted 1:20 in sample buffer and heated for 45 seconds at 100°C. 15 μl was loaded per well for mini gels.

2.2.2.2 Coomassie Staining Detection of Proteins

Staining of protein profiles with Coomassie Brilliant Blue G250 (CBB) Stain was performed according to the method of LKB Pharmacia (1986). The gel was placed in fixing solution for Coomassie and silver stained SDS-PAGE (Section 2.1.8) to allow the SDS to diffuse out of the gel and the proteins to precipitate (30 minutes to

overnight). The gel was then placed in the CBB staining solution (Section 2.1.8) for 30 minutes and was then destained in fixing solution for coomassie and silver stained SDS-PAGE gels (Section 2.1.8) until the background was clear. The gel was then washed several times with distilled water.

2.2.2.3 Silver Staining Detection of Proteins

Staining of protein profiles with Silver Stain was performed according to the method of LKB Pharmacia (1986). The gel was placed in fixing solution for silver stained SDS-PAGE gels (Section 2.1.8) for a minimum of 30 minutes to allow the SDS to diffuse out of the gel and the proteins to precipitate. The gel was then placed in incubation solution for a minimum of 30 minutes, after which it was washed several times with distilled water (at least 10 minutes per wash for three washes) and then placed in silver solution for 40 minutes allowing the proteins to react with the silver. The gel was then placed in developing solution until the protein bands become intensely dark. To stop further colour development via the reaction, the gel was placed in stop solution for silver staining SDS-PAGE gels (Section 2.1.8) for 15 minutes. The gel was then washed several times with distilled water.

2.2.2.4 SYPRO®Orange Staining Detection of Proteins

Staining of protein profiles with SYPRO®Orange stain was performed according to Bio-Rad Laboratories Pty Ltd Product Information (*SYPRO®Orange Protein Gel Stain Instruction Manual*) with some minor modifications. The gel was placed in SYPRO® Orange staining reagent (10 µl of SYPRO® Orange protein stain [Bio-Rad Laboratories Pty. Ltd., Catalogue Number: 170-3120] dissolved in 50 ml 7.5 % glacial acetic acid) for 30 minutes and then de-stained in 7.5 % glacial acetic acid for between 30 minutes to 1 hour.

2.2.3 Screening for pGEX-5X-3-PARP-1 Recombinants for Protein Expression in *E. coli*

pGEX-5X-3-PARP-1 recombinants were screened for GST-gene fusion protein expression according to GST Gene Fusion System, Second Edition, Pharmacia Biotech (1994a).

pGEX-5X-3-PARP-1 recombinants and pGEX-5X-3 (positive control) were grown overnight at 37°C in 2 ml LB broth containing 100 μg/ml ampicillin. The following morning, all the colonies were re-inoculated into LB broth containing 100 μg/ml ampicillin and grown with vigorous agitation at 37°C until their optical density at 600 nm reached between 0.6 and 0.8 (approximately 3 – 5 hours). Fusion protein expression was then induced by the addition of 100 mM IPTG to a final concentration of 0.1 mM. 1 ml samples were taken prior to the addition of IPTG and at 1, 2, 3, 5 and 24 hours after induction. Each sample was then centrifuged in a microfuge at 14,000 *g* for 2 minutes and the supernatant removed via aspiration. The pellet was then resuspended in 50 μl PBS and 50 μl 2x SDS-loading buffer and stored at –20°C for evaluation via SDS-PAGE electrophoresis (Section 2.2.2).

2.2.4 Protein Expression in Yeast cultures using pYES2.0

2.2.4.1 Growth Conditions for Yeast Cultures

Liquid cultures were grown at 28°C with rapid agitation in conical flasks, media volume being 1/5 of the flask volume.

2.2.4.2 Preparation and Transformation of Saccharomyces cerevisiae

Yeast cells were transformed by the alkali salt method for transformation of intact cells described by Ito *et al.* (1983). A 100 ml YEPD culture was grown overnight to late exponential phase. The cells were harvested by centrifugation at 4000 *g*, 5 minutes. The supernatant was removed by aspiration and the cell pellet washed twice with 20 ml of sterile TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA, pH 7.5), resuspended in 5 ml LiOAc (0.1 M lithium acetate in TE) buffer and incubated at the growth temperature for 30 minutes. After harvesting, the cells were resuspended in 0.5 ml LA. To 100 μl of these competent cells, 2 μg plasmid DNA and 100 μg single stranded carrier DNA (sheared, heat-denatured salmon sperm DNA) was added. 100 μl competent cells without the addition of plasmid DNA were also processed as a control. After incubation for 10 minutes on ice, 1 ml 70% Fision's PEG 4000 (7 g PEG 4000 in 3 ml TE) was added with mixing. Cells were incubated at the growth temperature for 45 minutes, then heat shocked for 5 minutes at 45°C (optional). After harvesting the cells were resuspended in 0.5 ml YEPD. After incubating at the

growth temperature for 60 minutes, 100 µl aliquots were plated on selective dry plates and incubated for between two to three days at 28°C.

2.2.4.3 *Screening for pYES2-PARP-1 Recombinants for Protein Expression in Saccharomyces cerevisiae*

pYES2-PARP-1 recombinants and pYES2-*lux*AB (positive control) were screened for PARP-1 and *lux*AB protein expression, respectively according to the manufacturer's (Invitrogen Inc.) instruction. pYES2-PARP-1 recombinants and pYES2-*lux*AB (positive control) destined for galactose induction were grown in media containing 4 % (w/v) raffinose (YNB Raffinose) or 0.1 % (w/v) glucose and 5 % (v/v) glycerol (YNB glucose and glycerol) as the carbon source. The pYES2-PARP-1 recombinants and pYES2-*lux*AB (positive control) were then grown overnight or until the optical density at 600 nm reached between 0.5 – 0.9. The cells were then collected via centrifugation and resuspended in fresh YNB media containing 2 % (w/v) galactose (YNB Galactose). Raffinose is a non-repressing carbon source and therefore cultures grown in raffinose usually show a rapid response to galactose induction. 1 ml aliquots were collected prior to induction and at 1, 3, 5, 12 and 24 hours after induction. Each sample was centrifuged in a microfuge at 14,000 *g* for 2 minutes and the supernatant removed via aspiration. The pellet was then resuspended and extracted in ice-cold protein extraction buffer (PEB – Section 2.1.8) containing a cocktail of various protease inhibitors and equal volume of glass beads. The cellular suspension was then vortexed at maximum speed for 30 seconds. This step was repeated at least three times. The glass beads and cellular debris was then removed from the cellular extract via centrifugation in a microfuge at 4000 g for 5 minutes at 4°C. The cellular extract was then stored at –20°C until evaluation via SDS-PAGE electrophoresis (Section 2.2.2).

2.2.5 Protein Expression using the BAC to BAC™ Baculovirus Expression System

2.2.5.1 *Preparation of Escherichia coli DH10Bac Competant Cells*

E. coli DH10 Bac competent cells were prepared as described in Section 2.2.1.1, however 50 µg/ml kanamycin and 10 µg/ml tetracycline was required in the medium throughout their preparation. These antibiotics were required in the medium to ensure

that the helper DNA and bacmid DNA was maintained in the host throughout the preparation of competent cells to enable effective transposition.

2.2.5.2 *Transposition Procedure for Generation of Recombinant Bacmid DNA*

The transposition procedure for the generation of recombinant bacmid DNA was performed according to Gibco-BRL-LifeTechnologies (1990). Approximately 1 μg recombinant donor plasmid (in 5 μl) was added to 50 μl *Escherichia coli* DH10Bac competant cells. The DNA was gently mixed with the cells by tapping the side of the eppendorf tube. The competant cell mix was incubated on ice for 30 minutes and then the mixture was heat shocked at 42°C in a water bath for approximately 2 minutes. After heat shock, 450 μl LB medium (pre-warmed to 37°C) was added to the competant cell mix and the mix was placed in a shaking incubator at 37°C with medium agitation (~200 rpm) for at least 4 hours. After incubation, 100 μl aliquots were plated on selective agar plates (LB containing Tet, Gent, Kan, X-gal and IPTG). The plates were then incubated for at least 24 hours at 37°C.

2.2.5.3 *Isolation of Recombinant Bacmid DNA*

The isolation of recombinant bacmid DNA was performed as described in Gibco-BRL-LifeTechnologies (1990). Approximately 10 white candidate colonies carrying recombinant bacmid DNA were re-inoculated to fresh LB plates containing Tet, Gent, Kan, X-gal and IPTG to verify the white phenotype. These plates were incubated overnight at 37°C. Once the white phenotype was confirmed, a single bacterial colony was inoculated in 2 ml LB broth containing Tet, Gent and Kan and grown overnight at 37°C, shaking at 200 rpm until the culture reached stationary phase (up to 16 hours). 1.5 ml of culture was transferred in to an Eppendorf tube and centrifuged at 14,000 *g* in a microfuge for 2 minutes. The supernatant was removed by vacuum aspiration and the pellet resuspended in 0.3 ml of Solution I for bacmid DNA preparation (Section 2.1.8). 0.3 ml of Solution II for bacmid DNA preparation (Section 2.1.8) was slowly added to the bacmid preparation, which was gently mixed and incubated at room temperature for 5 minutes. After incubation at room temperature, 0.3 ml 3 M potassium acetate (pH 5.5) was slowly added to the bacmid preparation and gently mixed. A thick white precipitate of protein and genomic *E.*

coli DNA formed. The bacmid preparation was then placed on ice between for 5 – 10 minutes and then centrifuged for 10 minutes at 14,000 *g* in a microfuge. During centrifugation, fresh eppendorf tubes were labeled and 0.8 ml of isopropanol was added to them. After centrifugation, the bacmid supernatant was gently transferred to the freshly labelled eppendorf tube containing the isopropanol. The bacmid preparation was gently mixed by inversion and then placed on ice for 5 – 10 minutes. The bacmid preparation was then centrifuged for 15 minutes at 14,000 *g* in a microfuge at room temperature and then the supernatant was removed by aspiration and 150 µl 70% ethanol added to each tube. The eppendorf tube was then inverted several times to wash the pellet and then centrifuged at 14,000 *g* in a microfuge at room temperature for 2 minutes. The supernatant was removed from the eppendorf tube and the pellet containing the bacmid DNA allowed to air-dry for 5 – 10 minutes at room temperature. The bacmid DNA was then dissolved in 20 µl sterile double-distilled H_2O and stored at -20°C. Preparations of recombinant bacmid DNA were then analysed via agarose gel electrophoresis (Section 2.2.5.4) and by PCR (Section 2.2.5.5).

2.2.5.4 *Analysis of Recombinant Bacmid DNA by Agarose Gel Electrophoresis*

Recombinant bacmid DNA was analysed by agarose gel electrophoresis according to Gibco-BRL-LifeTechnologies (1990). 5 µl recombinant bacmid DNA was electrophoresed in a 0.5 % (w/v) agarose gel, overnight at 13 V. The 0.5 % (w/v) agarose gel was prepared and visualised as described in Section 2.2.1.8.

2.2.5.5 *Analysis of Recombinant Bacmid DNA by PCR*

Insertion of PARP-1 cDNA into the bacmid vector was confirmed by PCR utilising pUC/M13 forward and reverse primers (Gibco-BRL-LifeTechnologies, 1990). The PCR reaction was prepared and analysed as described in Section 2.2.1.13.

2.2.5.6 *Transfection of Sf9 Cells with Recombinant Bacmid DNA*

The transfection of *Sf*-9 or *Sf*-21 cells with recombinant bacmid DNA was performed as described in Gibco-BRL-LifeTechnologies (1990). 9 x 10^5 *Sf-9* or *Sf-21* cells were seeded per 35 mm well in 2 ml of Sf-900 II SFM. The cells were allowed to attach to

the well at 27°C for at least 1 hour. The following solutions were prepared in sterile tubes; Solution A contained 5 µl of recombinant bacmid DNA diluted in 100 µl Sf-900II SFM and Solution B contained 6 µl CellFECTIN™ reagent diluted in 100 µl Sf-900II SFM. Once prepared these two solutions containing the DNA and lipid complex were combined and mixed gently and incubated at room temperature for a minimum of 15 minutes and a maximum of 45 minutes. Whilst the combined solution is incubating, the cells seeded previously were washed once with 1 ml of Sf-900 II SFM. To the combined DNA-lipid complex, 0.8 ml Sf-900 II SFM was added and gently mixed. The wash media was aspirated from cells and the 1 ml of the diluted lipid-DNA complexes was overlayed onto the cells. The cells were incubated for 5 hours in a 27°C incubator. After 5 hours incubation, the transfection mixture was removed and 1.3 ml of Sf-900 II SFM added to the cells. The cells were then placed in the incubator at 27°C for between 48 and 72 hours and the cells and virus were harvested as described in Section 2.2.5.7 and Section 2.2.5.8.

2.2.5.7 Harvesting of Infected Sf-9 or Sf-21 Insect Cells

Infected *Sf*-9 or *Sf*-21 insects cells were harvested and analysed for PARP-1 and hexahistidine-tagged PARP-1 (HT-PARP-1) protein expression as follows. The *Sf*-900 II SFM media was removed from the *Sf*-9 or *Sf*-21 cells via aspiration and the recombinant baculovirus contain in the media was collected as described in Section 2.2.5.8. Once the *Sf*-900II SFM media was removed, the *Sf*-9 or *Sf*-21 cells were washed once with 1 ml 1X PBS and then resuspended in 150 µl 1X PBS and then scraped with a cell scraper. The infected *Sf*-9 or *Sf*-21 cells were then stored at -20°C until evaluation via SDS-PAGE electrophoresis (Section 2.2.2).

2.2.5.8 Harvest/Storage of Recombinant Baculovirus

Recombinant baculovirus was harvested and stored as described in Gibco-BRL-LifeTechnologies (1990). 2 ml of recombinant baculovirus supernatent was transferred into a sterile, capped tube. The recombinant baculovirus supernatant was clarified by centrifugation for 5 minutes at 500 *g* to remove cells and/or cellular debris and the baculovirus-containing supernatant transferred into a fresh tube. The baculovirus was stored at 4°C and protected from light. For the long term storage of

baculovirus, the addition of foetal bovine serum (FBS) to a final concentration of at least 2 %. An aliquot of the baculoviral stock was also stored at -70°C.

2.2.5.9 Passaging Sf-9 Insect Cells

Insect cells (*Sf*-9 or *Sf*-21) were passaged and maintained as described in Gibco-BRL-LifeTechnologies (1990). Cells were passaged when the monolayer reached 80-100 % confluency. When passaged, flasks were seeded with 2 x 10^5 viable cells/ml. To achieve this, approximately 8 to 10 ml of pre-warmed fresh *Sf*-900II SFM media was filter sterilized into a fresh 75 cm^2 flask. The confluent monolayer was dislodged by tapping the 75 cm^2 flask on its side and between 2 – 3 ml of culture was added to the fresh 75 cm^2 flask. To ensure the cells were resuspended uniformly, the flask was rocked from side to side gently. The cells were incubated at 28°C with loose caps to allow gas exchange. Insect cells were passaged every three to four days.

2.2.5.10 Cryopreservation of Insect Cell Cultures

The cryopreservation of insect cells was performed as described in Gibco-BRL-LifeTechnologies (1990). Insect cells were harvested mid-log phase of growth with viability greater than 90 %. A viable cell count was performed and the required volume of cryopreservation medium was calculated to yield a final cell density of 1 x 10^7 to 2 x 10^7 cells/ml. The cryopreservation medium consisted of 7.5 % DMSO in 50 % fresh *Sf*-900 II SFM and 50 % conditioned medium (2 to 3 day conditioned *Sf*-900 II SFM from the cell line to be frozen; filter sterilized through a 0.22 μM filter) was prepared and chilled to 4°C and held at this temperature until use. The cells were centrifuged at 500 *g* for 5 minutes and the cell pellet resuspended in the required volume of chilled cryopreservation medium and transferred to cryovials. The cryovials were refrigerated at 4°C for 30 minutes prior to being placed at -80°C for two hours and then into liquid nitrogen for long term storage.

2.2.6 PARP Purification Method

Infected insect cells were lysed and homogenized by vortexing (3 x 30 seconds) at 4°C or sonication (3 x 5 seconds, Output 6.5, Duty Cycle 65 %, Branson Sonifer 450) on ice in ice-cold hypertonic homogenization buffer (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 0.2 % Tween-

20, 0.2 % Nonidet P-40 and 0.5 M NaCl). Cellular debris were then removed by centrifugation (14,000 g for 15 minutes at 4°C). Endogenous DNA was removed by the addition of 1 mg/ml PMS followed by centrifugation (14,000 g for 10 minutes at 4°C). The supernatant was then precipitated in two steps with 0-30 % followed by 30-70 % ammonium sulphate. The precipitated proteins were dissolved in ice-cold chromatography-extraction buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$ and 0.2 M NaCl) and applied to a DNA-cellulose column equilibrated with chromatography-extraction buffer A (flow rate 0.3 ml/min). PARP-1 and HT-PARP-1 were eluted from the affinity matrix using chromatography-extraction buffer B (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$ and 1.0 M NaCl). To remove unwanted salts from the preparations, fractions eluted with the highest amount of PARP activity were further purified by ultrafiltration using CentriPrep10 from Amicon Inc., USA in storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT). Samples were stored at -80°C until required.

2.2.7 Estimation of Protein Content

2.2.7.1 Amino Acid Analysis

Amino acid analysis was performed by the Australian Proteome Analysis Facility (APAF), Macquarie University, Sydney, NSW, Australia, 2109. A sample of PARP-1 was applied to a Prosorb kit to remove any unwanted components in the sample and then amino acid analysis was performed as per APAF standard methods.

2.2.7.2 The Lowry Protein Assay

A modified version of the Lowry method (Lowry *et al.* 1951) was used for this research. A calibration curve containing 10, 20, 30, 40, 50, 75 and 100 µg of standard protein (BSA, Sigma Biochemicals, Catalogue Number: A-7906) in a total volume of 500 µl in double-distilled water. A reagent blank and dilutions of the purified PARP-1 were also prepared to a final volume of 500 µl of double-distilled water. A volume of 500 µl of Lowry Protein Assay Solution A (Section 2.1.8) was added to each of the standards and the PARP-1 sample and mixed thoroughly by vortexing. The standards and the PARP-1 sample were then incubated at 37°C for 10 minutes. After

incubation, 1.5 ml of Lowry Protein Assay Solution B (Section 2.1.8) was added to each of the standards and the PARP-1 sample and mixed thoroughly by vortexing. The standards and the PARP-1 samples were then incubated at 52°C for 20 minutes. Absorbances of the standards and the PARP-1 samples were determined at 680 nm.

2.2.7.3 The Bradford Protein Assay

The Bradford Protein Assay for determining protein concentration was performed as described by Bradford (1976). A calibration curve was prepared by using a series of solutions containing 10, 20, 40, 70 and 100 µg of standard protein (BSA) in a total volume of 100 µl of double-distilled water. A reagent blank and dilutions of the purified PARP-1 were prepared to a final volume of 100 µl with double-distilled water as the diluent. A volume of 5.0 ml of Bradford protein reagent (Section 2.1.8) was added to each tube and then mixed thoroughly with the standards and the purified PARP-1 solutions in the respective tubes by inversion. Absorbances for the PARP-1 samples and the standards were determined at 595 nm.

2.2.7.4 Estimating Protein Content using Coomassie Brilliant Blue G250, Silver and SYPRO®Orange Stained SDS-PAGE Gels

Standards and dilutions of the PARP-1 solution were run on 7.5 % SDS-PAGE gels as described in the Bio-Rad Laboratories Pty Ltd Product Information (*Mini Protean II Dual Slab Cell Instruction Manual*) (Section 2.2.2). After electrophoresis, gels were stained either with CBB, silver or SYPRO® Orange (Sections 2.2.2.2, 2.2.2.3 and 2.2.2.4). The CBB, silver and SYPRO®Orange stained protein gels were photographed using a Fujifilm LAS1000 charged-coupled device (CCD) and analysed using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998).

Estimates of protein concentration from stained SDS-PAGE gels were based on duplicate gels. For the CBB and SYPRO®Orange-stained gel, the standard curve was generated using mean ODs from duplicate gels. All experiments were repeated at least once and gave similar results. The total amount of protein in a band was estimated by measuring the intensity across the whole area of the band, thus differences in band size were accounted for.

2.2.7.5 *NanoOrange® Reagent for determining Protein Content*

Protein content using the NanoOrange® reagent was performed as described by the manufacturer (NanoOrange® Protein Quantitation Kit (N-6666), Molecular Probes) (MolecularProbes 2001).

1X NanoOrange® Protein quantitation diluent and 1X NanoOrange® working solution was prepared prior to performing the NanoOrange® Protein Assay as described in Section 2.1.8. BSA was used as the standard reference protein and the following concentrations were prepared in 2.5 ml 1X NanoOrange® working solution: 0.01, 0.03, 0.06 and 0.10 μg/ml. PARP-1 samples were diluted to a final volume of 2.5 ml in 1X NanoOrange® working solution (Note: The sample volume was no more than 4 % of the 2.5 ml total volume). PARP-1 samples and the BSA standards were incubated at 90°C for 10 minutes and then allowed to cool to room temperature for 20 minutes. The fluorescence of the standards and the PARP-1 samples were determined at an excitation wavelength of 485 nm and emission wavelength of 590 nm.

2.2.8 Assay for PARP Activity

The assay used for this work was based on methods described by Benjamin and Gill (1980) and Tsopanakis *et al.* (1978); the standard reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0 (adjusted at 37°C), 4 mM $MgCl_2$, 0.2 mM DTT, 2 μg/ml histone H1, 2 μg/ml DNA, 200 μM NAD^+ (0.5 μCi [2,5',8-^{3}H]NAD^+, 6.66 x 10^4 dpm/pmol), and 200 ng of PARP-1 or HT-PARP-1 in a total volume of 100 μl.

The reaction mixture was incubated at 25°C for 10 minutes. The reaction was terminated by the addition of 1ml of ice-cold 20% (w/v) trichloroacetic acid (TCA) containing 4 mg/ml NAD^+. The precipitate was left on ice for 60 minutes and subsequently collected on GF/C glass fibre filters and washed twice with 10 % (w/v) TCA, followed by an acetone wash. The filter was then placed in an oven to dry and its radioactivity was measured in a Wallac 1410 Liquid Scintillation counter. The liquid scintillant cocktail (Section 2.1.8) used was 0.5 % (w/v) PPO in AnalaR™ Toluene.

One unit of activity (U) is defined as the amount of enzyme activity that incorporates 1 pmol of radioactive ADP-ribose from NAD^+ into an acid-insoluble material in 1 minute at 25°C.

3 A comparison of bacterial, yeast and insect-based expression systems for the production of Human PARP-1

3.1 Introduction

To study the relationship between structure and function in proteins usually requires the production of relatively large quantities of highly purified protein. In the modern biochemistry laboratory this is commonly achieved by expressing the relevant gene in a microbial or cell culture system and purifying the expression product using standard biochemical approaches. When selecting and/or developing a system for expression of a recombinant protein the choice of host and expression vector is critical and may have to accommodate requirements for further genetic manipulation following cloning. Additionally, not all proteins lend themselves well to standard expression and purification schemes. The aim of work described in this chapter was to develop an expression system for the production of human PARP-1.

Expression of PARP-1 in heterologous systems became possible following the cloning of PARP-1 cDNA from several sources including human (Alkhatib et al. 1987; Cherney *et al.* 1987; Uchida et al. 1987), bovine (Saito *et al.* 1990) and murine origins (Huppi *et al.* 1989). Full length and truncated versions of PARP-1 cDNA were subsequently expressed using a variety of host cells ranging from bacterial (i.e. *E. coli*) (Herzog *et al.* 1989; Gradwohl *et al.* 1990; Ikejima *et al.* 1990; Simonin *et al.* 1990; Cherney *et al.* 1991; Trucco *et al.* 1996; Rolli *et al.* 1997), yeast (Kaiser *et al.* 1992; Avila *et al.* 1994; Perrin *et al.* 2000) and insect cells using baculovirus expression systems (Giner *et al.* 1992; Jung *et al.* 1994; Miranda *et al.* 1997).

While PARP-1 has been expressed in numerous types of systems most of these have not been particularly useful for expressing high quality PARP-1 for structure - function studies. For example, many researchers have been successful in cloning and expressing the different domains of human PARP-1 in *E. coli* but to-date successful expression of full length protein has yielded relatively low amounts of PARP-1 protein, often in a truncated form or precipitated as intracellular aggregates called "inclusion bodies" (Alkhatib *et al.* 1987; Ikejima *et al.* 1990). Prof. de Murcia's laboratory (Ecole Superieure de Biotechnologie de Strasbourg, Centre National de la Recherche Scientifique, France) was successful in expressing full length PARP-1 in a prokaryotic expression system (Trucco *et al.* 1996; Rolli *et al.* 1997), but this system

(based on the plasmid pTG161) was not available for the work described in this thesis[1] and whether this system is able to produce high yields of active PARP-1 protein was unknown.

Several researchers have tried to express human PARP-1 in yeast (Kaiser *et al.* 1992; Avila *et al.* 1994; Perrin *et al.* 2000). Kaiser *et al.*(1992) for example tested the yeast, *Saccharomyces cerevisiae* for its capacity to produce human PARP-1 from a human PARP-1 cDNA under the control of either a constitutive alcohol dehydrogenase or inducible *GAL10* promoter. Immunoblot and activity gel analysis showed a single band with the expected molecular weight and activity of PARP-1 (Kaiser *et al.* 1992), however, there was in sufficient PARP-1 protein to be detected on a Coomassie blue stained gel suggesting that the yield was less than 1% of total cellular protein (Kaiser *et al.* 1992). Avila *et al.*(1994) utilised the fission yeast, *Schizosaccharomyces pombe* to express human PARP-1 under the control of the SV40 early promoter. However protein yields were again extremely low.

To date, most success has been achieved using the insect-based baculovirus expression system. Giner, *et al.* (1992) reported expression of relatively high yields of full length human PARP-1, whereas Jung, *et al.* (1994) and Miranda, *et al.* (1997) reported high levels of expression of the catalytic domain of chicken PARP-1 using baculovirus expression systems, but the quality of the products was not evaluated.

The major aim of work described in this chapter was to develop an efficient protein expression system to make quantities of high quality human PARP-1 to be used in subsequent structure – function studies. The product (after purification) would be required to have K_m and V_{max} values of human PARP-1 as reported in the literature. To achieve this, three expression systems were trialed to assess their potential: *E. coli*-based, yeast-based and insect cell-based.

[1] A request was made to obtain pTG161 prokaryotic expression vector and its host cell but these were not available commercially or as a gift.

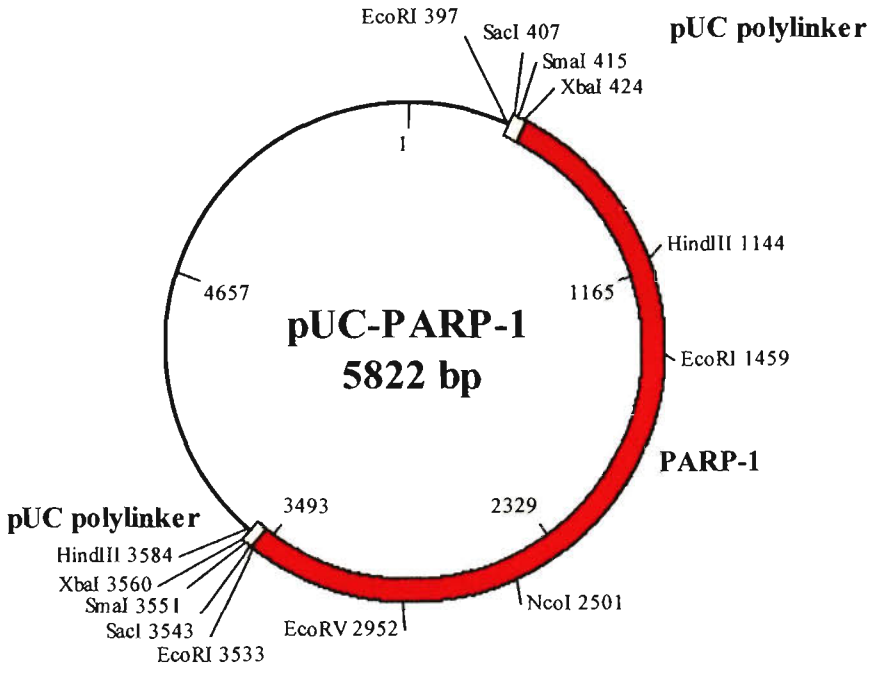
3.2 Results

3.2.1 Source and integrity of the human PARP-1 cDNA construct for work described in this thesis

The human PARP-1 cDNA used for work described throughout this thesis was a generous gift from Prof. G. de Murcia (Institut de Moleculaire et Cellularie, Centre National Recherche Scientifique, Strasbourg, France). Provided in a pUC cloning vector containing two multiple cloning sites (See Figure 3.1), the pUC-PARP-1 construct was transformed into *E. coli* TG2 cells. Plasmid DNA was subsequently extracted from several transformants and characterised by restriction enzyme digestion. A typical restriction pattern obtained from one clone is shown in Figure 3.2. The sizes of the restriction fragments obtained confirmed the integrity of the pUC-PARP-1 construct.

Automated DNA sequencing was used to confirm the integrity of the human PARP-1 cDNA. Sequencing reactions were prepared using DyeTerminator® and BigDye Terminator® according to the manufacturer's instructions as described in Section 2.2.1.15. Sequencing was performed by the DNA Sequencing Service at the Department of Microbiology, Monash University, Clayton campus, Clayton, Australia on an ABI 373A Sequencer (Applied Biosystems Inc., USA). The sequence data obtained was aligned with human PARP-1 cDNA, accession number M18112 (Uchida, *et al.* (1987), Appendix 1) using the BioEdit sequence alignment program, version 5.0.6 (Hall 1999). The PARP-1 sequence obtained was 100 % homologous to human PARP-1 cDNA, accession number M18112 and this data is attached in Appendix 2.

Figure 3.1: Restriction map of pUC-PARP-1. pUC-PARP-1 was obtained from Prof. G. de Murcia, Institut de Moleculaire et Cellulaire, Centre National Recherche Scientifique, Strasbourg, France.



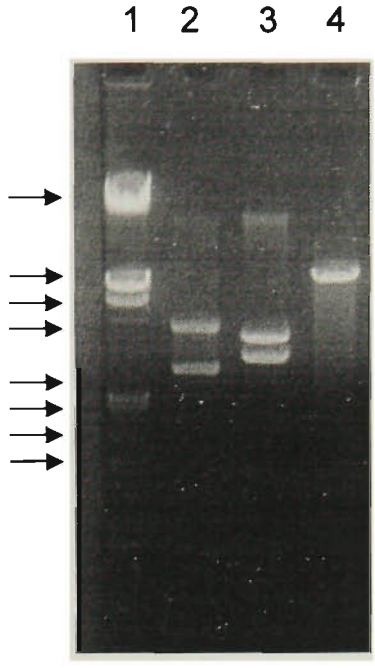


Figure 3.2: The integrity of a pUC-PARP-1 clone was confirmed by restriction enzyme digestion.

Restriction enzyme digests were performed according to the manufacturer's instructions and the products were separated using agarose (1.0% w/v) gel electrophoresis in 1X TAE as the running buffer. Lane 1 contains the λ/*Hind*III and *Eco*RV DNA marker (21227 bp, 5060 bp, 4269 bp, 3530 bp, 2027 bp, 1904 bp, 1587 bp, 1375 bp, 947 bp and 831 bp), Lanes 2 – 4 contain pUC-PARP-1 plasmid DNA digested with *Hind*III, *Sma*I and *Eco*RV respectively. The sizes of the restriction products obtained from each of the digests were as expected; *Hind*III – 3382 bp and 2440 bp; *Sma*I – 3136 bp and 2686 bp; and *Eco*RV – 5822 bp. These resultant restriction products confirm the integrity of the pUC-PARP-1 construct. **Please note:** Only the first eight bands of the λ/*Hind*III and *Eco*RV DNA marker are visible on the photographed gel and are indicated by the arrows.

3.2.2 Expression of Human PARP-1 in *E. coli* using pGEX-5X-3

Many laboratories have attempted to express the human PARP-1 gene in bacterial systems but these have met with limited success (Herzog *et al.* 1989; Gradwohl *et al.* 1990; Ikejima *et al.* 1990; Simonin *et al.* 1990; Cherney *et al.* 1991; Trucco *et al.* 1996; Rolli *et al.* 1997). Nonetheless it was decided to trial prokaryotic expression of PARP-1 because at least one such system has been reported to work well (Trucco *et al.* 1996; Rolli *et al.* 1997), and generally these systems are easy to maintain and handle (Brent 1997). Expression is also easy to regulate in *E. coli* (Schendel 1998) and can yield a large amount of recombinant protein (as much as 30% of total protein) (Brent 1997).

At the time of performing this work a prokaryotic expression system based on the vector pGEX-5X-3 (Pharmacia Biotech, Inc.) had been used by another scientist in the laboratory at Victoria University for the expression of a range of heterologous proteins. Thus it was decided to trial the same system for expression of PARP-1. pGEX-5X-3 belongs to a group of fusion protein expression vectors that have an integrated glutathione S-transferase (GST) gene from *Schistosoma japonicum.* Expression of GST-fusion products is chemically regulated by the *tac* promoter.

The expression of PARP-1 as a fusion product would have two desirable features: the fusion protein product could be readily purified by affinity chromatography under non denaturing conditions via affinity chromatography on Glutathione Sepharose 4B, and GST-fusion products could be readily detected by a colorimetric assay or immunoassay that targets the GST tag. Another advantage of using pGEX-5X-3 is that this vector contains the *lac* repressor allele. The *lac* repressor (the product of the *lac* I^q gene) binds to the *tac* promoter repressing the expression of GST fusion proteins (Smith and Corcoran 1994). This prevents leaky protein expression which can lead to the formation of inclusion bodies or cell death (PharmaciaBiotech 1994b). Upon induction with IPTG, derepression occurs, leading to the production of GST fusion products. However, using a GST-PARP-1 fusion product in structure-function studies may be problematic. It is not desirable to use fusion proteins in structure-

function studies and removal of the GST tag is not always straight forward. The vector map including all the salient features of pGEX-5X-3 is shown in Appendix 9.4.

Human PARP cDNA was ligated into the *Sma*I site in the polylinker region of pGEX-5X-3 (Figure 3.3). Ligation products were transformed into *E. coli* TG2 and transformants selected on LB plates containing ampicillin. Colonies were then picked and plasmid DNA extracted, and digested with restriction enzymes to identify transformants carrying the PARP-1 cloned into pGEX-5X-3 in the correct orientation. The diagnostic restriction digest which was used to confirm that the PARP-1 gene had been cloned in pGEX-5X-3 in the correct orientation was the *Apa*I and *Sac*I double digest. Where the PARP-1 gene was cloned in pGEX-5X-3 in the correct orientation, the size of the restriction digest products would be 2940 bp and 5170 bp (as shown in Figure 3.4, lane 9). If cloned in the wrong orientation, the size of the restriction digest products would be approximately 2060 bp and 6050 bp. A clone carrying PARP-1 in the correct orientation was chosen for further analysis. This pGEX-5X-3-PARP-1 construct was digested with a range of restriction enzymes as seen in Figure 3.4. The following enzymes were chosen *Apa*I, *Eco*RV, *Hind*III, *Sac*I and *Sma*I to digest the pGEX-5X-3-PARP-1 construct. These digests produced fragments with the following expected sizes: 8110 bp; 3771 bp and 4339 bp; 8110 bp; 8110 bp; and 3136 bp and 4974 bp, respectively. Two double digests were also performed on this construct: *Apa*I and *Hind*III and *Apa*I and *Sac*I. These double digests produced fragments with the following expected sizes: 5340 bp and 2770 bp; and 2940 and 5170 bp, respectively. This pGEX-5X-3-PARP-1 construct and along with several other constructs which contained the PARP-1 gene ligated in pGEX-5X-3 in the correct orientation were then sequenced over the cloning regions to confirm that PARP-1 was in the correct reading frame and without mutations (data not shown).

