The effect of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactobacillus acidophilus and Lactobacillus casei

J. Dairy Sci. 96:40–49
http://dx.doi.org/10.3168/jds.2012-5761

The effect of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus acidophilus* and *Lactobacillus casei*

M. M. Ayyash,* F. Sherkat,† and N. P. Shah‡[1]
*School of Biomedical and Health Sciences, Victoria University, Werribee Campus, PO Box 14428, Melbourne, Victoria 8001, Australia
†School of Applied Sciences, RMIT University, PO Box 2476, Melbourne, Victoria 3001, Australia
‡Food and Nutritional Science, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong

ABSTRACT

The aim of this study was to investigate the effect of substitution of NaCl with KCl at different pH levels and salt concentrations on proteinase activity of cell-free extract and cell-free supernatant of the probiotics *Lactobacillus acidophilus* and *Lactobacillus casei*. de Man, Rogosa, and Sharpe broth aliquots were mixed with 2 pure salts (NaCl and KCl) and 2 salt concentrations at 2 concentration levels (5 and 10%), inoculated with *Lactobacillus acidophilus* or *Lactobacillus casei*, and incubated aerobically at 37°C for 22 h. The cultures were then centrifuged at 4,000 × *g* for 30 min, and the collected cell pellets were used to prepare cell-wall proteinases and the supernatants used as a source of supernatant (extracellular) proteinases. The proteolytic activity and protein content of both portions were determined. After incubation of both portions with 3 milk caseins (α-, β-, κ-casein), the supernatants were individually subjected to analysis of angiotensin-converting enzyme (ACE)-inhibitory activity and proteolytic activity using the *o*-phthalaldehyde method. Significant differences were observed in ACE-inhibitory and proteolytic activities between salt substitution treatments of cell-free extract and cell-free supernatant from both probiotic strains at the same salt concentration and pH level.
Key words: salt substitution, *Lactobacillus acidophilus*, *Lactobacillus casei*, KCl

INTRODUCTION

High salt (sodium chloride) content in food products has emerged as a serious problem worldwide. High salt intake is associated with osteoporosis (Heaney, 2006), kidney stones (Massey, 2005), and hypertension, and the latter is directly related to cardiovascular diseases (Kotchen, 2005; Alderman, 2006). The World Health Organization recommends that food manufacturers gradually reduce salt content in their products (World Health Organization, 2007). Among dairy products, cheese contributes to about 4% of Na intake in the United Kingdom (Ash and Wilbey, 2010), 9.2% in France (Meneton et al., 2009), and 5% in Australia (National Health and Medical Research Council, 2003). A simple reduction of NaCl without substitution with other salts adversely affects cheese quality (Reddy and Marth, 1991). Substitution of NaCl with KCl has been used successfully to preserve cheese quality and safety (Reddy and Marth, 1991). Our previous studies on the effect of NaCl substitution with KCl on various cheese types, including Halloumi cheese (Ayyash and Shah, 2010, 2011a), Nabulsi cheese (Ayyash and Shah, 2011b), and low-moisture Mozzarella cheese (Ayyash and Shah, 2011c,d), showed that salt substitution with KCl affected proteinase activity of the bacterial culture, as measured by 5% phosphotungstic acid-soluble nitrogen (PTA-SN), confirming that bacterial proteinases play an important role during cheese ripening by contributing to flavor and texture of cheese (Fox, 2003; McSweeney, 2004). Information is lacking on the effects of salt substitution with KCl on bacterial proteinase activity. Therefore, growing cultures in pure broth (outside of cheese) to examine the effect of salt substitutions on their proteinase activities would provide knowledge valuable in future substitution studies. In a previous study, we evaluated the effects of salt substitution on *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, and a substantial link between salt substitution and proteinase activities was established for these organisms (Ayyash et al., 2012). The authors concluded that salt substitution affected the proteinase activity of starter cultures at each pH level and salt concentration. The aim of the current study was to investigate the effects of full and partial salt substitution on the proteinase activities of cell-free extract and supernatant from 2 probiotic cultures—*Lactobacillus acidophilus* and *Lactobacillus casei*—at different pH levels and salt concentrations, and on production of

Received May 23, 2012.
Accepted September 8, 2012.
[1]Corresponding author: npshah@hku.hk

angiotensin-converting enzyme (**ACE**)-inhibitory peptides after incubation with individual milk caseins at different pH levels and salt concentrations.

MATERIALS AND METHODS

Bacterial Cultivation

Lactobacillus acidophilus 2401 *and Lactobacillus casei* 290 were obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia) as cultures frozen at −80°C. After thawing the frozen stock, the cultures were activated by transferring loopfuls to de Man, Rogosa, and Sharpe (**MRS**) broth (Merck Pty. Ltd., Victoria, Australia) and incubating aerobically at 37°C for 24 h. Three subsequent cultures were carried out before starting the experiment.

Experiment Design

The experimental design of this study is illustrated in Figure 1. Briefly, *L. acidophilus* and *L. casei* were cultured, separately, in pH-modified MRS broth at pH 5.0, 5.5, and 6.0 without salt addition (control). For the salt treatments, MRS was mixed with 4 salt treatments: NaCl only, 1 NaCl:1 KCl, 1 NaCl:3 KCl, and KCl only, individually at 2 salt concentrations (5 and 10%; wt/vol). Each salt treatment batch was divided into 3 equal portions to adjust the pH to 6.0, 5.5, and 5.0. The MRS samples thus prepared were sterilized and aseptically distributed into 50-mL tubes and inoculated by 1% culture, separately. The inoculated tubes were incubated aerobically at 37°C for 22 h followed by centrifugation at 4,000 × *g* for 30 min at 4°C. The clear cell-free supernatant (**CFS**) from each tube was collected and stored individually at −80°C until used for assays. The cell pellets from each tube were washed with PBS (130 m*M* sodium chloride, 10 m*M* sodium phosphate, pH 7.4), and resuspended in 3 mL of PBS containing 20% glycerol and kept at −80°C until needed for assays. To prepare cell-free extract (**CFE**), the frozen cell pellets were thawed and mixed with 300 μL of Cellytic B 10× (Sigma, St. Louis, MO) to rupture the cell walls and extract the cell contents. According to the manufacturer, Cellytic B does not affect the activity of extracted proteins or interfere in further experiments. The mixture was kept at 4°C for 10 min followed by centrifugation at 4,000 × *g*. The collected supernatant was considered as the cell-free extract containing the cell-wall proteinases. All experiments were conducted in triplicate.

