Evaluation of in silico approach for prediction of presence of opioid peptides in wheat

Evaluation of *in silico* approach for prediction of presence of opioid peptides in wheat gluten

Abstract

Opioid like morphine and codeine are used for the management of pain, but are associated with serious side-effects limiting their use. Wheat gluten proteins were assessed for the presence of opioid peptides on the basis of tyrosine and proline within their sequence. Eleven peptides were identified and occurrence of predicted sequences or their structural motifs were analysed using BIOPEP database and ranked using PeptideRanker. Based on higher peptide ranking, three sequences YPG, YYPG and YIPP were selected for determination of opioid activity by cAMP assay against μ and κ opioid receptors. Three peptides inhibited the production of cAMP to varied degree with EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM for μ-opioid receptor, and 1.9 mM, 1.2 mM and 3.2 mM for κ-opioid receptor, respectively. The study showed that *in silico* approach can be used for the prediction of opioid peptides from gluten.

Keywords: opioid, tyrosine, proline, in-silico, BIOPEP, peptides, peptide ranking

1 Introduction

Opioids, such as morphine and codeine, are the most common clinically used drugs for pain management (Janecka, Fichna, & Janecki, 2004; Teschemacher, 2003; Trescot, Datta, Lee, & Hansen, 2008). These opioids bind to opioid receptors present in the central and peripheral nervous system. However, they are often associated with side-effects like sedation, dizziness, nausea, vomiting, constipation, addiction, tolerance and respiratory depression (Benyamin et al., 2008). Opioids were considered to be alkaloid (derived from opium) only until discovery of endogenous opioid peptides in 1975 (Goldstein, Goldstein, & Cox, 1975; Hughes et al., 1975). These endogenous peptides and their modified forms have shown activity similar to alkaloids (Giordano et al., 2010; Mollica et al., 2014; Mollica et al., 2005; Mollica et al., 2013b; Mollica et al., 2011). However, exogenous opioid peptides or exorphins are naturally derived from food proteins (Stefanucci et al., 2016; Yoshikawa, 2013). These exogenous peptides are of particular interest as they are naturally derived from food, have possibly less side-effects (compared to synthetic drugs) and are inexpensive to produce (Garg, Nurgali, & Mishra, 2016; Udenigwe, Gong, & Wu, 2013). Most known bioactive peptides are small and non-immunogenic, as compared to larger peptides (6-25 amino acids) (Wang, Mejia, & Gonzalez, 2005). Hence, small peptides are researched more for their bioactivity and considered safe (Shahidi & Zhong, 2008).

Generally, bioactive peptides, including opioids, are produced by hydrolysis of food protein during food processing (ripening, fermentation), storage (Choi, Sabikhi, Hassan, & Anand, 2012) and during gastrointestinal (GI) digestion (Garg et al., 2016; Stefanucci et al., 2016). The protein hydrolysate is then tested for bioactivity using *in vitro* and *in vivo* methods. Since these hydrolysates are mixtures of several peptides, their bioactivity results from the additive and synergistic effect of various components present. Bioactive hydrolysates containing mixtures of peptide needs to be fractionated, purified and then tested for bioactivity

(Udenigwe & Aluko, 2012). The whole process of preparing bioactive peptides from native proteins by hydrolysis, separation and fractionation, is tedious, time consuming and the yields are low (Udenigwe, 2014), limiting and/or delaying their use in clinical applications.

Alternatively, bioinformatics tools can be used for predicting the presence of bioactive peptides in proteins (*in silico* approach) (Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López, & Dávila-Ortiz, 2012; Holton, Pollastri, Shields, & Mooney, 2013; Lacroix & Li-Chan, 2012). Using this approach, one can search for potential precursors of bioactive peptides and develop efficient proteolytic enzymes for their release from native protein sequences (Carrasco-Castilla et al., 2012; Udenigwe et al., 2013). In this approach, protein databases, such as, UniProtKB, SwissProt and TrEMBL can be used to access sequences of a food protein, and presence of bioactive peptides can be predicted using peptide databases BIOPEP and Pepbank (Udenigwe, 2014). The BIOPEP application contains a database of biologically active peptide sequences and a program enabling construction of profiles of the potential biological activity of protein fragments, calculation of quantitative descriptors as measures of the value of proteins as potential precursors of bioactive peptides, and prediction of bonds susceptible to hydrolysis by endopeptidases in a protein (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). In fact, it has been successfully used for prediction of bioactive peptides from different food proteins having angiotensin converting enzyme inhibitory (ACE-I) activity (Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009; Dellafiora et al., 2015) and dipeptidyl peptidase-IV inhibitors (DPP-IV) (Lacroix & Li-Chan, 2012; Nongonierma, Mooney, Shields, & FitzGerald, 2014). PeptideRanker is a web based application and can predict the probability of a peptide being bioactive according to their score between 0 and 1 and can assist in the discovery of new bioactive peptides across many functional classes. Generally, any peptide over 0.5 threshold is labelled to be bioactive (Mooney, Haslam, Holton, Pollastri, & Shields,

2013; Mooney, Haslam, Pollastri, & Shields, 2012). Increasing the threshold from 0.5 to 0.8 reduces the number of false positive prediction from 16 to 6 %, however, true positive rates also decrease (Mooney et al., 2012). If predicted probability is close to 1, the probability of peptide to be bioactive is significantly high (Mooney et al., 2012).