Figure 3.3: Map of pGEX-5X-3-PARP-1 construct. PARP-1 was cloned into the *Sma*I site of pGEX-5X-3.

The GST tag is located at the N-terminal of the GST-PARP-1 fusion product. The vector map including all the salient features of the pGEX-5X-3 is shown in Appendix 9.4.

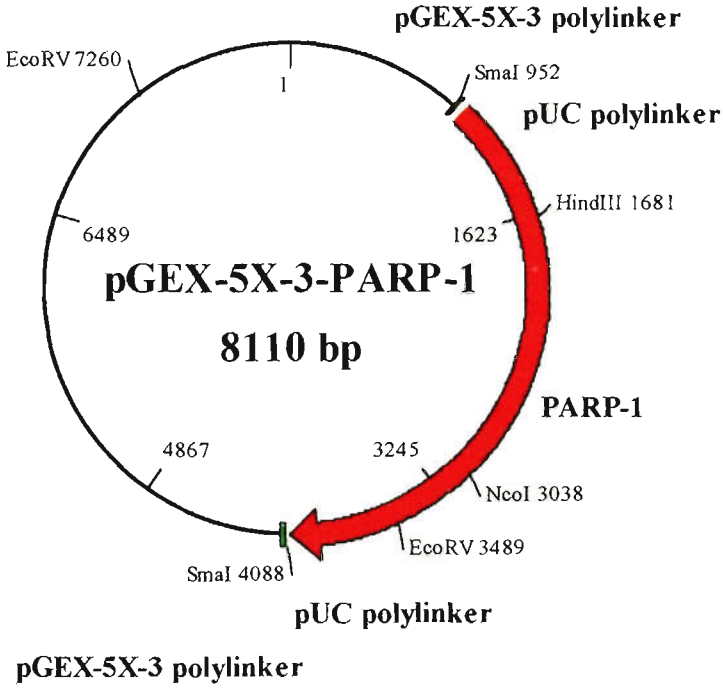
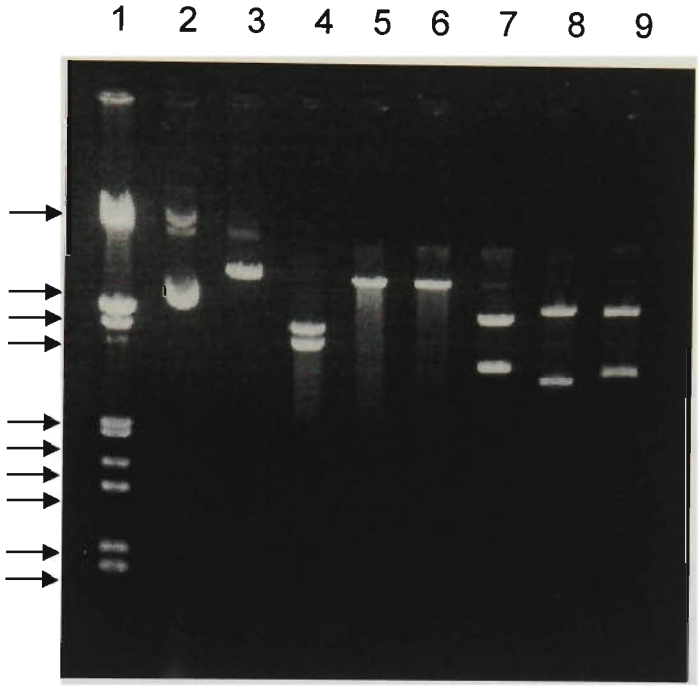


Figure 3.4: The integrity of a pGEX-5X-3-PARP-1 clone was confirmed by restriction enzyme digestion.

Restriction enzyme digests were performed according to the manufacturer's instructions and the products were separated using agarose (0.9 %) gel electrophoresis in 1X TAE running buffer. Lane 1: λ/*Hind*III and *Eco*RV DNA marker (21227 bp, 5060bp, 4269 bp, 3530 bp, 2027 bp, 1904 bp, 1587 bp, 1375 bp, 947 bp and 831 bp), Lane 2: undigested pGEX-5X-3-PARP-1 plasmid DNA, Lanes 3 to 7 contain pGEX-5X-3-PARP-1 plasmid digested with either *Apa*I, *Eco*RV, *Hind*III, *Sac*I or *Sma*I respectively; Lanes 8 and 9 contain pGEX-5X-3-PARP-1 digested with two restriction enzymes; *Apa*I and *Hind*III; and *Apa*I and *Sac*I respectively. The sizes of the restriction products obtained from the restriction digests were as expected; *Apa*I – 8110 bp; *Eco*RV – 3771 bp and 4339 bp; *Hind*III – 8110 bp; *Sac*I – 8110 bp; and *Sma*I – 3136 bp and 4974 bp. The sizes of the restriction products obtained from the double digests were as expected; *Apa*I and *Hind*III – 2770 bp and 5340 bp, and *Apa*I and *Sac*I – 2940 and 5170 bp. From the *Apa*I and *Sac*I double digest (lane 9), it is clear that the PARP-1 gene was successfully cloned into pGEX-5X-3 in the correct orientation.


1
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9

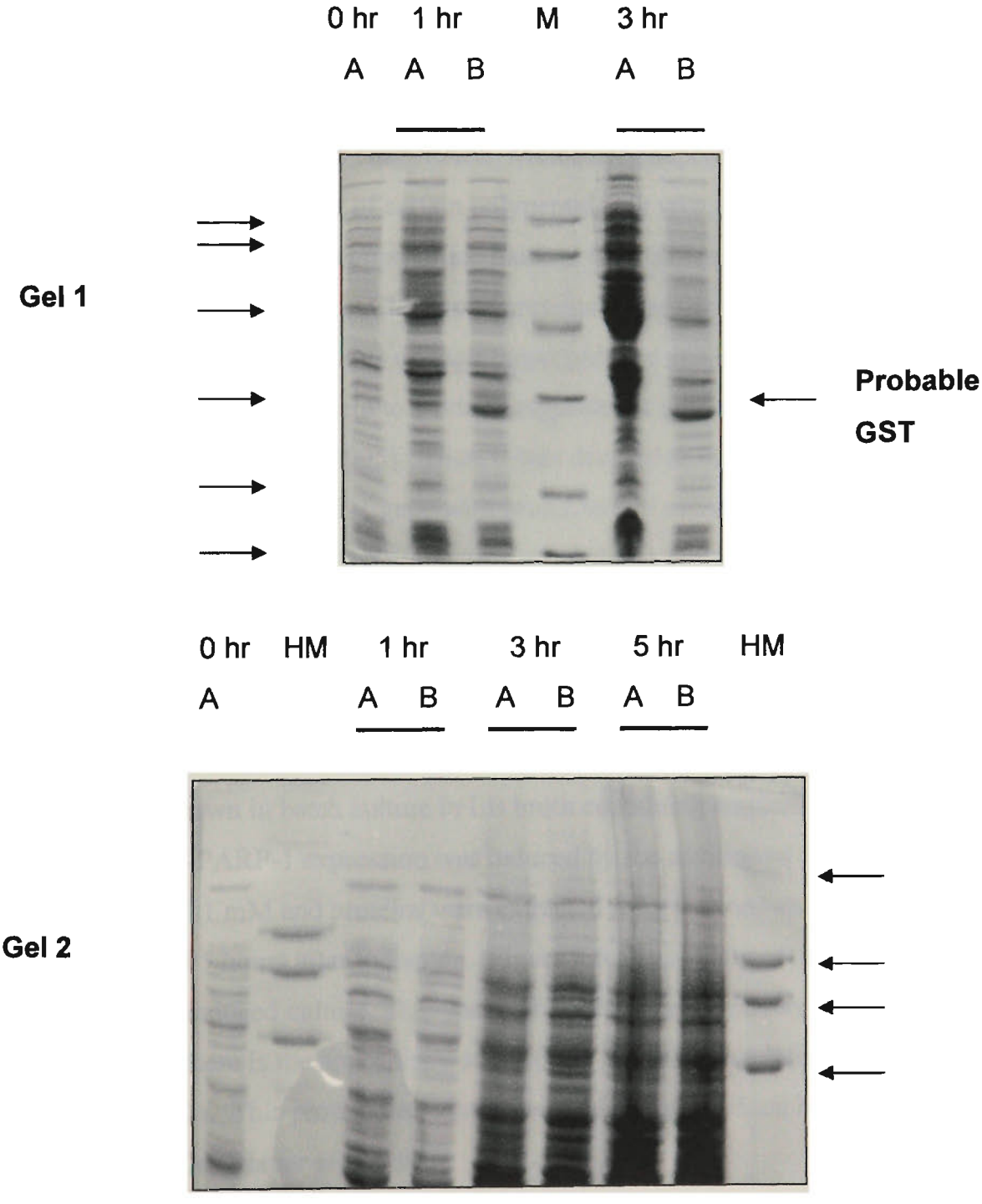
Initial attempts at expression of the successfully ligated PARP-1 gene into pGEX-5X-3 utilised the bacterial host strain *E. coli* BL21. Expression of GST with no PARP-1 insert (as a positive control) and GST-PARP-1 was induced by the addition of IPTG to a final concentration of 0.1 mM to a culture in mid exponential growth phase. Cells were collected at regular time intervals (i.e. 1, 2, 3, 5 and 24 hours after induction – as described in Section 2.2.3) to determine whether protein expression had been induced. It should be noted that the expression of some fusion-protein products required an overnight induction time-frame. The protein profiles from harvested cells was analysed using SDS-PAGE.

It is clear from Figure 3.5 that GST was successfully expressed from pGEX-5X-3 carried in *E. coli* BL21; an induced band of about 29.7 kDa was evident, and this is consistent with the M_r of GST which is 29 kDa (Product Information: Pharmacia Biotech Inc. (1994a)). However, no GST-PARP-1 fusion products were apparent (Figure 3.5, Gel 2), even after several attempts and trialling a different host strain of *E. coli* (MC1061) and various induction temperatures. This is consistent with the findings of many workers in the field (e.g. Kim, *et al.*(2000)). One possibility for the failure to express GST-PARP-1 fusion product maybe that it forms inclusion bodies in the cell. The presence of inclusion bodies in the insoluble protein fraction were not looked for. However, it is well documented that the PARP-1 expression in *E. coli* is likely to produce inclusion bodies (Personal communication: Prof. de Murcia, Institut de Moleculaire et Cellulaire, Centre National Recherche Scientifique, Strasbourg, France). This being the case, it was reasoned that co-expression of human PARP-1 with a chaperone protein that facilitates protein folding may promote expression. Thus, co-expression of human PARP-1 with the chaperone protein *groEL* was trialed.

Figure 3.5: Protein profiles following induced expression of pGEX-5X-3 (Gel 1) and pGEX-5X-3-PARP-1 (Gel 2) in *E. coli* BL21.

Gel 1: 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue. The protein profiles obtained from non-induced (A) and induced (B) pGEX-5X-3 *E. coli* BL21 cultures at 0, 1 and 3 hours. The low range molecular weight protein standards (M) are phosphorlyase B 97.4 kDa, bovine serum albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa and lysozyme 14.5 kDa. Induction of proteins of M_r equivalent to that of GST was apparent after induction at 3 hours.

Gel 2: 7.5% SDS-PAGE gel stained with Coomassie Brilliant Blue. The protein profiles from non-induced (A) and induced (B) pGEX-5X-3-PARP-1 at 0, 1, 3 and 5 hours. The high range molecular weight protein standards (HM) are myosin 205 kDa, β-galactosidase 116 kDa, phosphorlyase B 97.4 kDa and bovine serum albumin 66 kDa. There was no sign of an induced protein with a M_r equivalent to that of GST-human PARP-1 (~145 kDa).


0 hr
1 hr
M
3 hr
A
A
B
A
B
Gel 1
Probable
GST
0 hr
HM
1 hr
3 hr
5 hr
HM
A
A
B
A
B
A
B
Gel 2

3.2.2.1 *Co-expression of Human PARP-1 with GroEL*

Co-expression of recombinant proteins with *groEL* has been used in several industrial settings to produce proteins that have proven to be difficult to express using conventional approaches (personal communication: Dr. Ahmed Azad, CSIRO Biomolecular Engineering, Parkville, Victoria, Australia). *GroEL* belongs to a family of proteins known as molecular chaperones. Molecular chaperones appear to be essential for the correct folding of certain polypeptides *in vivo*, for their assembly into oligomers and for preventing inappropriate liaisons with other proteins during their synthesis, folding and transport. Chaperone proteins recognise exposed helices and other secondary structure elements on their target proteins and this initial interaction then allows the chaperone protein to guide or regulate the subsequent events of folding (Garrett and Grisham 1995). Thus, it was decided to investigate whether co-expression of the human PARP-1 gene with *groEL* would assist in the folding of the PARP-1 protein and eliminate the production of insoluble protein aggregrates (i.e. inclusion bodies).

pGEX-5X-3-PARP-1 was transformed into *E. coli* MC1061 containing pACYC-*groEL*. Transformants selected on ampicillin and kanamycin plates (to select for both plasmids) were grown in batch culture in LB broth containing ampicillin and kanamycin. GST-PARP-1 expression was induced by the addition of IPTG to a final concentration of 0.1 mM and proteins were extracted from induced and non-induced culture at 1, 3 and 5 hours after induction. Figure 3.6 shows the protein profiles of a non-induced and induced culture. In proteins from the induced culture (Figure 3.6: lanes 2, 5 and 7) there is the appearance of a protein band with a relative molecular weight of 63.2 kDa. This protein was almost certainly *groEL,* which has a molecular weight of ~ 60 kDa (Blatter *et al.* 1997).

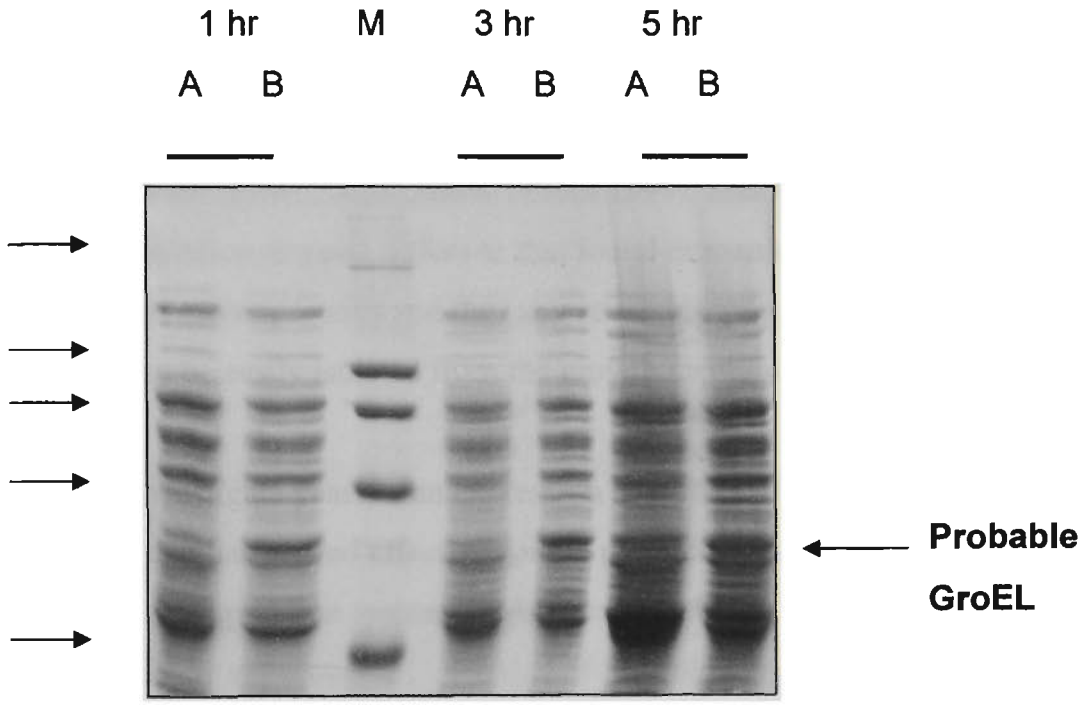
It is however possible (although unlikely) that the induced 63.2 kDa protein was a partial GST-PARP-1 fusion product. Other workers in the field have reported the expression of truncated PARP-1 protein products when expressing PARP-1 cDNA in *E. coli* (Herzog *et al.* 1989). These workers reported that under the control of the *lac* promoter in *E. coli*, the expression of two truncated PARP-1 protein products. These

truncated PARP-1 protein products had a relative molecular weight of approximately 99 kDa and 89 kDa and were immunoreactive with PARP-1 antibody and had catalytic properties very similar to that of the human enzyme. Microsequencing of these two truncated protein products revealed that translation was initiated at translation start signals located within the PARP-1 cDNA (Herzog *et al.* 1989). Thus, the findings of Herzog *et al.* (1989) suggest that the 63.2 kDa protein is unlikely to be a truncated PARP-1 protein product intiatied at a translation start signal located within the PARP-1 cDNA. Therefore, it is highly likely that the 63.2 kDa protein was *groEL*.

However, as this thesis required expression of full length PARP-1, and this was not apparent in the protein profile in Figure 3.5, further identification of this 63.2 kDa protein was not pursued. At about the time of concluding this work, expression using a eukaryotic expression system was looking promising, thus no further work was performed on the *E. coli* protein expression system.

Figure 3.6: Co-expression of pGEX-5X-3-PARP-1 with pACYC-*groEL* in *E. coli* MC1061.

Expression was performed at 28°C and was induced by the addition of 0.1 mM IPTG. Protein profiles are for non-induced (A) and induced (B) pGEX-5X-3-PARP-1 with pACYC-*groEL* in *E. coli* MC1061 at 1, 3 and 5 hours. High range molecular weight standards (M) are myosin 205 kDa, β-galactosidase 116 kDa, phosphorlyase B 97.4 kDa, bovine serum albumin 66 kDa and ovalbumin 45 kDa. An induced protein of 63.2 kDa is apparent (as indicted by the arrow on the right) at 1 and 3 hours. The same band is also apparent at 5 hours but also appears, at lower levels, in the uninduced sample (this is probably a native *E. coli* *groEL* protein). However, there was no sign of an induced protein with a M_r equivalent to that of GST-human PARP-1 (~145 kDa).


1 hr
M
3 hr
5 hr
A
B
A
B
A
B
Probable
GroEL

3.2.3 Expression of the human PARP-1 gene in yeast using pYES2

As mentioned in the introduction to this chapter (see section 3.1), three laboratories have had some, albeit limited success in expression of the human PARP-1 gene in yeast (Kaiser *et al.* 1992; Avila *et al.* 1994; Perrin *et al.* 2000).

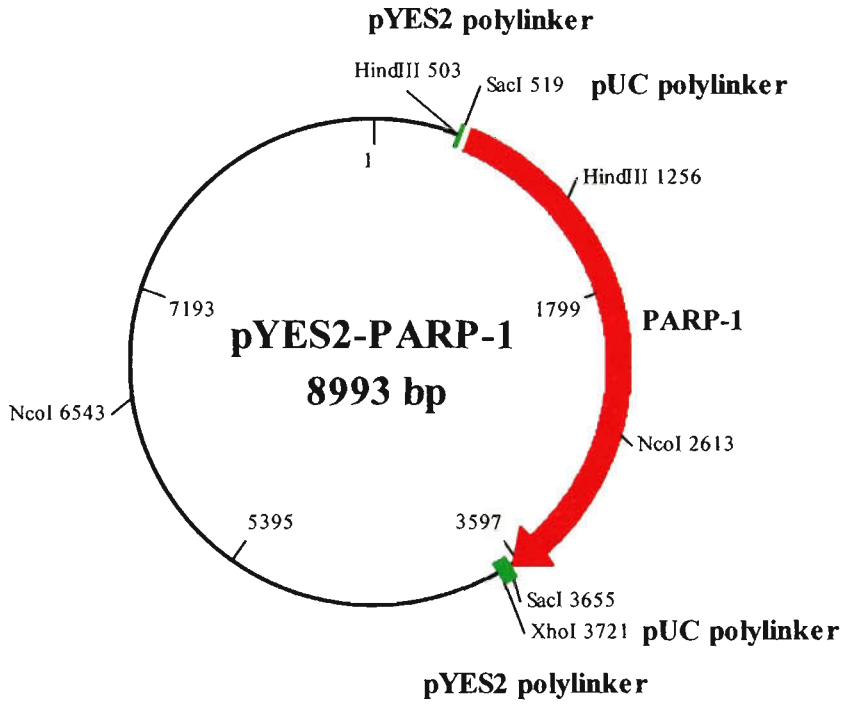
Yeast is an ideal host for the expression of many heterologous proteins and yeast protein expression systems are easily adapted for large scale production and downstream processing at a relatively low cost. In contrast to *E. coli*, yeast cells also facilitate glycosylation of the recombinant protein (Brent 1997), although it should be borne in mind that glycosylation in yeast differs to that found in mammals. Also, yeast only secrete 0.5% of native proteins and thus any recombinant proteins that are secreted from the cells can be easily isolated from the growth medium.

Thus, it was decided to investigate yeast as an expression host for PARP-1 using a yeast expression system that has proved effective for the production of heterologous proteins. The pYES2 yeast expression system (Invitrogen Inc., USA) has been successfully employed for the expression of a number of heterologous proteins including, for example, the *luxAB* protein in the laboratories at Victoria University (personal communication: Yaping Chen, currently at the Victoria Institute of Animal Science, Attwood, Victoria, Australia).

pYES2 (See Figure 3.7) is a high copy, episomal vector that replicates autonomously and is designed for inducible protein expression in *Saccharomyces cerevisiae*. Heterologous protein expression from this vector is driven by the *GAL*1 promoter and thus recombinant protein expression is induced when galactose is the sole carbon source (it is repressed by glucose). Transformation of yeast with pYES2 is straightforward using a standard yeast transformation protocol as described in section 2.2.4.2 and selection of transformants is based on rescue of uracil auxotrophy in *ura3*$^-$ cells; the plasmid carries a *URA3* gene. The vector map including all the salient features of pYES2 is shown in Appendix 9.4.

Figure 3.7: Map of pYES2-PARP-1 construct. PARP-1 was cloned into the *Sac*I restriction site in pYES2 expression vector (Invitrogen Inc.).

The vector map including all the salient features of pYES2 is shown in Appendix 9.4.



Human PARP-1 cDNA was ligated into the *Sac*I restriction site in the polylinker region of pYES2. Ligation products were transformed into *E.coli* TG2 and transformants selected on LB plates containing ampicillin. Plasmid DNA was extracted from four clones (A – D) and then digested with *Sac*I restriction enzymes to confirm that each clone carried the PARP-1 gene (3136 bp) in pYES2 (5857 bp) (Figure 3.8, Lanes 3 - 6). To confirm that PARP-1 had been cloned into pYES2 in the correct orientation, these clones were digested with *Eco*RV and *Hind*III (Figure 3.8, lanes 8 – 11) or *Cla*I and *Hind*III (Figure 3.8, lanes 12 – 15). Further confirmation that the PARP-1 gene had been successfully been ligated in pYES2 in the correct orientation came from sequencing the ligated products (data not shown).

pYES2-PARP-1 constructs and pYES2-luxAB (positive control) were then transformed into *S. cerevisiae* PMY 1.1 and subsequently screened for expression of PARP-1 for the former and *LuxAB* the latter. This was done by growing pYES2-PARP-1 and pYES2-luxAB transformants in minimal medium (grown in YNB containing raffinose or YNB containing glycerol and glucose – Table 2.3) to mid exponential phase. This ensured that the transformants were healthly and viable prior to induction. pYES2-PARP-1 and pYES2-luxAB transformants in *S. cerevisiae* PMY 1.1 were then collected by centrifugation and protein expression was induced by resuspending the transformants in YNB containing galactose (Table 2.3).

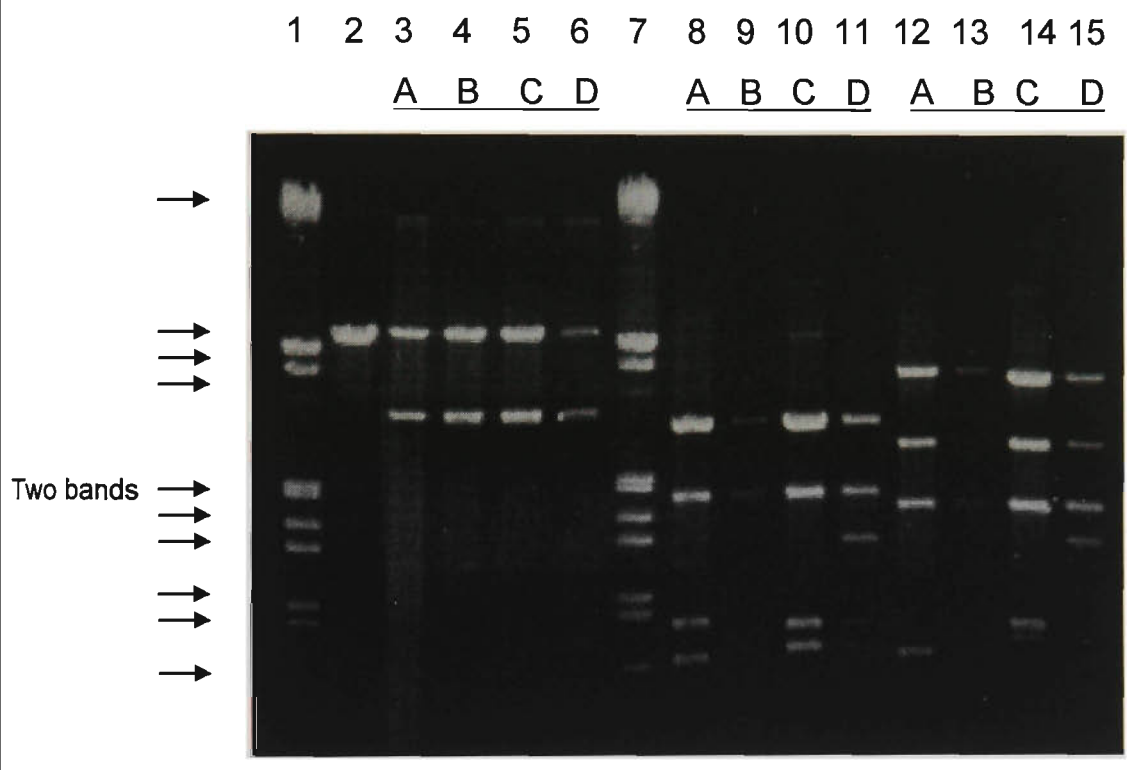
From the results, it was evident that induction of the *LuxAB* protein (positive control) was almost immediate in *S. cerevisiae*, PMY 1.1; an induced protein of the expected size (78.5 kDa) can be clearly seen on the SDS-PAGE gel (Figure 3.9) and there was an increase in bioluminescence in the induced culture (Figure 3.10). Protein profiles from harvested *S. cerevisiae* PMY 1.1 cells expressing pYES2-PARP-1 showed an over-expressed protein with the molecular weight of approximately 89 kDa (Figure 3.11) which is considerably lower than that expected for PARP-1. This protein product may have been a protease-digestion product of PARP-1 or perhaps a "partial-expression" product.

Another strain of *S. cerevisiae*, BJ 2168, was trialed as a host for expression of human PARP-1 using the same recombinant pYES2-PARP-1 construct. The protein profiles

from induced cultures of transformed cells showed an over-expressed product with the molecular weight of approximately 93 kDa (Figure 3.12). This was very similar to the molecular weight of the induced protein in PMY 1.1 cells and certainly not a full length human PARP-1 protein (116 kDa). Further characterisation of these truncated PARP-1 protein products was not persued however as this thesis was only interested in obtaining expression of full length human PARP-1 and at the time of generating the above results, I had begun to trial expression in an insect cell-based baculovirus system and the initial data looked promising.

Figure 3.8: The integrity of four different pYES2-PARP-1 clones (A – D) was confirmed by restriction enzyme digestion.

Restriction enzyme digests were performed according to the manufacturer's instructions and the products were separated using agarose (1.0% w/v) gel electrophoresis in 1X TAE running buffer. Lane 1 and lane 7 contains λ/*Hind*III and *Eco*RV DNA marker (21227 bp, 5060bp, 4269 bp, 3530 bp, 2027 bp, 1904 bp, 1587 bp, 1375 bp, 947 bp, 831 bp and 564 bp), lane 2 contains pYES2 digested with *Sac*I, lanes 3 – 6 contain pYES2-PARP-1 clones (A – D) digested with *Sac*I, lanes 8 – 11 contain pYES2-PARP-1 clones (A – D) digested with *Eco*RV and *Hind*III, and lanes 12 – 15 contains pYES2-PARP-1 clones (A – D) digested with *Cla*I and *Hind*III. The sizes of the restriction products obtained from the restriction digests were as expected; *Sac*I – 3136 bp and 5857 bp. The sizes of the restriction products obtained from the *Eco*RV and *Hind*III double digest were expected to be 2932 bp, 2864 bp, 1808 bp, 753 bp and 636 bp if PARP-1 was cloned into pYES2 in the correct orientation. Whereas the sizes of the restriction products obtained from the *Cla*I and *Hind*III double digest were expected to be 3926 bp, 2510 bp, 1672 bp, 753 bp and 132 bp (this last band is not visible on this gel) if PARP-1 was cloned into pYES2 in the correct orientation. It is evident from the restriction patterns obtained from pYES2-PARP-1 clones A and C that the PARP-1 gene was successfully ligated in the correct orientation in pYES2 because of the resultant fragment sizes obtained from the *Eco*RV and *Hind*III, and *Cla*I and *Hind*III double digests. Sequence data obtained from clones A and C confirmed that the PARP-1 gene had been successfully been ligated in pYES2 in the correct orientation, in the correct reading frame and without mutation (data not shown).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A B C D
A B C D
A B C D
Two bands

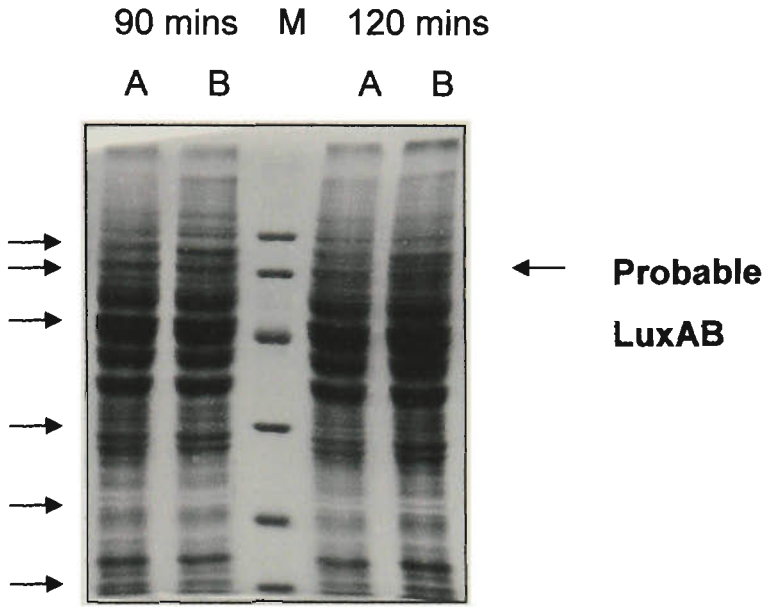


Figure 3.9: *LuxAB* expression in *S. cerevisiae*, PMY1.1.

The protein profiles obtained from induced pYES2 (A) and pYES2-*LuxAB* (B) cultures grown in YNB containing galactose, at 90 minutes and 120 minutes were separated on 12.5 % SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The arrow to the right of the gel indicates the position of an induced 78.5 kDa protein band which is consistent with that of *LuxAB* protein. The low range molecular weight protein markers (M), indicated by arrows to the left of the gel, are phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa and lysozyme 14.5 kDa.

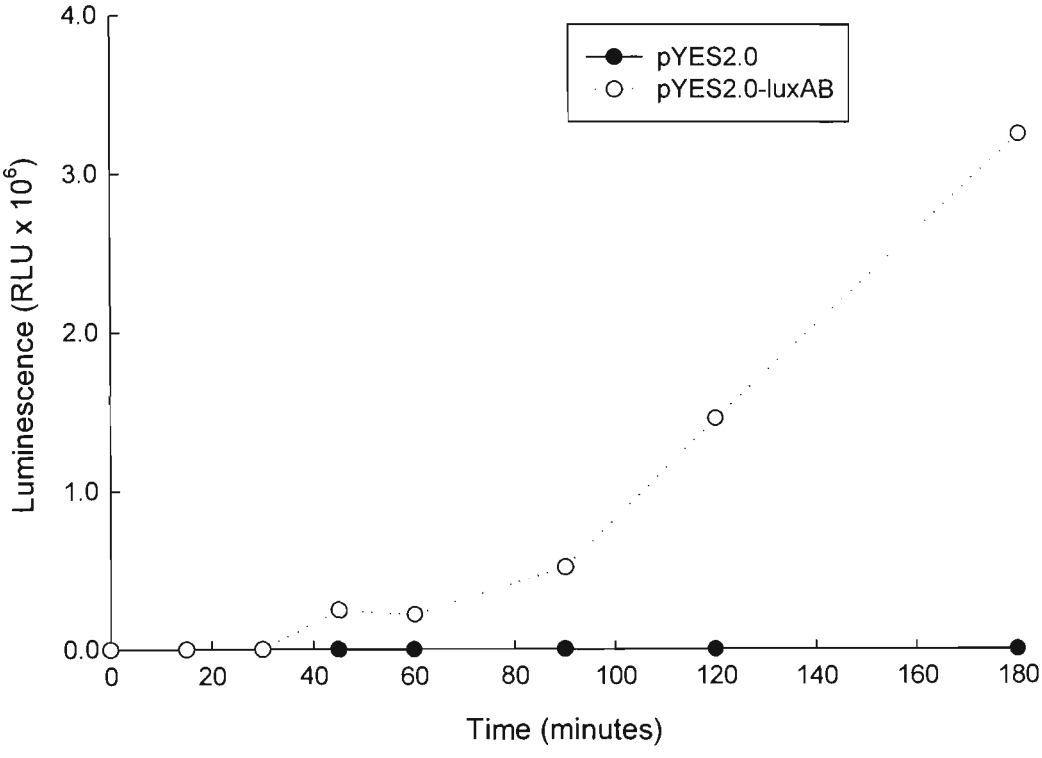


Figure 3.10: To confirm the expression of *LuxAB* in *S. cerevisiae*, PMY1.1, bioluminescence was measured in a yeast culture containing the yeast expression plasmid alone (pYES2.0) and the yeast expression plasmid containing the luxAB gene (pYES2.0-luxAB) following induction in YNB media containing galactose.

This was achieved by taking a 1 ml sample of each culture at various intervals after induction in galactose (0, 15, 30, 45, 60, 90, 120 and 180 minutes) and measuring the luminescence following the addition of substrate (1 µl of aldehyde).

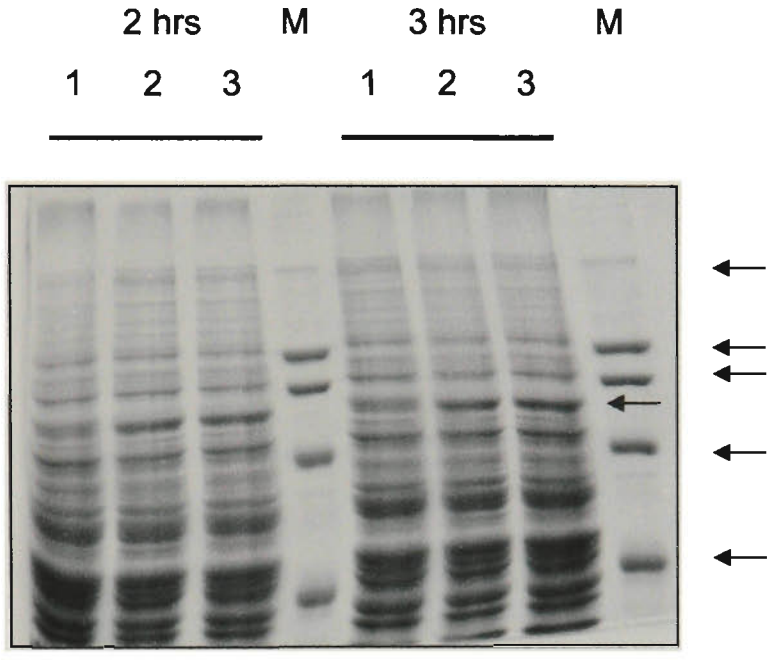


Figure 3.11: Induced expression from pYES2-PARP-1 in *S. cerevisiae*, PMY1.1.