Protein Content

The protein contents of CFE and CFS were determined using Bradford reagent and a 96-well plate assay protocol according to the manufacturer's instructions (Sigma-Aldrich, 2011). Briefly, 5 μL of each sample (CFE or CFS) was placed in a well of the 96-well plate (Cellstar, Greiner Bio-One, Monroe, NC) followed by

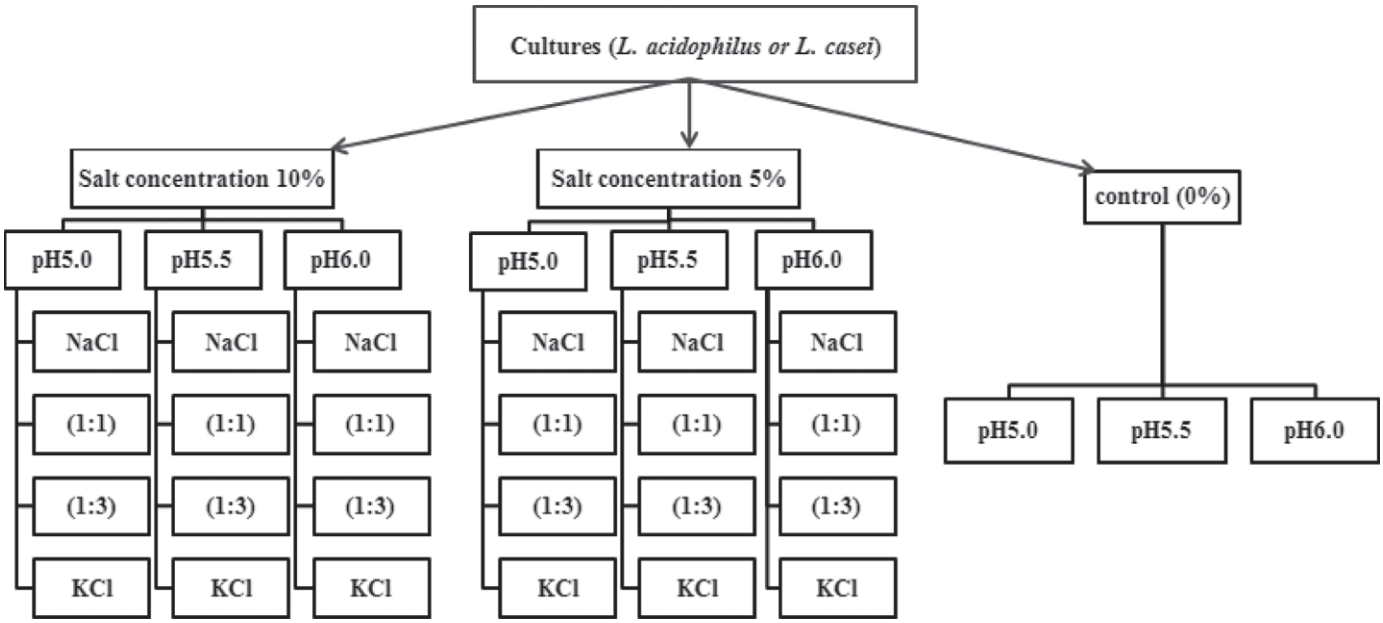

Figure 1. The experimental design of *Lactobacillus acidophilus* and *Lactobacillus casei* culture at 3 pH levels (5.0, 5.5, and 6.0), 2 salt concentrations (5 and 10%), and 4 salt treatments: Control = no salt addition; (1:1) = 1 NaCl:1 KCl; (1:3) = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

250 μL of Bradford reagent (Sigma). The 96-well plate was shaken for 30 s followed by incubation at room temperature for 10 min. Absorbance was measured at 595 nm using a microplate absorbance reader (iMark, Bio-Rad Laboratories Pty., Victoria, Australia). An external protein standard curve ranging from 0.1 to 1.4 mg/mL was prepared using a BSA standard (Sigma).

Proteinase Activity Assay

The proteinase activity of the CFE and CFS was assayed according to Shin et al. (2004) using the chromogenic substrate azocasein. One hundred microliters of specimen was mixed with 100 μL of 2% (wt/vol) azocasein solution prepared in 50 m*M* sodium phosphate buffer (pH 7.0) followed by incubation at 37°C for 16 h, as the enzyme activity was linear over a 16-h assay. Afterward, the reaction was terminated by the addition of 500 μL of 12% (wt/vol) TCA solution and mixed thoroughly. After 15 min at room temperature (21°C), the mixture was centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant (500 μL) was mixed with 500 μL of 1.0 *M* NaOH, and absorbance was measured at 440 nm against a blank without sample. One unit of proteinase activity was defined as the amount of the enzyme that resulted in an increase of 0.01 absorbance unit per hour at 440 nm.

Casein Hydrolysis

An aliquot (100 μL) of CFE or CFS was incubated with 100 μL of α-, β-, or κ-casein (Sigma; 4 mg/mL of 20 m*M* phosphate buffer, pH 6.0) individually and incubated at 37°C for 24 h. Then, the samples were immersed for 2 min into ice to minimize proteinase activity, followed by centrifugation at 12,000 × *g* for 5 min at 4°C. Two 50-μL aliquots were used for subsequent determination of proteolytic activity by *o*-phthalaldehyde (**OPA**) reagent and ACE-inhibitory activity.