Bioinformatics approach is used for identification of structural patterns of peptides of known bioactivities. Presence of tryptophan in a peptide is associated with antioxidant activity (Chuan-Hsiao, Yin-Shiou, Shyr-Yi, & Wen-Chi, 2014) and carboxyl terminal alanine or proline containing peptides are DPP-IV inhibitors (Lacroix & Li-Chan, 2012). However, there is general lack of information for screening opioid peptides using bioinformatics approach. Wheat gluten contains exorphins; A5, A4, B5, B4 and C, having sequences GYYPT, GYYP, YGGW, YGGWL and YPISL, respectively (Fukudome & Yoshikawa, 1992; Zioudrou, Streaty, & Klee, 1979). Most of food derived opioid peptides have tyrosine and proline residues within them (Yoshikawa, 2013). Tyrosine (Y) is present either at the amino terminal or at the second position (as in gluten exorphins GYYPT and GYYP) and acts as part of the message domain to anchor the opioid peptide within the receptor (Heyl et al., 2003; Li et al., 2005). At position 1, Y acts as a dual hydrogen bond donor/acceptor with less acidic hydroxyl groups exhibiting stronger binding to opioid receptors. Moreover, steric bulk in the Y strengthens receptor binding by either a ligand conformational effect or enhanced van der Waals interactions with a loose receptor site (Heyl et al., 2003). Proline (P) acts as a spacer that fixes the peptide shape and induces other residues to assume proper spatial orientation for interacting with the opioid receptor (Cardillo, Gentilucci, Qasem, Sgarzi, & Spampinato, 2002). Peptides containing P also exhibit enhanced resistance to hydrolysis by enzymes of GI tract (Cardillo et al., 2002; Trivedi et al., 2014) and are therefore more likely to be active upon oral administration (Yang et al., 2001).

For peptides to exert opioid activity, they must bind to opioid receptors present within the central and enteric nervous systems. Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and on activation by opioid ligands, they inhibit adenylate cyclase enzyme (Garg et al., 2016; Gupta, Décaillot, & Devi, 2006) thus decreasing the production of cyclic adenosine monophosphate (cAMP) in the cells (Gupta et al., 2006). This decrease in concentration of cAMP in cells is used for screening opioid ligands (Huang, Kehner, Cowan, & Liu-Chen, 2001). This forms the basis of using cell lines transfected with opioid receptors for assaying the activity of peptides and using it for confirmation of peptides selected using bioinformatics approach.

The objective of this study was to search for opioid peptides in wheat gluten proteins based on the presence of tyrosine and proline, and use bioinformatics tools, BIOPEP and PeptideRanker to identity and rank these peptides for likelihood of having opioid activity. The identified peptides were then assayed for opioid activity by cAMP assay for confirmation of their bioactivity.

2 Materials and Methods

2.1 Chemicals and reagents

Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), antibiotic-antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) - pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabiliser and optiplate were from Perkin Elmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin (DAMGO) and dynorphin A and all other

chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated. Custom peptides were synthesized from Mimotopes (Melbourne, Australia) at > 95% purity.

2.2 Cell lines

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding either human μ or κ receptors were a kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent and hygromycin-B (200 μg/mL) was used as a selection agent (Burford et al., 2015).

2.3 In silico analysis

2.3.1 Sequences of wheat gluten proteins

The sequences of wheat storage proteins high molecular weight (HMW) and low molecular weight (LMW) glutenins and gliadins (alpha, gamma and omega) were accessed from UniProt database at http://www.uniprot.org/uniprot/ (Boutet, Lieberherr, Tognolli, Schneider, & Bairoch, 2007). These sequences were then searched for the presence of tri and oligo-peptides containing Y and P amino acids either consecutively or separated by a single amino acid.

2.3.2 Peptide ranking and bioactivity prediction

Occurrence of predicted sequences or their structural motif thereof were analysed in known opioids using BIOPEP database (Minkiewicz et al., 2008). The PeptideRanker (Bioware.ucd.ie) was used to rank the predicted sequences according to bioactivity. A peptide having ranking closer to 1, increases its chances to be bioactive so they were selected to be tested for opioid activity.

2.4 Opioid activity assay

Opioid activity of the peptides was determined on the basis of cAMP assay. Cells were grown and maintained at 37°C in a humidified incubator containing 5% CO_2 in DMEM, 10% FBS, 1% antibiotic-antimycotic and 200 µg/mL hygromycin-B (Burford et al., 2015). Cells were grown to 90% confluency, harvested and resuspended at $2x10^6$ cells/mL in the media (DMEM + FBS + hygromycin B) and 100 µL of cells were seeded into sterile 96 well plates and incubated at 37°C and 5% CO_2 overnight. The culture media in all the wells were replaced with stimulation buffer consisting of PBS, 50 mM IBMX and BSA stabiliser and incubated for 30 minutes before stimulation. Cells were stimulated at different concentrations of peptides in the presence of 10 µM forskolin for 30 minutes. The stimulation buffer containing peptides was then removed and 50 µL of ice cold 100% ethanol was added to each well. Ethanol was then evaporated and 75 µL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1 % BSA) was added to each well and the change in concentration of cAMP in the lysate was determined using Lance cAMP detection kit. 5 µL of lysate containing cAMP was mixed with 5 µL of Alexa flour-647 anti-cAMP antibody (stock antibody diluted at 1:100 in detection buffer provided in kit). Detection mix containing Europium W8044 labelled streptavidin and biotin-cAMP was prepared according to kit instructions and kept at room temperature for 15 minutes. The detection mix (10 µL) was added to each well and incubated for 1 hour before reading. Time-resolved fluorescence (TRF) was detected using an Envision plate reader (Perkin Elmer, Cambridge, MA) with excitation at 337 nm and emission read at 615 nm and 665 nm. DAMGO and dynorphin A were used as positive controls for µ and κ opioid receptors, respectively. Data were analysed and EC_{50} values determined using nonlinear regression analysis to fit a logistic equation using Graph Pad Prism, version 7 (Graph Pad San Diego, California, US).

3 Results and Discussions

3.1 Prediction of opioid peptide sequences in wheat gluten

Wheat is one of the most important cereals consumed globally providing protein and carbohydrates to the diet. The main storage protein of wheat gluten consists of glutenins and gliadins. Table 1 shows the sequence of gluten proteins and the relevant sequences containing Y and P are highlighted in each of the wheat proteins. Peptides sequences YPG, YPTSP, YYPG (from HMW glutenin), YIPP, YPH, YPQ, YPS (from alpha-gliadin), YPH, YVPP (from gamma gliadin) and YPN (from omega gliadin) are identified to have opioid activity (Table 1). The occurrence frequencies of these opioid peptides are 17, 7, 2 and 1 in HMW glutenin, alpha gliadin, gamma gliadin and omega gliadin, respectively. Based on this observation, HMW glutenin is by far a superior source of opioid peptides than the rest of tested proteins.