Expression was induced by growth in YNB containing galactose. Proteins extracted from cells carrying pYES2 (lane 1) and cells carrying pYES2-PARP-1 (lanes 2 and 3) at 2 hours and 3 hours were resolved on 7.5% SDS-PAGE gels and stained with Coomassie Brilliant Blue. The arrow on the gel indicates the location of an induced 89 kDa protein band. High range molecular weight protein markers (M), indicated by the arrows to the right of the gel are myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa and ovalbumin 45 kDa.

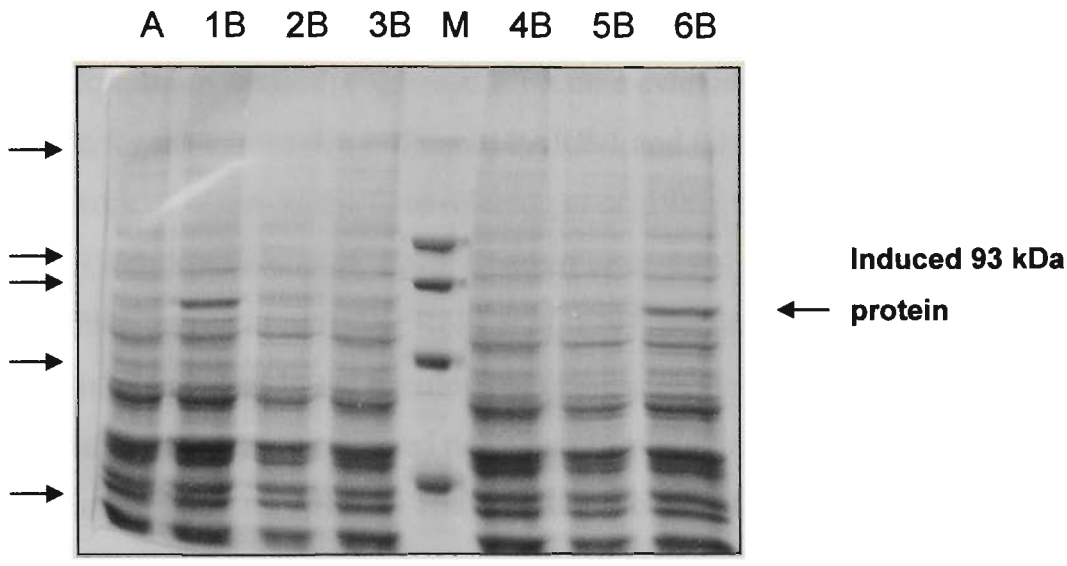


Figure 3.12: pYES2-PARP-1 expression in *S. cerevisiae*, BJ 2168.

Expression was induced by growth in YNB containing galactose. Proteins extracted from the expressed pYES2 (A) and various pYES2-PARP-1 transformants (1B – 6B), 24 hours after induction, were separated on 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. The arrow to the right of the gel indicates the location of an induced 93 kDa protein band. High range molecular weight protein markers (M), indicated by arrows on the left of the gel are myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa and ovalbumin 45 kDa.

3.2.4 Expression of the human PARP-1 gene in Insect Cells using the Bac to Bac™ Baculovirus Expression System

When starting the work described in this chapter, it was hoped that a bacterial and/or yeast expression system would be suitable for the expression of human PARP-1 as these systems are generally straightforward to use and are inexpensive. However, as bacterial and yeast expression studies progressed it become evident that these systems might be problematic for the expression of human PARP-1 and this was largely consistent with the work of others in the field (Herzog *et al.* 1989; Gradwohl *et al.* 1990; Ikejima *et al.* 1990; Simonin *et al.* 1990; Cherney *et al.* 1991; Kaiser *et al.* 1992; Avila *et al.* 1994; Trucco *et al.* 1996; Rolli *et al.* 1997; Perrin *et al.* 2000). Thus the introduction of an insect-based baculovirus expression system to the laboratory at Victoria University was deemed necessary to trial its effectiveness as a host for the production of human PARP-1. The reported success of Giner *et al.*(1992) in producing fully active human PARP-1 protein and Jung *et al.* (1994) and Miranda *et al.* (1997) in producing the catalytic domain of chicken PARP-1from baculovirus expression systems further endorsed our decision to trial such a system as a host for the production of human PARP-1.

While traditional baculovirus expression systems are more costly and demanding than bacterial and yeast protein expression systems, these systems have a number of advantages, particularly for the expression of eukaryotic proteins. Baculovirus expression systems:

- are considered to be very safe to use as baculoviral DNA infects only a small number of arthropod hosts (O'Reilly *et al.* 1994);
- usually produce large amounts of soluble protein,which are easy to purify (O'Reilly *et al.* 1994);
- possess post-translational processing mechanisms similar to many higher eukaryotes that are essential for activity and/or stability of many mammalian proteins (Brent 1997);
- produce recombinant proteins that are similar in function and antigenic properties to the native protein (O'Reilly *et al.* 1994; Richardson 1995); and

- are easily adaptable to suspension culture for the large-scale production of recombinant proteins in bioreactors (Gibco-BRL-LifeTechnologies 1990).

A drawback with traditional baculovirus expression systems is the need to produce viral DNA (baculoviral DNA) for co-transfection with recombinant constructs; this can be very time-consuming and tedious, particularly for a novice at baculovirus expression. Thus, for work described in the following, an alternative method for producing recombinant baculoviral DNA was sought. The Bac to Bac™ Baculovirus Expression System developed by Monsanto Inc. (USA) eliminates the need to produce viral DNA for co-transfection with recombinant constructs. This system utilises site-specific transposition of an expression cassette (carrying the gene to be expressed) into a baculovirus shuttle vector (Bacmid DNA) propogated in *E. coli* (Gibco-BRL-LifeTechnologies 1990) which leads to the production of a recombinant bacmid DNA construct (see Figure 3.13). Recombinant bacmid DNA constructs can then be rapidly isolated from small-scale cultures and transfected into insect cells. Virus stocks are then harvested from transfected cells and used to infect fresh insect cells for subsequent protein expression. Thus, it was decided to investigate the use of the Bac to Bac™ baculovirus expression system for the expression of human PARP-1. Human PARP-1 cDNA was ligated into the *Xba*I site in the polylinker region of pFastBac1 from GibcoBRL-Life Technologies Inc. (Figure 3.14), ligation products were transformed into *E.coli* TG2 and transformants selected on LB plates containing ampicillin. Plasmid DNA was extracted and digested with *Hind*III restriction enzymes to identify transformants carrying the PARP-1 gene in the correct orientation in pFastBac1 (Figure 3.15). A vector map including all the salient features of pFastBac1 is shown in Appendix 9.4.

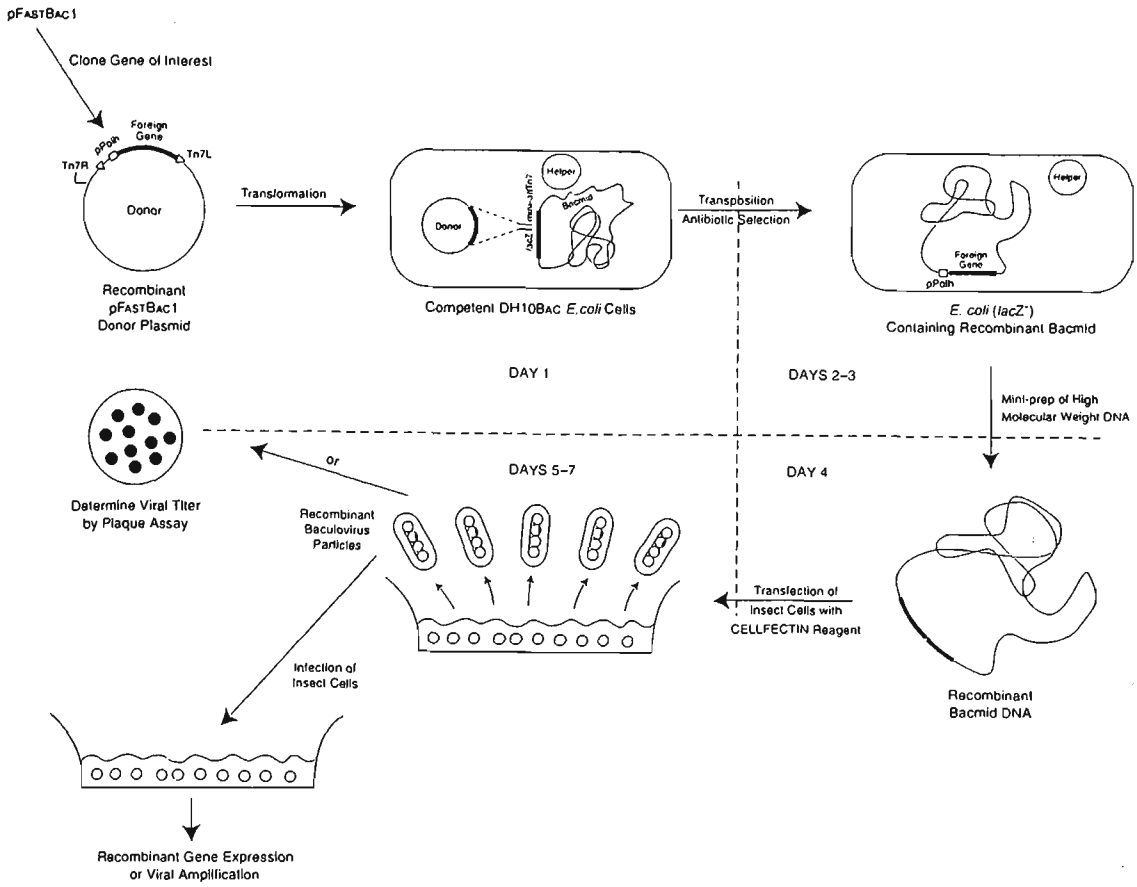
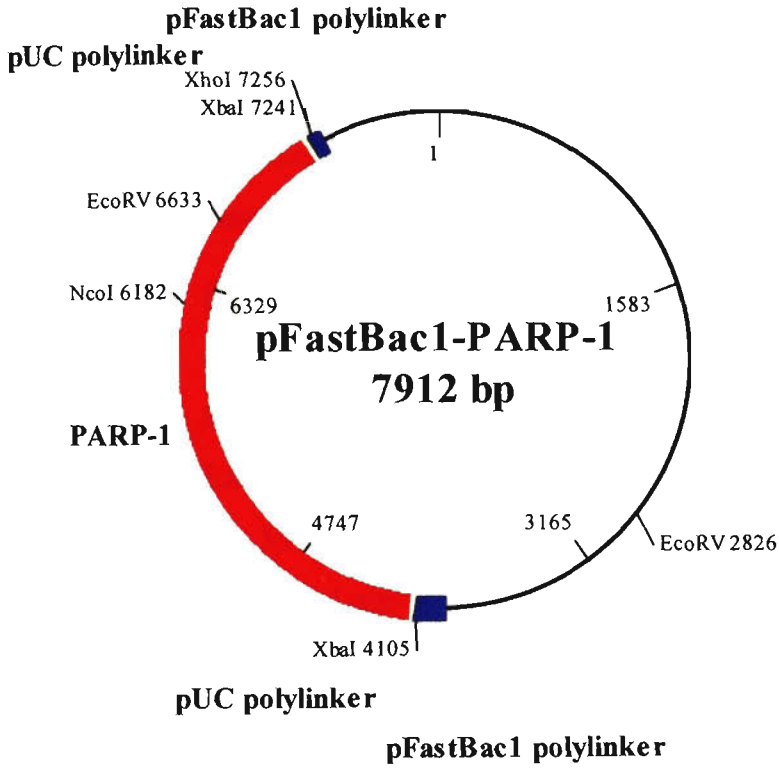


Figure 3.13: Schematic representation of the generation of recombinant baculoviruses and protein expression utilising the Bac to Bac™ Baculovirus Expression System (from Product Information: Gibco-BRL-LifeTechnologies (1990)).

Figure 3.14: Map of pFastBac1-PARP-1 construct. PARP-1 was cloned into the *Xba*I restriction site in pFastBac1 Baculovirus expression vector (GibcoBRL-Life Technologies, Inc.).

The vector map including all the salient feactures of pFastBac1 is shown in Appendix 9.4.



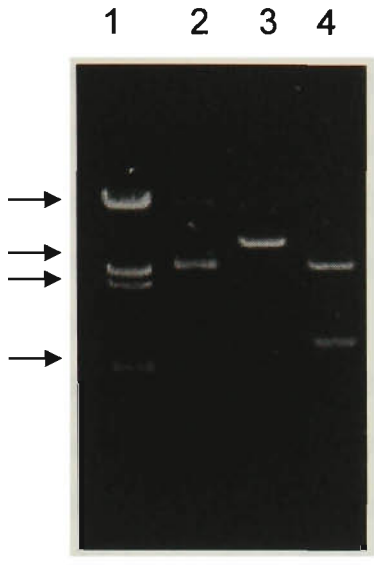


Figure 3.15: The integrity of a pFastBac1-PARP-1 clone was confirmed by restriction enzyme digestion.

Restriction enzyme digests were performed according to the manufacturer's instructions and the products were separated using agarose (1.0% w/v) gel electrophoresis in 1X TAE running buffer. Lane 1: λ/*Hind*III and *Eco*RV DNA marker (21227 bp, 5060bp, 4269 bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp and 564 bp), Lane 2: undigested pFastBac1-PARP-1 plasmid DNA, Lanes 3 and 4 contain pFastBac1-PARP-1 plasmid DNA digested with *Xho*I or *Hind*III respectively. The size of the restriction products obtained from the restriction digests were as expected; *Xho*I – 7912 bp and *Hind*III – 5460 bp and 2450 bp. It was evident from the restriction pattern obtained that the PARP-1 gene had been successfully cloned in the correct orientation in pFastBac1 because of the resultant restriction products from the *Hind*III digest (Lane 4). This digest confirms that PARP-1 was integrated in pFastBac1 in the correct orientation. **Please note:** Not all the DNA marker bands in the λ/*Hind*III and *Eco*RV DNA marker are apparent in this photograph, and the DNA marker bands indicated by the arrows which are visible are 21 227 bp, 5060 bp, 4269 bp and 2027 bp.

Where the PARP-1 gene was cloned in pFastBac1 in the correct orientation, the size of the *Hind*III restriction products would be approximately 5460 bp and 2450 bp (as shown in Figure 3.15, lane 4); whereas, PARP-1 cloned in the wrong orientation, would produce *Hind*III fragments of approximately 7190 bp and 720 bp. A clone carrying PARP-1 in the correct orientation was chosen for further analysis. This pFastBac1-PARP-1 construct was digested with *Xho*I and *Hind*III (as seen in Figure 3.15). The *Xho*I digest produced one fragment with the expected size of 7912 bp; whereas the *Hind*III digest produced two fragments with the expected sizes of 5460 bp and 2450 bp. This pFastBac1-PARP-1 construct, along with several other pFastBac1-PARP-1 constructs which contained the PARP-1 gene ligated in pFastBac1 in the correct orientation, were then sequenced over the cloning regions to confirm that PARP-1 was in the correct reading frame and without mutations (data not shown).

pFastBac1-PARP-1 was then prepared for baculovirus expression according to Product information: GibcoBRL-Life Technologies Inc., *Bac to Bac™ Baculovirus Expression Systems Instruction Manual*. Recombinant bacmid DNA was generated by transposing pFastBac1-PARP-1 into bacmid propagated *E. coli* DH10Bac cells (see Figure 3.13). Colonies where the PARP-1 gene had successfully been transposed into the bacmid DNA were identified as white colonies on colour selection plates. White colonies were then subcultured in flasks containing fresh medium with the appropriate antibiotics, and grown overnight at 37°C. Recombinant bacmid DNA was then isolated from cells in these cultures. Verification that the transposition of the PARP-1 cDNA into the bacmid DNA was successful was achieved by PCR analysis. Utilising M13/pUC forward and reverse amplification primers, the resultant size of the PCR products verified whether the transposition was successful. Successful transposition of the PARP-1 cDNA into the bacmid DNA resulted in a PCR product of approximately 5100 bp, whereas failure to transpose the PARP-1 cDNA resulted in a PCR product of approximately 300 bp (see Figure 3.16). Recombinant bacmid DNA isolated from the colonies where PARP-1 cDNA had been successfully transposed into the bacmid DNA was then transfected into *Sf-9* or *Sf-21* insect cells using Cellfectin™ as the transfection reagent (as described in section 2.2.5.6).

Transfected *Sf-9* or *Sf-21* insect cells were then screened for expression of human PARP-1. Expression studies involved harvesting *Sf-9* or *Sf-21* insect cells infected with recombinant bacmid DNA containing PARP-1 cDNA and separating protein extracts from these cells on a SDS-PAGE gel. The results of PARP-1 protein expression using the Bac to Bac™ Baculovirus expression system are shown in Figure 3.17. From these results, it is clear that a protein band of 116 kDa was produced only in insect cells carrying the human PARP-1 gene. The size of this band was consistent with the molecular weight of PARP-1. Thus it was decided to stay with this expression system and subsequent work (covered in the following chapters) confirmed that this band was indeed PARP-1.

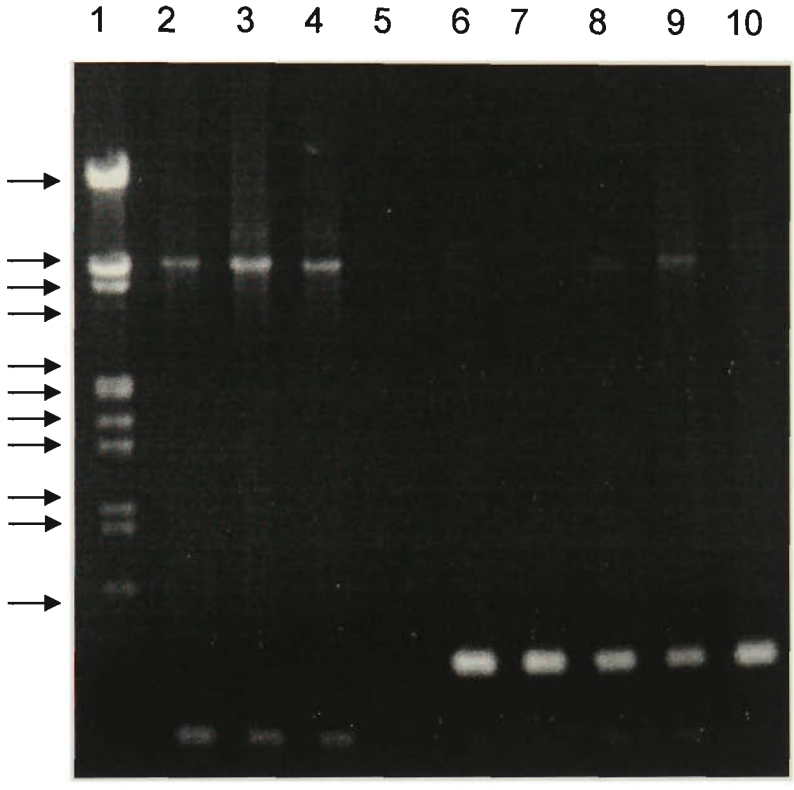


Figure 3.16: Verification of transposition of PARP-1 into bacmid DNA.

Lane 1: λ/*Hind*III and *Eco*RV DNA marker (21227 bp, 5060 bp, 4269 bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp and 564 bp). Lane 2 to 10: various recombinant bacmid DNA preparations for pFastBac1-PARP-1 and Lane 11: recombinant bacmid DNA preparation for pFastBac1-CAT. Utilising M13/pUC forward and reverse amplification primers, a successful transposition resulted in a PCR product of approximately 5100 bp for pFastBac1-PARP-1; whereas a failed transposition resulted in a PCR product of approximately 300 bp. Lanes 2, 3 and 4 show products from a successful transposition, whereas lanes 6 through to 10 show products from a failed transposition.

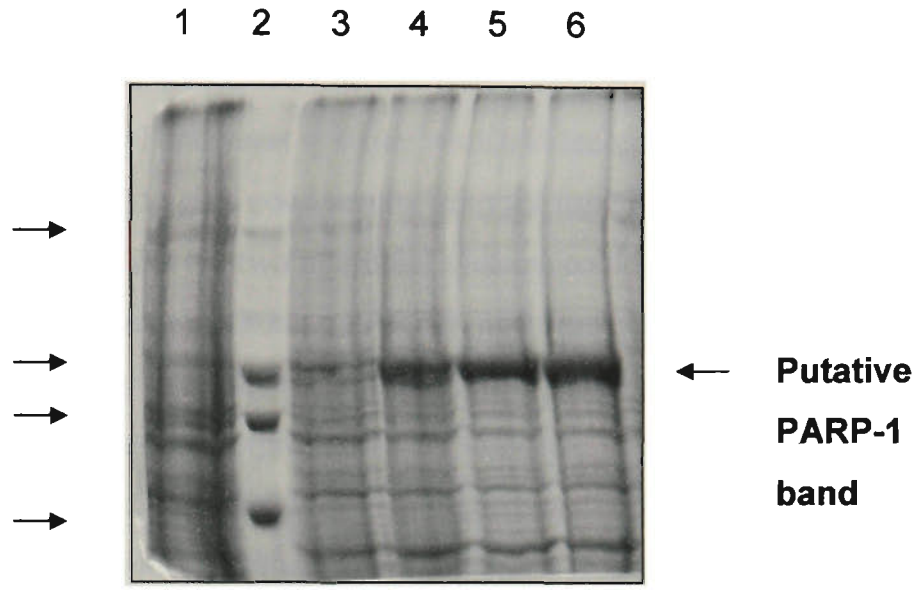


Figure 3.17: Baculovirus expression of pFastBac1-PARP-1.

Sf-9 cells (~95% viability) were infected with recombinant bacmid DNA. Seventy-two hours post baculovirus infection, the cells (~2 ml of *Sf-9* cells) were collected by centrifugation and resuspended in 150 µl 1X PBS. The protein profiles (10 µl aliquots of the cell pellet) of control insect cells (Lane 1) and insect cells infected with recombinant bacmid DNA from pFastBac1 (Lane 3) and recombinant bacmid DNA from several pFastBac1-PARP-1 isolates (Lanes 3 – 6) were then separated in 7.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 2: High range molecular weight protein standards, indicated by arrows on the left of the gel are myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa and bovine serum albumin 66 kDa. In all subsequent experiments, the recombinant bacmid DNA from the pFastBac1-PARP-1 isolate in lane 6 was used because this isolate produced the highest amount of PARP-1 protein. This result was consistent for all recombinant bacmid DNA infections.

3.3 Discussion

This chapter describes how three expression systems, bacterial, yeast and baculovirus, were trialed for the production of human PARP-1 from a cDNA of the human PARP-1 gene. Work on the bacterial and yeast systems remained incomplete as the baculovirus system appeared to give good results from the time it was applied. It is difficult to know whether or not the two microbial systems could have been developed and optimized to express human PARP-1, and time constraints meant that there was not the opportunity to explore this further. It is possible that the 89 – 93 kDa proteins produced in the yeast expression were truncated versions of PARP-1 and if this were the case it is possible that this system might have been adapted to produce the full-length protein. Certainly, if time had permitted, these truncated protein products would have been analysed further for PARP-1 activity and in western blots using anti-PARP-1 antibody.

Nonetheless, the work described here was successful in that an expression system, namely the Bac to Bac™ baculovirus expression system, was found for the production of human PARP-1. Like the traditional baculovirus expression system utilised by Giner *et al.* (1992), the Bac to Bac™ baculovirus expression system was able to yield a authentic PARP-1 protein in amounts suitable for further studies, such as, structure – function analyses including crystallographic determination of 3-dimensional structure. A crude estimate of the overall amount of human PARP-1 produced was approximately 400 – 500 μg of PARP-1 protein per 10 ml of insect cells culture (approximately 10 x 10^6 *Sf-9* cells). This estimate was obtained by comparing the amount of the high molecular weight standards loaded on the gel (Figure 3.17, Lane 2) and the amount of PARP-1 protein produced (Figure 3.17, Lane 6). The amount of PARP-1 protein produced was substantially higher than might be expected for bacterial- and/or yeast-based protein expression systems and probably similar to that produced by the baculovirus expression system reported by Giner *et al.* (1992)[2]. It should be noted that spinner flasks regularly achieves higher protein yields than tissue culture flasks, due to better aeration and growth of *Sf-9* or *Sf-21* cells (O'Reilly *et al.*

[2] Note that estimating PARP-1 protein concentration was later found to be problematic, and thus the above estimate may not have been accurate. This issue is addressed in Chapter 5.

1994). Thus, the recombinant bacmid DNA from the pFastBac1-PARP-1 isolate in Figure 3.17, lane 6 was amplified for the large scale production of human PARP-1. This PARP-1 protein was further purified and studied in the subsequent chapters.

4 Development of an efficient extraction and purification process for the production of Human PARP-1

4.1 Introduction

With the successful expression of human PARP-1 in the baculovirus expression system now in place (See Chapter 3), the next stage of this project was to develop an efficient extraction and purification procedure to generate high yields of PARP-1 with high specific activity. While there is a range of protocols described in the literature for the production and purification of human PARP-1 none was thought to be adequate for structure – function studies of the enzyme, particularly if one were aiming to use such techniques as X-ray crystallography on the purified product. With this in mind a protocol was developed that utilised features of several key techniques from existing approaches but introduced important modifications.

One of these important modifications was the introduction of a vortexing or sonication step to disrupt cells following expression of the cloned human PARP-1 gene in the baculovirus system. Vigorous vortexing or sonication made the purification protocol more rapid thus minimising potential losses of the PARP-1 enzyme due to protease activity.

It is well documented that salts and ions present in PARP-1 preparations can severely affect the activity of the enzyme (Sallmann *et al.* 1998). Previous studies removed unwanted salts and ions from the preparation using dialysis (Yoshihara *et al.* 1978; Giner *et al.* 1992; Ruscetti *et al.* 1998) or hydroxyapatite column chromatography (Burstcher *et al.*, 1986). The method developed in this thesis introduced an ultrafiltration (CentriPrep10, Amicon Inc.) to remove unwanted salts and ions from the preparation of PARP-1. Ultrafiltration had the advantage of concentrating the PARP-1 protein.

The resultant protocol developed here is relatively simple and rapid and is well suited to making PARP-1 in quantities and of a quality suitable for structure – function studies. This work described in this chapter has been published in Knight and Chambers (2001).

4.1.1 Background Literature

PARP-1 has been isolated and purified from a range of animal tissues including human tonsils (Carter and Berger 1982), rat liver (Okayama *et al.* 1977), calf and lamb thymus (Mandel *et al.* 1977; Yoshihara *et al.* 1978; Petzold *et al.* 1981) and human placenta (Burtscher *et al.* 1986) but over the past ten years or so most researchers working on this enzyme have used recombinant sources of the gene expressed in a range of backgrounds including *E. coli* (e.g. Simonin *et al.*(1993a); Trucco *et al.*(1996)), yeast (e.g. Kaiser *et al.*(1992); Avila *et al.*(1994); Perrin *et al.*(2000)) and baculovirus expression systems (e.g. Giner *et al.*(1992); Jung *et al.*(1994)).

The isolation and purification protocols developed to extract PARP-1 from the above sources have been quite variable. Key papers in the field (Tsopanakis *et al.* 1978; Yoshihara *et al.* 1978; Burtscher *et al.* 1986; Thibodeau *et al.* 1990; Giner *et al.* 1992; Jung *et al.* 1994; Ruscetti *et al.* 1998) that describe the production of purified PARP-1 (albeit in limited quantities) have several features that, whilst not common to all, were thought to be important for the development of the protocol described in this chapter. Five key steps were identified: homogenisation, removal of DNA, ammonium sulphate precipitation, affinity chromatography and ultrafiltration/dialysis. It was decided from the outset that the protocol developed for this thesis would be built around these steps.

From the work of others in the field (Tsopanakis *et al.* 1978; Yoshihara *et al.* 1978; Burtscher *et al.* 1986; Giner *et al.* 1992) it is clear that the extraction buffer used to release PARP-1 from its source must contain protease inhibitors; PARP-1 is relatively unstable when released from chromatin, and this is almost certainly due to the action of proteases (Shall 1984). Thus, any procedure developed to successfully extract and purify PARP-1 must use buffers carrying such components as PMSF and sodium dithionite ($NaHSO_3$), and sulphydryl-reducing agents (e.g. DTT and β-mercaptoethanol) to minimise proteolytic degradation and to protect the thiol groups from oxidation. The extraction buffer must also contain a minimum of 0.3 M sodium chloride as this facilitates the separation of PARP-1 from chromatin (Gill 1972;

Yoshihara *et al.* 1978) and helps in removal of DNA in later stages of the preparation (Giner *et al.* 1992).

The first step in extraction and purification protocols of PARP-1 is homogenisation of the tissue-source or cell culture-source. When purifying PARP-1 from tissue, homogenisation procedures are relatively time consuming, as large amounts of tissue are required and have to be carefully prepared and then vigourously homogenised in a blender (Tsopanakis *et al.* 1978; Yoshihara *et al.* 1978; Zahradka and Ebisuzaki 1984) followed by separation, usually using centrifugation. Purification of PARP-1 from cell culture-sources however eliminates the need for vigorous homogenisation. For example, Giner *et al.*(1992) isolated PARP-1 from baculovirus-infected insect cells using a hypertonic buffer and slight agitation on ice for 20 minutes to break open the cells. Cellular debris was then removed from the preparation using centrifugation.

The second step for extraction and purification PARP-1 is the removal of endogenous DNA from the protein extract (Tsopanakis *et al.* 1978; Yoshihara *et al.* 1978; Burtscher *et al.* 1986; Thibodeau *et al.* 1990; Giner *et al.* 1992; Jung *et al.* 1994; Sallmann *et al.* 1998); PARP-1 is a DNA binding protein and therefore DNA tends to co-purify with PARP-1, necessitating its removal. DNA can be removed from PARP-1 preparations using hydroxylapatite column chromatography (e.g., Yoshihara *et al.* (1978)) or protamine sulphate precipitation (e.g., Giner *et al.* (1992)). The removal of DNA by hydroxylapatite column chromatography was considered to be time consuming and in the laboratory at Victoria University had previously been found to give highly variable yields (unpublished data). Thus, protamine sulphate precipitation as described by Giner *et al.* (1992) was utilised. The protamine sulphate - DNA complex was subsequently removed via centrifugation at 4°C.

The third step common to many PARP-1 extraction and purification protocols is step-wise ammonium sulphate precipitation. Step-wise ammonium sulphate precipitation is believed to stabilise the enzyme preparation, as well as minimises the loss of protein due to protease digestion (Yoshihara *et al.* 1978; Althaus and Richter 1987). To date, a range of ammonium sulphate concentrations has been reported in the literature on PARP-1 purification: Yoshihara *et al.* (1978) used 40 – 80% saturation,

Giner *et al.* (1992) and Panzeter *et al.* (1994) used 30 – 70% saturation, whereas Jung *et al.* (1994) used 40 – 65% saturation to purify the catalytic domain of chicken PARP-1 from infected *Sf*-9 insect cells. For work described in this thesis it was decided to trial 30 – 70% ammonium sulphate precipitation as described by Giner *et al.* (1992) and Panzeter *et al.* (1994).

The fourth and perhaps the most important step identified in the extraction and purification protocols of PARP-1 is affinity chromatography. A variety of affinity matrices have been utilised by many investigators including DNA-cellulose (Yoshihara *et al.* 1978), 3-aminobenzamide (3-AB) affinity matrix (Burtscher *et al.* 1986; Giner *et al.* 1992) and more recently heparin-sepharose CL-6B (Sallmann *et al.* 1998).

Burstcher *et al.* (1986) and Giner *et al.* (1992) successfully purified PARP-1 utilising 3-AB, a very potent competitive inhibitor of PARP-1, on an affinity column, and eluted this with 3-methoxybenzamide (3-mBA), another potent inhibitor of PARP-1. The 3-mBA was subsequently removed from the preparation by dialysis. The use of competitive inhibitors to affinity purify proteins may however cause problems if the inhibitor is carried over in the purified product. Whilst Burstcher *et al.* (1986) and Giner *et al.* (1992) were able to produce PARP-1 with kinetic parameters similar to those reported in the literature, and therefore presumably successfully separated PARP-1 from inhibitors, it was considered to be preferable to avoid the risks associated with the use of competitive inhibitors for purification work where the product is to be used in structure – function studies. Therefore, the use of DNA-cellulose affinity matrix chromatography (as described in Yoshihara *et al.* (1978)), which targets the zinc fingers in PARP-1 and their affinity for DNA was used. PARP-1 is easily eluted from the DNA-cellulose affinity matrix at 1.0 M sodium chloride (Yoshihara *et al.* 1978).

The fifth and final step used in many PARP-1 purification protocols is dialysis (e.g. Yosihara *et al.* (1978); Burstcher *et al.* (1986); Giner *et al.* (1992); Ruscetti *et al.* (1998)). Dialysis is required to remove unwanted salts and ions from PARP-1 preparations along with potent PARP-1 inhibitors used to elute PARP-1 from 3-AB

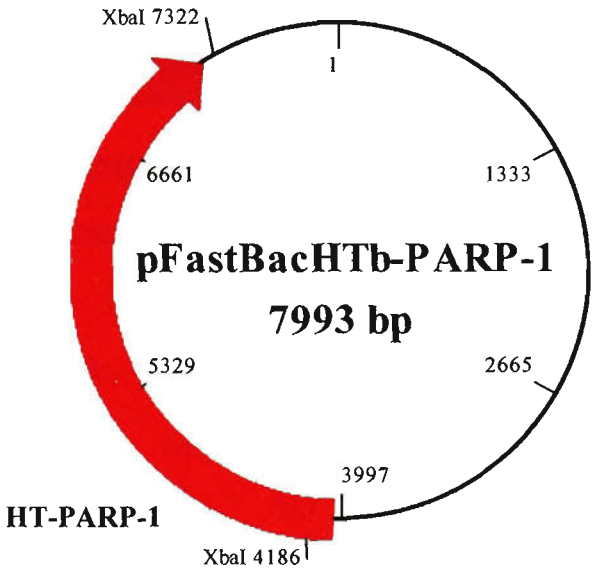
affinity column. It is necessary to remove these compounds from PARP-1 preparations as they might otherwise influence PARP-1 structure and/or function in the purified protein product. Thus a desalting step is important to the purification protocol. However it was decided to trial ultrafiltration instead of dialysis for work described in this thesis as dialysis had previously been found to give rise to variable yields in work performed in the laboratory at Victoria University (unpublished data).

Section 4.2 details the extraction and purification protocol developed to rapidly isolate human PARP-1 and hexahistidine-tagged human PARP-1 (HT-PARP-1) from infected *Sf*-9 or *Sf*-21 insect cells.

HT-PARP-1 was produced by ligating human PARP-1 cDNA into the *Xba*I site in the polylinker region of pFastBacHTb from GibcoBRL-Life Technologies Inc. (See Figure 4.1). The vector map including all the salient features of pFastBacHTb expression vector is shown in Appendix 9.4 and schematic representation of HT-PARP-1 is shown in Figure 4.2. Plasmid DNA was extracted and digested with *Hind*III restriction enzyme to identify pFastBacHTb-PARP-1 transformants carrying the PARP-1 gene in the correct orientation in pFastBacHTb (data not shown). Transformants carrying the PARP-1 gene in the correct orientation were then prepared for baculovirus expression according to the *Bac to Bac*™ *Baculovirus Expression Systems Instruction Manual* (GibcoBRL-Life Technologies Inc.). HT-PARP-1 was expressed at similar levels to PARP-1 following baculovirus infection of *Sf-9* or *Sf-21* cells. HT-PARP-1 was generated primarily to determine whether the presence of a hexahistidine tag would affect the purification of PARP-1.