OPA Assay

The OPA assay was performed according to Church et al. (1983). Briefly, 50 μL of the casein hydrolysis supernatant was placed into a 1.5-mL cuvette and mixed with 1 mL of OPA reagent [prepared in a 50-mL volumetric flask by dissolving 25 mL of 100 m*M* disodium tetraborate (Merck Pty. Ltd.), 2.5 mL of 20% (wt/wt) SDS (Merck Pty. Ltd.), 40 mg of OPA (Sigma) in 1 mL of methanol (Merck Pty. Ltd.), and 100 μL of β-mercaptoethanol (Sigma), and then making up the volume to 50 mL with Milli-Q water (Millipore, Billerica, MA)]. The cuvette was inverted twice and kept at room temperature (21°C) for 2 min before reading the absorbance at 340 nm using a UV-VIS spectrophotometer [Shimadzu Scientific Instruments (Oceania) Pty. Ltd., Rydalmere, New South Wales, Australia].

ACE-Inhibitory Activity

The ACE-inhibitory activity was measured according to Ayyash and Shah (2011d) using an HPLC method. Angiotensin-converting enzyme and hippuryl-histidyl-leucine were purchased from Sigma and prepared in Tris buffer (50 m*M*, pH 8.3) containing 300 m*M* NaCl. A mixture consisting of 50 μL of 3.0 m*M* hippuryl-histidyl-leucine, 50 μL of 1.25 mU/mL of ACE (from rabbit lung), and 50 μL of pre-hydrolyzed casein supernatant was placed in a glass tube. The mixture was incubated for 30 min at 37°C in a water bath without mixing, followed by an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop the ACE activity. The reaction mixture was kept at −20°C for further analysis by HPLC. The hippuric acid (**HA**) resulting from the previous reaction was determined by HPLC. An external standard curve of HA was prepared to quantify the resultant HA in samples. An aliquot (20 μL) of the mixture was injected onto the HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV-visible tunable absorbance detector, and a 730 data module (Varian Inc., Santa Clara, CA). The system was fitted with a Luna column (C18, 300 mm × 4.6 mm, 3 μm; Phenomenex Australia Pty. Ltd., New South Wales, Australia) with a guard column (C18 4 × 3.0 mm; Phenomenex). The separation was conducted at room temperature (~22°C) at a flow rate of 0.8 mL/min. The mobile phase was an isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck) in MilliQ water (Millipore), and the pH was adjusted to pH 3.0 using glacial acetic acid. The detection device was an UV-visible detector set at 228 nm. The control reaction mixture contained 50 μL of buffer solution instead of the assay sample and was expected to liberate the maximum amount of HA from the substrate due to uninhibited ACE activity. The percentage inhibition of enzyme activity was calculated as follows:

$$\text{Inhibition} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100\%.$$

Statistical Analysis

One-way ANOVA was performed to investigate the effect of salt treatment on proteinase activity of CFE and CFS at the same salt concentration and pH level. Fisher's test (least significant difference) was used to examine the difference between salt treatment means at

Table 1. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus acidophilus* 2401 grown in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 5% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.11 ± 0.00[a]	0.11 ± 0.01[b]	1.11 ± 0.27[a]	0.27 ± 0.01[a]
	1:1	0.09 ± 0.00[a]	0.13 ± 0.00[a]	1.24 ± 0.37[a]	0.27 ± 0.01[a]
	3:1	0.09 ± 0.00[a]	0.13 ± 0.01[a]	1.27 ± 0.03[a]	0.26 ± 0.01[a]
	KCl	0.11 ± 0.00[a]	0.09 ± 0.00[b]	1.26 ± 0.02[a]	0.24 ± 0.01[a]
	NaCl	0.13 ± 0.04[a]	0.11 ± 0.01[b]	1.12 ± 0.07[a]	0.26 ± 0.01[a]
5.5	Control	0.09 ± 0.00[b]	0.13 ± 0.01[a]	0.72 ± 0.18[b]	0.28 ± 0.01[a]
	1:1	0.09 ± 0.00[b]	0.14 ± 0.00[a]	1.12 ± 0.29[ab]	0.27 ± 0.03[a]
	3:1	0.10 ± 0.00[b]	0.14 ± 0.00[a]	1.15 ± 0.21[ab]	0.26 ± 0.00[a]
	KCl	0.12 ± 0.00[a]	0.12 ± 0.00[b]	1.27 ± 0.02[ab]	0.29 ± 0.01[a]
	NaCl	0.09 ± 0.00[b]	0.12 ± 0.00[b]	1.32 ± 0.03[a]	0.29 ± 0.00[a]
6.0	Control	0.10 ± 0.00[ab]	0.14 ± 0.01[ab]	0.89 ± 0.28[a]	0.26 ± 0.01[a]
	1:1	0.09 ± 0.00[c]	0.16 ± 0.02[a]	1.05 ± 0.25[a]	0.26 ± 0.00[a]
	3:1	0.09 ± 0.00[b]	0.14 ± 0.00[ab]	1.10 ± 0.30[a]	0.26 ± 0.00[a]
	KCl	0.08 ± 0.00[d]	0.10 ± 0.00[c]	1.29 ± 0.02[a]	0.27 ± 0.00[a]
	NaCl	0.10 ± 0.00[a]	0.12 ± 0.01[bc]	1.09 ± 0.07[a]	0.26 ± 0.00[a]

[a–c]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

the same salt concentration and pH level. As a factorial design, 2-way and 3-way ANOVA were performed to investigate the effect of interactions of salt treatment × pH, salt treatment × salt concentration, salt concentration × pH, and salt treatment × salt concentration × pH.

RESULTS AND DISCUSSION

Azocasein and Protein Content

The effect of NaCl substitution with KCl on the proteolytic activity and protein content of CFE and CFS from *L. acidophilus* 2401 and *L. casei* 290 obtained at different pH and salt concentrations are shown in Tables 1, 2, 3, and 4.