Table 1. Sequence of wheat storage proteins as obtained by UniProt

Wheat protein	**UniProt accession number**	**Amino acid (AA) sequence**	**AA residue**
HMW glutenin subunit	Q41553	MTKRLVLFAAVVVALVALTAAEGEASGQLQCEREL QEHSLKACRQVVDQQLRDVSPECQPVGGGPVARQY EQQVVVPPKGGSFYPGETTPPQQLQQSILWGIPALLR RYYLSVTSPQQVSYYPGQASSQRPGQGQQEYYLTSP QQSGQWQQPGQGQSGYYPTSPQQSGQKQPGYYPTS PWQPEQLQQPTQGQQRQQPGQGQQLRQGQQGQQS GQGQPRYYPTSSQQPGQLQQLAQGQQGQQPERGQQ GQQSGQGQQLGQGQQGQQPGQKQQSGQGQQGYY PISPQQLGQGQQSGQGQLGYYPTSPQQSGQGQSGY YPTSAQQPGQLQQSTQEQQLGQEQQDQQSGQGRQG	815

		QQSGQRQQDQSGQGQQPGQRQPGYYSTSPQQLGQ GQPRYYPTSPQQPGQEQQPRQLQQPEQGQQGQQPE QGQQGQQQRQGEQGQQPGQGQQGQQPGQGQPGY YPTSPQQSGQGQPGYYPTSPQQSGQLQQPAQGQQP GQEQQGQQPGQGQQPGQGQPGYYPTSPQQSGQEQ QLEQWQQSGQGQPGHYPTSPLQPGQGQPGYYPTSP QQIGQGQQPGQLQQPTQGQQGQQPGQGQQGQQPG EGQQGQQPGQGQQPGQGQPGYYPTSLQQSGQGQQ PGQWQQPGQGQPGYYPTSSLQPEQGQQGYYPTSQQ QPGQGPQPGQWQQSGQGQQGYYPTSPQQSGQGQQ PGQWLQPGQWLQSGYYLTSPQQLGQGQQPRQWLQ PRQGQQGYYPTSPQQSGQGQQLGQGQQGYYPTSPQ QSGQGQQGYDSPYHVSAEHQAASLKVAKAQQLAA QLPAMCRLEGGDALLASQ	
LMW glutenin	Q8W3V5	MKTFLVFALIAVVATSAIAQMETSCISGLERPWQQQPLPP QQSFSQQPPFSQQQQQPLPQQPSFSQQQPPFSQQQPILSQQ PPFSQQQQPVLPQQSPFSQQQQLVLPPQQQQQQLVQQQIP IVQPSVLQQLNPCKVFLQQQCSPVAMPQRLARSQMWQQ SSCHVMQQQCCQQLQQIPEQSRYEAIRAIIYSIILQEQQQG FVQPQQQQPQQSGQGVSQSQQQSQQQLGQCSFQQPQQQ LGQQPQQQQQQVLQGTFLQPHQIAHLEAVTSIALRTLPT MCSVNVPLYSATTSVPFGVGTGVGAY	303
alpha-gliadin	A0A0E3Z527	MKTFLILALLAIVATTATIAVRVPVPQLQPQNPSQQQ PQEQVPLMQQQQQFPGQQEQFPPQQPYPHQQPFPSQ QPYPQPQPFPPQLPYPQTQPFPPQQPYPQPQPQYPQP QQPISQQQAQQQQQQQQILQQILQQQLIPCRDVVLQ QHNIAHASSQVLQQSTYQLVQQLCCQQLWQIPEQS RCQAIHNVVHAIILHQQQQQQQQQQQQPLSQVSFQ QPQQQYPSGQGSFQPSQQNPQAQGSVQPQQLPQFEE IRNLALETLPAMCNVYIPPYCTIAPVGIFGTN	284
gamma gliadin	P21292	MKTLLILTILAMATTIATANMQVDPSGQVQWPQQQPFPQ PQQPFCQQPQRTIPQPHQTFHHQPQQTFPQPQQTYPHQPQ QQFPQTQQPQQPFPQPQQTFPQQPQLPFPQQPQQPFPQPQ QPQQPFPQSQQPQQPFPQPQQQFPQPQQPQQSFPQQQQPA	302

		IQSFLQQQMNPCKNFLLQQCNHVSLVSSLVSIILPRSDCQ VMQQQCCQQLAQIPQQLQCAAIHSVAHSIIMQQEQQQG VPILRPLFQLAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCN VYVPPDCSTINVPYANIDAGIGGQ	
omega gliadin	Q6PNA3	MKPHHDGYKYTCSIIVTFHYPNFKHQDQKHQFQESIKHK SKMKTFIIFVLLSMPMSIVIAARHLNPSDQELQSPQQQFLE KTIISAATISTSTIFTTTTISHTPTIFPPSTTTTISPTPTTNPPTT TMTIPLATPTTTTTFSPAPTTISLATTTTISLAPTTNSPITTT TIPAATPETTTTIPPATRTNNYASTATTISLLTATTTPPATP TTILSATTTTISPAPTIISPATRTNNSLATPTTIPPATATTIPP ATRTNNSPATATTIPPAPQQRFPHTRQKFPRNPNNHSLCS THHFPAQQPFPQQPGQIIPQQPQQPLPLQPQQPFPWQPEQ RSSQQPQQPFSLQPQQPFS	354