Figure 4.1: Map of pFastBacHTb-PARP-1 construct. PARP-1 was cloned into the *Xba*I restriction site in pFastBacHTb Baculovirus expression vector (GibcoBRL-Life Technologies, Inc.).

The vector map including all the salient feactures of pFastBacHTb is shown in Appendix 9.4.



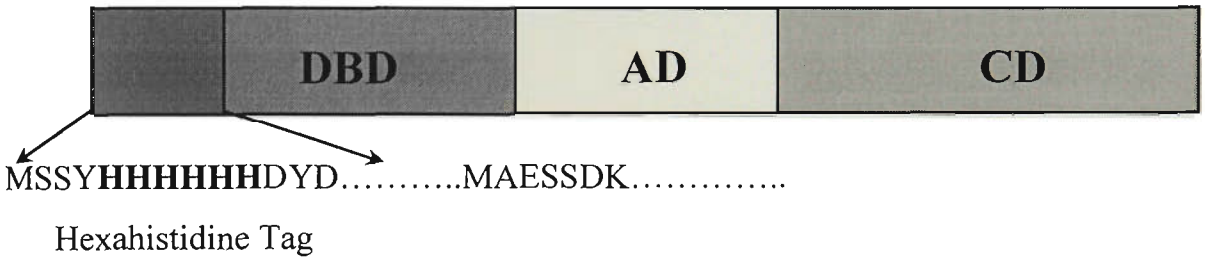


Figure 4.2: Schematic of Hexahistidine tagged PARP-1 (HT-PARP-1).

where DBD – DNA-binding domain,
AD – Automodification domain, and
CD – Catalytic domain.

4.2 Results and Discussion

Biologically active PARP-1 has previously been successfully purified to homogeneity from a variety of sources using a range of methods. Table 4.1 identifies the key features of the extraction and purification protocol used to purify human PARP-1 for research presented in this thesis. The extraction and purification was primarily based on methods described by Yoshihara *et al.* (1978) and Giner *et al.* (1992), however, the procedure was adapted to incorporate several important modifications, which improved PARP-1 isolation making the purification procedure rapid, simple and efficient.

The homogenisation step for extraction of PARP-1 from insect cells used the same hypertonic homogenisation buffer as described by Giner *et al.* (1992). The hypertonic homogenisation buffer contained the required cocktail of protease inhibitors including PMSF, sodium dithionite ($NaHSO_3$) and sulphydryl-reducing agents such as DTT and β-mercaptoethanol. However, where Giner *et al.* (1992) used slight agitation for 20 minutes at 4°C to lyse baculovirus infected insect cells, this thesis investigated the use of more rapid approaches including vigorous vortexing (3 x 30 seconds) and sonication (3 x 5 seconds) to lyse the baculovirus-infected insect cells. This was seen as a means of speeding up the method and minimizing losses of PARP-1 protein due to lengthy agitation times and associated protease digestion. Cellular debris was then removed from the PARP-1 preparation via centrifugation (14,000 *g*, 15 minutes at 4°C). The yield of PARP-1 obtained was qualitatively and quantitatively the same for the vortexing and sonication as determined by Coomassie stained SDS-PAGE (Figure 4.3).

The second step identified in the extraction and purification of PARP-1 involved the removal of endogenous DNA from the protein extract. As in Giner *et al.* (1992), protamine sulphate precipitation was utilised for this and the protamine sulphate - DNA complex was removed via centrifugation at 14,000 *g* for 10 minutes at 4°C. It should be noted however that one of the reviewers of Knight and Chambers (2001) commented that the quality of protamine sulphate is notoriously variable and they

suggested another possible way to accomplish the removal of endogenous DNA using phenylethyleneimine. This will be tested in future purification studies.

Following protamine sulphate precipitation, the third step in the extraction and purification of PARP-1 was ammonium sulphate precipitation. In this thesis, the PARP-1 protein extract was precipitated in a two-step ammonium sulphate precipitation: 0 – 30% and 30 – 70% (see Figure 4.4). PARP-1 was successfully fractionated in the 30 – 70% ammonium sulphate fraction[3]. Ammonium sulphate precipitated proteins were dissolved in ice-cold chromatography-extraction buffer A which contained 0.2 M NaCl (See Section 2.2.6).

One of the most critical stages in the extraction and purification of PARP-1 is undoubtedly the affinity chromatography step. As discussed in Section 4.1.1, it was decided for this thesis to avoid the use of competitive inhibitors for this step; a DNA-cellulose affinity column was used instead.

[3] It should be noted that bovine PARP-1 has also been successfully fractionated at 40 – 80% in past work in laboratories at Victoria University with similar success (unpublished data).

Table 4.1: Summary of the protocol developed to extract and purify high quality human PARP-1 for this thesis.

Extraction and purification method developed in this thesis to isolate high quality PARP-1	Key features of the extraction and purification protocol
Homogenisation Hypertonic Homogenisation Buffer: 25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 0.2 % Tween-20, 0.2 % Nonidet P-40 and 0.5 M NaCl. To ensure complete cellular lysis, cells were either vortexed or sonicated and cleared by centrifugation (14,000 g, 15 minutes at 4°C).	Hypertonic Homogenisation Buffer was used by Giner *et al.* (1992) to lyse insect cells infected with baculovirus containing the PARP-1 gene. It was necessary for any homogenisation buffer to contain at least 0.2 M NaCl (Gill 1972; Yoshihara *et al.* 1978). Vortexing or sonication was used to ensure the cells were completely lysed.
↓	
Removal of DNA DNA was removed from the preparation by precipitation with 1 mg/ml protamine sulphate and pelleted by centrifugation (14,000 *g*, 10 minutes).	The method used to remove endogenous DNA from the PARP-1 preparation was described by Giner *et al.* (1992).
↓	
30 – 70% Ammonium Sulphate Precipitation Proteins precipitated at 30-70% saturation were dissolved in ice-cold chromatography-extraction buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$ and 0.2 M NaCl).	Ammounium sulphate precipitation stabilizes the enzyme preparation. A range of ammonium sulphate concentrations has used by several authors when extracting and purifying PARP-1 (see Yoshihara *et al.* 1978; Burstcher *et al.* 1986; Giner *et al.* 1992; Jung *et al.* 1994; Panzeter *et al.* 1994).
↓	
Affinity Chromatography using DNA-Cellulose PARP-1 was affinity purified from other proteins using step-wise elution from a DNA cellulose column with ice-cold chromatography-extraction buffer B (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$ and 1.0 M NaCl).	DNA-Cellulose affinity chromatography was previously successfully used by Yoshihara, *et al.* (1978) and Zahradka and Ebisuzaki (1982) to affinity purify PARP-1 from bovine thymus.
↓	
Ultrafiltration Fractions containing the highest amount of PARP-1 protein were pooled together and concentrated using ultrafiltration column from Amicon.	

The DNA-cellulose affinity matrix was pre-equilibrated with ice-cold chromatography-extraction buffer A and the ammonium sulphate precipitated proteins loaded onto the matrix (as described in Yoshihara *et al.* 1978). PARP-1 was eluted from the column step-wise with ice-cold chromatography-extraction buffer B which contained 1 M NaCl (See Section 2.2.6). The elution profiles for the DNA-cellulose affinity matrix column are shown in Figure 4.5.

The fifth and final step in the extraction and purification of PARP-1 requires the removal of unwanted ions and salts that might otherwise influence PARP-1 structure and/or function in the purified protein product. For example, Sallmann *et al.*(1998) found that salts could severely affect the detection of PARP-1 in an enzyme-linked immunosorbent assay (ELISA). These unwanted ions and salts are most commonly removed using dialysis (e.g. Yosihara *et al.* (1978); Burstcher *et al.* (1986); Giner *et al.* (1992); Ruscetti *et al.* (1998)) or hydroxylapatite column chromatography (e.g. Burstcher *et al.* (1986)). However, ultrafiltration using a Centriprep-10 centrifugal concentrator (Amicon) was used for this thesis as dialysis had been found to deliver variable yields of PARP-1 as previous work in the laboratory at Victoria University (unpublished data) and it was thought ultrafiltration would be more rapid than using a chromatography column. Ultrafiltration had the additional advantage of concentrating the PARP-1 sample. As can be seen from Figure 4.6, the final product of this purification protocol was a single band (on a sliver stained gel) of appropriate molecular weight. This gel also shows a hexahistidine-tagged PARP-1 protein that was produced using the same approach as for PARP-1 indicating that the method developed may be suitable for purifying variants of PARP-1.

At the time of performing this work samples were taken at each stage of purification for protein and enzyme activity assays to determine the yield and purity of the preparation. However problems encountered with assaying protein concentration in purified preparations of PARP-1 required a systematic approach to finding an assay that would work for PARP-1. This is described in the following chapter along with kinetic properties of this preparation in Chapter 6.

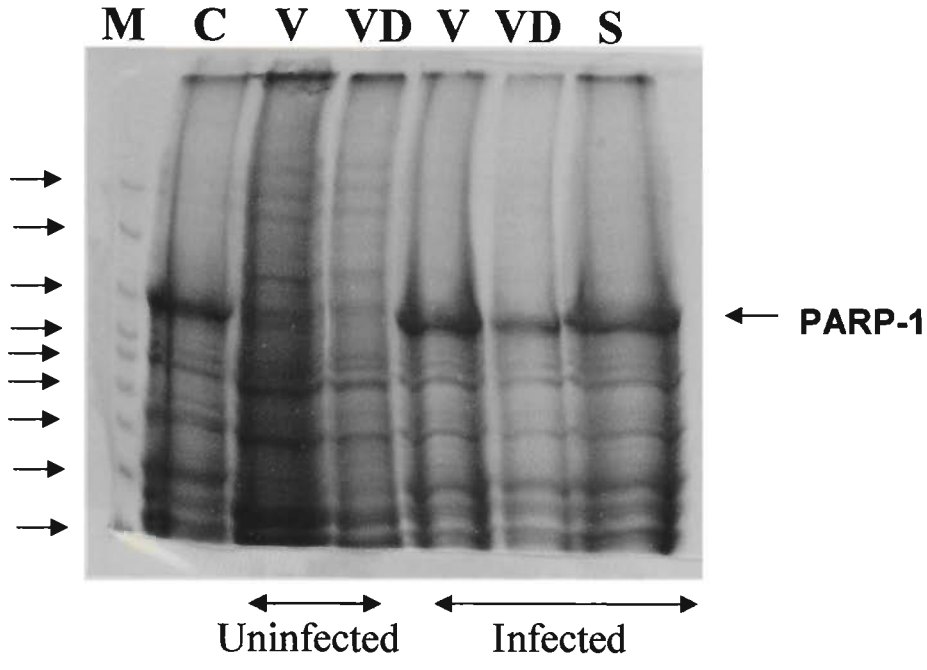


Figure 4.3: 7.5% SDS-PAGE protein profile of the initial stages of PARP-1 extraction and purification from baculovirus-infected and uninfected cells.

The first stage of PARP-1 extraction and purification involved cellular lysis in a hypertonic lysis buffer and extraction by vortexing or sonication. The above gel shows crude protein preparations from insect cells that were either vortexed or sonicated. The gel was stained with Coomassie Brillant Blue R250. The molecular weights of BenchMark Protein Markers® (M) (Gibco-Life Technologies, Inc.), indicated by the arrows are 220 kDa, 160 kDa, 120 kDa, 100 kDa, 90 kDa, 80 kDa, 70 kDa, 60 kDa and 50 kDa. C = control infected insect cells before disruption, V = proteins from vortexed cells, VD = cellular debris from vortexed cells and S = proteins from sonicated cells.
Note: While the identification of PARP-1 in this gel was based on its molecular weight, the identity of the band was validated in subsequent experiments described in Chapters 5 and 6.

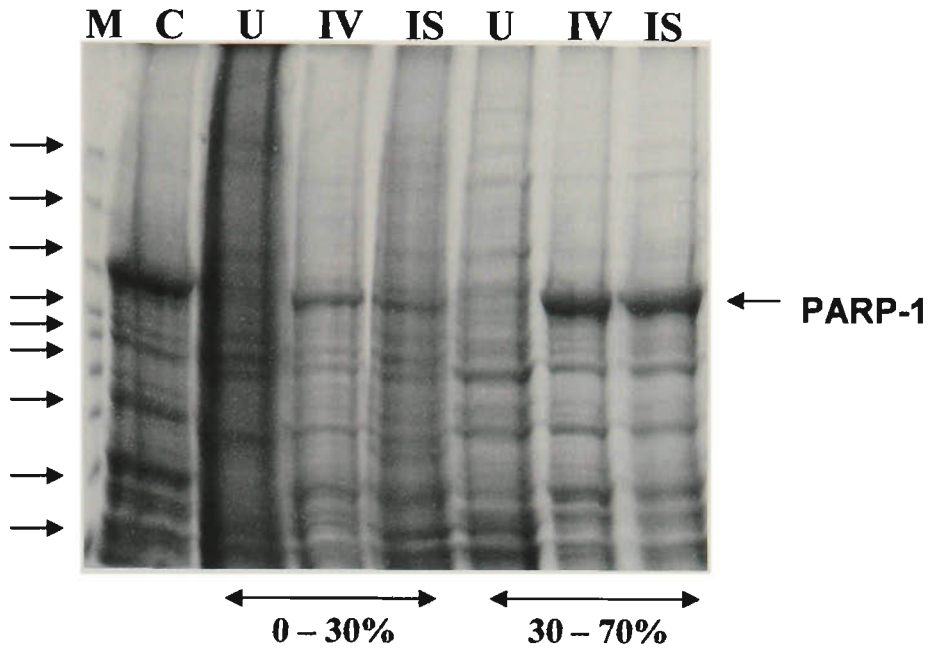


Figure 4.4: 7.5% SDS-PAGE protein profile of the initial stages of PARP-1 extraction and purification from baculovirus-infected and uninfected cells.

Step-wise ammonium sulphate precipitation of proteins from baculovirus-infected and uninfected cells. The gel was stained with Coomassie Brilliant Blue. The molecular weights of the BenchMark Protein Markers® (M), indicated by the arrows are 220 kDa, 160 kDa, 120 kDa, 100 kDa, 90 kDa, 80 kDa, 70 kDa, 60 kDa and 50 kDa. C = control infected insect cells before sonication and vortexing, U = proteins from disrupted, uninfected, insect cells and IV = proteins from vortexed disrupted, infected, insect cells and IS = proteins from sonicated disrupted, infected, insect cells. **Note:** While the identification of PARP-1 in this gel was based on its molecular weight, the identity of the band was validated in subsequent experiments described in Chapters 5 and 6.

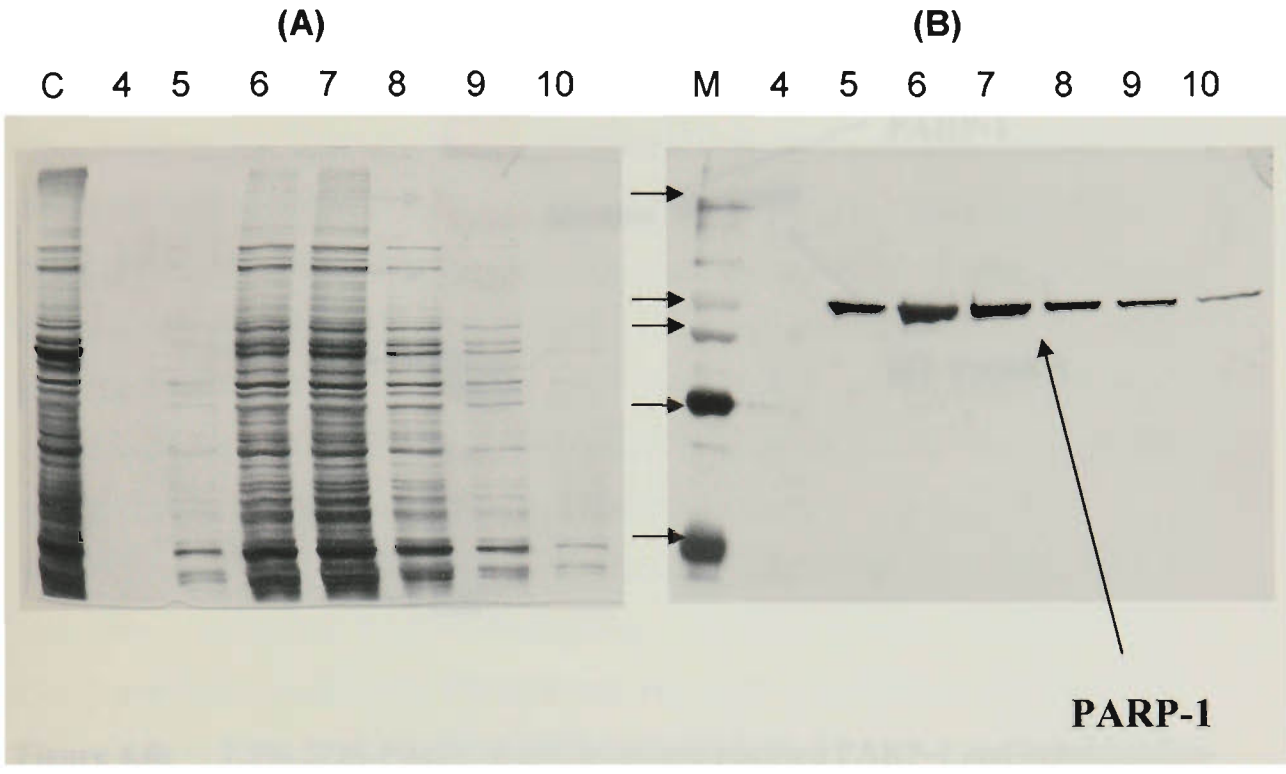


Figure 4.5: 7.5% SDS-PAGE protein profile of PARP-1 fractions prior to elution **(A)** and after elution **(B)** from DNA-cellulose column.

The gel was silver stained. The SDS-PAGE molecular weight markers (M) are indicated by the arrows are myosin 205 kDa, β-galactosidase 116 kDa, phosphorlyase B 97.4 kDa, bovine serum albumin 66 kDa and egg albumin 45 kDa. C = crude PARP-1 preparation prior to loading on the DNA-cellulose column. **Note:** While the identification of PARP-1 in this gel was based on its molecular weight, the identity of the band was validated in subsequent experiments described in Chapters 5 and 6.

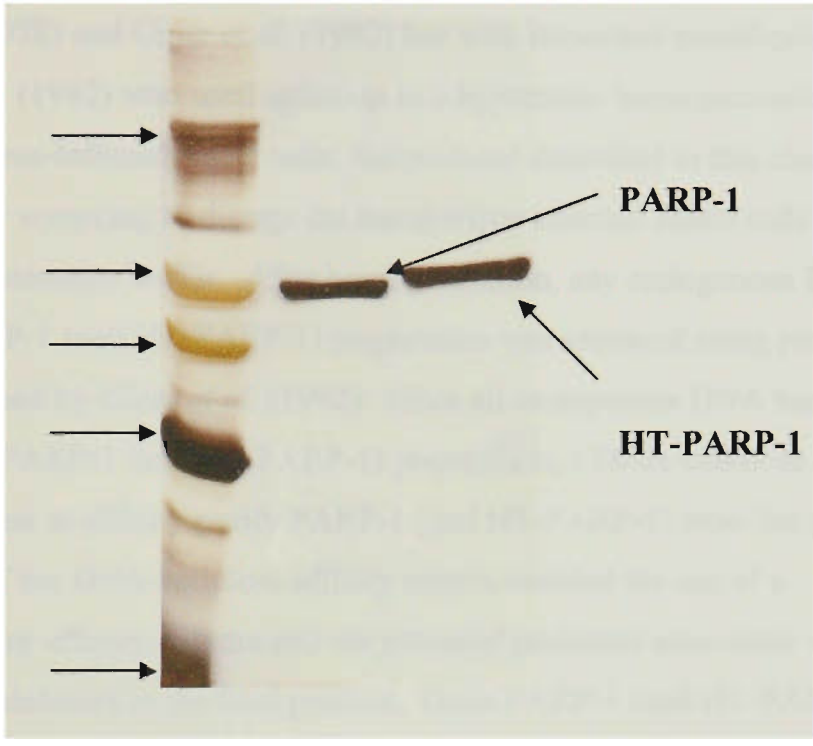


Figure 4.6: 7.5% SDS-PAGE of extracted and purified PARP-1 and hexahistidine-tagged PARP-1 (HT-PARP-1).

Fifteen microlitres (~150ng) aliquots of purified PARP-1 and HT-PARP-1 were loaded onto the 7.5% SDS-PAGE gel. The total volume of the purified PARP-1 and HT-PARP-1 solution was 20 ml (The protein concentration of each stock was 10 μg/ml – See Chapter 5). The gel was silver stained and the SDS-PAGE molecular weight markers indicated by the arrows are myosin 205 kDa, β-galactosidase 116 kDa, phosphorlyase B 97.4 kDa, bovine serum albumin 66 kDa and egg albumin 45 kDa. **Note:** While the identification of PARP-1 in this gel was based on its molecular weight, the identity of the band was validated in subsequent experiments described in Chapters 5 and 6.

In summary, this chapter describes a rapid and efficient method for extraction and purification PARP-1 (and HT-PARP-1) from baculovirus-infected *Sf*-9 or *Sf*-21 insect cells. The method developed here utilises aspects of procedures described by Yoshihara *et al.* (1978) and Giner *et al.* (1992) but with important modifications. Unlike Giner *et. al.* (1992) who used agitation in a hypertonic homogenisation buffer to disrupt baculovirus-infected insect cells, the protocol described in this chapter used either sonication or vortexing to disrupt the baculovirus-infected insect cells in a hypertonic homogenisation buffer. After homogenisation, any endogenous DNA present in the PARP-1 (and HT-PARP-1) preparation was removed using protamine sulphate, as described by Giner *et al.* (1992). Once all endogenous DNA had been removed from the PARP-1 (and HT-PARP-1) preparation, a DNA-cellulose affinity matrix was then used to affinity purify PARP-1 (and HT-PARP-1) from the protein extract. The use of the DNA-cellulose affinity matrix avoided the use of a competitive inhibitor affinity column and the potential problems associated with carry-over of the inhibitors to the final product. Once PARP-1 (and HT-PARP-1) was affinity purified, a simple ultrafiltration step was used to concentrate and dialyse the purified protein, removing unwanted salts and ions from the preparation that could dramatically affect PARP-1 activity. Thus, the method developed to extract and purify PARP-1 (and HT-PARP-1) for this thesis was relatively simple and rapid. Purified PARP-1 (and HT-PARP-1) migrated as a unique 116 kDa band (and a unique 120 kDa band) on a SDS-PAGE (see Figure 4.5 and Figure 4.6).

The K_m and V_{max} values for the human PARP-1 purified in this chapter were subsequently found to be consistent with the kinetic properties of other PARP-1's reported in the literature; this is reported in chapter 6.

5 Quantitation of Human PARP-1

5.1 Introduction

To determine the efficiency of the purification protocol developed in the previous chapter and to ascertain the quality (i.e. specific activity) of the final product required a reproducible protein assay that gave an accurate measurement of PARP-1 protein concentration. However, it is not unusual to get conflicting results when using different protein assays to quantify purified proteins (Ausubel *et al.* 1996) and thus it is prudent, if not essential, to test the reliability of the protein assay one intends to use to determine protein concentration.

In preliminary experiments for the work described in this chapter, it was decided that the Lowry protein assay (Lowry *et al.* 1951) may be suitable for estimating levels of PARP-1 proteins in purified preparations as it is generally reliable, reproducible and rapid. To validate use of the Lowry protein assay for determining PARP-1 concentrations it was decided to compare it with a method that relies on a different principle for detecting proteins, and thus the Bradford assay (Bradford 1976) was chosen. It was clear from the outset that the two approaches gave very different estimates, with an order of magnitude or more difference between the two for the same PARP-1 solution (data not shown). Thus a more extensive, systematic approach was required to find a method for determining the concentration of PARP-1 in solution. This chapter describes how this was achieved and then presents data on the purity and specific activity of the PARP-1 product from the previous chapter.

This work is published in Knight, M.I. and Chambers, P.J. (2003) Problems associated with determining protein concentration: A comparison of techniques for protein estimations. *Molecular Biotechnology,* **23**, 19-28.

5.1.1 Methods for determining Protein Concentration

Many methods are available for determining protein concentration (Table 5.1 provides a list of commonly used assays), the criteria for a suitable assay usually being sufficient sensitivity, accuracy and reproducibility. The following is a brief overview of some of the most commonly used techniques.

5.1.1.1 Amino Acid Analysis

This is the most accurate and sensitive method for determining protein concentration. Whilst access to this approach in the past was restricted to specialized laboratories, it is now readily available, usually as a service provided by specialist laboratories. Additionally, significant advances in the technologies underpinning this approach have improved the precision and the sensitivity of amino acid analysis (Ausubel *et al.* 1996). Amino acid analysis requires proteins to be quantitatively broken down to their constituent amino acids by chemical treatments that lead to peptide bond hydrolysis; this is most commonly achieved by acid hydrolysis. The amino acids are then resolved and quantitated using HPLC or GC-MS.

5.1.1.2 The Lowry Protein Assay

The most widely used protein assay in the literature, the Lowry Protein Assay (Lowry *et al.* 1951), detects the phenolic group on tyrosine residues in proteins and the sensitivity of this assay is between 2 to 100 μg/ml of protein (Ausubel *et al.* 1996). As the Lowry protein assay detects tyrosine residues in a protein and the number of tyrosine residues varies between proteins it is important that the protein used as a standard has a similar proportion of tyrosine to the protein being assayed. Another critical component of the Lowry protein assay is the length of the incubation time to develop the product of the Lowry reaction; differences in incubation times between samples in a Lowry protein assay will lead to non-reproducible results. The Lowry protein assay is also subject to interference from a wide range of components such as Tris and EDTA (Markwell *et al.* 1981), both of which are components of many buffers used for the purification of recombinant proteins. However assuming there is sufficient protein the effect of these chemicals can be minimized by diluting the protein sample.

5.1.1.3 The Bradford Protein Assay

The Bradford protein assay is both rapid and accurate; indeed Ausubel *et al.* (1996) proposed that the Bradford protein assay is "the method of choice" for accurately determining protein concentration. Practical advantages of this method are that the Bradford protein reagent is simple to prepare and the color develops rapidly and is stable. The assay relies on the binding of Coomassie Brilliant Blue G250 to protein

(Bradford 1976). The dye binds in its anionic form to basic amino acids within the protein (particularly arginine residues) and when bound produces a complex that has an Absorbance peak at 595nm (Compton and Jones 1985). The amount of dye that binds is dependent on the content of basic amino acid residues in the protein. Thus, the proportion of basic residues in the protein standard for the assay and the protein to be assayed should be similar. The sensitivity range for the Bradford Assay is between 0.2 and 20 μg/ml of protein (Stoscheck 1990).

5.1.1.4 Using SDS-PAGE Gels to determine Protein Concentration

Protein concentration can also be estimated using laser densitometry of stained SDS-PAGE gels; for some stains the intensity of a stained band of protein in such a gel is proportional to the concentration of protein in the band. One of the most commonly used stains for protein gels is Coomassie Brilliant Blue G250 (CBB). Whilst this stain can be used to detect relatively small amounts of protein in an SDS-PAGE gel (it can detect as little as 40 – 50 ng of protein in a band on a gel (Promega 1993)), it is generally regarded as, at best, semi quantitative.

Silver staining provides a significant advantage over the traditional CBB stain as it is reported to be between 20 to 200 times more sensitive (Patel *et al.* 1988) and thus able to detect as little as 1 – 5 ng of protein in a band on an SDS-PAGE gel (Rabilloud 1992; Promega 1993). However, the precise chemical interactions between protein and silver salts in silver staining proteins are unknown (for more detail see Patel (1988) and Rabilloud (1992)) and silver staining is generally regarded as non-quantitative.

It is claimed that fluorometric methods for quantifying protein concentration outperform all existing routine methods for determining protein concentration (see Molecular Probes (2001)). The newly developed SYPRO® Orange fluorescent stain (Bio-Rad Laboratories Pty. Ltd., Catalogue Number: 170-3120) for SDS-PAGE gels can detect as little as 1 ng of protein in a band on an SDS-PAGE gel (Steinberg *et al.* 1996).

5.1.2 Determining Human PARP-1 Protein Concentration

It is evident from the literature that a variety of methods have been used to determine the concentration of PARP-1. For example, Molinete *et al.* (1993) determined the concentration of over-expressed and purified PARP-1 DNA-binding domain by absorbance at 280 nm. Simonin *et al.* (1993b) and Rolli *et al.*. (1997) generated a standard curve by immunoblotting, using a polyclonal antibody directed against the second zinc finger of PARP-1 to determine the amount of PARP-1 in cleared cell lysates using purified PARP-1 from calf thymus and over-expressed and purified human PARP-1 produced in insect cells, respectively. The intensity was determined by densitometry. Ruscetti *et al.* (1998) quantified the concentration of Histidine tagged DNA binding domain of human PARP-1 using a standard Bicinchoninic Acid (BCA) reagent assay as described by Pierce (Pierce Inc., Rockford, Illinois, USA).

For this thesis, we used amino acid analysis to get an accurate estimation of protein concentration of a stock solution of purified human PARP-1. This stock was then used as a standard to test a range of other assays for their sensitivity, accuracy and reproducibility. The aim of this work was to find a simple and reliable method for quantifying PARP-1 that could use a readily available protein standard such as bovine serum albumin (BSA) as a reference.

Table 5.1: Comparisons of routinely used methods for quantitating protein concentration.

Method	Detection Range	Active Residues Detected	Comments	Reference
Lowry Protein Assay	2-100 µg/ml	The phenolic group on tyrosine residues	Choice of standard is critical Incompatible with some detergents and some reducing agents Tris and EDTA can interfere with assay	Lowry *et al.* 1951. Markwell *et al.* 1981.
Bradford Protein Assay	0.2-20 µg/ml	Basic amino acids residues, particularly arginine	High protein to protein variation Incompatible with some detergents	Bradford 1976. Stoscheck 1990.
Laser Densitometry of SDS-PAGE Gels				
i. Coomassie Brilliant Blue G250 staining:	40-50 ng band	Detects basic amino acids on the same principle as Bradford protein method	Time consuming but can give reasonable estimate of protein concentration	Promega 1993.
ii. SYPRO® Orange staining:	1 ng band	Binds to SDS-coat around protein	Low protein to protein variation Detection not influenced by nucleic acids and other contaminants	Steinberg *et al.* 1996. Biorad 1998 and Molecular Probes 2001.
iii. Silver staining:	1-5 ng band	Relies on the reduction of ionic silver to its metallic form on binding proteins	Qualitative method Time consuming	Rabilloud 1992. Patel *et al.* 1988. Promega 1993.
Amino acid analysis		All amino acid residues are detected	Specialised technique Requires GC-MS or HPLC Very accurate quantitation method	Ausubel *et al.* 1996.

5.2 Results and Discussion

5.2.1 Quantitation of Human PARP-1

A stock solution of human PARP-1, expressed from a cloned source and purified as described in the previous chapter and Knight and Chambers (2001) was used to compare a range of protein assays to determine which would be most reliable for accurate determination of PARP-1 concentrations for future work in our laboratory. The concentration of the stock PARP-1 solution was determined by amino acid analysis (performed at Australian Proteome Facility, Macquarie University, Sydney, NSW, Australia, 2109) and was estimated to be 10 μg/ml. This was consistent with the level of PARP-1 enzyme activity associated with the preparation (see Chapter 6). This estimate was therefore assumed to be a reasonable measure of PARP-1 concentration in the stock solution.

5.2.1.1 Quantitation of human PARP-1 using the Lowry Protein Assay

As mentioned previously the Lowry protein assay detects tyrosine residues in a protein and therefore it is important that the protein used as a standard has a similar proportion of tyrosine residues to the protein being assayed. The proportion of tyrosine residues in BSA and human PARP-1 is 3.5% and 3.3% respectively and thus BSA should be a suitable standard for assaying PARP-1 concentration.

The Lowry protein assay is also subject to interference from a range of chemicals such as Tris and EDTA (Markwell *et al.* 1981), both of which are components of the buffer used to make the standard stock solution of human PARP-1. However, the concentration of these components in the PARP-1 preparation used here was minimal and would be unlikely to have interfered with the assay. This was shown to be the case in an experiment where a range of concentrations of BSA was dissolved in PARP-1 storage buffer (see section 2.2.6) and in water and the two sets of solutions were put through a Lowry protein assay. The resultant absorbance values were the same for PARP-1 buffer as for water (data not shown).

The Lowry protein assay was performed in duplicate (as described in Section 2.2.7.2) and repeated, with similar results in replicates and across experiments. With BSA

used as a standard, the stock solution of human PARP-1 used for this work was estimated to be at a concentration of 2.6 mg/ml (Figure 5.1 is the standard curve used to determine the concentration of human PARP-1). Thus, the modified Lowry protein assay gave an estimate of protein concentration that was two orders of magnitude higher than the concentration of the stock solution of human PARP-1 as determined by amino acid analysis. Therefore, the Lowry protein assay is clearly not a suitable method for determining the concentration of PARP-1, at least when BSA is used as a standard.

5.2.1.2 Quantitation of human PARP-1 using the Bradford Protein Assay

The Bradford protein assay detects the amount of CBB dye binding to basic amino acids in proteins, thus the relative proportions of basic amino acid in the standard and the protein being assayed is of critical importance. The percentage of basic amino acid residues in BSA protein and PARP-1 is 17.0% and 17.5% respectively. Therefore, BSA was considered to be a suitable standard when determining the protein concentration of PARP-1.

The Bradford protein assay was performed in duplicate (as described in Section 2.2.7.3) and repeated giving similar results in replicates and across experiments. With BSA used as the standard, the Bradford protein assay gave a concentration for the stock human PARP-1 of 220 – 240 μg/ml (Figure 5.2 is the standard curve used to determine the concentration of human PARP-1); a value that is 10 – 20 times greater than that obtained by amino acid analysis. Although this result is better than the Lowry assay, the result is still more than an order of magnitude greater than the expected value. Therefore the Bradford protein assay is not suitable for estimating PARP-1 concentration, at least when BSA is used as a standard.