At a salt concentration of 10% and the same pH level, proteolytic activity of *L. acidophilus* CFE and CFS differed insignificantly ($P > 0.05$) between salt treatments, except for CFS at pH 5.0 (Table 2). However, the differences were significant ($P < 0.05$) at 5% salt and the same pH level (Table 1). This implies that salt concentration affected the proteinase activity of both CFE and CFS of *L. acidophilus*. The azocasein absorbance values of CFS of *L. acidophilus* at both

Table 2. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus acidophilus* 2401 grown in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 10% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.11 ± 0.00[a]	0.11 ± 0.01[b]	1.11 ± 0.27[ab]	0.27 ± 0.01[a]
	1:1	0.08 ± 0.00[a]	0.12 ± 0.00[ab]	0.70 ± 0.38[b]	0.30 ± 0.02[a]
	3:1	0.07 ± 0.00[a]	0.12 ± 0.00[ab]	1.37 ± 0.01[a]	0.29 ± 0.01[a]
	KCl	0.09 ± 0.01[a]	0.11 ± 0.00[b]	1.37 ± 0.03[a]	0.27 ± 0.02[a]
	NaCl	0.27 ± 0.20[a]	0.14 ± 0.01[a]	1.45 ± 0.03[a]	0.29 ± 0.01[a]
5.5	Control	0.09 ± 0.00[a]	0.13 ± 0.01[a]	0.72 ± 0.18[b]	0.28 ± 0.01[a]
	1:1	0.07 ± 0.00[a]	0.12 ± 0.01[a]	1.36 ± 0.03[a]	0.28 ± 0.01[a]
	3:1	0.07 ± 0.00[a]	0.16 ± 0.01[a]	1.36 ± 0.02[a]	0.27 ± 0.01[a]
	KCl	0.10 ± 0.01[a]	0.12 ± 0.00[a]	1.36 ± 0.05[a]	0.27 ± 0.02[a]
	NaCl	0.20 ± 0.13[a]	0.20 ± 0.06[a]	1.47 ± 0.01[a]	0.28 ± 0.01[a]
6.0	Control	0.10 ± 0.00[a]	0.14 ± 0.01[a]	0.89 ± 0.28[b]	0.26 ± 0.01[b]
	1:1	0.08 ± 0.00[a]	0.15 ± 0.01[a]	1.36 ± 0.02[a]	0.27 ± 0.01[b]
	3:1	0.07 ± 0.00[a]	0.13 ± 0.01[a]	1.33 ± 0.09[a]	0.31 ± 0.01[a]
	KCl	0.09 ± 0.00[a]	0.14 ± 0.00[a]	1.38 ± 0.06[a]	0.31 ± 0.01[a]
	NaCl	0.09 ± 0.02[a]	0.16 ± 0.02[a]	1.40 ± 0.02[a]	0.27 ± 0.00[b]

[a,b]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 3. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus casei* 290 growth in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 5% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.09 ± 0.00[a]	0.15 ± 0.01[a]	1.21 ± 0.04[a]	0.28 ± 0.01[a]
	1:1	0.09 ± 0.00[a]	0.13 ± 0.00[a]	1.20 ± 0.08[a]	0.27 ± 0.01[a]
	3:1	0.09 ± 0.00[a]	0.15 ± 0.03[a]	1.27 ± 0.03[a]	0.26 ± 0.01[a]
	KCl	0.09 ± 0.00[a]	0.12 ± 0.01[a]	0.85 ± 0.18[b]	0.27 ± 0.00[a]
	NaCl	0.09 ± 0.00[a]	0.12 ± 0.01[a]	1.24 ± 0.05[a]	0.26 ± 0.00[a]
5.5	Control	0.09 ± 0.00[b]	0.18 ± 0.00[a]	0.29 ± 0.01[b]	0.29 ± 0.01[a]
	1:1	0.10 ± 0.00[ab]	0.14 ± 0.00[b]	1.18 ± 0.09[a]	0.24 ± 0.00[b]
	3:1	0.10 ± 0.00[a]	0.14 ± 0.01[b]	1.30 ± 0.26[a]	0.25 ± 0.01[b]
	KCl	0.10 ± 0.01[a]	0.13 ± 0.00[b]	1.05 ± 0.13[a]	0.27 ± 0.01[a]
	NaCl	0.10 ± 0.00[ab]	0.13 ± 0.01[b]	1.09 ± 0.13[a]	0.28 ± 0.01[a]
6.0	Control	0.09 ± 0.00[ab]	0.19 ± 0.00[a]	1.08 ± 0.12[a]	0.28 ± 0.01[a]
	1:1	0.09 ± 0.00[ab]	0.14 ± 0.00[b]	1.06 ± 0.02[a]	0.25 ± 0.00[c]
	3:1	0.09 ± 0.00[ab]	0.13 ± 0.00[bc]	1.18 ± 0.05[a]	0.25 ± 0.01[c]
	KCl	0.09 ± 0.00[b]	0.11 ± 0.01[d]	0.99 ± 0.12[a]	0.27 ± 0.00[ab]
	NaCl	0.10 ± 0.00[a]	0.12 ± 0.01[cd]	0.96 ± 0.09[a]	0.26 ± 0.01[bc]

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

salt concentrations were lower compared with those of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 (Ayyash et al., 2012).

We observed significant ($P < 0.05$) differences in protein content of *L. acidophilus* CFE and CFS between salt treatments at the same salt concentration and pH level (Tables 1 and 2), except for protein content of CFS at 5% salt (Table 1). It was clear that salt treatment and salt concentration significantly affected protein content; however, further investigation is needed to confirm these findings.

Similar trends were observed for proteolytic activity and protein content of CFE and CFS of *L. casei* (Tables 3 and 4). Significant ($P < 0.05$) differences were observed in proteolytic activity of CFE and CFS of *L. casei* between salt treatments at the same salt concentration and pH level. Thus, substitution of NaCl with KCl significantly affected the proteolytic activity of both enzymes obtained from CFE and CFS. We hypothesize that salt treatment might affect the activity of proteinases or enzyme production by *L. casei*. Armenteros et al. (2009) reported that the presence of