181 **:** predicted opioid peptide sequences

182

183 **3.2 Selection of opioid peptides using BIOPEP database and PeptideRanker**

184 BIOPEP is a database of biologically active peptide sequences and a tool for evaluation of
185 proteins as the precursor of bioactive peptides. The database was used to compare the
186 presence of structural motifs comprising of di or tripeptides in identified peptides in
187 comparison to known opioid peptides (Table 2). The peptides were classified according to
188 different search terms and for every sequence within the class, EC_{50} values are reported. YYP
189 (part of YYPG) structural motif is present in eight opioid peptides with EC_{50} as low as 60 µM
190 (gluten A5 exorphin) (Table 2). Similarly, five known opioid peptides contain structural
191 motif IP (part of YIPP), whereas Casoxin C, containing YIP (part of YIPP) is an antagonist.
192 YVP is present in only one known opioid peptide. YP (part of YPG, YPQ, YPH, and YPN)
193 was present in 92 out of 156 (nearly 60%) opioid peptides present. It is also realised that YP
194 can be present at amino or carboxyl terminals or in the middle of peptide sequences and may
195 be followed either by aromatic or non-aromatic amino acids. Majority of opioid peptides have
196 aromatic amino acid tryptophan (W), phenylalanine (F) or tyrosine (as in YPYY) and had
197 EC_{50} value as low as 0.01 µM (YPFGFR, YPFGFRG) and 0.02 µM (YPFGFS, YPFGFK,

198 YPFGFG, YPFGFGG, YPFGFKG and YPFGFSG) making these very effective opioids.
199 Non-aromatic amino acids are also present as isoleucine (I) in YPISL, leucine (L) in YPLG
200 and YPLSL, serine (S) in YPSYGLN, YPSF and YPS, valine (V) in YPVSL, alanine (A) in
201 YPASL, threonine (T) in YPTSL and YPTS (Table 2). EC_{50} values of YPISL, YPVSL and
202 YPASL were 13.50 µM, 200 µM and 200 µM, respectively. This implies that peptides having
203 YP structural motif followed by non-aromatic amino acids as predicted in Table 1 (YPG,
204 YPQ, YPH, YPT, YPS and YPN) may have opioid activity. With limited information
205 available of EC_{50} value of the sequences (Table 2), it is also evident that within the search
206 term, the opioid activity is dependent on chain length of the peptide sequence. For example,
207 high EC_{50} values of sequences YYPT (1000µM) and YYP (800µM) are suggestive of their
208 weak or negligible opioid activity. Similarly, it can also be stated that casein are superior to
209 gluten as a protein source for the opioid peptides as the EC_{50} values of peptides containing
210 YP motif of casomorphin are significantly lower.
211
212
213
214

215 **Table 2 Opioid peptides as predicted by BIOPEP database**

	Name	Sequence	Molecular mass	EC_{50} (μM)
Search term – YYP by sequence				
1	gluten A5 exorphin	GYYPT	599.61	60
2	gluten A4 exorphin	GYYP	498.50	70
3		YYPT	542.56	800
4		YYP	441.45	1000
5		RYYP	597.64	190
6		WYYP	627.67	n.d
7		SYYP	528.53	200
8		GYYPTS	686.69	72
Search term – IP by sequence				
1		FGGFTGRIPKLWDNQ	1735.95	n.d
2		YPFVEPIP	961.10	n.d
3		YPFPGPIP	887.02	n.d
4	casoxin C *	YIPIQYVLSR	1251.46	50
5	β-casomorphin-11 (60-70)	YPFPGPIPNSL	1201.37	10
Search term – YVP by sequence				
1	fragment of human as1-casein	YVPFP	621.71	n.d
Search term - YP by sequence				
1	VV-hemorphin-7	VVYPWTQRF	1194.60	34.3
2	VV-hemorphin-5	VVYPWTQ	891.43	78.2
3	oryzatensin*	GYPMYPLPR	1092.52	n.d
4		YPFT	526.23	n.d
5		LVVYPWTQR	1160.62	n.d

6		YPFVEP	750.34	n.d
7		YPFV	524.25	n.d
8		YPFVEPIP	960.48	n.d
9		YPFVE	653.29	n.d
10	opioid peptide	YPFP	522.23	n.d
11	opioid peptide	YPFPGPIP	886.44	n.d
12	opioid peptide	YPF	425.18	n.d
13	opioid fragment of dermorphin	YAFGYPS	803.33	n.d
14	opioid fragment of β-lipotropin β-neoendorphin	YGGFLRKYP	1099.56	n.d
15	opioid fragment of β-lipotropin β/α-neoendorphin	YGGFLRKYPK	1227.66	n.d
16	LVV-hemorphin-7	LVVYPWTQRF	1307.68	29.1
17	LVV-hemorphin-5	LVVYPWTQ	1004.51	80.5
18	hemorphin-8	YPWTQRFF	1143.53	4.6
19	hemorphin-7	YPWTQRF	996.46	2.9
20	hemorphin-6	YPWTQR	849.40	4.3
21	hemorphin-5	YPWTQ	693.29	46.3
22	hemorphin-4	YPWT	565.24	45.2
23	gluten C exorphin	YPISL	591.31	13.5
24	gluten A5 exorphin	GYYPT	599.24	60
25	gluten A4 exorphin	GYYP	498.19	70
26	gliadin 2 exorphin	YPLG	448.22	n.d
27	casoxin from bovine kappa-casein fr: 35-41	YPSYGLN	812.35	n.d
28	casoxin (fr.33-38 of bovine kappa-casein) *	SRYPSY	771.34	n.d
29	Casoxin	YPYY	604.24	n.d
30	β-casomorphin	YPSF	512.21	n.d

31	β-casomorphin-11 (60-70)	YPFPGPIPNSL	1200.60	10
32	β-casomorphin-7 (60-66)	YPFPGPI	789.39	14
33	β-casomorphin-5 (60-64)	YPFPG	579.25	1.1
34		YPFGFF~	775.35	0.06
35		YPFGFE~	757.33	0.12
36		YPFGFW~	814.36	0.09
37		YPFGFCQ~	756.34	0.08
38		YPFGFD~	743.31	0.07
39		YPFGFV~	727.35	0.05
40		YPFGFS~	715.32	0.02
41		YPFGFL~	741.37	0.05
42		YPFGFT~	729.33	0.04
43		YPFGFI~	741.37	0.04
44		YPFGFY~	791.35	0.04
45		YPFGFH~	765.34	0.04
46		YPFGFM~	759.32	0.03
47		YPFGFQ~	756.34	0.03
48		YPFGFP~	725.34	0.03
49		YPFGFA~	699.32	0.03
50		YPFGFK~	756.38	0.02
51		YPFGFG~	685.30	0.02
52		YPFGFR~	784.38	0.01
53		YPFGFN~	742.33	0.03
54		YPFGFGG	743.31	0.02
55		YPFGFNG	800.33	0.03
56		YPFGFAG	757.33	0.03