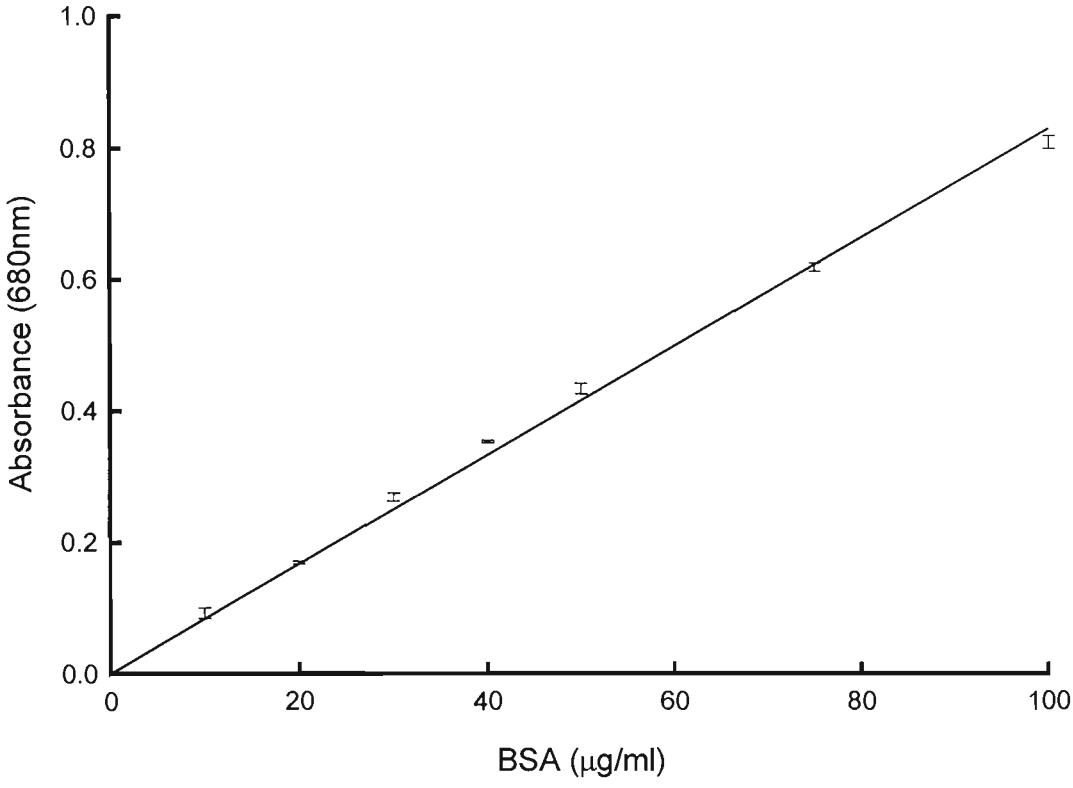


Figure 5.1: Standard curve used for the Lowry protein assay.

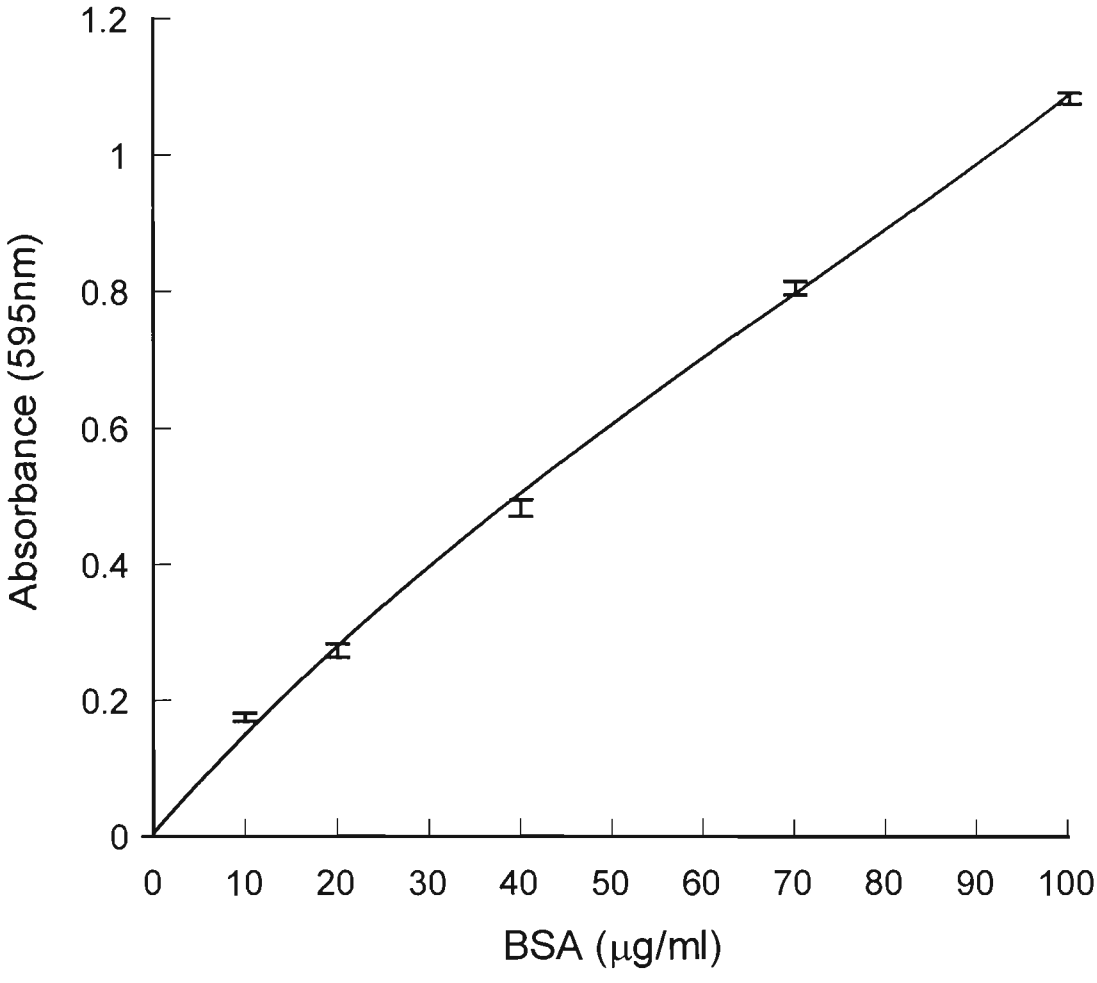


Figure 5.2: Standard curve used for the Bradford protein assay.

5.2.1.3 Quantitation of human PARP-1 using stained SDS-PAGE gels

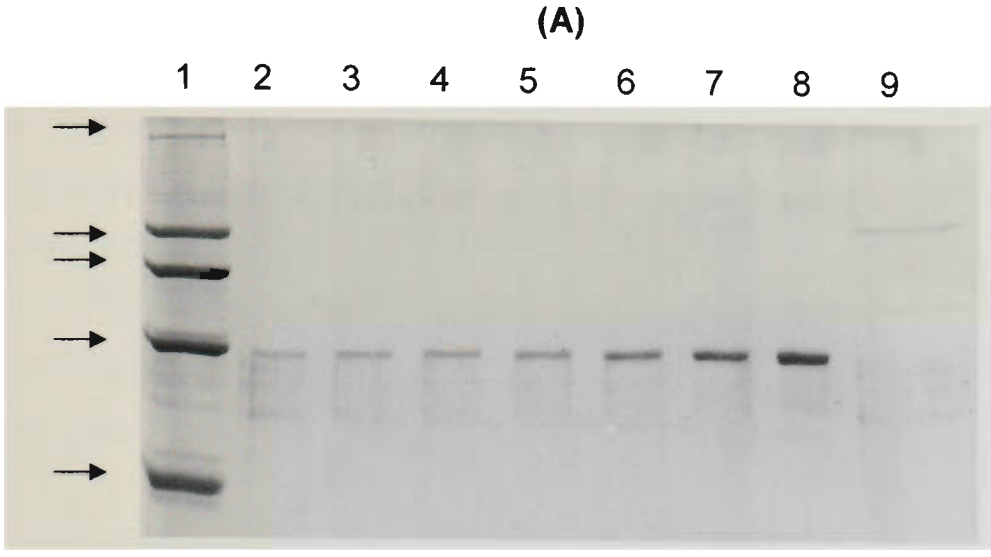
Protein concentration can be estimated using laser densitometry of stained SDS-PAGE gels and CBB is undoubtedly one of the most commonly used stains for this purpose. A range of BSA standards (50 ng to 400 ng) was resolved on a 7.5% SDS-PAGE gel and the gel was stained using CBB. The stained CBB protein gel image was photographed using Fujifilm LAS1000 CCD (Figure 5.3A) and the optical density of the stained bands of BSA was determined with ImageGauge Analysis densitometry software, version 3.121 (Fujifilm Ltd., 1998). The intensity of CBB-stained protein bands was found to be directly proportional to the concentration of protein in the respective bands; from approximately 50 to 300 ng of BSA, the relationship between intensity and protein concentration was almost linear (Figure 5.3B).

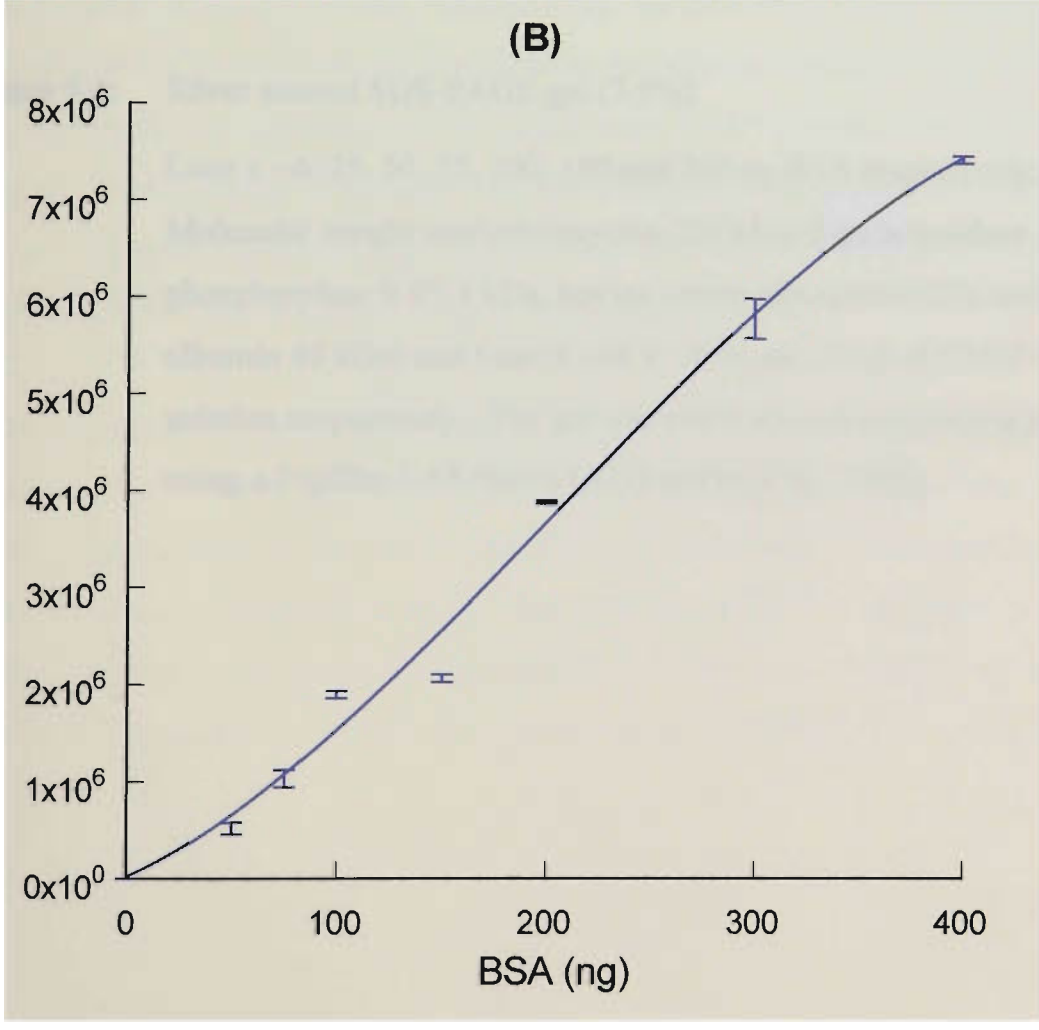
The concentration of PARP-1 in the stock solution appeared to be at the detection limits for CBB-stained gels. Nonetheless, ImageGauge densitometry analysis, using complete area of each band on the gel (See Figure 5.3B), gave a value of 60 ng of protein. This equated to a concentration of approximately 6 μg/ml in the PARP-1 stock solution, a value that more closely reflects that determined by amino acid analysis (10 μg/ml).

Silver stain can detect as little as 1 ng of protein in a band on an SDS-PAGE gel and is therefore much more sensitive than traditional CBB stains (Rabilloud 1992). However, silver stain does not have a large linear dynamic range for quantifying protein concentration and there is huge protein to protein variation which makes silver staining generally a poor choice of method for estimating protein concentration (Jacobs *et al.* 2000). Nonetheless, silver staining can, for some proteins, be used for crude estimates of protein concentration. From visual inspection of the silver-stained gel for this work, the estimated amount of human PARP-1 present in a band of this protein to be 50 – 100 ng (See Figure 5.4). This would equate to 5 – 10 μg/ml in the stock PARP-1 solution, a result that is consistent with that obtained from the CBB-stained gel and close to the estimate from amino acid analysis.

Figure 5.3: CBB-stained SDS-PAGE gel (7.5%).

Lane 1: Molecular weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa and egg albumin 45 kDa) Lanes 2 – 8; 50, 75, 100, 150, 200, 300 and 400 ng BSA respectively; and Lane 9: 10 µl of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1 and molecular weight standards were artifacts of electrophoresis **(A)**. The optical density (OD) across the whole area of the stained bands of BSA was determined using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998). A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution using mean ODs obtained from duplicate gels **(B)**. All experiments were repeated at least once and gave similar results.


(A)
1 2 3 4 5 6 7 8 9


(B)
Intensity of Protein Band - Background (AU)
8x10^6
7x10^6
6x10^6
5x10^6
4x10^6
3x10^6
2x10^6
1x10^6
0x10^0
0
100
200
300
400
BSA (ng)

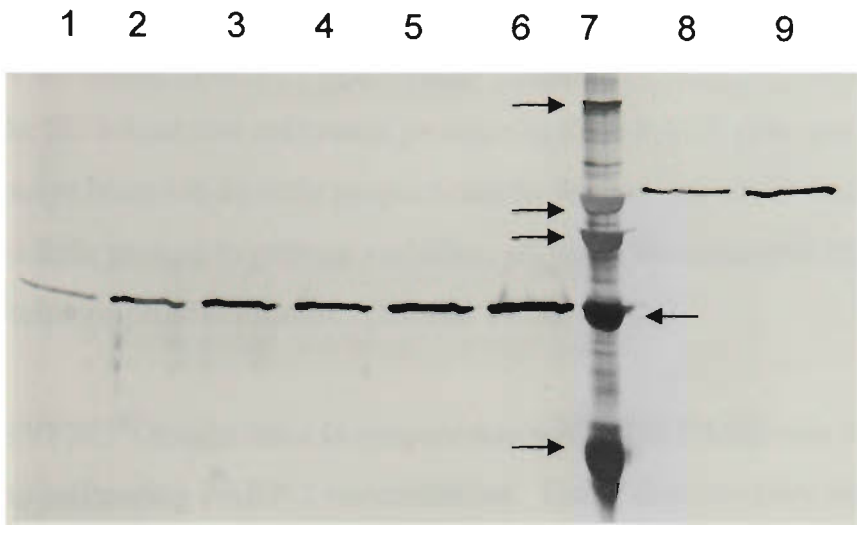


Figure 5.4: Silver stained SDS-PAGE gel (7.5%).

Lane 1 - 6: 25, 50, 75, 100, 150 and 200 ng BSA respectively; Lane 7: Molecular weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa and egg albumin 45 kDa) and Lane 8 and 9: 10 µl and 20 µl of PARP-1 stock solution respectively. The gel was silver stained and photographed using a Fujifilm LAS1000 CCD (Fujifilm Ltd., 1998).

Fluorescent stains are reported to provide the sensitivity of silver staining but with the added advantage of a large linear dynamic range for determining protein concentration (MolecularProbes 2001). The newly developed SYPRO®Orange fluorescent stain (Bio-Rad Laboratories Pty. Ltd., Catalogue Number: 170-3120) has been reported to detect as little as 1 ng of protein in a band on an SDS-PAGE gel (Steinberg *et al.* 1996). The SYPRO®Orange reagent becomes fluorescent upon binding to the SDS-coat that surrounds proteins in SDS-PAGE gels and the amount of SYPRO®Orange bound is directly proportional to the amount of SDS-coated protein. Thus there is little protein to protein variation, allowing accurate quantitation of purified proteins or protein mixtures (Biorad 1998).

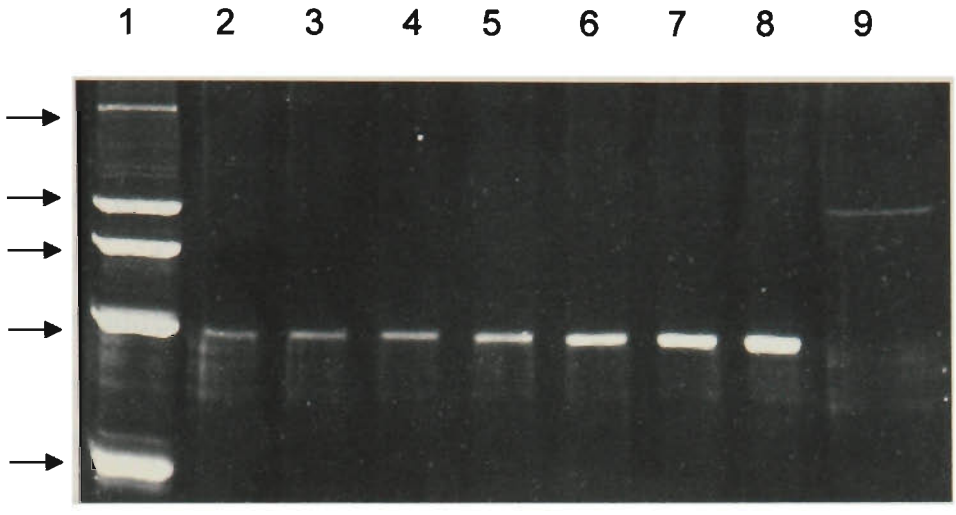
The use of SYPRO®Orange stain in conjunction with SDS-PAGE was found to be well suited to estimating PARP-1 concentration. Using densitometry and ImageGauge analysis software, version 3.121, the amount of human PARP-1 present in the SYPRO®Orange-stained protein band was approximately 100 ng, which would equate to a final concentration of 10 μg/ml in the PARP-1 stock solution (Figure 5.5A and B), a value similar to that provided by densitometry analysis of CBB-stained protein gels (see Figure 5.3A and B) and consistent with silver stained protein gels (see Figure 5.4) and with the results of amino acid analysis.

Determination of protein concentration in the PARP-1 stock solution using the NanoOrange® Protein Quantitation Kit from Molecular Probes (MolecularProbes 2001) was also tested. However it was found that the levels of fluorescence obtained were extremely variable for both the stock solution of PARP-1 and BSA standards at concentrations between 10 – 50 ng/ml (data not shown) thus making it extremely difficult to accurately determine the concentration of human PARP-1 in the stock solution.

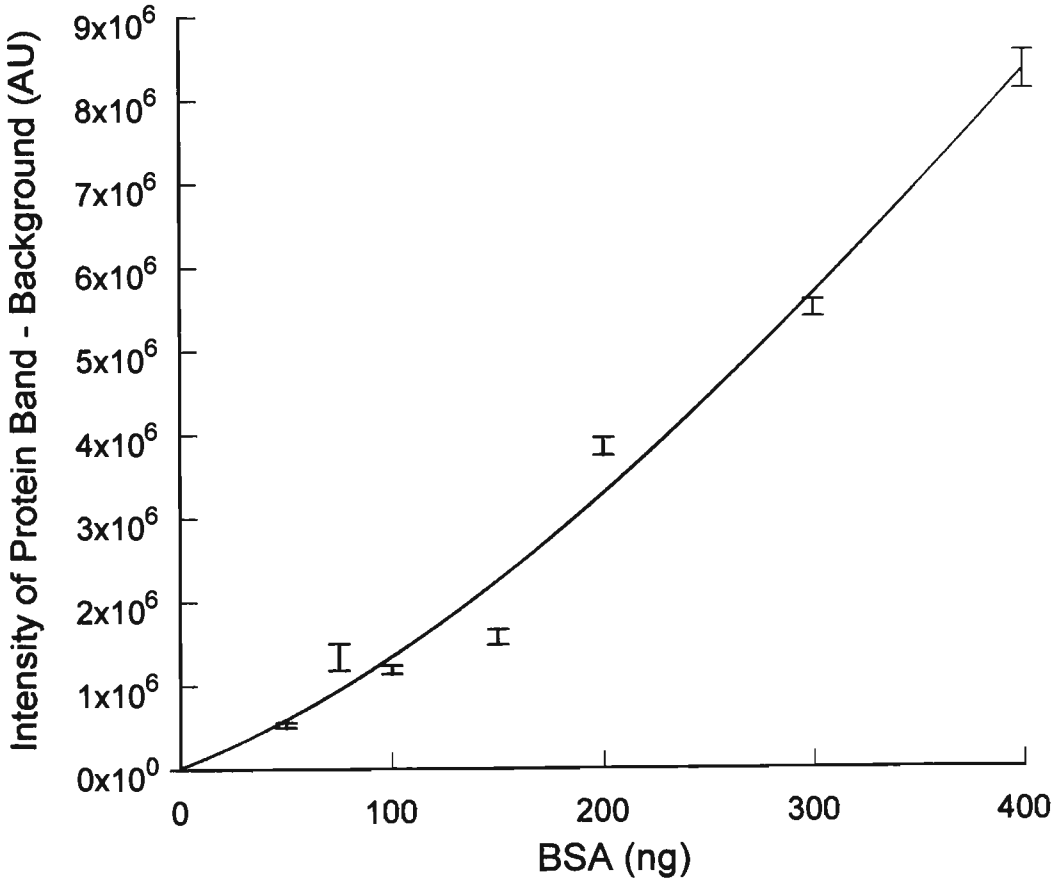
Figure 5.5: SYPRO®Orange-stained SDS-PAGE gel (7.5%).

Lane 1: Molecular weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa and egg albumin 45 kDa) Lanes 2 – 8; 50, 75, 100, 150, 200, 300 and 400 ng BSA respectively; and Lane 9: 10 µl of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1 and molecular weight markers were artifacts of electrophoresis **(A)**. The OD across the whole area of stained bands of BSA was determined using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998). A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution, using mean ODs obtained from duplicate gels **(B)**. All experiments were repeated at least once and gave similar results.

(A)


1
2
3
4
5
6
7
8
9

(B)


Intensity of Protein Band - Background (AU)
9x10^6
8x10^6
7x10^6
6x10^6
5x10^6
4x10^6
3x10^6
2x10^6
1x10^6
0x10^0
0
100
200
300
400
BSA (ng)

This chapter highlights the potential to get enormous variation in estimates of protein concentration for the same protein solution across a range of commonly used techniques when using BSA as a standard (See Table 5.2). It also highlights the importance of trialing more than one technique and/or potential protein standard for one's research or for routine analytical work. Amino acid analysis provides one of the most sensitive and accurate approaches for estimating protein concentration but the technology required for this is probably not suitable or appropriate for routine analysis in most laboratories. The next best approach would be to use amino acid analysis to determine the concentration of a stock of the protein to be estimated in future work, and to then use this stock as a standard. If however, making a stock of known standard is not a viable option then one should consider testing and comparing a range of techniques and potential protein standards to determine which combination is best suited to work to be undertaken.

From results presented here I would suggest that for laboratories lacking access to amino acid analysis, the best method for estimating concentration of human PARP-1 is quantitation from an SDS-PAGE protein gel stained with SYPRO®Orange with BSA as a standard. An added advantage of estimating protein concentration from SDS-PAGE gels is that one also gets information on the quality of the protein.

Table 5.2: Estimates of protein concentration of stock human PARP-1 solution as determined using several different protein assays.

Type of Protein Assay	Estimated Concentration of Standard PARP-1 Solution (μg/ml)	Magnitude Difference compared to amino acid analysis
Amino acid analysis	10	-
Lowry assay	2600	260 x
Bradford assay	220 – 240	22 – 24 x
CBB gel*	6	0.6 x
SYPRO®Orange gel**	10	1.0 x

*Coomassie Brilliant Blue stained SDS-PAGE gel

**SYPRO®Orange stained SDS-PAGE gel

6 Characterisation of Recombinant Human PARP-1 Enzymatic Activity

6.1 Introduction

With the successful expression of a 116 kDa protein from insect-cells infected with baculovirus carrying the human PARP-1 gene (see Chapter 3), a method for purifying this protein (see Chapter 4), and a means of quantifying levels of PARP-1 protein (see Chapter 5) all successfully in place, the next step was to ascertain the quality (and yield) of the PARP-1 protein produced.

A standard assay for determining PARP-1 activity was proposed at an International Meeting in 1978. Many workers agreed and initially adopted the method described by Tsopanakis *et al.* (1978) as the standard assay. The standard enzymatic reaction mixture for this assay contains 100 mM Tris-HCl, pH 8.0 (adjusted at 37°C), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μg DNA (calf thymus, Sigma Type I), 10 μg histone (calf thymus, lysine rich, Sigma Type V-S), 4 mM radioactive NAD^+ (^3H- or ^{14}C-labeled) and enzyme in a reaction volume of 500 μl. The reaction proceeds for 10 minutes at 37°C and is terminated by the addition of 20% (w/v) trichloroacetic acid, which precipitates acid-insoluble material. Precipitated protein (including PARP-1) is collected on a Whatman glass fibre filter (Whatman, GF/C) or Millipore filter (Type HA, 0.45 μm) and counted in a liquid scintillation counter.

However, variations in the standard activity assay soon began to appear in the literature. For example, Niedergang *et al.* (1979) used a reaction volume of 100 μl instead of 500 μl and a reaction temperature of 25°C instead of 37°C. Further modifications were made as described in Giner *et al.* (1992), Simonin *et al.* (1993a) and Ruf *et al.* (1998) leading to the development of the following assay: 50mM Tris HCl buffer, pH 8.0 (adjusted at 37°C), 4 mM $MgCl_2$, 0.2 mM DTT, 2 μg/ml DNA, 2 μg/ml histone H1, 200 μM NAD^+ (0.5 μCi [2,5',8-^3H] NAD^+) and 200 ng PARP-1 in a reaction volume of 100 μl. The reaction proceeds for 10 minutes at 25°C and is terminated by the addition of 20% (w/v) trichloroacetic acid, which precipitates acid-insoluble material. The precipitated protein (including PARP-1) is collected on a Whatman glass fibre filter (Whatman GF/C) and counted in a liquid scintillation counter (as described by Tsopanakis *et al.* (1978)). This modified PARP-1 enzymatic assay has been routinely used by many laboratories for determining PARP-1 activity

and the K_m and V_{max} values of various preparations of PARP-1 (see Simonin *et al.* (1993a); Giner *et al.* (1992); and Ruf *et al.* (1998)).

The modified assay reported by Giner *et al.* (1992), Simonin *et al.* (1993a) and Ruf *et al.* (1998) was used to determine K_m and V_{max} for the purified enzyme produced as described in Chapter 4. The assay was also used to analyse fractions from the purification protocol described in Chapter 4 to determine the purity of the products at each stage in the purification protocol.

6.2 Results and Discussion

6.2.1 K_m and V_{max} values of expressed and purified PARP-1

PARP-1 enzymatic activity followed typical Michaelis-Menton kinetics with enzyme activity increasing with respect to substrate concentration (See Figure 6.1). Lineweaver-Burke analysis of this data showed the purified enzyme had a K_m of 42 μM and V_{max} of 480 pmol/min/μg (Figure 6.2), which is consistent with values reported in the literature by a range of workers as in Table 1.1 (see page 20) (i.e. K_m values of around 50 μM with a range of 20 to 80 μM and the majority of V_{max} values ranging from 500 to 1500 pmol/min/μg). For example, Yoshihara, *et al.* (1978) reported a K_m of 60 μM and V_{max} of 910 pmol/min/μg for PARP-1 isolated from bovine thymus and Giner, et al. (1992) reported a K_m 50 μM and V_{max} of 1010 pmol/min/μg for PARP-1 isolated from baculovirus-infected insect cells.

While the K_m and V_{max} values of PARP-1 produced varies a little from what is published in the literature it is important to remember that estimating the concentration of PARP-1 is problematic and this may have impacted on the data generated by other laboratories. The work described here gives the first estimates of K_m and V_{max} for PARP-1 for which the problems associated with determining PARP-1 concentration was taken into account.

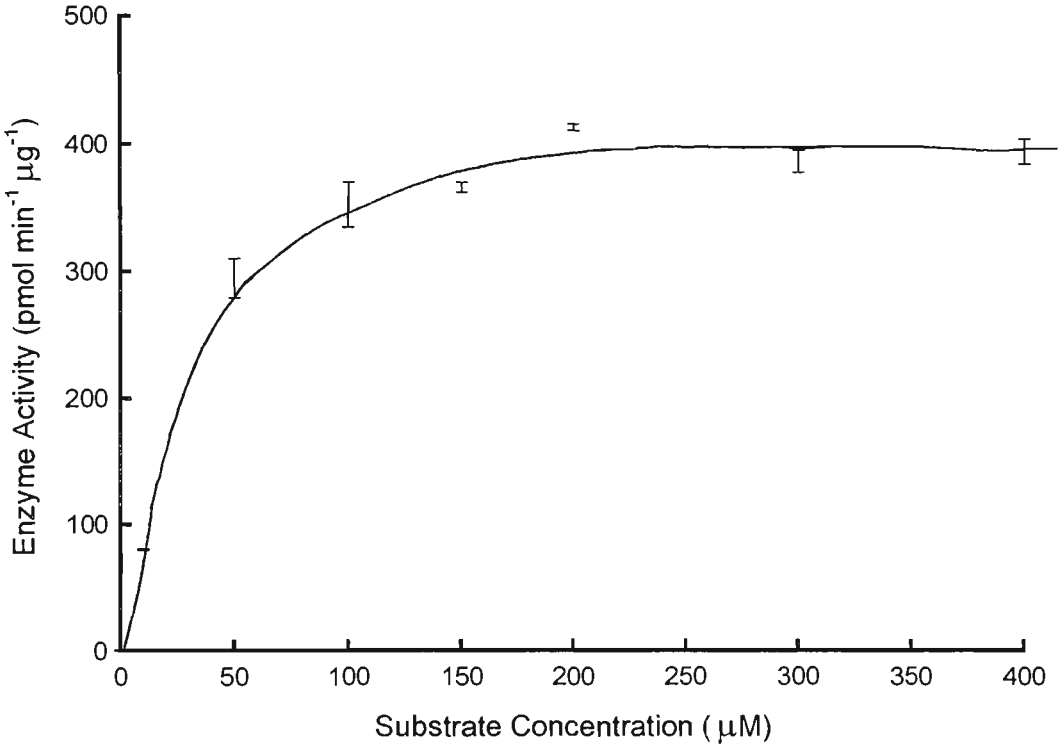


Figure 6.1: The affect of substrate (NAD^+) concentration on PARP-1 activity.

The PARP-1 enzyme assay contained 50 mM Tris-HCl buffer, pH 8.0, 4 mM $MgCl_2$, 0.2 mM DTT, 2 µg/ml DNA, 2 µg/ml histone H1, 200 ng of PARP-1 and a range of substrate (NAD^+) concentrations ranging from 0 – 400 µM containing 0.5 µCi [2,5',8-^{3}H] NAD^+ in a total reaction volume of 100 µl. This experiment was repeated and gave similar results for various purified PARP-1 preparations.

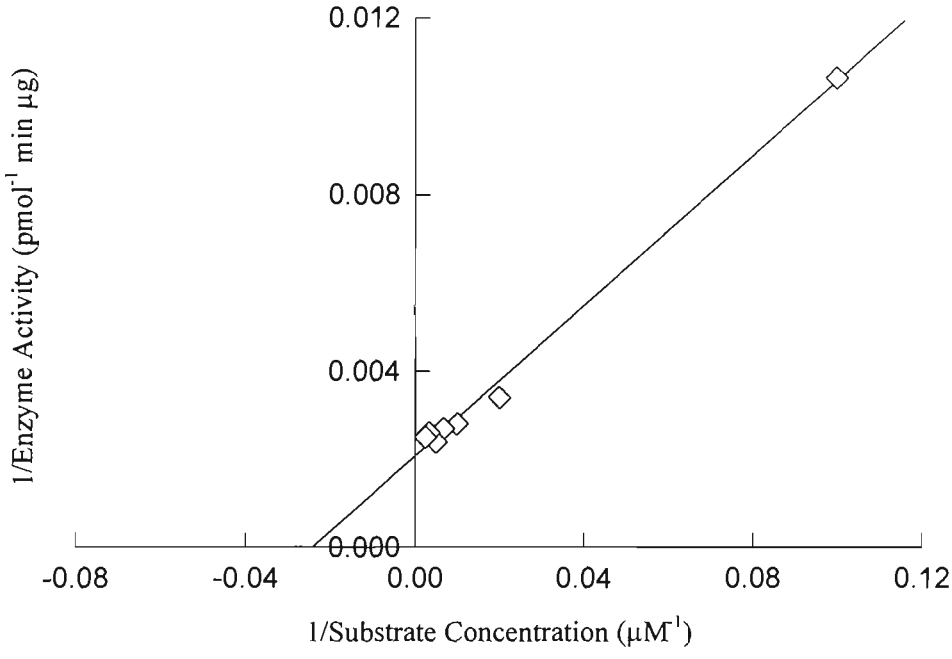


Figure 6.2: Lineweaver-Burke plot for extracted and purified human PARP-1.

The above graph is a double reciprocal plot of the data presented in Figure 6.1.

6.2.2 The effect of histones on PARP-1 activity

It is well known that histone proteins act as acceptors of ADP-ribose polymers (Althaus and Richter 1987; Althaus 1992; Althaus *et al.* 1994). The first report that histones were an important component of the PARP-1 reaction was shown by Tsopanakis *et al.* (1978), who found a histone-free PARP-1 preparation, isolated from pig nuclei, had virtually no enzymatic activity in the absence of histones. However, Yoshihara *et al.* (1978) showed that the omission of histone from the reaction mixture merely suppressed PARP-1 enzymatic activity.

While histones are incorporated into the standard and modified PARP-1 activity assays as described previously in this chapter, it was of interest to our laboratory to test whether PARP-1 purified in this research was dependent on the presence of histones in the reaction mix. This is particularly important for our laboratory because we are interested in investigating the automodifcation reaction of PARP-1 which is thought to require PARP-1 homodimer formation; the presence of target proteins other than PARP-1 in the reaction would interfere with this.

Thus, the need for the presence of histone in the modified PARP-1 assay was investigated. This was done by comparing reactions with or without 2 μg/ml added histone over a range of substrate (NAD^+) concentrations; 2 μg/ml histone was used for this work as this is the amount routinely used by other laboratories (Tsopanakis et al. 1978; Yoshihara et al. 1978; Simonin et al. 1993b).

Figure 6.3 shows that PARP-1 activity was not entirely dependent on histones in the reaction mix. However, the omission of histone from the modified PARP-1 assay did reduce activity by approximately 50%. This finding is similar to the observations by Yoshihara, *et al.* (1978), who found that when histones were omitted from the PARP-1 enzymatic reaction, PARP-1 activity was reduced to 52%. Therefore, the ability of PARP-1 purified in this thesis to ADP-ribosylate itself was confirmed. Thus, the PARP-1 protein produced in this thesis will serve well for structure – function studies that focus on the nature of PARP-1 – PARP-1 homodimer formation.