Table 4. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus casei* 290 growth in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 10% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.09 ± 0.00[a]	0.15 ± 0.01[a]	1.21 ± 0.04[b]	0.28 ± 0.01[a]
	1:1	0.07 ± 0.00[b]	0.11 ± 0.00[c]	0.31 ± 0.02[c]	0.27 ± 0.01[ab]
	3:1	0.08 ± 0.00[b]	0.13 ± 0.00[b]	1.29 ± 0.04[b]	0.28 ± 0.01[a]
	KCl	0.09 ± 0.00[a]	0.12 ± 0.00[bc]	1.41 ± 0.01[a]	0.24 ± 0.01[b]
	NaCl	0.07 ± 0.00[b]	0.13 ± 0.00[b]	1.41 ± 0.01[a]	0.27 ± 0.01[ab]
5.5	Control	0.09 ± 0.00[a]	0.18 ± 0.00[a]	0.29 ± 0.01[b]	0.29 ± 0.01[a]
	1:1	0.08 ± 0.00[b]	0.13 ± 0.01[b]	1.36 ± 0.02[a]	0.26 ± 0.01[ab]
	3:1	0.07 ± 0.00[b]	0.14 ± 0.01[b]	1.37 ± 0.02[a]	0.27 ± 0.01[ab]
	KCl	0.09 ± 0.00[a]	0.12 ± 0.00[b]	1.41 ± 0.09[a]	0.23 ± 0.02[b]
	NaCl	0.07 ± 0.00[b]	0.14 ± 0.00[b]	1.44 ± 0.01[a]	0.28 ± 0.01[a]
6.0	Control	0.09 ± 0.00[a]	0.19 ± 0.00[a]	1.08 ± 0.12[b]	0.28 ± 0.01[a]
	1:1	0.09 ± 0.00[a]	0.15 ± 0.00[c]	1.38 ± 0.02[a]	0.25 ± 0.00[b]
	3:1	0.08 ± 0.00[a]	0.13 ± 0.01[d]	1.33 ± 0.07[a]	0.28 ± 0.01[ab]
	KCl	0.10 ± 0.01[a]	0.13 ± 0.00[d]	1.38 ± 0.04[a]	0.28 ± 0.01[ab]
	NaCl	0.11 ± 0.04[a]	0.18 ± 0.00[b]	1.41 ± 0.03[a]	0.27 ± 0.01[ab]

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 5. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.47 ± 0.07^{ab}	0.51 ± 0.02^{b}	0.51 ± 0.06^{b}	0.64 ± 0.02^{c}	0.65 ± 0.00^{b}	0.60 ± 0.02^{a}
	1:1	0.43 ± 0.06^{b}	0.50 ± 0.01^{b}	0.49 ± 0.01^{b}	0.73 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.02^{a}
	3:1	0.42 ± 0.02^{b}	0.45 ± 0.00^{c}	0.47 ± 0.02^{b}	0.68 ± 0.02^{bc}	0.70 ± 0.01^{a}	0.68 ± 0.02^{a}
	KCl	0.59 ± 0.00^{a}	0.57 ± 0.01^{a}	0.66 ± 0.02^{a}	0.71 ± 0.00^{ab}	0.68 ± 0.00^{ab}	0.49 ± 0.21^{a}
	NaCl	0.45 ± 0.01^{b}	0.40 ± 0.01^{d}	0.47 ± 0.03^{b}	0.69 ± 0.01^{ab}	0.69 ± 0.02^{a}	0.67 ± 0.00^{a}
5.5	Control	0.45 ± 0.06^{b}	0.47 ± 0.07^{b}	0.45 ± 0.08^{b}	0.62 ± 0.01^{b}	0.68 ± 0.01^{b}	0.61 ± 0.01^{c}
	1:1	0.41 ± 0.06^{b}	0.48 ± 0.01^{b}	0.44 ± 0.01^{b}	0.75 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.00^{b}
	3:1	0.42 ± 0.04^{b}	0.47 ± 0.01^{b}	0.43 ± 0.02^{b}	0.76 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.01^{b}
	KCl	0.61 ± 0.00^{a}	0.58 ± 0.01^{a}	0.68 ± 0.01^{a}	0.80 ± 0.02^{a}	0.72 ± 0.01^{a}	0.75 ± 0.02^{a}
	NaCl	0.47 ± 0.01^{b}	0.44 ± 0.01^{b}	0.45 ± 0.02^{b}	0.68 ± 0.01^{b}	0.67 ± 0.01^{b}	0.67 ± 0.01^{b}
6.0	Control	0.55 ± 0.03^{a}	0.54 ± 0.00^{a}	0.64 ± 0.00^{a}	0.63 ± 0.01^{c}	0.65 ± 0.01^{a}	0.59 ± 0.00^{c}
	1:1	0.42 ± 0.06^{b}	0.48 ± 0.01^{c}	0.45 ± 0.00^{d}	0.71 ± 0.02^{b}	0.62 ± 0.00^{b}	0.65 ± 0.01^{b}
	3:1	0.42 ± 0.06^{b}	0.48 ± 0.00^{c}	0.44 ± 0.00^{d}	0.76 ± 0.01^{a}	0.62 ± 0.00^{b}	0.67 ± 0.01^{b}
	KCl	0.49 ± 0.03^{ab}	0.54 ± 0.00^{a}	0.60 ± 0.02^{b}	0.73 ± 0.02^{ab}	0.68 ± 0.01^{a}	0.73 ± 0.01^{a}
	NaCl	0.51 ± 0.01^{ab}	0.50 ± 0.01^{b}	0.50 ± 0.00^{c}	0.69 ± 0.00^{b}	0.67 ± 0.02^{a}	0.67 ± 0.01^{b}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

KCl activated some aminopeptidases and inactivated others in meat products.

The azocasein absorbance values of CFS were numerically higher than those of CFE at the same salt concentration, salt treatment, and pH level (Tables 3 and 4). This suggests that CFS may have a greater number of specific proteinases acting on the azocasein substrate compared with CFE.

The protein content of CFE and CFS of *L. casei* showed significant ($P < 0.05$) differences between salt treatments at the same salt concentration and pH level (Tables 3 and 4). As expected, the protein contents of CFE were higher compared with the CFS of *L. casei*. The mechanism of the effect of salt treatment on protein content needs more investigation to explore whether that effect occurred because of salt treatment or other factors.

OPA Activity of Casein Hydrolysis

The OPA absorbance values of the CFE and CFS of *L. acidophilus* with individual milk caseins at 37°C for 24 h are presented in Tables 5 and 6. We observed significant ($P < 0.05$) differences in OPA absorbance values of CFE and CFS from *L. acidophilus* between salt treatments at the same salt concentration and pH level. This suggests that substitution of NaCl with KCl significantly affected the proteinases of both cell wall and supernatant. This result is in agreement with Armenteros et al. (2009), who reported that KCl activated some aminopeptidases and inactivated others.