57	YPFGFQG	814.35	0.03
58	YPFGFKG	814.38	0.02
59	YPFGFMG	817.33	0.03
60	YPFGFRG	842.39	0.01
61	YPFGFSG	773.32	0.02
62	YPFGFDG	801.31	0.07
63	YPFGFEG	815.33	0.12
64	YPFGFPG	783.34	0.03
65	YPFGFCQG	814.35	0.08
66	YPFGFFG	833.36	0.06
67	YPFGFVG	785.36	0.05
68	YPFGFLG	799.37	0.05
69	YPFGFTG	787.34	0.04
70	YPFGFIG	799.37	0.04
71	YPFGFYG	849.35	0.04
72	YPFGFHG	823.35	0.04
73	YPFGFWG	872.37	0.09
74	YYPT	542.22	800
75	YYP	441.17	1000
76	YPVSL	577.29	200
77	YPLSL	591.31	200
78	YPASL	549.26	n.d
79	YPTSL	579.27	n.d
80	YPFSL	625.29	70
81	RYYP	597.27	190
82	YPWSL	664.31	70

83		WYYP	627.25	n.d
84		SYYP	528.21	200
85		GYYPTS	686.27	72
86		YPFW~	610.27	n.d
87		YPFWG	668.28	n.d
88		YPFF~	571.26	n.d
89		YPFFG	629.27	n.d
90	soymorphin-5	YPFVV	623.31	6
91	soymorphin-6	YPFVVN	737.36	9.2
92	soymorphin-7	YPFVVNA	808.39	13

* Opioid antagonist, n.d. = not defined

PeptideRanker is a web based application that predicts probability of the peptide to be bioactive (Mooney et al., 2013). In this study, PeptideRanker was used to rank bioactivity of peptides as identified from Table 1 and compared to known gluten exorphins (opioid peptides from wheat) and presented in Table 3. PeptideRanking of known gluten exorphins varied from 0.55 to 0.96 (Table3) and exorphins A4, B4 and B5 show higher likelihood of bioactivity based on high ranking than exorphins A5 and C. The ranking for the unknown 11 peptides as identified from Table 1 varied between 0.38 - 0.83. Only 3 out of 11 peptides, YPG, YYPG and YIPP were ranked > 0.77. These 3 peptides were used for confirmation of opioid activity.

227 **Table 3.** Ranking of the predicted peptide sequences obtained by PeptideRanker
228 (bioware.ucd.in) as compared to known exorphins from gluten

Known peptides	**Peptide ranking**
GYYPT (exorphin A5)	0.58
GYYP (exorphin A4)	0.8
YGGWL (exorphin B5)	0.96
YGGW (exorphin B4)	0.96
YPISL (exorphin C)	0.55
Predicted sequences from Table 1	
YPG	0.83
YYPG	0.78
YIPP	0.77
YVPP	0.52
YPH	0.59
YPISP	0.52
YPTSP	0.41
YPQ	0.47
YPS	0.44
YPN	0.55
YPT	0.38

229

230 **3.3 Assessment of wheat protein derived peptides for opioid activity**

231 Based on the *in silico* analysis, peptides YPG, YYPG and YIPP are opioid and should bind to
232 opioid receptors to exert bioactivity. The activity of these peptides was confirmed by using

cyclic AMP assay, which is based on inhibition of the adenylate cyclase enzyme. The decreasing concentration of cAMP within the cells is taken to be an indication of a positive test and used in determination of activity expressed as EC_{50} values. All three peptides - YPG, YYPG and YIPP inhibited production of cAMP in the presence of 10 µM forskolin in cells expressing µ and κ opioid receptors (Figures 1, 2). Decreases in concentration of cAMP is graphed against concentration of peptides using GraphPad Prism 7 software and EC_{50} of the peptides were calculated from a sigmoid response curve. Calculated EC_{50} values of all tested peptides was greater than 1.0 mM for both µ and κ receptors indicating that a high dose of these peptides is required for them to exert opioid activity. For µ opioid receptor, EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM respectively, while for κ opioid receptor, EC_{50} values were 1.8 mM, 1.2 mM and 3.2 mM, respectively. For both receptors, YYPG had the lowest EC_{50} value, and is more potent opioid peptide than either YPG or YIPP which can be due to presence of 2 Y residues in the peptide. Also, these peptides have higher affinity to κ opioid receptors than for µ opioid receptors. As shown in Table 2, YYP has EC_{50} of 1 mM, and presence of glycine (G) at the amino terminal end in GYYP (gluten exorphin A4) decreased its EC_{50} value to 70µM making it more effective peptide than YYP. Presence of non-aromatic amino acid (Threonine, T) at the carboxyl terminal (YYPT) also decreased its EC_{50} value to 800 µM but presence of G at the carboxyl terminal (YYPG) did not decrease EC_{50} value. Despite of these peptides binding to opioid receptors (µ and κ), they are not as effective (higher EC_{50} values) in their native form and therefore need modification to improve their binding and agonistic activities. For example, Torino et al., (2010) reported improvement in opioid activity though modification of native endomorphine-2. Further research is required to find if these peptides are adsorbed intact or pass the blood brain barrier (BBB) to make them effective for clinical application. Small size of predicted peptide assures that these peptides can pass the GI tract (De Noni et al., 2009). Use of D-amino acids, β-

amino acids, various types of synthetic residues and backbone cyclization can improve stability against enzymatic hydrolysis (Mollica et al., 2013b) and therefore modify their fate in human system. However, even if these peptides may not be able to pass BBB or absorbed, they can also stimulate the brain by brain-gut axis (Stefanucci et al., 2016). They are still worth further investigation for further pharmaceutical development because of their high selectivity and low toxicity (Garg et al., 2016; Mollica, Pinnen, Azzurra, & Costante, 2013a; Stefanucci et al., 2016).