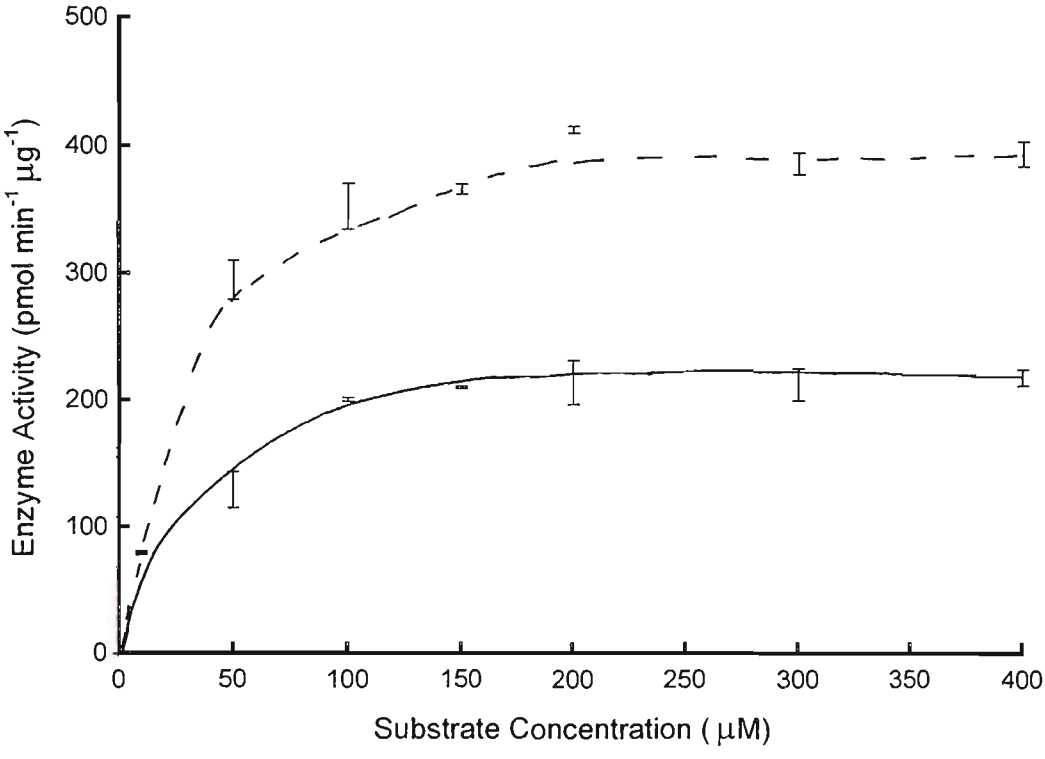


Figure 6.3: The affect of histone on PARP-1 activity at various substrate (NAD^+) concentrations.

The PARP-1 enzyme assay contained 50 mM Tris-HCl buffer, pH 8.0, 4 mM $MgCl_2$, 0.2 mM DTT, 2 µg/ml DNA, 2 µg/ml histone H1 (with [-----] or without [——] histone H1), 200 ng of PARP-1 and a range of NAD^+ concentrations from 0 – 400 µM containing 0.5 µCi [2,5',8-^{3}H] NAD^+ in a total reaction volume of 100 µl. This experiment was repeated and gave similar results for various PARP-1 preparations.

6.2.3 Quality and yield of products of the PARP-1 purification protocol described in Chapter 4

Using the protein assay recommended in Section 5.2.1.3 and the enzyme assay described in this chapter, the protein fractions produced as described in the extraction and purification protocol developed in Chapter 4 were assayed for yield and purity of PARP-1. A summary of the stages of preparation of human PARP-1 is given in Table 6.1 with activity and protein content given for each stage of extraction and purification.

6.2.4 Conclusion

It is clear from work described in this chapter that the PARP-1 protein produced using the method developed is high quality native PARP-1 with a high specific activity and K_m and V_{max} values similar to what is reported by other workers in the field. It is evident that this PARP-1 preparation can be used in assays without the need for additional target proteins such as histones, thus making it ideally suited for investigating the role of PARP-1 – PARP-1 homodimer formation in the activity of the enzyme. This protocol will therefore be particularly well suited to making human PARP-1 in a quantity and of a quality suitable for structure-function studies.

Table 6.1: Purification of human PARP-1 from baculovirus-infected insect cells.

Stage of Purification	Total Protein (mg)	Total Activity[a] (pmol)	Specific Activity[b] (pmol/min/μg)	% Yield[c]	Fold Purification[d]
Crude Extract	20	70,300	3.52	100	-
Crude Lysate (Figure 4.3 and Figure 4.4)	18	75,200	4.18	100	-
Protamine Sulphate	17	73,400	4.32	100	-
Ammonium Sulphate (Figure 4.4)	10	40,100	4.01	57	-
DNA-Cellulose (Figure 4.5)	0. 25	9,700	38.8	14	11.0
Ultrafiltration (Figure 4.6)	0. 20	10,200	50.8	14	14.5

[a]1 unit of activity is the number of pmol of ADP-ribose incorporated into TCA precipitable counts in 1 minute.

[b]Specific acitivity is the data in column [a] divided by total protein for the same fraction.

[c]Data calculated by dividing total activity with the total activity of the initial fraction (crude extract).

[d]Ratio of the specific activity of a given step versus the specific activity of the initial fraction (crude extract).

7 Concluding Statement and Future Studies

7.1 Concluding Statement

The work described in this thesis makes a significant contribution to the field of PARP-1 research. Results presented describe the development of an efficient production and purification protocol for human PARP-1 with high specific activity. Unlike traditional PARP-1 purification protocols, the method described requires only one chromatography step thus minimising losses of the enzyme and also avoids the use of a competitive inhibitor-based affinity chromatography step, which is common to several other protocols described in the literature. The method described produced high-quality native PARP-1 with K_m and V_{max} values similar to that reported by other researchers in the field. This high-quality native PARP-1 is particularly well suited for future structure-function studies. This protocol was also used to successfully purify hexahistidine-tagged PARP-1 (HT-PARP-1).

To determine the efficiency of the purification protocol developed for this thesis and to ascertain the quality (i.e. specific activity) of the PARP-1 protein product, a protein assay that gave an accurate measurement of PARP-1 protein concentration was sought. This thesis described this quest and identified many problems associated with determining PARP-1 protein concentration in purified preparations when using traditional protein assays. Based on these findings, it was concluded that amino acid analysis was the most sensitive and accurate approach for determining PARP-1 protein concentration. However, this approach is not very accessible for routine analysis in most laboratories. Thus it was recommended that a stock solution of the protein be made and assayed to determine its concentration using amino acid analysis. This stock could then be used as a standard for future quantitation. In the case of PARP-1, I recommend the best method for quantitation is using an SDS-PAGE gel stained with SYPRO Orange.

These findings have been published in two papers:

- Knight, M.I. and Chambers, P.J. (2001) Production, extraction, and purification of human poly(ADP-ribose) polymerase-1 (PARP-1) with high specific activity. *Prot. Expr. Purif.*, **23,** 453-8.
- Knight, M.I. and Chambers, P.J. (2003) Problems associated with determining protein concentration: A comparison of techniques for protein estimations. *Mol. Biotech.*, **23**, 19-28.

7.2 Proposed PARP-1 Structure-Function Studies

It is well documented that PARP-1 activity is dependent on DNA strand breaks and that substrate concentration is extremely important when investigating auto-mono or poly(ADP-ribosylation) reactions. Also, extensive kinetic and biochemical studies by numerous laboratories has showed that the PARP-1 automodification reaction is a bimolecular reaction (Bauer *et al.* 1990; Mendoza-Alvarez and Alvarez-Gonzalez 1993; Alvarez-Gonzalez et al. 1994; Mendoza-Alvarez and Alvarez-Gonzalez 1999) requiring homodimer formation. However, very little is known about how the different functional domains of PARP-1 (the DNA binding domain (DBD), the automodification domain (AD) and the catalytic domain (CD)) affect homodimer formation at a molecular level. Future studies aim to elucidate the role of the different functional domains in PARP-1-PARP-1 homodimer formation.

To determine the role of DNA binding in homodimer formation, DBD mutants would be generated that do not bind DNA strand breaks. Several point mutations have been described in the literature that meet this requirement. Molinete *et al.* (1990) reported that cysteine residues at positions 21 (zinc finger I) and 125 (zinc finger II) are essential for binding DNA strand breaks. Replacing these cysteines with glycine residues in a double mutant substituting at amino acids 21 and 125 or a single mutant at position 125 completely prevents PARP-1 binding to DNA strand breaks.

To determine the functional role of the CD in homodimer formation. CD mutants would be generated that do not initiate or elongate poly(ADP-ribose) polymer synthesis. Two point mutations have been described in the literature that meet this requirement. Simonin *et al.* (1990) reported that lysine 893 is essential for the initiation reaction and that replacing this residue with isoleucine completely

inactivated the covalent attachment of PARP to acceptor proteins (i.e. the initiation reaction). Whereas, Marsischky *et al.* (1995) reported that glutamate 988 is essential for the synthesis of the poly(ADP-ribose) polymers (i.e. the elongation reaction) and that replacing this residue with aspartate completely inhibited this reaction.

All of the mutations described above would be introduced into the PARP-1 gene using a PCR based site-directed *in vitro* mutagenesis protocol. Mutated PARP-1 protein would be produced and purified using the method described in this thesis. It should be noted that the DBD mutants would be expressed in the construct carrying a hexahistidine tag. The hexahistidine tag would then be used to purify these constructs on a standard nickel column because these mutants are not able to bind the DNA-cellulose affinity column used in this thesis. However, prior to structure-function analyses, the hexahistidine tag must be removed because it may impact on the structure of the protein.

Production of these mutants will enable an investigation into the roles of two functional domains of PARP-1 in PARP-1-PARP-1 interactions. To achieve this aim, each mutant will be used in complementation assays, BIAcore assays and gel shift assays to determine the impact of the mutation on PARP-1-PARP-1 interactions and how this affects PARP-1 activity. Questions these experiments will address include:

- The role of DNA binding and the DBD in PARP-1-PARP-1 interactions; and
- The role of substrate binding and the CD in PARP-1-PARP-1 interactions.

8 References

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9 Appendices

9.1 Appendix 1

PARP-1 cDNA and protein sequence obtained from GenBank, accession number M18112 (Uchida *et al.* 1987).

```
1     atg gcg gag tct tcg gat aag ctc tat cga gtc gag tac gcc aag   45
1      M   A   E   S   S   D   K   L   Y   R   V   E   Y   A   K    15

46    agc ggg cgc gcc tct tgc aag aaa tgc agc gag agc atc ccc aag   90
16     S   G   R   A   S   C   K   K   C   S   E   S   I   P   K    30

91    gac tcg ctc cgg atg gcc atc atg gtg cag tcg ccc atg ttt gat   135
31     D   S   L   R   M   A   I   M   V   Q   S   P   M   F   D    45

136   gga aaa gtc cca cac tgg tac cac ttc tcc tgc ttc tgg aag gtg   180
46     G   K   V   P   H   W   Y   H   F   S   C   F   W   K   V    60

181   ggc cac tcc atc cgg cac cct gac gtt gag gtg gat ggg ttc tct   225
61     G   H   S   I   R   H   P   D   V   E   V   D   G   F   S    75

226   gag ctt cgg tgg gat gac cag cag aaa gtc aag aag aca gcg gaa   270
76     E   L   R   W   D   D   Q   Q   K   V   K   K   T   A   E    90

271   gct gga gga gtg aca ggc aaa ggc cag gat gga att ggt agc aag   315
91     A   G   G   V   T   G   K   G   Q   D   G   I   G   S   K   105

316   gca gag aag act ctg ggt gac ttt gca gca gag tat gcc aag tcc   360
106    A   E   K   T   L   G   D   F   A   A   E   Y   A   K   S   120

361   aac aga agt acg tgc aag ggg tgt atg gag aag ata gaa aag ggc   405
121    N   R   S   T   C   K   G   C   M   E   K   I   E   K   G   135

406   cag gtg cgc ctg tcc aag aag atg gtg gac ccg gag aag cca cag   450
136    Q   V   R   L   S   K   K   M   V   D   P   E   K   P   Q   150

451   cta ggc atg att gac cgc tgg tac cat cca ggc tgc ttt gtc aag   495
151    L   G   M   I   D   R   W   Y   H   P   G   C   F   V   K   165

496   aac agg gag gag ctg ggt ttc cgg ccc gag tac agt gcg agt cag   540
166    N   R   E   E   L   G   F   R   P   E   Y   S   A   S   Q   180

541   ctc aag ggc ttc agc ctc ctt gct aca gag gat aaa gaa gcc ctg   585
181    L   K   G   F   S   L   L   A   T   E   D   K   E   A   L   195

586   aag aag cag ctc cca gga gtc aag agt gaa gga aag aga aaa ggc   630
196    K   K   Q   L   P   G   V   K   S   E   G   K   R   K   G   210

631   gat gag gtg gat gga gtg gat gaa gtg gcg aag aag aaa tct aaa   675
211    D   E   V   D   G   V   D   E   V   A   K   K   K   S   K   225

676   aaa gaa aaa gac aag gat agt aag ctt gaa aaa gcc cta aag gct   720
226    K   E   K   D   K   D   S   K   L   E   K   A   L   K   A   240

721   cag aac gac ctg atc tgg aac atc aag gac gag cta aag aaa gtg   765
241    Q   N   D   L   I   W   N   I   K   D   E   L   K   K   V   255

766   tgt tca act aat gac ctg aag gag cta ctc atc ttc aac aag cag   810
256    C   S   T   N   D   L   K   E   L   L   I   F   N   K   Q   270

811   caa gtg cct tct ggg gag tcg gcg atc ttg gac cga gta gct gat   855
271    Q   V   P   S   G   E   S   A   I   L   D   R   V   A   D   285

856   ggc atg gtg ttc ggt gcc ctc ctt ccc tgc gag gaa tgc tcg ggt   900
286    G   M   V   F   G   A   L   L   P   C   E   E   C   S   G   300

901   cag ctg gtc ttc aag agc gat gcc tat tac tgc act ggg gac gtc   945
301    Q   L   V   F   K   S   D   A   Y   Y   C   T   G   D   V   315
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946   act gcc tgg acc aag tgt atg gtc aag aca cag aca ccc aac cgg   990
316    T   A   W   T   K   C   M   V   K   T   Q   T   P   N   R   330

991   aag gag tgg gta acc cca aag gaa ttc cga gaa atc tct tac ctc   1035
331    K   E   W   V   T   P   K   E   F   R   E   I   S   Y   L   345

1036  aag aaa ttg aag gtt aaa aag cag gac cgt ata ttc ccc cca gaa   1080
346    K   K   L   K   V   K   K   Q   D   R   I   F   P   P   E   360

1081  acc agc gcc tcc gtg gcg gcc acg cct ccg ccc tcc aca gcc tcg   1125
361    T   S   A   S   V   A   A   T   P   P   P   S   T   A   S   375

1126  gct cct gct gct gtg aac tcc tct gct tca gca gat aag cca tta   1170
376    A   P   A   A   V   N   S   S   A   S   A   D   K   P   L   390

1171  tcc aac atg aag atc ctg act ctc ggg aag ctg tcc cgg aac aag   1215
391    S   N   M   K   I   L   T   L   G   K   L   S   R   N   K   405

1216  gat gaa gtg aag gcc atg att gag aaa ctc ggg ggg aag ttg acg   1260
406    D   E   V   K   A   M   I   E   K   L   G   G   K   L   T   420

1261  ggg acg gcc aac aag gct tcc ctg tgc atc agc acc aaa aag gag   1305
421    G   T   A   N   K   A   S   L   C   I   S   T   K   K   E   435

1306  gtg gaa aag atg aat aag aag atg gag gaa gta aag gaa gcc aac   1350
436    V   E   K   M   N   K   K   M   E   E   V   K   E   A   N   450

1351  atc cga gtt gtg tct gag gac ttc ctc cag gac gtc tcc gcc tcc   1395
451    I   R   V   V   S   E   D   F   L   Q   D   V   S   A   S   465

1396  acc aag agc ctt cag gag ttg ttc tta gcg cac atc ttg tcc cct   1440
466    T   K   S   L   Q   E   L   F   L   A   H   I   L   S   P   480

1441  tgg ggg gca gag gtg aag gca gag cct gtt gaa gtt gtg gcc cca   1485
481    W   G   A   E   V   K   A   E   P   V   E   V   V   A   P   495

1486  aga ggg aag tca ggg gct gcg ctc tcc aaa aaa agc aag ggc cag   1530
496    R   G   K   S   G   A   A   L   S   K   K   S   K   G   Q   510

1531  gtc aag gag gaa ggt atc aac aaa tct gaa aag aga atg aaa tta   1575
511    V   K   E   E   G   I   N   K   S   E   K   R   M   K   L   525

1576  act ctt aaa gga gga gca gct gtg gat cct gat tct gga ctg gaa   1620
526    T   L   K   G   G   A   A   V   D   P   D   S   G   L   E   540

1621  cac tct gcg cat gtc ctg gag aaa ggt ggg aag gtc ttc agt gcc   1665
541    H   S   A   H   V   L   E   K   G   G   K   V   F   S   A   555

1666  acc ctt ggc ctg gtg gac atc gtt aaa gga acc aac tcc tac tac   1710
556    T   L   G   L   V   D   I   V   K   G   T   N   S   Y   Y   570

1711  aag ctg cag ctt ctg gag gac gac aag gaa aac agg tat tgg ata   1755
571    K   L   Q   L   L   E   D   D   K   E   N   R   Y   W   I   585

1756  ttc agg tcc tgg ggc cgt gtg ggt acg gtg atc ggt agc aac aaa   1800
586    F   R   S   W   G   R   V   G   T   V   I   G   S   N   K   600

1801  ctg gaa cag atg ccg tcc aag gag gat gcc att gag cac ttc atg   1845
601    L   E   Q   M   P   S   K   E   D   A   I   E   H   F   M   615

1846  aaa tta tat gaa gaa aaa acc ggg aac gct tgg cac tcc aaa aat   1890
616    K   L   Y   E   E   K   T   G   N   A   W   H   S   K   N   630

1891  ttc acg aag tat ccc aaa aag ttt tac ccc ctg gag att gac tat   1935
631    F   T   K   Y   P   K   K   F   Y   P   L   E   I   D   Y   645
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1936  ggc cag gat gaa gag gca gtg aag aag ctc aca gta aat cct ggc   1980
646    G   Q   D   E   E   A   V   K   K   L   T   V   N   P   G    660

1981  acc aag tcc aag ctc ccc aag cca gtt cag gac ctc atc aag atg   2025
661    T   K   S   K   L   P   K   P   V   Q   D   L   I   K   M    675

2026  atc ttt gat gtg gaa agt atg aag aaa gcc atg gtg gag tat gag   2070
676    I   F   D   V   E   S   M   K   K   A   M   V   E   Y   E    690

2071  atc gac ctt cag aag atg ccc ttg ggg aag ctg agc aaa agg cag   2115
691    I   D   L   Q   K   M   P   L   G   K   L   S   K   R   Q    705

2116  atc cag gcc gca tac tcc atc ctc agt gag gtc cag cag gcg gtg   2160
706    I   Q   A   A   Y   S   I   L   S   E   V   Q   Q   A   V    720

2161  tct cag ggc agc agc gac tct cag atc ctg gat ctc tca aat cgc   2205
721    S   Q   G   S   S   D   S   Q   I   L   D   L   S   N   R    735

2206  ttt tac acc ctg atc ccc cac gac ttt ggg atg aag aag cct ccg   2250
736    F   Y   T   L   I   P   H   D   F   G   M   K   K   P   P    750

2251  ctc ctg aac aat gca gac agt gtg cag gcc aag gtg gaa atg ctt   2295
751    L   L   N   N   A   D   S   V   Q   A   K   V   E   M   L    765

2296  gac aac ctg ctg gac atc gag gtg gcc tac agt ctg ctc agg gga   2340
766    D   N   L   L   D   I   E   V   A   Y   S   L   L   R   G    780

2341  ggg tct gat gat agc agc aag gat ccc atc gat gtc aac tat gag   2385
781    G   S   D   D   S   S   K   D   P   I   D   V   N   Y   E    795

2386  aag ctc aaa act gac att aag gtg gtt gac aga gat tct gaa gaa   2430
796    K   L   K   T   D   I   K   V   V   D   R   D   S   E   E    810

2431  gcc gag atc atc agg aag tat gtt aag aac act cat gca acc aca   2475
811    A   E   I   I   R   K   Y   V   K   N   T   H   A   T   T    825

2476  cac aat gcg tat gac ttg gaa gtc atc gat atc ttt aag ata gag   2520
826    H   N   A   Y   D   L   E   V   I   D   I   F   K   I   E    840

2521  cgt gaa ggc gaa tgc cag cgt tac aag ccc ttt aag cag ctt cat   2565
841    R   E   G   E   C   Q   R   Y   K   P   F   K   Q   L   H    855

2566  aac cga aga ttg ctg tgg cac ggg tcc agg acc acc aac ttt gct   2610
856    N   R   R   L   L   W   H   G   S   R   T   T   N   F   A    870

2611  ggg atc ctg tcc cag ggt ctt cgg ata gcc ccg cct gaa gcg ccc   2655
871    G   I   L   S   Q   G   L   R   I   A   P   P   E   A   P    885

2656  gtg aca ggc tac atg ttt ggt aaa ggg atc tat ttc gct gac atg   2700
886    V   T   G   Y   M   F   G   K   G   I   Y   F   A   D   M    900

2701  gtc tcc aag agt gcc aac tac tgc cat acg tct cag gga gac cca   2745
901    V   S   K   S   A   N   Y   C   H   T   S   Q   G   D   P    915

2746  ata ggc tta atc ctg ttg gga gaa gtt gcc ctt gga aac atg tat   2790
916    I   G   L   I   L   L   G   E   V   A   L   G   N   M   Y    930

2791  gaa ctg aag cac gct tca cat atc agc aag tta ccc aag ggc aag   2835
931    E   L   K   H   A   S   H   I   S   K   L   P   K   G   K    945

2836  cac agt gtc aaa ggt ttg ggc aaa act acc cct gat cct tca gct   2880
946    H   S   V   K   G   L   G   K   T   T   P   D   P   S   A    960

2881  aac att agt ctg gat ggt gta gac gtt cct ctt ggg acc ggg att   2925
961    N   I   S   L   D   G   V   D   V   P   L   G   T   G   I    975
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2926  tca tct ggt gtg ata gac acc tct cta cta tat aac gag tac att   2970
976    S   S   G   V   I   D   T   S   L   L   Y   N   E   Y   I    990

2971  gtc tat gat att gct cag gta aat ctg aag tat ctg ctg aaa ctg   3015
991    V   Y   D   I   A   Q   V   N   L   K   Y   L   L   K   L   1005

3016  aaa ttc aat ttt aag acc tcc ctg tgg   3042
1006   K   F   N   F   K   T   S   L   W   1014
```

9.2 Appendix 2

The PARP-1 gene was cloned in a pUC cloning vector between two multiple cloning sites (see Figure 3.1). The pUC-PARP-1 construct was obtained from Prof. G. de Murcia, Institut de Moleculaire et Cellulaire, Centre National Recherche Scientifique, France. The PARP-1 cDNA consisted of a total 3042 nucleotides between position 447 and 3488 of the pUC-PARP-1 construct. To obtain sequence data on the PARP-1 gene, several forward and reverse sequencing primer pairs (see Table 9.1) were designed to enable sequencing of the PARP-1 cDNA in to six sequencing cassettes of approximately 500 – 550 nucleotides in length. The sequencing cassettes were then amplified using the polymerase chain reaction (PCR) as described in section 2.2.1.13. Sequencing cassette 1 was between nucleotides 424 – 1001 and used P1 forward and reverse primers; sequencing cassette 2 was between nucleotides 987 – 1521 and used P2 forward and reverse primers; sequencing cassette 3 was between nucleotides 1502 – 2035 and used P3 forward and reverse primers; sequencing cassette 4 was between nucleotides 1989 – 2537 and used P4 forward and reverse primers; sequencing cassette 5 was between nucleotides 2488-3037 and used P5 forward and reverse primers; and sequencing cassette 6 was between nucleotides 2990 – 3488 and used P6 forward and reverse primers. After PCR amplification, the sequencing cassettes (PCR products) were purified from contaminants, primer-dimers and excess dNTPs using mini columns prepared by Wizard Preparations (Promega Inc., USA) (see section 2.2.1.14). Once purified, each sequencing cassette was sequenced in the forward and reverse direction at the Department of Microbiology, Monash University, Clayton Campus, Melbourne, Australia. Sequencing reactions were prepared as described in section 2.2.1.15. The sequence data obtained was then aligned against the PARP-1 cDNA sequence (M18112) (Uchida *et al.* 1987) using the BioEdit software progam, version 5.0.6 (Hall 1999) and the result is shown on the following pages.

Table 9.1: Primers generated to sequence the PARP-1 gene in pUC-PARP-1.

Primers	Sequence (5'→3')	'mer	Melting Temperature (T_m) °C	Position on pUC-PARP-1 sequence (bp)
P1 Forward	CTA GAG TCG ACC TGC AGG	18	58	424-441
P1 Reverse	GCT GAA GCC CTT GAG CTG AC	20	64	982-1001
P2 Forward	CTC AAG GGC TTC AGC CTC CTT	21	66	987-1007
P2 Reverse	ACG GAG GCG CTG GTT TCT GG	20	68	1540-1521
P3 Forward	GCA GGA CCG TAT ATT CCC	18	56	1502-1519
P3 Reverse	CCT CCT TTA AGA GTT AAT TTC	21	56	2035-2015
P4 Forward	GGT ATC AAC AAA TCT GAA AAG AG	23	62	1989-2011
P4 Reverse	GGG CAT CTT CTG AAG GTC G	19	60	2537-2519
P5 Forward	GTA TGA AGA AAG CCA TGG TG	20	58	2488-2507
P5 Reverse	GAC CCG TGC CAC AGC AAT CT	20	64	3037-3018
P6 Forward	CAA GCC CTT TAA GCA GCT TC	20	60	2990-3009
P6 Reverse	CCA CAG GGA GGT CTT AA	17	50	3488-3472

The six sequencing cassettes were aligned against the PARP-1 cDNA sequence (M18112) (Uchida *et al.* 1987) using the BioEdit software progam, version 5.0.6 (Hall 1999). The nucleotide sequence obtained for PARP-1 (**Seq cass 1 – 6**) was 100 % homologous to the human PARP-1 cDNA sequence, accession number M18112 (**PARP-1**). Conserved nucleotide residues are indicated by a dot (.).

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
PARP-1 seq      ~~~~~~~~~~ ~~~~~~~~~~ ~~~atggcgg agtcttcgga taagctctat
Seq Cass 1      ctagagtcga cctgcagggg agg..... ..........  ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
PARP-1 seq      cgagtcgagt acgccaagag cgggcgcgcc tcttgcaaga aatgcagcga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
PARP-1 seq      gagcatcccc aaggactcgc tccggatggc catcatggtg cagtcgccca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
PARP-1 seq      tgtttgatgg aaaagtccca cactggtacc acttctcctg cttctggaag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
PARP-1 seq      gtgggccact ccatccggca ccctgacgtt gaggtggatg ggttctctga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
PARP-1 seq      gcttcggtgg gatgaccagc agaaagtcaa gaagacagcg gaagctggag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
PARP-1 seq      gagtgacagg caaaggccag gatggaattg gtagcaaggc agagaagact
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
PARP-1 seq      ctgggtgact ttgcagcaga gtatgccaag tccaacagaa gtacgtgcaa
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
PARP-1 seq      ggggtgtatg gagaagatag aaaagggcca ggtgcgcctg tccaagaaga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
PARP-1 seq      tggtggaccc ggagaagcca cagctaggca tgattgaccg ctggtaccat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
PARP-1 seq      ccaggctgct ttgtcaagaa cagggaggag ctgggtttcc ggcccgagta
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
PARP-1 seq      cagtgcgagt cagctcaagg gcttcagcct ccttgctaca gaggataaag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      ~~~~~~~~~~ ~~~........ .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
PARP-1 seq      aagccctgaa gaagcagctc ccaggagtca agagtgaagg aaagagaaaa
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
PARP-1 seq      ggcgatgagg tggatggagt ggatgaagtg gcgaagaaga aatctaaaaa
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
PARP-1 seq      agaaaaagac aaggatagta agcttgaaaa agccctaaag gctcagaacg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
PARP-1 seq      acctgatctg gaacatcaag gacgagctaa agaaagtgtg ttcaactaat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
PARP-1 seq      gacctgaagg agctactcat cttcaacaag cagcaagtgc cttctgggga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
PARP-1 seq      gtcggcgatc ttggaccgag tagctgatgg catggtgttc ggtgccctcc
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
PARP-1 seq      ttccctgcga ggaatgctcg ggtcagctgg tcttcaagag cgatgcctat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
PARP-1 seq      tactgcactg gggacgtcac tgcctggacc aagtgtatgg tcaagacaca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1010       1020       1030       1040       1050
PARP-1 seq      gacacccaac cggaaggagt gggtaacccc aaaggaattc cgagaaatct
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1060       1070       1080       1090       1100
PARP-1 seq      cttacctcaa gaaattgaag gttaaaaagc aggaccgtat attccccca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~.. .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1110       1120       1130       1140       1150
PARP-1 seq      gaaaccagcg cctccgtggc ggccacgcct ccgccctcca cagcctcggc
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1160       1170       1180       1190       1200
PARP-1 seq      tcctgctgct gtgaactcct ctgcttcagc agataagcca ttatccaaca
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
PARP-1 seq      tgaagatcct gactctcggg aagctgtccc ggaacaagga tgaagtgaag
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
PARP-1 seq      gccatgattg agaaactcgg ggggaagttg acggggacgg ccaacaaggc
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
PARP-1 seq      ttccctgtgc atcagcacca aaaaggaggt ggaaaagatg aataagaaga
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1360       1370       1380       1390       1400
PARP-1 seq      tggaggaagt aaaggaagcc aacatccgag ttgtgtctga ggacttcctc
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1410       1420       1430       1440       1450
PARP-1 seq      caggacgtct ccgcctccac caagagcctt caggagttgt tcttagcgca
Seq Cass 1      .......... .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1460       1470       1480       1490       1500
PARP-1 seq      catcttgtcc ccttgggggg cagaggtgaa ggcagagcct gttgaagttg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550
PARP-1 seq      tggccccaag agggaagtca ggggctgcgc tctccaaaaa aagcaagggc
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1560       1570       1580       1590       1600
PARP-1 seq      caggtcaagg aggaaggtat caacaaatct gaaaagagaa tgaaattaac
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      ~~~~~~~~~~ ~~~~~..... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1610       1620       1630       1640       1650
PARP-1 seq      tcttaaagga ggagcagctg tggatcctga ttctggactg gaacactctg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1660       1670       1680       1690       1700
PARP-1 seq      cgcatgtcct ggagaaaggt gggaaggtct tcagtgccac ccttggcctg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1710       1720       1730       1740       1750
PARP-1 seq      gtggacatcg ttaaaggaac caactcctac tacaagctgc agcttctgga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1760       1770       1780       1790       1800
PARP-1 seq      ggacgacaag gaaaacaggt attggatatt caggtcctgg ggccgtgtgg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1810       1820       1830       1840       1850
PARP-1 seq      gtacggtgat cggtagcaac aaactggaac agatgccgtc caaggaggat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1860       1870       1880       1890       1900
PARP-1 seq      gccattgagc acttcatgaa attatatgaa gaaaaaaccg ggaacgcttg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1910       1920       1930       1940       1950
PARP-1 seq      gcactccaaa aatttcacga agtatcccaa aaagttttac cccctggaga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1960       1970       1980       1990       2000
PARP-1 seq      ttgactatgg ccaggatgaa gaggcagtga agaagctcac agtaaatcct
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2010       2020       2030       2040       2050
PARP-1 seq      ggcaccaagt ccaagctccc caagccagtt caggacctca tcaagatgat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2060       2070       2080       2090       2100
PARP-1 seq      ctttgatgtg gaaagtatga agaaagccat ggtggagtat gagatcgacc
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      ~~~~~~~~~~ ~~~~.....  .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2110       2120       2130       2140       2150
PARP-1 seq      ttcagaagat gcccttgggg aagctgagca aaaggcagat ccaggccgca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2160       2170       2180       2190       2200
PARP-1 seq      tactccatcc tcagtgaggt ccagcaggcg gtgtctcagg gcagcagcga
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2210       2220       2230       2240       2250
PARP-1 seq      ctctcagatc ctggatctct caaatcgctt ttacaccctg atcccccacg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2260       2270       2280       2290       2300
PARP-1 seq      actttgggat gaagaagcct ccgctcctga acaatgcaga cagtgtgcag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2310       2320       2330       2340       2350
PARP-1 seq      gccaaggtgg aaatgcttga caacctgctg gacatcgagg tggcctacag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2360       2370       2380       2390       2400
PARP-1 seq      tctgctcagg ggagggtctg atgatagcag caaggatccc atcgatgtca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2410       2420       2430       2440       2450
PARP-1 seq      actatgagaa gctcaaaact gacattaagg tggttgacag agattctgaa
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2460       2470       2480       2490       2500
PARP-1 seq      gaagccgaga tcatcaggaa gtatgttaag aacactcatg caaccacaca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2510       2520       2530       2540       2550
PARP-1 seq      caatgcgtat gacttggaag tcatcgatat ctttaagata gagcgtgaag
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2560       2570       2580       2590       2600
PARP-1 seq      gcgaatgcca gcgttacaag ccctttaagc agcttcataa ccgaagattg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      ~~~~~~~~~~ ~~~~~~.... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2610       2620       2630       2640       2650
PARP-1 seq      ctgtggcacg ggtccaggac caccaacttt gctgggatcc tgtcccaggg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2660       2670       2680       2690       2700
PARP-1 seq      tcttcggata gccccgcctg aagcgcccgt gacaggctac atgtttggta
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2710       2720       2730       2740       2750
PARP-1 seq      aagggatcta tttcgctgac atggtctcca agagtgccaa ctactgccat
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2760       2770       2780       2790       2800
PARP-1 seq      acgtctcagg gagacccaat aggcttaatc ctgttgggag aagttgccct
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2810       2820       2830       2840       2850
PARP-1 seq      tggaaacatg tatgaactga agcacgcttc acatatcagc aagttaccca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2860       2870       2880       2890       2900
PARP-1 seq      agggcaagca cagtgtcaaa ggtttgggca aaactacccc tgatccttca
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2910       2920       2930       2940       2950
PARP-1 seq      gctaacatta gtctggatgg tgtagacgtt cctcttggga ccgggatttc
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2960       2970       2980       2990       3000
PARP-1 seq      atctggtgtg atagacacct ctctactata taacgagtac attgtctatg
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       3010       3020       3030       3040       3050
PARP-1 seq      atattgctca ggtaaatctg aagtatctgc tgaaactgaa attcaatttt
Seq Cass 1      .......... .......... .......... .......... ..........
Seq Cass 2      .......... .......... .......... .......... ..........
Seq Cass 3      .......... .......... .......... .......... ..........
Seq Cass 4      .......... .......... .......... .......... ..........
Seq Cass 5      .......... .......... .......... .......... ..........
Seq Cass 6      .......... .......... .......... .......... ..........