Tables 5 and 6 show that, at the same pH level and salt treatment, the OPA absorbance values of CFE obtained at 5% salt and incubated with milk caseins were higher ($P < 0.05$) compared with those at 10% salt. This implies that the proteinase activities (especially aminopeptidases) of *L. acidophilus* were significantly ($P < 0.05$) affected by salt concentration (5 and 10%), which in turn affected the free AA produced by these enzymes.

The OPA absorbance values of CFS were significantly ($P < 0.05$) higher with all milk caseins than those of CFE (Tables 5 and 6). This may be attributed to 2 factors: the residual AA in the supernatant after *L. acidophilus* propagation may interfere with the OPA readings, and the proteases of CFS may have greater specificity toward milk caseins compared with CFE proteinases. This could explain the higher free AA produced after incubation with CFS compared with CFE.

Similar trends were observed for OPA absorbance values of *L. casei* (Tables 7 and 8) compared with *L. acidophilus* (Tables 5 and 6). Salt treatment significantly ($P < 0.05$) affected the OPA readings of CFE and CFS at the same salt concentration and pH level (Tables 7 and 8). The control treatment (without salt) at the same pH level with all caseins had higher OPA absorbance compared with other treatments., implying that the proteolytic activities of the proteinases obtained from CFE were significantly affected by salt concentration.

The OPA absorbance values of CFS with all milk caseins were significantly ($P < 0.05$) higher than those of CFE at the same salt concentration, salt treatment, and pH level (Tables 7 and 8). Furthermore, the OPA readings of CFE obtained at 10% salt were lower ($P < 0.05$) compared with those obtained at 5% salt (Tables 7 and 8).

Table 6. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.47 ± 0.07[a]	0.51 ± 0.02[a]	0.51 ± 0.06[a]	0.64 ± 0.02[b]	0.65 ± 0.00[b]	0.60 ± 0.02[c]
	1:1	0.31 ± 0.01[b]	0.29 ± 0.01[b]	0.26 ± 0.01[b]	0.67 ± 0.01[b]	0.65 ± 0.01[b]	0.64 ± 0.01[bc]
	3:1	0.29 ± 0.01[b]	0.29 ± 0.01[b]	0.22 ± 0.01[b]	0.66 ± 0.01[b]	0.64 ± 0.01[b]	0.65 ± 0.01[b]
	KCl	0.45 ± 0.04[a]	0.45 ± 0.04[a]	0.39 ± 0.07[a]	0.82 ± 0.01[a]	0.78 ± 0.01[a]	0.69 ± 0.00[a]
	NaCl	0.28 ± 0.01[b]	0.28 ± 0.00[b]	0.20 ± 0.00[b]	0.66 ± 0.01[b]	0.65 ± 0.00[b]	0.64 ± 0.00[b]
5.5	Control	0.45 ± 0.06[a]	0.47 ± 0.07[a]	0.45 ± 0.08[a]	0.62 ± 0.01[ab]	0.68 ± 0.01[b]	0.61 ± 0.01[c]
	1:1	0.32 ± 0.00[b]	0.31 ± 0.00[b]	0.27 ± 0.01[b]	0.67 ± 0.01[ab]	0.66 ± 0.02[b]	0.64 ± 0.02[bc]
	3:1	0.29 ± 0.01[b]	0.28 ± 0.00[b]	0.22 ± 0.01[b]	0.49 ± 0.20[b]	0.65 ± 0.01[b]	0.65 ± 0.01[b]
	KCl	0.52 ± 0.00[a]	0.51 ± 0.00[a]	0.48 ± 0.01[a]	0.82 ± 0.01[a]	0.78 ± 0.00[a]	0.69 ± 0.00[a]
	NaCl	0.28 ± 0.01[b]	0.27 ± 0.00[b]	0.23 ± 0.01[b]	0.66 ± 0.01[ab]	0.68 ± 0.01[b]	0.63 ± 0.00[bc]
6.0	Control	0.55 ± 0.03[a]	0.54 ± 0.00[a]	0.64 ± 0.00[a]	0.63 ± 0.01[c]	0.65 ± 0.01[b]	0.59 ± 0.00[d]
	1:1	0.31 ± 0.01[b]	0.24 ± 0.01[e]	0.29 ± 0.03[c]	0.66 ± 0.01[b]	0.65 ± 0.01[b]	0.67 ± 0.02[ab]
	3:1	0.27 ± 0.00[b]	0.26 ± 0.00[d]	0.20 ± 0.00[d]	0.65 ± 0.01[bc]	0.63 ± 0.01[b]	0.64 ± 0.01[bc]
	KCl	0.53 ± 0.02[a]	0.52 ± 0.01[b]	0.50 ± 0.01[b]	0.83 ± 0.00[a]	0.78 ± 0.01[a]	0.70 ± 0.01[a]
	NaCl	0.28 ± 0.01[b]	0.29 ± 0.00[c]	0.23 ± 0.01[d]	0.68 ± 0.01[b]	0.66 ± 0.00[b]	0.63 ± 0.00[c]

[a–e]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

ACE-Inhibitory Activity

The ACE-inhibitory activity of peptides released after 24-h incubation of CFE and CFS of *L. acidophilus* (obtained from different salt concentrations, pH, and salt treatments) with 3 milk caseins (α-, β-, and κ-caseins) at 37°C are presented in Tables 9 and 10.

The least significant difference test showed a significant ($P < 0.05$) difference in ACE-inhibitory activity of both CFE and CFS of *L. acidophilus* between salt treatments at the same salt concentration and pH level with all milk caseins. This suggests that substitution of NaCl with KCl may have affected the activity of proteinases of CFE and CFS, which in turn affected the ACE-inhibitory peptides quantitatively and qualitatively. Although changing the pH level significantly affected ACE-inhibitory activity at the same salt concentration and salt treatment, it was inconsistent. Changing salt concentration showed a nonsignificant ($P > 0.05$) effect on ACE-inhibitory activity of both CFE and CFS of *L. acidophilus* with all caseins at the same pH level and salt treatment (Tables 9 and 10).