Conclusion

Bioinformatics approach was used in identification of opioid peptides from wheat gluten proteins. Structural motifs, as particular amino acids or their combinations responsible for opioid bioactivity were identified. Using tyrosine and proline residues in the peptide sequences as predictors of opioid peptides, HMW glutenin was found to be the best source of opioid peptides. It was predicted that eleven peptides from wheat gluten could have opioid activity, out of which YPG, YYPG and YIPP were selected as these showed higher ranking of 0.83, 0.78 and 0.77, respectively. The activity of these predicted peptides were determined using cAMP assay in cell lines expressing opioid receptors. Based on the lowest EC_{50} value, YYPG is more potent opioid than YPG or YIPP. The study shows that bioinformatics tools can assist in screening opioid peptides from gluten proteins. This approach can be a cost effective for selection and comparison of proteins as source for production of opioid peptides.

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'Conflicts of interest: none'.

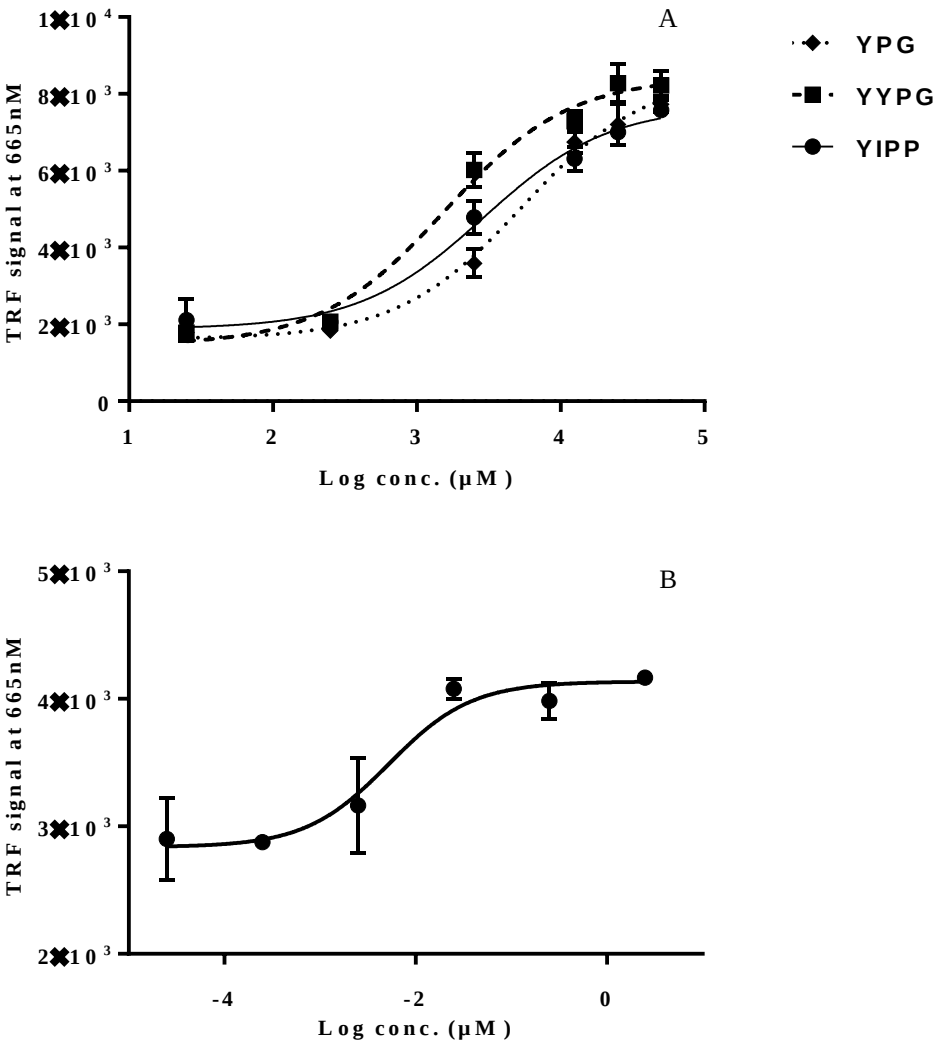

Figure 1. Dose response curves of three peptides (A) and DAMGO (B) against μ-opioid receptors based on inhibition of adenylate cyclase as depicted by TRF signal measured at 665 nm.