                ....|....| ....|
                       3060
PARP-1 seq      aagacctccc tgtgg
Seq Cass 1      .......... .....
Seq Cass 2      .......... .....
Seq Cass 3      .......... .....
Seq Cass 4      .......... .....
Seq Cass 5      .......... .....
Seq Cass 6      .......... .....
```

9.3 Appendix 3

The overlapping regions of the DNA sequence data obtained from the six DNA sequencing cassettes (see Appendix 2) were removed and the PARP-1 DNA sequencing cassettes were linked together to make one continuous PARP-1 sequence. This continuous PARP-1 DNA sequence was then virtually translated into protein and aligned against the protein sequence of human PARP-1 sequence, accession number P09874 (Uchida *et al.* 1987) using the BioEdit software program (BioEdit, version 5.0.6, 2001) (Hall 1999). As shown below, the protein sequence obtained (**PARP-1 pro**) is 100 % homologous to the human PARP-1 sequence, accession number P09874 (**P09874**). Amino acid residues that are conserved are indicated by a dot (.).

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
P09874          MAESSDKLYR VEYAKSGRAS CKKCSESIPK DSLRMAIMVQ SPMFDGKVPH
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
P09874          WYHFSCFWKV GHSIRHPDVE VDGFSELRWD DQQKVKKTAE AGGVTGKGQD
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
P09874          GIGSKAEKTL GDFAAEYAKS NRSTCKGCME KIEKGQVRLS KKMVDPEKPQ
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
P09874          LGMIDRWYHP GCFVKNREEL GFRPEYSASQ LKGFSLLATE DKEALKKQLP
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
P09874          GVKSEGKRKG DEVDGVDEVA KKKSKKEKDK DSKLEKALKA QNDLIWNIKD
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
P09874          ELKKVCSTND LKELLIFNKQ QVPSGESAIL DRVADGMVFG ALLPCEECSG
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
P09874          QLVFKSDAYY CTGDVTAWTK CMVKTQTPNR KEWVTPKEFR EISYLKKLKV
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
P09874          KKQDRIFPPE TSASVAATPP PSTASAPAAV NSSASADKPL SNMKILTLGK
PARP-1 pro      .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
P09874          LSRNKDEVKA MIEKLGGKLT GTANKASLCI STKKEVEKMN KKMEEVKEAN
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
P09874          IRVVSEDFLQ DVSASTKSLQ ELFLAHILSP WGAEVKAEPV EVVAPRGKSG
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
P09874          AALSKKSKGQ VKEEGINKSE KRMKLTLKGG AAVDPDSGLE HSAHVLEKGG
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
P09874          KVFSATLGLV DIVKGTNSYY KLQLLEDDKE NRYWIFRSWG RVGTVIGSNK
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
P09874          LEQMPSKEDA IEHFMKLYEE KTGNAWHSKN FTKYPKKFYP LEIDYGQDEE
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
P09874          AVKKLTVNPG TKSKLPKPVQ DLIKMIFDVE SMKKAMVEYE IDLQKMPLGK
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
P09874          LSKRQIQAAY SILSEVQQAV SQGSSDSQIL DLSNRFYTLI PHDFGMKKPP
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
P09874          LLNNADSVQA KVEMLDNLLD IEVAYSLLRG GSDDSSKDPI DVNYEKLKTD
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
P09874          IKVVDRDSEE AEIIRKYVKN THATTHNAYD LEVIDIFKIE REGECQRYKP
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
P09874          FKQLHNRRLL WHGSRTTNFA GILSQGLRIA PPEAPVTGYM FGKGIYFADM
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
P09874          VSKSANYCHT SQGDPIGLIL LGEVALGNMY ELKHASHISK LPKGKHSVKG
PARP-1 pro      .......... .......... .......... .......... ..........
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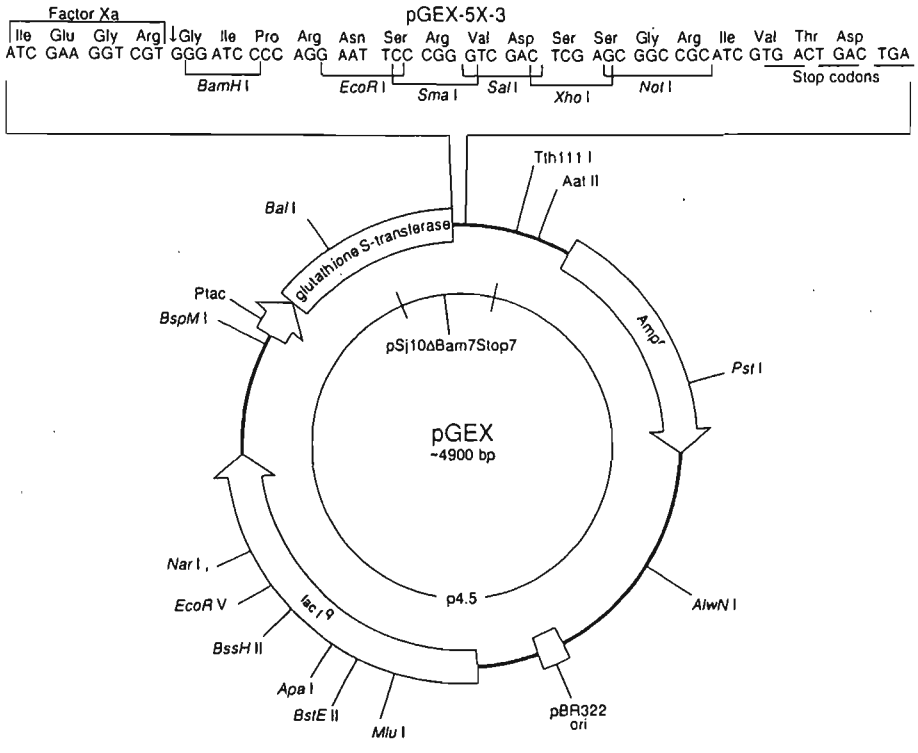
```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990        1000
P09874          LGKTTPDPSA NISLDGVDVP LGTGISSGVI DTSLLYNEYI VYDIAQVNLK
PARP-1 pro      .......... .......... .......... .......... ..........