Tables 9 and 10 show that ACE-inhibitory activity of CFE of *L. acidophilus* with all milk caseins were nu-

Table 7. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.54 ± 0.05[a]	0.51 ± 0.05[a]	0.55 ± 0.05[a]	0.70 ± 0.01[a]	0.64 ± 0.02[a]	0.63 ± 0.00[bc]
	1:1	0.46 ± 0.01[a]	0.46 ± 0.03[ab]	0.37 ± 0.03[b]	0.65 ± 0.00[b]	0.62 ± 0.00[b]	0.62 ± 0.00[c]
	3:1	0.49 ± 0.02[a]	0.45 ± 0.01[ab]	0.41 ± 0.00[b]	0.65 ± 0.01[b]	0.65 ± 0.01[a]	0.63 ± 0.01[bc]
	KCl	0.49 ± 0.00[a]	0.47 ± 0.02[ab]	0.44 ± 0.01[b]	0.72 ± 0.01[a]	0.66 ± 0.06[a]	0.72 ± 0.01[a]
	NaCl	0.49 ± 0.02[a]	0.43 ± 0.01[b]	0.44 ± 0.01[b]	0.66 ± 0.02[b]	0.67 ± 0.01[a]	0.64 ± 0.01[b]
5.5	Control	0.46 ± 0.03[b]	0.48 ± 0.02[b]	0.41 ± 0.03[b]	0.68 ± 0.01[b]	0.63 ± 0.01[b]	0.66 ± 0.01[b]
	1:1	0.50 ± 0.01[ab]	0.48 ± 0.02[b]	0.41 ± 0.03[b]	0.67 ± 0.02[b]	0.62 ± 0.00[c]	0.62 ± 0.00[b]
	3:1	0.50 ± 0.01[ab]	0.49 ± 0.00[ab]	0.42 ± 0.01[b]	0.70 ± 0.02[b]	0.62 ± 0.00[c]	0.62 ± 0.01[b]
	KCl	0.55 ± 0.01[a]	0.52 ± 0.00[a]	0.51 ± 0.01[a]	0.76 ± 0.02[a]	0.72 ± 0.01[a]	0.72 ± 0.02[a]
	NaCl	0.52 ± 0.03[a]	0.49 ± 0.01[b]	0.46 ± 0.02[ab]	0.66 ± 0.01[b]	0.64 ± 0.01[b]	0.62 ± 0.01[b]
6.0	Control	0.44 ± 0.06[c]	0.42 ± 0.01[b]	0.37 ± 0.06[b]	0.69 ± 0.00[b]	0.61 ± 0.01[d]	0.65 ± 0.00[ab]
	1:1	0.46 ± 0.01[bc]	0.46 ± 0.02[ab]	0.39 ± 0.05[ab]	0.69 ± 0.02[b]	0.62 ± 0.00[c]	0.63 ± 0.00[b]
	3:1	0.50 ± 0.01[abc]	0.49 ± 0.00[ab]	0.39 ± 0.03[ab]	0.67 ± 0.01[bc]	0.62 ± 0.00[c]	0.62 ± 0.01[b]
	KCl	0.54 ± 0.02[ab]	0.50 ± 0.02[ab]	0.49 ± 0.01[a]	0.73 ± 0.01[a]	0.68 ± 0.00[a]	0.69 ± 0.03[a]
	NaCl	0.55 ± 0.00[a]	0.52 ± 0.03[a]	0.50 ± 0.00[a]	0.64 ± 0.01[c]	0.66 ± 0.01[b]	0.63 ± 0.01[b]

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 8. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.54 ± 0.05^{a}	0.51 ± 0.04^{a}	0.55 ± 0.05^{a}	0.70 ± 0.01^{b}	0.64 ± 0.02^{b}	0.63 ± 0.00^{b}
	1:1	0.34 ± 0.03^{b}	0.28 ± 0.01^{b}	0.25 ± 0.01^{c}	0.64 ± 0.02^{c}	0.63 ± 0.00^{b}	0.60 ± 0.01^{c}
	3:1	0.29 ± 0.00^{b}	0.26 ± 0.00^{b}	0.20 ± 0.01^{c}	0.63 ± 0.00^{c}	0.63 ± 0.01^{b}	0.60 ± 0.02^{c}
	KCl	0.50 ± 0.00^{a}	0.46 ± 0.01^{a}	0.42 ± 0.01^{b}	0.82 ± 0.01^{a}	0.78 ± 0.00^{a}	0.68 ± 0.01^{a}
	NaCl	0.27 ± 0.00^{b}	0.26 ± 0.00^{b}	0.23 ± 0.03^{c}	0.66 ± 0.00^{c}	0.65 ± 0.02^{b}	0.62 ± 0.01^{bc}
5.5	Control	0.46 ± 0.03^{a}	0.48 ± 0.01^{a}	0.41 ± 0.03^{a}	0.68 ± 0.01^{ab}	0.63 ± 0.01^{b}	0.66 ± 0.01^{ab}
	1:1	0.34 ± 0.00^{b}	0.31 ± 0.00^{b}	0.27 ± 0.00^{b}	0.64 ± 0.01^{ab}	0.63 ± 0.00^{b}	0.59 ± 0.01^{d}
	3:1	0.29 ± 0.01^{c}	0.27 ± 0.01^{c}	0.22 ± 0.01^{bc}	0.47 ± 0.19^{b}	0.63 ± 0.00^{b}	0.63 ± 0.00^{c}
	KCl	0.50 ± 0.02^{a}	0.47 ± 0.02^{a}	0.41 ± 0.02^{a}	0.82 ± 0.01^{a}	0.77 ± 0.01^{a}	0.68 ± 0.01^{a}
	NaCl	0.29 ± 0.01^{c}	0.27 ± 0.00^{c}	0.21 ± 0.02^{c}	0.66 ± 0.01^{ab}	0.64 ± 0.01^{b}	0.63 ± 0.00^{bc}
6.0	Control	0.44 ± 0.06^{a}	0.42 ± 0.06^{a}	0.37 ± 0.06^{a}	0.69 ± 0.00^{b}	0.61 ± 0.01^{b}	0.65 ± 0.00^{b}
	1:1	0.32 ± 0.01^{b}	0.29 ± 0.01^{b}	0.27 ± 0.01^{b}	0.64 ± 0.00^{c}	0.63 ± 0.01^{b}	0.64 ± 0.00^{bc}
	3:1	0.32 ± 0.01^{b}	0.27 ± 0.01^{b}	0.21 ± 0.00^{b}	0.63 ± 0.01^{c}	0.63 ± 0.00^{b}	0.60 ± 0.01^{d}
	KCl	0.51 ± 0.02^{a}	0.48 ± 0.02^{a}	0.44 ± 0.02^{a}	0.81 ± 0.00^{a}	0.76 ± 0.00^{a}	0.68 ± 0.01^{a}
	NaCl	0.28 ± 0.00^{b}	0.27 ± 0.00^{b}	0.24 ± 0.01^{b}	0.65 ± 0.01^{c}	0.62 ± 0.01^{b}	0.62 ± 0.00^{c}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