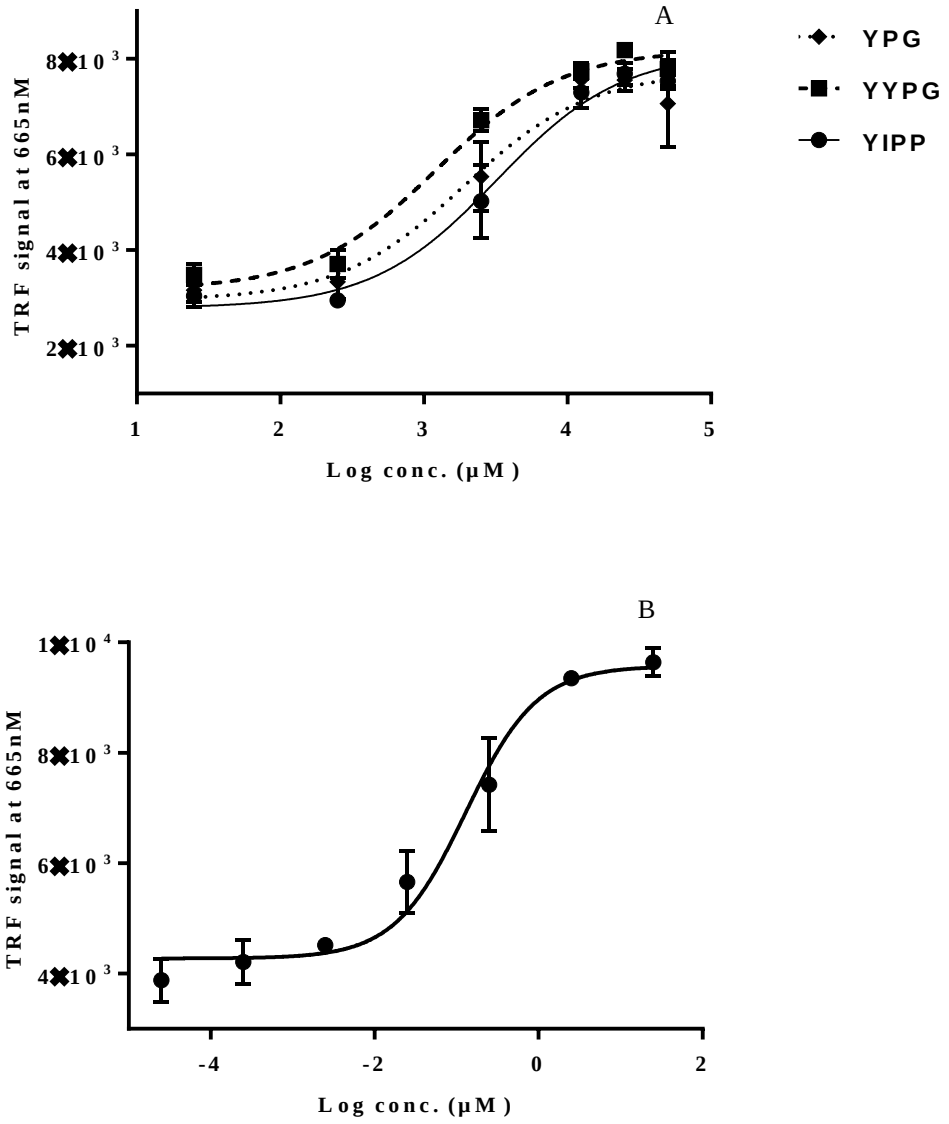

Figure 2. Dose response curves of three peptides (A) and dynorphin A (B) against κ-opioid receptors based on inhibition of adenylate cyclase as depicted by TRF signal measured at 665 nm.

References

Benyamin, R., Trescot, A. M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., Glaser, S. E., & Vallejo, R. (2008). Opioid complications and side effects. *Pain Physician, 11*(2 Suppl), S105-S120.

Boutet, E., Lieberherr, D., Tognolli, M., Schneider, M., & Bairoch, A. (2007). UniProtKB/Swiss-Prot: the manually annotated section of the UniProt KnowledgeBase. *Plant Bioinformatics: Methods and Protocols*, 89-112.

Burford, N. T., Livingston, K. E., Canals, M., Ryan, M. R., Budenholzer, L. M. L., Han, Y., Shang, Y., Herbst, J. J., O'Connell, J., Banks, M., Zhang, L., Filizola, M., Bassoni, D. L., Wehrman, T. S., Christopoulos, A., Traynor, J. R., Gerritz, S. W., & Alt, A. (2015). Discovery, synthesis, and molecular pharmacology of selective positive allosteric modulators of the δ-opioid receptor. *Journal of Medicinal Chemistry, 58*(10), 4220-4229.

Cardillo, G., Gentilucci, L., Qasem, A. R., Sgarzi, F., & Spampinato, S. (2002). Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhanced enzymatic hydrolysis resistance. *Journal of Medicinal Chemistry, 45*(12), 2571-2578.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Gutiérrez-López, G. F., & Dávila-Ortiz, G. (2012). Use of proteomics and peptidomics methods in food bioactive peptide science and engineering. *Food Engineering Reviews, 4*(4), 224-243.

Cheung, I. W., Nakayama, S., Hsu, M. N., Samaranayaka, A. G., & Li-Chan, E. C. (2009). Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (Avena sativa) proteins by in silico and in vitro analyses. *Journal of Agricultural and Food Chemistry, 57*(19), 9234-9242.

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12.

Chuan-Hsiao, H., Yin-Shiou, L., Shyr-Yi, L., & Wen-Chi, H. (2014). Antioxidant and antiglycation activities of the synthesised dipeptide, Asn-Trp, derived from computer-aided simulation of yam dioscorin hydrolysis and its analogue, Gln-Trp. *Food Chemistry, 147*, 195-202.

De Noni, I., FitzGerald, R. J., Korhonen, H. J., Le Roux, Y., Livesey, C. T., Thorsdottir, I., Tomé, D., & Witkamp, R. (2009). Review of the potential health impact of β-casomorphins and related peptides. *EFSA Science Report, 231*, 1-107.

Dellafiora, L., Paolella, S., Dall'Asta, C., Dossena, A., Cozzini, P., & Galaverna, G. (2015). Hybrid in silico/in vitro approach for the identification of angiotensin I converting enzyme inhibitory peptides from Parma dry-cured ham. *Journal of Agricultural and Food Chemistry, 63*(28), 6366–6375.

Fukudome, S.-i., & Yoshikawa, M. (1992). Opioid peptides derived from wheat gluten: their isolation and characterization. *FEBS Letters, 296*(1), 107-111.

Garg, S., Nurgali, K., & Mishra, V. K. (2016). Food proteins as source of ppioid peptides-A review. *Current Medicinal Chemistry, 23*(9), 893-910.

Giordano, C., Sansone, A., Masi, A., Lucente, G., Punzi, P., Mollica, A., Pinnen, F., Feliciani, F., Cacciatore, I., & Davis, P. (2010). Synthesis and activity of

endomorphin-2 and morphiceptin analogues with proline surrogates in position 2. *European Journal of Medicinal Chemistry, 45*(10), 4594-4600.

Goldstein, A., Goldstein, J. S., & Cox, B. M. (1975). A synthetic peptide with morphine-like pharmacologic action. *Life Sciences, 17*(11), 1643-1654.

Gupta, A., Décaillot, F. M., & Devi, L. A. (2006). Targeting opioid receptor heterodimers: strategies for screening and drug development. *The AAPS Journal, 8*(1), E153-E159.