                ....|....| ....
                        1010
P09874          YLLKLKFNFK TSLW
PARP-1 pro      .......... ....
```

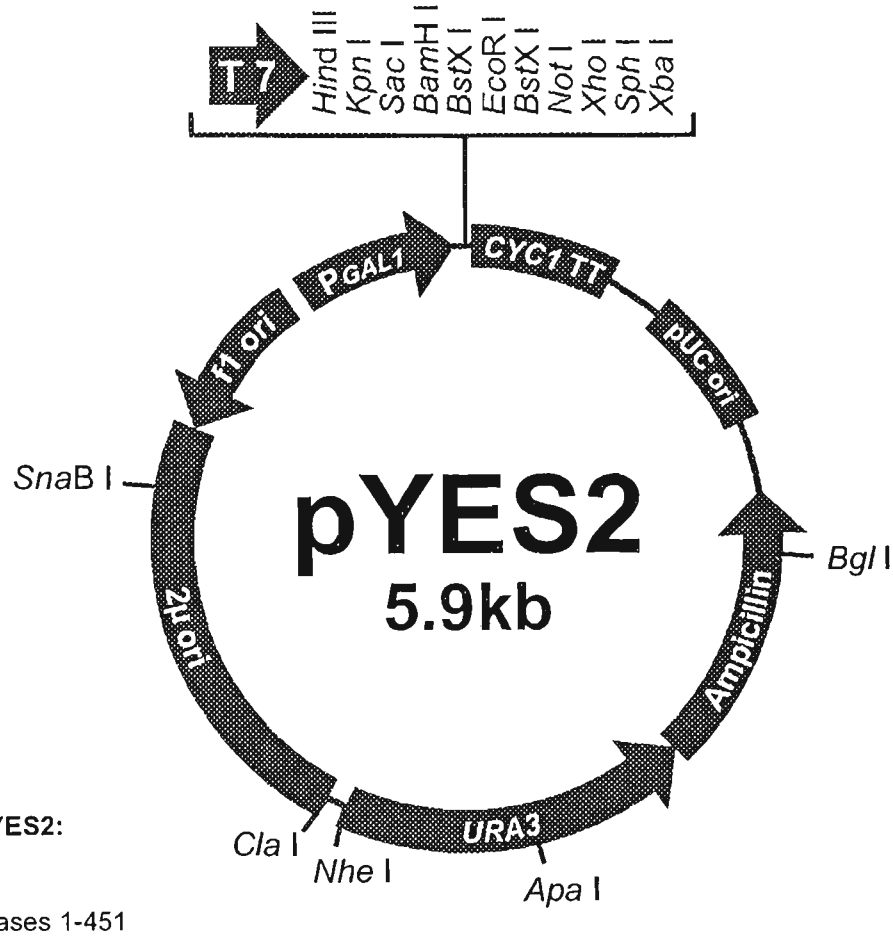
9.4 Appendix 4

Please find a copy of each of the expression vectors and their salient features: pGEX-5X-3, pYES2, pFastBac1 and pFastBacHTb.

(a.) pGEX-5X-3 (Amersham Biosciences Inc.)



(b.) pYES2 (Invitrogen Inc.)

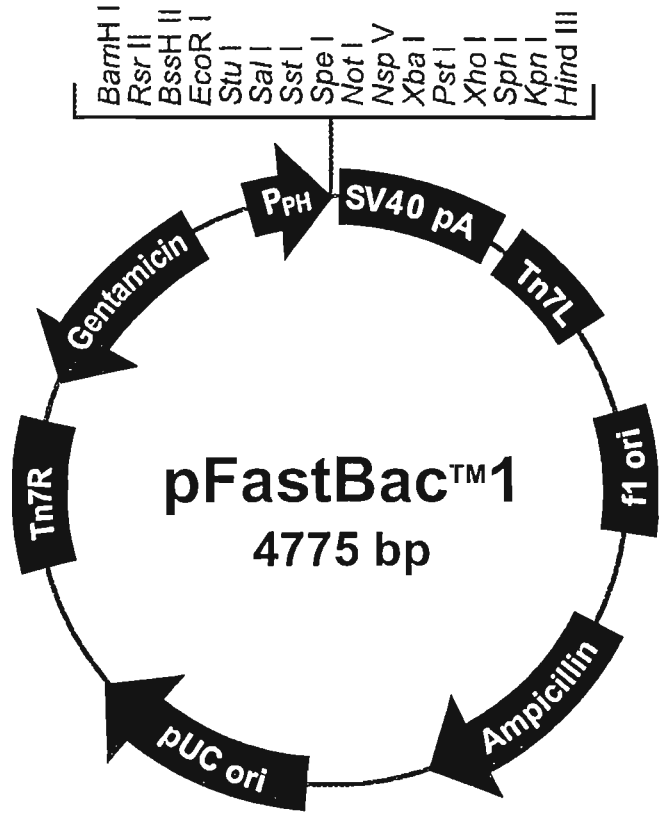


Comments for pYES2:
5856 nucleotides

GAL1 promoter: bases 1-451
T7 promoter/priming site: bases 475-494
Multiple cloning site: bases 501-600
CYC1 transcription terminator: bases 608-856
pUC origin: bases 1038-1711
Ampicillin resistance gene: bases 1856-2716 (C)
URA3 gene: bases 2734-3841 (C)
2 micron (µ) origin: bases 3845-5316
f1 origin: bases 5384-5839 (C)
(C) = complementary strand

Invitrogen™
life technologies

(c.) pFastBac1 (GibcoBRL-Life Technologies Inc.)

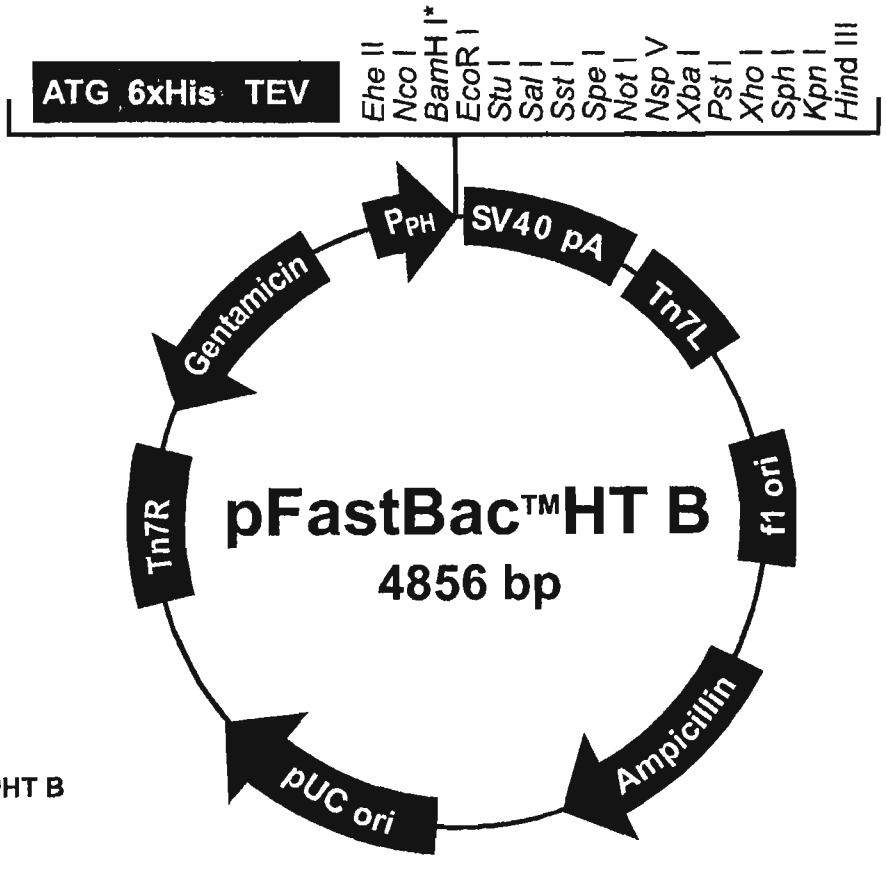


Comments for pFastBac™1
4775 nucleotides

f1 origin: bases 2-457
Ampicillin resistance gene: bases 589-1449
pUC origin: bases 1594-2267
Tn7R: bases 2511-2735
Gentamicin resistance gene: bases 2802-3335 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 3904-4032
Multiple cloning site: bases 4037-4142
SV40 polyadenylation signal: bases 4160-4400
Tn7L: bases 4429-4594

Invitrogen™
life technologies

(d.) pFastBacHTb (GibcoBRL-Life Technologies Inc.)



Comments for pFastBac™HT B
4856 nucleotides

f1 origin: bases 2-457
Ampicillin resistance gene: bases 589-1449
pUC origin: bases 1594-2267
Tn7R: bases 2511-2735
Gentamicin resistance gene: bases 2802-3335 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 3904-4032
Initiation ATG: bases 4050-4052
6xHis tag: bases 4062-4079
TEV recognition site: bases 4101-4121
Multiple cloning site: bases 4119-4222
SV40 polyadenylation signal: bases 4240-4480
Tn7L: bases 4509-4674

*Frameshift occurs at the *Bam*H I site in each vector

Invitrogen™
life technologies

9.5 Appendix 5

Please find attached a copy of each of the manuscripts accepted for publication as a result of data generated in this thesis for your interest.

Protein Expression and Purification 23, 453–458 (2001)
doi:10.1006/prep.2001.1513, available online at http://www.idealibrary.com on IDEAL®

Production, Extraction, and Purification of Human Poly(ADP-ribose) Polymerase-1 (PARP-1) with High Specific Activity

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Received May 29, 2001, and in revised form July 17, 2001

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme involved in a range of activities associated with DNA metabolism and plays a key role in maintaining the integrity of DNA and chromatin structure. As such, this enzyme is likely to provide a useful target when using a rational drug design approach to develop pharmaceutical reagents, including cancer therapeutics. However, there is still a great deal to learn about the mode of action of PARP-1 and therefore efforts are being directed at gaining a better understanding of the relationship between its structure and function. To this end we have developed a rapid and relatively simple approach to producing and purifying PARP-1. Unlike traditional PARP-1 purification protocols, the method described here requires only one chromatography step thus minimizing losses of the enzyme and also avoids the use of a competitive inhibitor-based affinity chromatography step, which is common to several other protocols in the literature. The product of the method described here is high-quality native PARP-1 with a high specific activity and K_m and V_{max} values similar to what is reported by other workers in the field. This protocol is particularly well suited to making PARP-1 in a quantity and of a quality suitable for structure–function studies.

Poly(ADP-ribose) polymerase-1 (PARP-1)[2] is a member of a family of ADP-ribosylating enzymes found in the nucleus of most eukaryotic cells. Human PARP-1 is a monomer of 116 kDa composed of three functional domains: a DNA-binding domain (DBD), an automodification domain, and a catalytic or nicotinamide adenine dinucleotide (NAD^+) binding domain (Fig. 1A) (1–3). PARP-1 is believed to be part of the cells extensive DNA surveillance network, involved in locating and repairing DNA damage, but researchers have also implicated PARP-1 activity in most other areas of DNA metabolism including DNA replication, differentiation, sister chromatid exchange, and cellular proliferation (4, 5).

PARP-1 activity is entirely dependent on binding to DNA strand breaks via its zinc fingers in the amino-terminal DBD (6). Once activated PARP-1 catalyzes the covalent attachment and subsequent polymerization of the ADP-ribose moiety from NAD^+ to a limited number of nuclear proteins (heteromodification) and PARP-1 itself (automodification). However, the exact molecular mechanism of PARP-1 activity remains unknown.

PARP-1 has been isolated and purified from a range of animal tissues including human tonsils (7), rat liver (8), calf and lamb thymus (9–11), and human placenta (12) but over the past 10 years or so most researchers working on this enzyme have used recombinant sources of the gene expressed in a range of backgrounds including *E. coli* (e.g. 13, 14), yeast (15–17), and baculovirus

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[2] Abbreviations used: PARP-1, poly(ADP-ribose) polymerase-1; DBD, DNA-binding domain; NAD^+, nicotinamide adenine dinucleotide; 3-AB, 3-aminobenzamide; HT-PARP-1, hexahistidine PARP-1; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; TCA, trichloroacetic acid; 3-mBA, 3-methoxybenzamide.

1046-5928/01 $35.00

expression systems (e.g., 18, 19). Successful strategies for purifying PARP-1 from homogenized tissues or cell culture usually include the use of salt extractions followed by affinity chromatography. Burtscher *et al.* (12), for example, reported an elegant three-step protocol that used 3-aminobenzamide (3-AB), a competitive inhibitor of PARP-1 (20), in an affinity column. In this approach, separation was achieved using hydroxylapatite followed by phosphocellulose column chromatography and then a 3-AB affinity column. This approach did not require an initial salt extraction step.

The method described in this paper utilizes aspects of extraction and purification procedures described by Yoshihara *et al.* (10) and Giner *et al.* (18) but with important modifications. The method described by Yoshihara *et al.* (10) was designed to extract PARP-1 from bovine tissue and involves several purification steps that are redundant when extracting PARP-1 from a baculovirus-infected cell culture. However, several key features of the Yoshihara method (10) have been retained, including the use of DNA–cellulose affinity chromatography as this approach had previously been used in our laboratories to successfully purify PARP-1 from bovine thymus (unpublished data).

Giner *et al.* (18) developed a protocol for purification of PARP-1 from a baculovirus expression system. In this method, agitation in a hypotonic lysis buffer was used to lyse baculovirus-infected cells. However, instead of using DNA–cellulose affinity chromatography to purify PARP-1, these authors used a 3-AB column and protamine sulfate instead of hydroxylapatite to remove endogenous DNA from the preparation. The method described in the following work utilizes the Giner *et al.* (18) approach for lysing cells and protamine sulfate to remove endogenous DNA; however, it avoids the use of a competitive inhibitor affinity column thus avoiding potential problems associated with carryover of the inhibitors to the final product. Additionally, the method we have developed minimizes the number of chromatography steps, thus reducing potential losses of product and reducing the time needed for purification.

MATERIALS AND METHODS

Cloning. All cloning steps followed standard molecular biological techniques as described in Sambrook *et al.* (21). In brief, complete PARP-1 cDNA (a generous gift from Professor G. de Murcia, Institut de Moleculaire et Cellulaire, Centre National Recherche Scientifique, Strasbourg, France) was cloned directly into the *Xba*I site of pFastBac1 or pFastBacHTb baculovirus expression vectors from Gibco Life Technologies. pFastBacHTb vector carries a coding sequence for six histidine residues at the amino terminus of the expression product. The resulting constructs, pFastBac1–PARP-1 and pFastBacHTb–PARP-1, were confirmed by restriction digestion and sequencing.

Standard protein expression. PARP-1 and hexahistidine PARP-1 (HT-PARP-1) were prepared following expression of pFastBac1–PARP-1 and pFastBacHTb–PARP-1 in a BAC-TO-BAC baculovirus expression system in *Sf*9 or *Sf*21 insect cells according to the "BAC-TO-BAC Baculovirus Expression Systems Instruction Manual" (22). In brief, recombinant baculovirus containing human PARP-1 cDNA was generated by transposing pFastBac1–PARP-1 and pFastBacHTb–PARP-1 into bacmid propagated in *Escherichia coli* DH10Bac cells. Insertion of the PARP-1 cDNA into the bacmid vector by transposition was confirmed by PCR utilizing M13/pUC forward and reverse amplification primers. Positive clones were then transfected into *Sf*9 or *Sf*21 insect cells using Cellfectin as the transfection reagent. Tissue culture medium from *Sf*9 or *Sf*21 insect cells infected with recombinant baculovirus containing PARP-1 cDNA was collected by centrifugation at 500*g* for 5 min in a benchtop microfuge. Medium containing viral supernatant had BSA added to a final concentration of 2% and was stored in a light-proof container at 4°C. PARP-1 and HT-PARP-1 expressions were accomplished by infecting *Sf*9 or *Sf*21 insect cells with viral supernatant containing recombinant baculovirus to achieve a multiplicity of infection of 5. Infected insect cells were harvested 72 h postinfection by centrifugation (500*g*, 5 min at 4°C) and the cells were immediately stored at −80°C until purification.

PARP-1 and HT-PARP-1 protein purification. Following expression of PARP-1 and HT-PARP-1, infected insect cells were lysed and homogenized by vortexing (3 × 30 s) at 4°C or sonication (3 × 5 s, output 6.5, duty cycle 65%, Branson sonifer 450) on ice in ice-cold hypotonic homogenization buffer (25 mM Tris–HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 0.2% Tween 20, 0.2% Nonidet P-40, and 0.5 M NaCl). Cellular debris was then removed by centrifugation (14,000*g* for 15 min at 4°C). Endogenous DNA was removed by the addition of 1 mg/ml salmon sperm protamine sulfate (Lot No. 91592, ICN Biomedicals) followed by centrifugation (14,000*g* for 10 min at 4°C). The supernatant was then precipitated in two steps with 0–30% (164 g of solid ammonium sulfate was added to 1000 ml to give 30% saturation) followed by 30–70% saturated ammonium sulfate (249 g of solid ammonium sulfate was added to 1000 ml to give 30–70% saturation). Ammonium sulfate precipitation was performed at 4°C and the samples were stirred overnight. The precipitated proteins were dissolved in ice-cold chromatography–extraction buffer A (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$, and 0.2 M NaCl) and applied to a DNA–cellulose column equilibrated

with the chromatography–extraction buffer A (flow rate 0.3 ml/min). PARP-1 and HT-PARP-1 were eluted from the affinity matrix using chromatography–extraction buffer B (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT, 50 mM $NaHSO_3$, and 1.0 M NaCl). To remove unwanted salts from the preparations, fractions eluted with the highest amount of PARP activity were further purified by ultrafiltration using CentriPrep10 (Amicon Inc.) in dialysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM DTT). Samples were stored at −80°C until required.

Product analysis. The purification product was analyzed for identity and quality using two approaches. The relative molecular weight of the product was determined by SDS–PAGE analysis as described by LKB Pharmacia (23) on a 7.5% gel.

The activity of the enzyme was determined using an approach based on methods described by Giner *et al.* (18) and Niedergang *et al.* (25). The standard reaction mixture contained 50 mM Tris–HCl buffer, pH 8.0 (adjusted at 37°C), 4 mM $MgCl_2$, 0.2 mM DTT, 2 μg/ml histone H1, 2 μg/ml DNA, 200 μM NAD^+ (0.5 μCi [2,5′,8-^{3}H]NAD^+, 6.66 × 10^4 dpm/pmol), and 200 ng of total protein from each of the various stages of the purification protocol of PARP-1 or HT-PARP-1 in a total reaction volume of 100 μl.

The reaction mixture was incubated at 25°C for 10 min after which it was terminated by the addition of 1 ml of ice-cold 20% (w/v) trichloroacetic acid (TCA) containing 4 mg/ml NAD^+. The resultant precipitate was left on ice for 60 min and subsequently collected on GF/C glass fiber filters and washed twice with 10% (w/v) TCA, followed by an acetone wash. The filter was then placed in an oven to dry and its radioactivity was measured in a Wallac 1410 liquid scintillation counter. The liquid scintillant used was 0.5% (w/v) PPO in AnalaR toluene.

RESULTS AND DISCUSSION

Studying the relationship between structure and function in proteins usually requires the production of relatively large (mg) quantities of highly purified protein. In the modern biochemistry laboratory this is commonly achieved by expressing the relevant gene in a microbial or cell culture system and purifying the expression product using standard biochemical approaches. We are currently studying the relationship between structure and function of PARP-1 using a cloned source of human PARP-1 expressed in a BAC-TO-BAC baculovirus expression system from *Sf*9 and *Sf*21 cells. To obtain a highly purified protein product we have developed an extraction and purification procedure utilizing aspects of previously published methods (10, 18) but with some important modifications.

A cDNA encoding the complete human PARP-1 protein (Fig. 1A) was cloned into the *Xba*I site of pFastBac1 and pFastBacHTb. PARP-1 and HT-PARP-1 coding sequences were then transposed into Bacmid vectors in Bacmid-propagated *E. coli* DH10Bac cells and then transfected into *Sf*9 or *Sf*21 cells. A schematic of the structure of HT-PARP-1 is shown in Fig. 1B.

In the Giner *et al.* (18) method, baculovirus-infected cells were lysed in a hypotonic buffer and to ensure that lysis was complete cells were agitated on ice for 30 min. We used the same buffer as these authors and homogenized cells by either vortexing or sonication. Cellular debris was then removed by centrifugation. The yield was qualitatively and quantitatively the same for sonication and vortexing as determined by Coomassie-stained SDS–PAGE (Fig. 2).

To minimize loss of expressed protein by protease activity and oxidation, several strategies consistent with other PARP-1 purification protocols were employed including the use of a number of protease inhibitors including PMSF and sodium dithionite ($NaHSO_3$) and sulfydryl-reducing agents such as DTT and β-mercaptoethanol.

As PARP-1 is a DNA binding protein, DNA tends to copurify with it thus necessitating the removal of this contaminant. This can be achieved using hydroxylapatite column chromatography (e.g., Ref. 10) or protamine sulfate precipitation (e.g., Ref. 18). The removal of DNA by hydroxylapatite column chromatography is time-consuming and in our laboratory has often led to reduced and highly variable yields (unpublished data). The method of PARP-1 and HT-PARP-1 purification described in this paper utilized protamine sulfate as described by Giner *et al.* (18).

Following protamine sulfate precipitation the supernatant was precipitated in a two-step ammonium

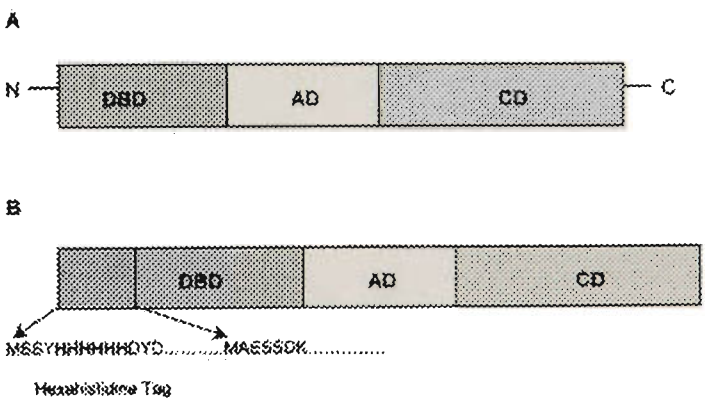

FIG. 1. (A) Schematic of full-length PARP-1 protein showing the three functional domains: DNA-binding domain (DBD), automodification domain (AD), and the catalytic or NAD^+ binding domain (CD). (B) Schematic of hexahistidine-tagged PARP-1 (HT-PARP-1).

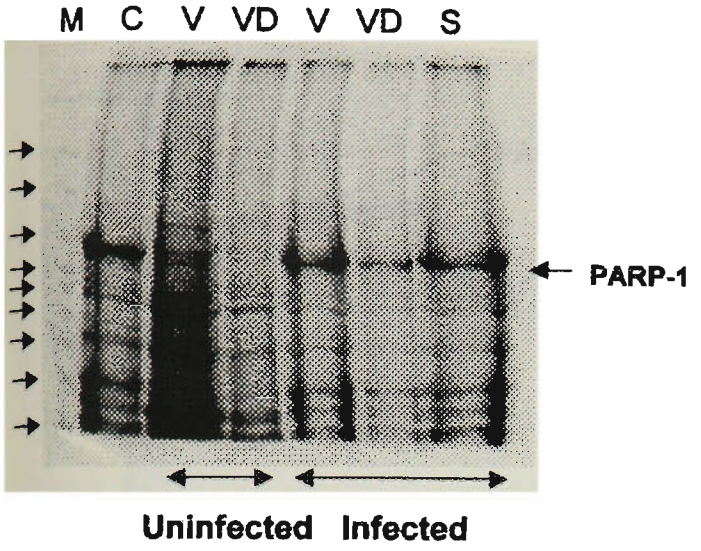

FIG. 2. 7.5% SDS–PAGE protein profile of the initial stages of PARP-1 extraction and purification from baculovirus-infected and uninfected cells. The first stage of PARP-1 extraction and purification involved cellular lysis in a hypotonic lysis buffer and extraction by vortexing or sonication. The gel was stained with Coomassie blue. The molecular weights of the BenchMark protein markers (M) (Gibco-Life Technologies) indicated by the arrows are 220, 160, 120, 100, 90, 80, 70, 60, and 50 kDa. C, control infected insect cells before sonication and vortexing; V, proteins from vortexed cells, S, proteins from sonicated cells; VD, cellular debris from vortexed cells.

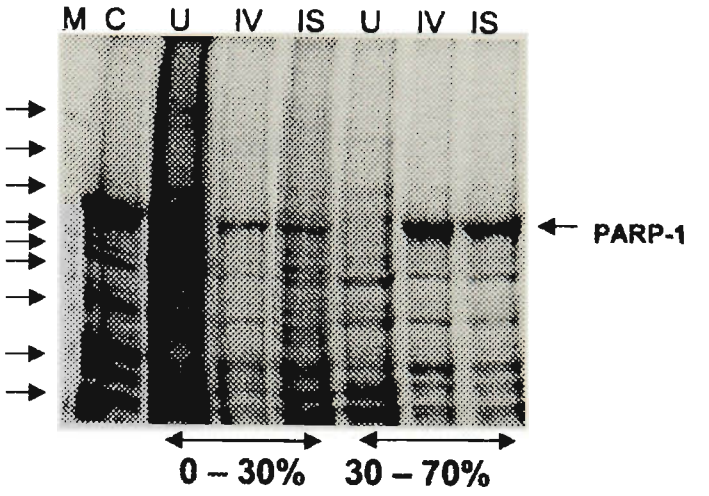

FIG. 3. 7.5% SDS–PAGE protein profile of the initial stages of PARP-1 extraction and purification from baculovirus-infected and uninfected cells. Stepwise ammonium sulfate precipitation of baculovirus-infected and uninfected cells. The gel was stained with Coomassie blue. The molecular weights of the BenchMark protein markers (M) indicated by the arrows are 220, 160, 120, 100, 90, 80, 70, 60, and 50 kDa. C, control infected insect cells before sonication and vortexing; U, proteins from disrupted uninfected insect cells; IV, proteins from vortexed disrupted infected insect cells; IS, proteins from sonicated disrupted infected insect cells.

sulfate precipitation. Stepwise ammonium sulfate precipitation has been utilized in PARP-1 purification procedures described in the literature to stabilize the enzyme preparation as well as minimize protease digestion (25). However, the range of ammonium sulfate concentrations used has varied: Yoshihara *et al.* (10) used 40–80% saturation, Giner *et al.* (18) and Panzeter *et al.* (26) used 30–70%, whereas Jung *et al.* (19) used 40–65% to purify the catalytic domain of chicken PARP-1 from infected *Sf*9 insect cells. We have used 30–70% ammonium sulfate for the work described in this paper (see Fig. 3), although we have used 40–80% in past work with similar success (data not shown).

Burstcher *et al.* (12) successfully purified PARP-1 utilizing 3-AB, a very potent competitive inhibitor of PARP-1, on an affinity column. PARP-1 was eluted from the 3-AB affinity matrix with 3-methoxybenzamide (3-mBA), another potent inhibitor of PARP-1. Giner *et al.* (18) also utilized 3-AB affinity chromatography and elution with 3-mBA to purify PARP-1 expressed in a baculovirus expression system. The 3-mBA was subsequently removed from the preparation by dialysis. The use of competitive inhibitors to affinity purify proteins may cause problems if the inhibitor is carried over in the purified product. While Burstcher *et al.* (12) was able to produce PARP-1 with kinetic parameters similar to those reported in the literature, and therefore presumably fully separated from the inhibitors, we thought that it would be preferable to avoid some of the risks associated with this approach. Thus we opted for the use of DNA–cellulose affinity matrix chromatography (as described in 10), which targets the zinc fingers in PARP-1 and their affinity for DNA.

The elution profiles for the DNA–cellulose affinity matrix column are shown in Fig. 4. Fractions containing PARP-1 were pooled together and then dialyzed and concentrated by ultrafiltration. This modification to previously reported PARP-1 purification procedures was important, as it removed salts and ions that might otherwise influence PARP-1 structure and/or function in the purified protein product. Sallmann *et al.* (27), for example, found that salts can severely affect the

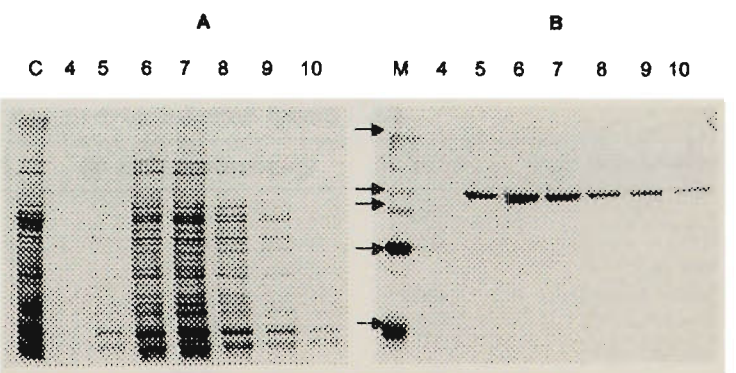

FIG. 4. 7.5% SDS–PAGE protein profile of PARP-1 fractions prior to elution (A) and after elution (B) from DNA–cellulose column. The gel was silver stained. The SDS–PAGE molecular weight markers (M) are indicated by the arrows are myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorlyase B, 97.4 kDa; bovine serum albumin, 66 kDa; and egg albumin, 45 kDa. C, crude PARP-1 preparation prior to loading on the DNA–cellulose column.

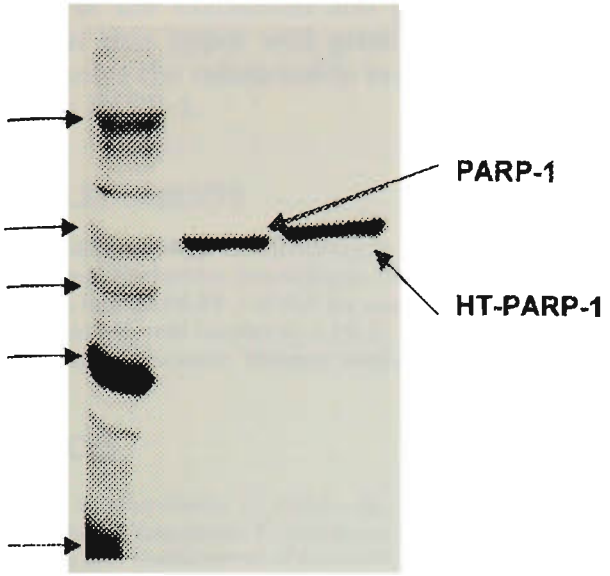

FIG. 5. 7.5% SDS–PAGE of extracted and purified PARP-1 and hexahistidine-tagged PARP-1 (HT-PARP-1). The gel was silver stained and the SDS–PAGE molecular weight markers indicated by the arrows are myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorlyase B, 97.4 kDa; bovine serum albumin, 66 kDa; and egg albumin, 45 kDa.

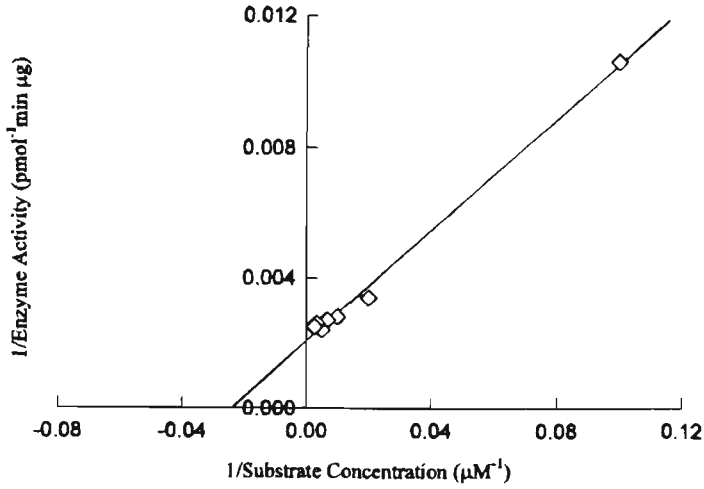

FIG. 6. Lineweaver–Burke plot for extracted and purified PARP-1.

detection of PARP-1 in an enzyme-linked immunosorbent assay.

Ruscetti *et al.* (28) recently described a protocol for the purification of a bacterially expressed hexahistidine-tagged PARP-1 DNA binding domain. These authors described some of the difficulties they encountered in developing the purification strategy that they eventually adopted. These difficulties included probable misfolding of the protein product, protein precipitation and protein degradation. While the authors were able to resolve the problems they encountered, the purification method still relied on the presence of a histidine tag for purification. In contrast, the method described in this paper relies only on the presence of a functional DBD and was successfully used to purify histidine-tagged or nontagged PARP-1 (Fig. 5). It is reasonable to assume that the same approach could be used to purify histidine-tagged (or nontagged) PARP-1 DBD.

Burtscher *et al.* (12) reported that PARP-1 preparations are unstable during purification with considerable losses of activity at each stage. These authors believed that this instability of the protein was due to proteolytic degradation. Work conducted in our laboratories suggests that valuable protein is lost primarily during column purification (unpublished data). Thus we have made every effort to minimize losses by the use of protease inhibitors and developing a method that requires only one column chromatography step.

In summary we report here a rapid and efficient method for extraction and purification PARP-1 and HT-PARP-1 from baculovirus-infected *Sf*9 or *Sf*21 insect cells (see Table 1). A simple ultrafiltration step was used to concentrate and dialyze the purified protein, thus removing unwanted salts from the preparation that could dramatically affect PARP activity. Purified PARP-1 migrated as a unique 116-kDa band on a SDS–PAGE (see Figs. 4 and 5). The purified enzyme had a

TABLE 1

Purification of Human PARP-1 from Baculovirus-Infected Insect Cells

Stage of purification	Total protein (mg)	Total activity[a] (pmol)	Sp act[b] (pmol/min/μg)	% Yield[c]	Fold purification[d]
Crude extract	20	70,300	3.52	100	—
Crude lysate (Fig. 2)	18	75,200	4.18	100	—
Protamine sulfate	17	73,400	4.32	100	—
Ammonium sulfate (Fig. 3)	10	40,100	4.01	57	—
DNA–cellulose (Fig. 4)	0.25	9,700	38.0	14	11.0
Ultrafiltration	0.20	10,200	50.8	14	14.5

[a] 1 unit of activity is the number of pmol of ADP-ribose incorporated into TCA-precipitable counts in 1 min.
[b] Specific activity is the data in column *a* divided by total protein for the same fraction.
[c] Data calculated by dividing total activity with the total activity of the initial fraction (crude extract).
[d] Ratio of the specific activity of a given step versus the specific activity of the initial fraction (crude extract).

K_m of 42 μM and V_{max} of 480 pmol/min/μg (see Fig. 6), consistent with values reported in the literature. The simplicity of the extraction and purification protocol reported in this paper will greatly enhance our endeavor to solve the relationship between structure and function in PARP-1.

ACKNOWLEDGMENTS

Professor Gilbert de Murcia's (Institut de Moleculaire et Cellulaire, Centre National Recherche Scientifique, Strasbourg, France) generous gift of the human PARP-1 cDNA for use in this study was greatly appreciated. M.I.K. was funded by a Ph.D. scholarship from Faculty of Engineering and Science, Victoria University.

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RESEARCH

Problems Associated with Determining Protein Concentration:

A Comparison of Techniques for Protein Estimations

Matthew I. Knight and Paul J. Chambers*

Abstract

Although a range of methods are available for determining protein concentration, many scientists encounter problems when quantifying proteins in the laboratory. The most commonly used methods for determining protein concentration in a modern biochemistry laboratory would probably be the Lowry and/or the Bradford protein assays. Other techniques, including direct spectrophotometric analysis and densitometry of stained protein gels, are applied, but perhaps to a lesser extent. However, the reliability of all of the above techniques is questionable and dependent to some extent on the protein to be assayed. In this paper we describe problems we encountered when using some of the foregoing techniques to quantify the concentration of poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1), a nuclear enzyme found in most eukaryotes. We also describe how, by using a fluorescence-based assay and amino acid analysis, we overcame the problems we encountered.

Index Entries: Bradford protein assay; Lowry protein assay; poly(ADP-ribose)polymerase-1.

1. Introduction

Our laboratory is currently investigating the relationship between structure and function in poly(adenosine diphosphate [ADP]-ribose) polymerase-1 (PARP-1). PARP-1 is a nuclear enzyme involved in a range of activities associated with DNA metabolism, and plays a key role in maintaining the integrity of DNA and chromatin structure *(1,2)*. We have successfully produced and purified human PARP-1 with high specific activity by using the BAC-TO-BAC Baculovirus expression system (Invitrogen, Inc.) *(3)*, work that required a reproducible and accurate assay to estimate protein concentration.

Many methods are available for determining protein concentration (**Table 1** provides a list of commonly used assays), and the criteria for a suitable assay usually include sufficient sensitivity, accuracy, and reproducibility. Several methods with the potential to meet these criteria were tested in the work described in this paper.

The most widely used protein assay in the literature, the Lowry protein assay *(4)*, detects the phenolic group on tyrosine residues in proteins, and has a sensitivity of 2–100 µg/mL of protein *(5)*. Because the Lowry protein assay detects tyrosine residues in a protein, and the number of tyrosine residues varies between proteins, it is important that the protein used as a standard have a similar proportion of tyrosine to the protein being assayed. Another critical component of the Lowry protein assay is the length of the incubation time to develop the product of the Lowry reaction; differences in incubation times between samples in a Lowry protein assay will lead to nonreproducible results. The Lowry protein assay is also subject to interference from a wide range of components such as Tris and ethylene diamine tetraacetic acid (EDTA) *(6)*, both of which are components of many buffers used for the purification of recombinant proteins. However, if one assumes that there is sufficient protein, the effect of these

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Table 1
Comparisons of Routinely Used Methods for Quantitating Protein Concentration

Method	Detection Range	Active Residues Detected	Comments	Reference
Lowry protein assay	2–100 µg/mL	Phenolic group on tyrosine residues	• Choice of standard is critical	*(4)*
			• Incompatible with some detergents and some reducing agents	*(6)*
			• Tris and EDTA can interfere with assay	
Bradford protein assay	0.2–20 µg/mL	Basic amino acid residues, particularly arginine	• High protein to protein variation	*(8)*
			•Incompatible with some detergents	*(10)*
Laser densitometry of SDS–PAGE gels				
Coomassie brilliant blue G250 staining	40–50 ng band	Detects basic amino acids on the same principle as the Bradford protein method	• Time consuming but can give reasonable estimate of protein concentration	*(11)*
SYPRO Orange staining[d]	1-ng band	Binds to SDS coat around proteins	• Low protein to protein variation	*(18)*
			• Detection not influenced by nucleic acids or other contaminants	*(14,17)*
Silver staining	1–5 ng band	Relies on the reduction of ionic silver to its metallic form on binding to proteins	• Qualitative method	*(13)*
			• Time consuming	*(12)*
				(11)
Amino acid analysis		Detects most amino acid residues	• Specialized techniques	*(5)*
			• Requires GC–MS or HPLC	
			• Very accurate quantitation method	

chemicals can be minimized by diluting the protein sample.

The Bradford protein assay is both rapid and accurate *(7)*. Ausubel et al. *(5)* proposed that the Bradford protein assay is "the method of choice" for accurately determining protein concentration. Practical advantages of this method are that the Bradford protein reagent is simple to prepare and that the color develops rapidly and is stable. The assay relies on the binding of Coomassie Brilliant Blue G250 (CBB) to protein *(8)*. The dye binds in its anionic form to basic amino acids within the protein (particularly arginine residues), and when bound produces a complex that has an absorbance peak at 595 nm *(9)*. The amount of dye that binds depends on the content of basic amino acid residues in the protein. Thus, the proportion of basic residues in the protein standard for the assay should be similar to that in the protein to be assayed. The sensitivity range for the Bradford assay is between 0.2 and 20 µg/mL of protein *(10)*.

Protein concentration can also be estimated through laser densitometry of stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels; for some stains the intensity of a stained band of protein in such a gel is

proportional to the concentration of protein in the band. CBB is one of the most commonly used stains for protein gels. Although this stain can be used to detect relatively small amounts of protein in an SDS-PAGE gel (it can detect as little as 40–50 ng of protein in a band on a gel *[11]*), it is generally regarded as at best semiquantitative (Personal communication: John Walker, Hertfordshire University, Hatfield, UK).

Silver staining provides a significant advantage over the traditional CBB stain, since it is reported to be between 20 and 200 times more sensitive *(12)*, and thus able to detect as little as 1–5 ng of protein in a band on an SDS-PAGE gel *(11,13)*. However, the chemical interactions between the protein and the silver salts in silver staining are unknown *(12,13)*, and silver staining is generally regarded as nonquantitative.

It is claimed that fluorometric methods for quantifying protein concentration outperform all existing routine methods for determining protein concentration *(14)*. The newly developed SYPRO Orange fluorescent stain (Bio-Rad Laboratories Pty. Ltd.) for SDS-PAGE gels can detect as little as 1 ng of protein in a band on a gel *(15)*.

The most accurate and sensitive method for determining protein concentration is amino acid analysis. Although access to this approach in the past was restricted to specialized laboratories, it is now readily available, usually as a service provided by specialist laboratories. Additionally, significant advances in the technologies underpinning this approach have improved the precision and the sensitivity of amino acid analysis *(5)*. Amino acid analysis requires proteins to be quantitatively broken down to their constituent amino acids by chemical treatments that lead to peptide-bond hydrolysis; this is most commonly achieved by acid hydrolysis. The amino acids are then resolved and quantitated with high-pressure liquid chromatography or gas chromatography–mass spectrometry.

In our study, we used amino acid analysis to get an accurate estimation of the protein concentration of a stock solution of purified human PARP-1. This stock solution was then used to test a range of other assays for their sensitivity, accuracy, and reproducibility. From the results obtained it is clear that two of the most commonly used protein assays, the Lowry and the Bradford methods, should be used with caution, at least when determining PARP-1 concentration. In contrast to this, determining human PARP-1 concentration from stained SDS-PAGE gels when using bovine serum albumin (BSA) as a standard gave accurate estimates of protein concentration. An added advantage of estimating protein concentration from SDS-PAGE gels is that one also gets information about the quality of the protein.

2. Materials and Methods

2.1. Stock Solution of Human PARP-1

Human PARP-1 was prepared from the BAC-TO-BAC Baculovirus expression system as described by Knight and Chambers *(3)*. A stock solution of human PARP-1 was made by dissolving purified human PARP-1 in 50 m*M* Tris HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* NaN_3, 1 m*M* glutathione and 0.5 m*M* dithiothreitol (DTT).

2.2. Amino Acid Analysis

Amino acid analysis was performed by the Australian Proteome Analysis Facility at Macquarie University, Sydney, Australia.

2.3. Lowry Protein Assay Determination of PARP-1 Concentration

A modified version of the Lowry method *(4)* was used for this work. A calibration curve was prepared by using a series of solutions containing 10, 20, 30, 40, 50, 75, and 100 µg of standard protein (BSA, Fraction V, cat. no. A-7906 [Sigma]) in a total volume of 500 µL of double-distilled water. A reagent blank and dilutions of the PARP-1 stock solution were also prepared to a final volume of 500 µL of double- distilled water. A volume of 500 µL of Lowry protein assay solution A [1 mL 5% (w/v) $CuSO_4$, 9 mL 1% (w/v) potassium tartrate, and 100 mL 10% (w/v) Na_2CO_3 in 0.5 *M* NaOH] was added to each of the standards and PARP-1 dilutions, and mixed thoroughly into them by vortexing. The standards and the PARP-1 dilutions were then incubated at 37°C for 10 min. After incubation, 1.5 mL of Lowry protein

assay solution B (1 mL Folin–Ciocalteau's reagent and 10 mL double-distilled water) were added to each of the standards and the PARP-1 dilutions, and mixed thoroughly into them by vortexing. The standards and the PARP-1 dilutions were then incubated at 52°C for 20 min. Absorbances of standards and the PARP-1 dilutions were determined at 680 nm.

2.4. Bradford Protein Assay Determination of PARP-1 Concentration

The Bradford protein assay for determining protein concentration was performed as described by Bradford *(8)*. A calibration curve was prepared by using a series of solutions containing 10, 20, 40, 70, and 100 µg of standard protein (BSA) in a total volume of 100 µL double-distilled water. A reagent blank and appropriate dilutions of the PARP-1 stock solution were prepared to a final volume of 100 µL with double-distilled water as the diluent. A volume of 5.0 mL of Bradford protein reagent (100 mg CBB dissolved in 50 mL of 95% ethanol, mixed with 100 mL of 85% orthophosphoric acid and made up to a final volume of 1.0 L with distilled water) was added to each tube and then mixed thoroughly with the standards and PARP-1 solutions in the respective tubes by inversion. Absorbances for the PARP-1 dilutions and the standards were determined at 595 nm.

2.5. Estimating Protein Content with CBB, Silver, and SYPRO Orange-Stained SDS–PAGE Gels

Standards and dilutions of the stock PARP-1 solution were run on 7.5% SDS-PAGE gels as described in the Bio-Rad Laboratories (Mini Protean II Dual Slab Cell Instruction Manual). After electrophoresis, gels were stained either CBB, silver, or SYPRO Orange.

CBB staining was performed according to the method described in the LKB-Pharmacia laboratory manuals *(16)*. The gel was placed in fixing solution (80 mL ethanol and 20 mL glacial acetic acid, made up to a final volume of 200 mL with distilled water) for a minimum of 30 min. The gel was then placed in CBB staining solution (1.25 g CBB dissolved in 230 mL methanol and 230 mL distilled water). The solution was stirred for an hour, and 40 mL glacial acetic acid was then added. If any particles appeared, the solution was filtered through Whatman 3M filter paper. The gel was left in the stain for 30 min and was then destained in fixing solution until the background was clear. The gel was then washed several times with distilled water.

Silver staining was performed according to the method described in the LKB Pharmacia laboratory manuals *(16)*. Gels were placed in fixing solution (80 mL ethanol and 20 mL glacial acetic acid, made up to a final volume of 200 mL with distilled water) for a minimum of 30 min to allow the SDS to diffuse out of the gel and the proteins to precipitate. The gel was then placed in incubation solution (60 mL ethanol, 13.6 g sodium acetate·$3H_2O$, 0.4 g sodium thiosulfate, and 1.04 mL glutaraldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) for a minimum of 30 min, after which it was washed several times with distilled water (at least 10 min per wash for three washes), placed in silver solution (0.2 g silver nitrate and 40 µL of formaldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) and allowed to shake in the latter for 40 min. The gel was then placed in developing solution (5.0 g sodium carbonate and 20 µL of formaldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) until the protein bands became intensely dark. To stop further color development via the reaction, the gel was placed in stop solution (2.92 g EDTA dissolved in distilled water to a final volume of 200 mL) for 15 min.

Staining of SDS-PAGE gels with SYPRO Orange was performed according to the Bio-Rad Laboratories SYPRO Orange Protein Gel Stain Instruction Manual *(17)* with some minor modifications. The gel was placed in SYPRO Orange staining reagent (10 µL of SYPRO Orange protein stain, dissolved in 50 mL of 7.5% glacial acetic acid) for 30 min and then destained in 7.5% glacial acetic acid for 30 min to 1 h.

The staining intensities of the CBB-, silver-, and SYPRO Orange-stained protein gels were

captured with a Fujifilm LAS1000 charged-coupled device (CCD) and analyzed by using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998).

Estimates of protein concentration from stained SDS-PAGE gels were based on densitometry of duplicate gels. For the CBB- and SYPRO Orange-stained gels, the standard curve was generated from mean optical densities (ODs) for duplicate gels. All experiments were repeated at least once and gave similar results. The total amount of protein in a band was estimated by measuring the intensity across the whole area of the band, thus compensating for differences in band size.

3. Results and Discussion

We used a stock solution of human PARP-1, expressed from a cloned source and purified as described in Knight and Chambers *(3)*, to compare several different quantitative protein assays so as to determine which assay would be most reliable for accurate determination of PARP-1 concentrations for future work in our laboratory. We determined the concentration of the stock PARP-1 solution used in this comparison by amino acid analysis, and was estimated to be 10 µg/mL. This was consistent with the level of PARP-1 enzyme activity associated with the preparation (data not shown). This estimate was therefore assumed to be a reasonable measure of the PARP-1 concentration in the stock solution.

The Lowry protein assay detects tyrosine residues in a protein, and because the number of tyrosine residues varies among proteins, it is important that the protein used as a standard has a similar proportion of tyrosine residues to the protein being assayed. The percentages of tyrosine residues in BSA and human PARP-1 are 3.5% and 3.3%, respectively, and BSA should therefore be a suitable standard for assaying PARP-1 concentration.

The Lowry protein assay is also subject to interference from a range of chemicals, such as Tris and EDTA *(6)*, both of which are components of the buffer used to make the standard stock solution of human PARP-1 that was used in our study. However, the concentration of these components in the PARP-1 buffer was minimal and was therefore unlikely to have interfered with the assay. This was shown to be the case in an experiment in which a range of concentrations of BSA was dissolved in PARP-1 buffer and in water, and the two sets of solutions were subjected to the Lowry protein assay. The resultant absorbance values were not affected by the buffer (data not shown).

The Lowry protein assay was performed in duplicate and repeated at least once, and gave similar results. With BSA used as a standard, the stock solution of human PARP-1 used for this work was estimated to have a protein concentration of 2.6 mg/mL (**Fig. 1**). Thus, the modified Lowry protein assay gave an estimate of protein concentration that was two orders of magnitude higher than the concentration of the stock solution of human PARP-1 as determined by amino acid analysis. Therefore, the Lowry protein assay is clearly not a suitable method for determining the concentration of PARP-1 when BSA is used as a standard.

The Bradford protein assay detects the amount of CBB dye that binds to base amino acids in proteins; consequently, the relative proportions of basic amino acids in the standard (BSA) and the protein being assayed are of critical importance. The percentages of basic amino acid residues in BSA and PARP-1 are 17.0% and 17.5%, respectively. Therefore, BSA was considered to be a suitable standard when determining the protein concentration of PARP-1.

The Bradford protein assay was performed in duplicate and repeated at least once and gave similar results. With BSA used as the standard, the Bradford protein assay gave a protein concentration for the stock human PARP-1 solution of 220–240 µg/mL (**Fig. 2**), a value that is 10–20 times greater than that obtained by amino acid analysis. Although this result is better than that given by the Lowry assay, it is still more than an order of magnitude greater than the expected value. Therefore, the Bradford assay, is not suitable for estimating PARP-1 concentration when BSA is used as a standard.

Protein concentration can be estimated through laser densitometry of stained SDS-PAGE gels, and CBB is undoubtedly one of the most com-

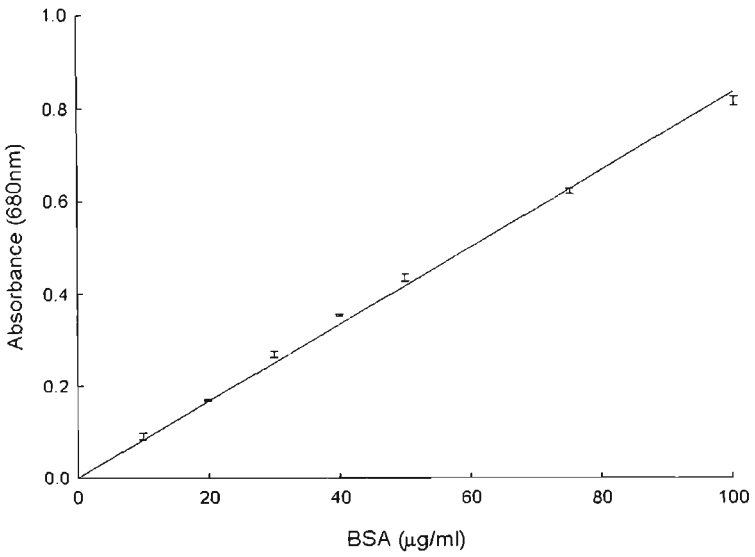

Fig. 1. Standard curve used for the Lowry protein assay.

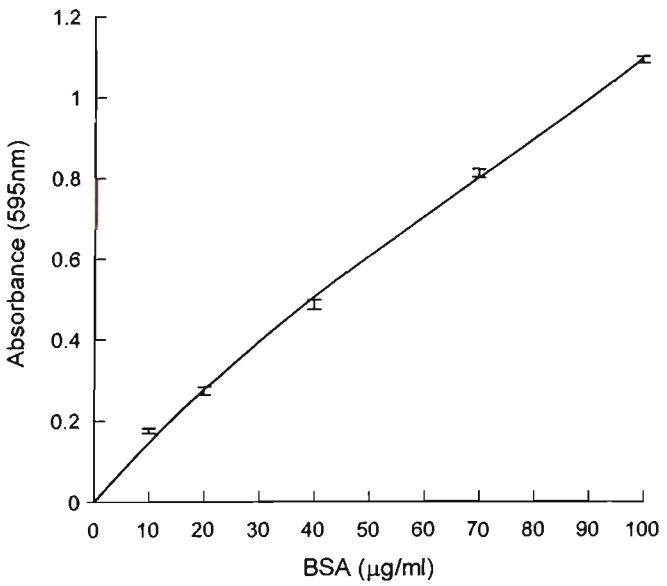

Fig. 2. Standard curve used for the Bradford protein assay.

monly used stains for this purpose. A range of BSA standards (50–400 ng) was resolved on a 7.5% SDS-PAGE gel and the gel was stained with CBB. The CBB-stained protein gel image was captured on Fujifilm LAS1000 CCD (**Fig. 3A**), and the OD of the stained bands of BSA was determined with ImageGauge Analysis densitometry software, version 3.121. The intensity of CBB-stained BSA protein bands was found to be proportional to the concentration of protein in the respective bands, and from approximately 50–300 ng of BSA, the relationship between intensity and protein concentration was almost linear (**Fig. 3B**).

The concentration of PARP-1 stock solution appeared to be at the detection limits for CBB-stained gels. Nonetheless, ImageGauge densitometry analysis, using the complete area of each band on the gel (**Fig. 3B**), gave a value of 60 ng of protein. This would equate to a concentration of approximately 6 µg/mL in the PARP-1 stock solution, a value that more closely reflects that determined by amino acid analysis. Thus, the estimated concentration of PARP-1 in the stock solution was considerably lower than had previously been estimated with the Lowry and Bradford protein assays for the same preparation.

Silver stain can detect as little as 1 ng of protein in a band on an SDS-PAGE gel, and is therefore much more sensitive than the CBB stain *(13)*. However, silver stain does not have a large linear dynamic range for quantifying protein concentration, and there is huge protein-to-protein variation that makes silver staining generally a poor choice of method for estimating protein concentration *(15)*. Nonetheless, silver staining can be useful as a means of obtaining crude estimates of protein concentration. From visual inspection of the silver-stained gel used for work described in this paper, we estimated the amount of human PARP-1 present in the band representing this protein to be 50–100 ng (**Fig. 4**). This would equate to 5–10 µg/mL in the stock solution of PARP-1, a result that is consistent with that obtained from the CBB stained gel.

Fluorescent stains are reported to provide the sensitivity of silver staining, but with the added advantage of a large linear dynamic range for determining protein concentration (Product Information. NanoOrange Protein Quantitation Kit). The newly developed SYPRO Orange fluorescent stain has been reported to detect as little as 1 ng of protein in a band on an SDS-PAGE gel *(18)*. The SYPRO Orange reagent becomes fluorescent upon binding to the SDS coat that surrounds proteins in SDS-PAGE gels, and the amount of SYPRO Orange that becomes bound is directly proportional to the amount of SDS-coated protein. Thus, there is little protein-to-protein variation,

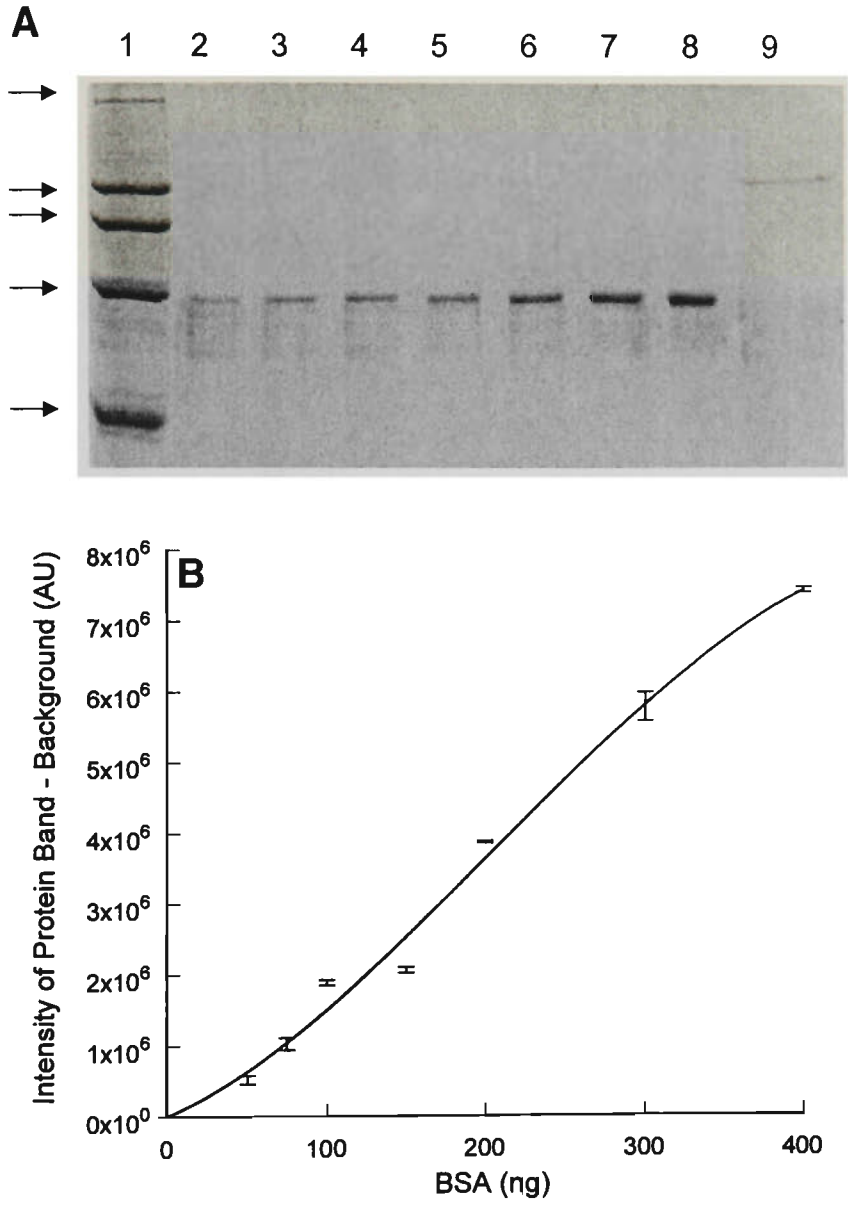

Fig. 3. CBB-stained SDS-PAGE gel (7.5%). Lane 1: Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa). Lanes 2–8: 50, 75, 100, 150, 200, 300, and 400 ng BSA, respectively. Lane 9: 10 µL of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1, and molecular-weight standards were artifacts of electrophoresis (**A**). The OD across the whole area of stained bands of BSA was determined with ImageGauge densitometry analysis software, version 3.121. A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution, using mean ODs obtained from duplicate gels (**B**). All experiments were repeated at least once and gave similar results.

allowing accurate quantitation of purified proteins or protein mixtures *(17)*.

We found that use of the SYPRO Orange stain in conjunction with SDS–PAGE was well suited to estimating PARP-1 concentration. Using densitometry and ImageGauge analysis software version 3.121, we estimated the amount of human PARP-1 present in the SYPRO Orange-stained protein band to be 100 ng, which would equate to a concentration of 10 µg/mL in the PARP-1 stock solution (**Fig. 5A,B**), a value similar to that provided by densitometry analysis of CBB-stained

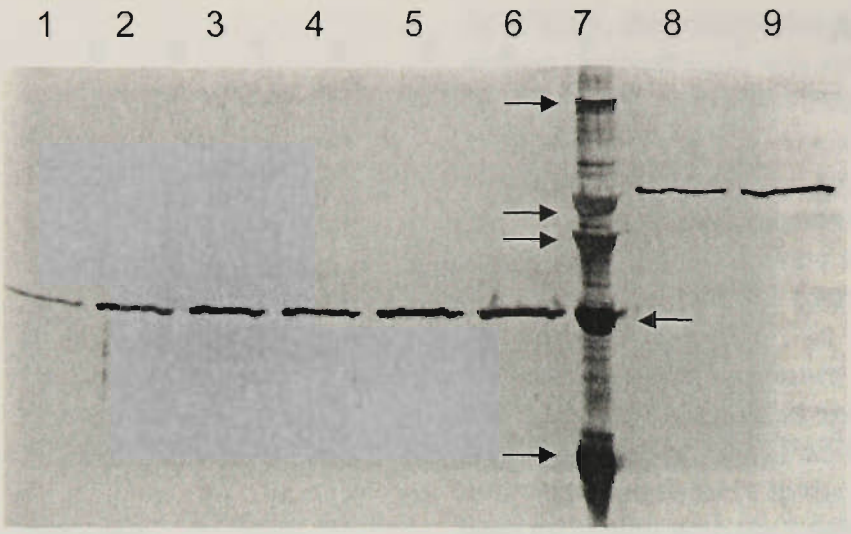

Fig. 4. Silver-stained SDS-PAGE gel (7.5%). *Lanes 1–6:* 25, 50, 75, 100, 150, and 200 ng BSA, respectively. *Lane 7:* Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa. *Lanes 8 and 9:* 10 µL and 20 µL pf PARP-1 stock solution, respectively. The gel was silver stained and captured with a Fujifilm LAS 1000 CCD.

protein gels (*see* **Fig. 3A,B**) and consistent with a visual comparison with silver-stained protein gels (*see* **Fig. 4**) and with the results of amino acid analysis.

We also tried to determine the protein concentration in the PARP-1 stock solution by using the NanoOrange Protein Quantitation Kit (Molecular probes, Eugene, OR). However we found that the levels of fluorescence obtained were extremely variable for both the stock solution of PARP-1 and the BSA standards at concentrations of 10–50 ng/mL (data not shown), thus making it difficult to accurately determine the concentration of human PARP-1 in the stock solution.

4. Conclusions

In this paper we have highlighted the potential for obtaining enormous variation in estimates of protein concentration for a specific protein solution with a range of commonly used techniques when using a different protein as a standard (**Table 2**). This highlights the importance of testing more than one technique for one's research or for routine analytical work. Amino acid analysis provides one of the most sensitive and accurate approaches for estimating protein concentration, but the technology required for this is probably not suitable or appropriate for routine analysis in most laboratories. The next best approach would be to use amino acid analysis to determine the concentration of a stock solution of the protein to be estimated in future work, and to then use this stock solution as a standard. If, however, preparing a stock solution of a known standard is not a viable option, one should consider testing and comparing a range of techniques and possible standards to determine which combination is best suited to the work that is to be undertaken. This report highlights the problem of assuming that any one of the

Table 2
Protein Concentration of Stock Human PARP-1 Solution as Determined with Several Different Assays

Type of Protein Assay	Estimated Concentration of Standard PARP-1 Solution (µg/mL)	Magnitude Difference Compared to Amino Acid Analysis
Amino acid analysis	10	—
Lowry assay	2600	260-fold
Bradford assay	220–240	22–24-fold
CBB gel[a]	6	0.6-fold
SYPRO Orange gel[b]	10	1.0-fold

[a]Coomassie Brilliant Blue stained SDS–PAGE gel.
[b]SYPRO Orange-stained SDS–PAGE gel.

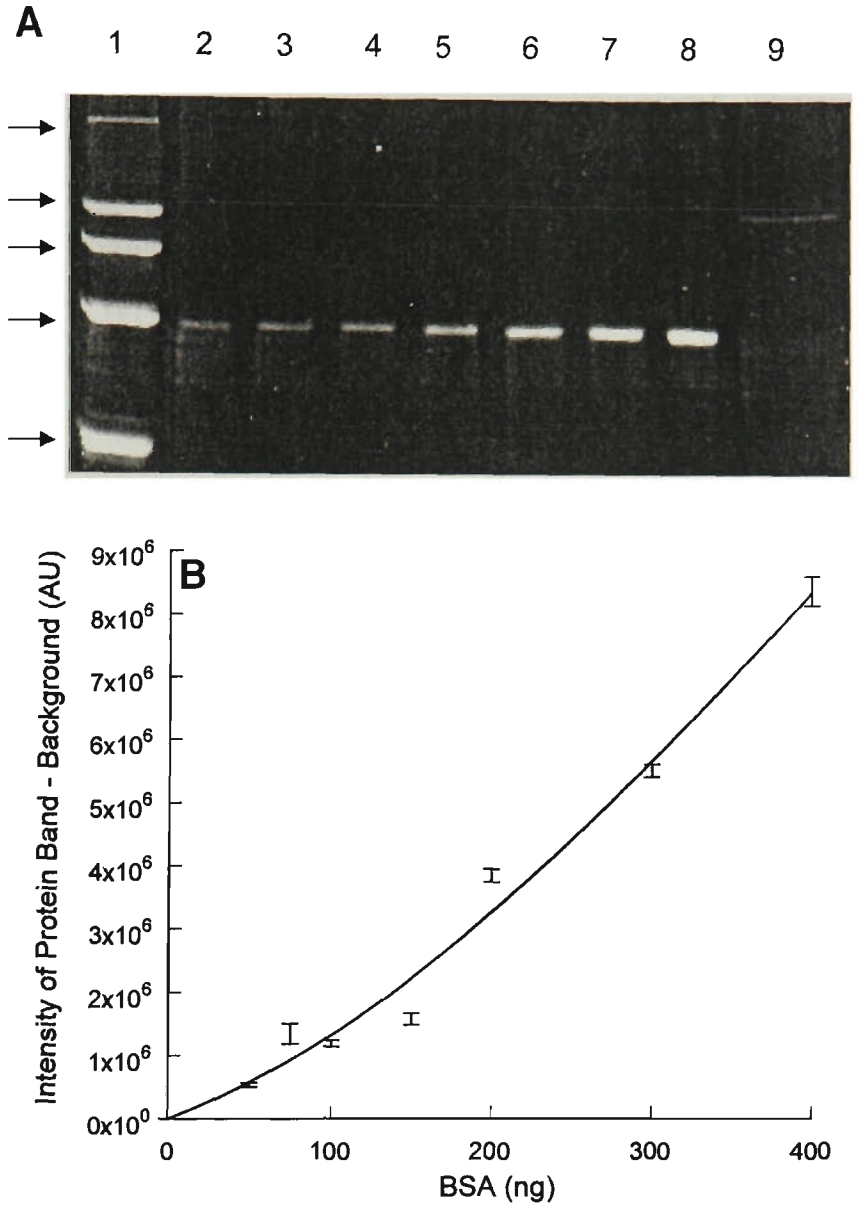

Fig. 5. SYPRO Orange-stained SDS-PAGE gel (7.5%). *Lane 1:* Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa). *Lanes 2–8:* 50, 75, 100, 150, 200, 300, and 400 ng BSA, respectively. *Lane 9:* 10 µL of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1, and molecular weight markers were artifacts of electrophoresis (**A**). The OD across the whole area of stained bands of BSA was determined with ImageGauge densitometry analysis software, version 3.121. A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution, using the mean ODs obtained from duplicate gels (**B**). All experiments were repeated at least once and gave similar results.

commonly used approaches will suffice. On the basis of our results, we suggest that for laboratories lacking access to amino acid analysis, the best method for determining low concentrations of human PARP-1 is quantitation from an SDS–PAGE protein gel stained with SYPRO Orange.

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