Heyl, D. L., Schullery, S. E., Renganathan, K., Jayamaha, M. N., Rodgers, D. W., & Traynor, J. R. (2003). pKa and volume of residue one influence δ/μ opioid binding: QSAR analysis of tyrosine replacement in a nonselective deltorphin analogue. *Bioorganic and Medicinal Chemistry, 11*(17), 3761-3768.

Holton, T. A., Pollastri, G., Shields, D. C., & Mooney, C. (2013). CPPpred: prediction of cell penetrating peptides. *Bioinformatics, 29*(23), 3094-3096.

Huang, P., Kehner, G. B., Cowan, A., & Liu-Chen, L.-Y. (2001). Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *Journal of Pharmacology and Experimental Therapeutics, 297*(2), 688-695.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., & Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature, 258*(5536), 577-580.

Janecka, A., Fichna, J., & Janecki, T. (2004). Opioid receptors and their ligands. *Current Topics in Medicinal Chemistry, 4*(1), 1-17.

Lacroix, I. M., & Li-Chan, E. C. (2012). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an in silico approach. *Journal of Functional Foods, 4*(2), 403-422.

Li, T., Fujita, Y., Tsuda, Y., Miyazaki, A., Ambo, A., Sasaki, Y., Jinsmaa, Y., Bryant, S. D., Lazarus, L. H., & Okada, Y. (2005). Development of potent μ-opioid receptor ligands using unique tyrosine analogues of endomorphin-2. *Journal of Medicinal Chemistry, 48*(2), 586-592.

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., & Darewicz, M. (2008). BIOPEP database and other programs for processing bioactive peptide sequences. *Journal of AOAC International, 91*(4), 965-980.

Mollica, A., Carotenuto, A., Novellino, E., Limatola, A., Costante, R., Pinnen, F., Stefanucci, A., Pieretti, S., Borsodi, A., & Samavati, R. (2014). Novel cyclic biphalin analogue with improved antinociceptive properties. *ACS Medicinal Chemistry Letters, 5*(9), 1032-1036.

Mollica, A., Davis, P., Ma, S.-W., Lai, J., Porreca, F., & Hruby, V. J. (2005). Synthesis and biological evaluation of new biphalin analogues with non-hydrazine linkers. *Bioorganic & Medicinal Chemistry Letters, 15*(10), 2471-2475.

Mollica, A., Pinnen, F., Azzurra, S., & Costante, R. (2013a). The Evolution of Peptide Synthesis: From Early Days to Small Molecular Machines. *Current Bioactive Compounds, 9*(3), 184-202.

Mollica, A., Pinnen, F., Costante, R., Locatelli, M., Stefanucci, A., Pieretti, S., Davis, P., Lai, J., Rankin, D., & Porreca, F. (2013b). Biological active analogues of the opioid

peptide biphalin: mixed α/β3-peptides. *Journal of Medicinal Chemistry*, *56*(8), 3419-3423.

Mollica, A., Pinnen, F., Feliciani, F., Stefanucci, A., Lucente, G., Davis, P., Porreca, F., Ma, S.-W., Lai, J., & Hruby, V. J. (2011). New potent biphalin analogues containing p-fluoro-L-phenylalanine at the 4, 4′ positions and non-hydrazine linkers. *Amino Acids, 40*(5), 1503-1511.

Mooney, C., Haslam, N. J., Holton, T. A., Pollastri, G., & Shields, D. C. (2013). PeptideLocator: Prediction of bioactive peptides in protein sequences. *Bioinformatics, 29*(9), 1120-1126.

Mooney, C., Haslam, N. J., Pollastri, G., & Shields, D. C. (2012). Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PLOS One, 7*(10), 1-12.

Nongonierma, A. B., Mooney, C., Shields, D. C., & FitzGerald, R. J. (2014). In silico approaches to predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV) inhibitors. *Peptides, 57*, 43-51.

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International, 91*(4), 914-931.

Stefanucci, A., Mollica, A., Macedonio, G., Zengin, G., Ahmed, A. A., & Novellino, E. (2016). Exogenous opioid peptides derived from food proteins and their possible uses as dietary supplements: A critical review. *Food Reviews International*, 1-17.

Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design, 9*(16), 1331-1344.

Torino, D., Mollica, A., Pinnen, F., Feliciani, F., Lucente, G., Fabrizi, G., Portalone, G., Davis, P., Lai, J., & Ma, S.-W. (2010). Synthesis and evaluation of new endomorphin-2 analogues containing (Z)-α, β-didehydrophenylalanine (ΔZPhe) Residues. *Journal of Medicinal Chemistry, 53*(11), 4550-4554.

Trescot, A. M., Datta, S., Lee, M., & Hansen, H. (2008). Opioid pharmacology. *Pain Physician, 11*(2 Suppl), S133-S153.

Trivedi, M. S., Shah, J. S., Al-Mughairy, S., Hodgson, N. W., Simms, B., Trooskens, G. A., Criekinge, W. V., & Deth, R. C. (2014). Food-derived opioid peptides inhibit cysteine uptake with redox and epigenetic consequences. *The Journal of Nutritional Biochemistry, 25*(10), 1011-1018.

Udenigwe, C. C. (2014). Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science & Technology*, *36*(2), 137-143.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science, 77*(1), R11-24.

Udenigwe, C. C., Gong, M., & Wu, S. (2013). In silico analysis of the large and small subunits of cereal Rubiso as precursors of cryptic bioactive peptides. *Process Biochemistry, 48*(11), 1794-1799.

Wang, W., Mejia, D., & Gonzalez, E. (2005). A new frontier in soy bioactive peptides that may prevent age- related chronic diseases. *Comprehensive Reviews in Food Science and Food Safety, 4*(4), 63-78.

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., & Yoshikawa, M. (2001). Rubiscolin, a δ selective opioid peptide derived from plant Rubisco. *FEBS Letters*, *509*(2), 213-217.

Yoshikawa, M. (2013). Exorphins *Handbook of Biologically Active Peptides* (pp. 1570-1576).

Zioudrou, C., Streaty, R. A., & Klee, W. A. (1979). Opioid peptides derived from food proteins. The exorphins. *Journal of Biological Chemistry*, *254*(7), 2446-2449.