merically higher ($P > 0.05$) compared with CFS at the same salt concentration, pH level, and salt treatment. This may suggest that the proteolytic action of the cell-wall proteinases releases extra ACE-inhibitory peptides compared with that of CFS. Further investigation is required to explore cell-wall proteinases and their effect on production of ACE-inhibitory peptides.

Tables 11 and 12 illustrate the ACE-inhibitory activity of CFE and CFS of *L. casei* (obtained at different salt concentrations, pH values, and salt treatments) incubated with milk caseins for 24 h at 37°C. The ACE-inhibitory activity of CFE with the 3 caseins was higher ($P < 0.05$) compared with CFS at the same salt concentration, pH level, and salt treatment (Tables 11 and 12). This may be due to the higher activity of cell-wall proteinases compared with those of CFS, or may indicate that CFE proteinases had more specificity toward milk caseins compared with those obtained from CFS.

We observed significant ($P < 0.05$) differences in ACE-inhibitory activities of CFE and CFS of *L. casei* between salt treatments at the same salt concentra-

Table 9. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.47 ± 0.86^{d}	58.71 ± 0.37^{b}	60.95 ± 0.22^{a}	45.46 ± 0.47^{b}	47.78 ± 0.26^{ab}	48.74 ± 0.36^{ab}
	1:1	74.97 ± 1.10^{a}	63.04 ± 0.56^{a}	51.11 ± 0.96^{c}	42.99 ± 0.93^{c}	49.19 ± 0.22^{a}	48.78 ± 0.35^{ab}
	3:1	68.79 ± 0.54^{b}	56.69 ± 1.71^{bc}	49.17 ± 0.78^{c}	45.94 ± 0.44^{b}	45.41 ± 0.32^{b}	46.28 ± 0.28^{c}
	KCl	60.60 ± 0.89^{c}	57.13 ± 0.48^{bc}	53.63 ± 0.93^{b}	48.31 ± 0.35^{a}	49.29 ± 0.91^{a}	50.02 ± 0.60^{a}
	NaCl	60.16 ± 0.68^{c}	56.00 ± 0.23^{c}	50.36 ± 0.73^{c}	44.96 ± 0.45^{b}	46.54 ± 1.49^{b}	47.44 ± 1.15^{bc}
5.5	Control	55.05 ± 0.37^{d}	56.67 ± 0.76^{c}	58.29 ± 1.62^{a}	43.85 ± 0.31^{b}	48.77 ± 0.36^{ab}	47.56 ± 0.20^{ab}
	1:1	78.56 ± 0.52^{a}	65.36 ± 0.16^{a}	52.16 ± 0.76^{b}	46.43 ± 0.86^{a}	49.78 ± 0.14^{a}	50.35 ± 0.16^{a}
	3:1	72.66 ± 0.55^{b}	61.20 ± 0.24^{b}	49.75 ± 0.20^{b}	40.45 ± 1.31^{c}	45.57 ± 0.10^{c}	45.91 ± 1.80^{b}
	KCl	62.05 ± 1.13^{c}	56.60 ± 0.50^{c}	52.13 ± 0.58^{b}	47.38 ± 0.48^{a}	47.61 ± 1.03^{b}	48.66 ± 0.76^{ab}
	NaCl	61.13 ± 0.59^{c}	55.85 ± 0.39^{c}	52.57 ± 2.10^{b}	47.17 ± 0.07^{a}	47.59 ± 0.60^{b}	48.79 ± 0.30^{a}
6.0	Control	54.61 ± 1.61^{c}	72.05 ± 2.13^{a}	52.22 ± 0.82^{ab}	42.80 ± 0.83^{b}	46.97 ± 0.15^{b}	47.96 ± 0.26^{c}
	1:1	57.26 ± 1.28^{c}	55.64 ± 0.85^{c}	54.01 ± 0.81^{a}	46.85 ± 0.43^{a}	49.47 ± 0.30^{a}	49.08 ± 0.16^{ab}
	3:1	75.60 ± 0.33^{a}	63.81 ± 0.83^{b}	52.03 ± 1.53^{ab}	41.22 ± 0.24^{c}	48.00 ± 0.36^{ab}	47.53 ± 0.34^{c}
	KCl	66.24 ± 1.58^{b}	54.84 ± 1.84^{c}	50.37 ± 0.96^{b}	46.82 ± 0.32^{a}	47.00 ± 0.64^{b}	48.41 ± 0.40^{bc}
	NaCl	62.20 ± 1.33^{b}	56.48 ± 0.39^{c}	51.45 ± 0.27^{ab}	47.05 ± 0.26^{a}	48.41 ± 1.14^{ab}	49.54 ± 0.30^{a}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 10. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.47 ± 0.86^{d}	58.71 ± 0.37^{a}	60.95 ± 0.22^{ab}	45.46 ± 0.47^{ab}	47.78 ± 0.26^{a}	48.74 ± 0.36^{a}
	1:1	77.05 ± 0.63^{c}	53.59 ± 0.23^{bc}	55.94 ± 3.64^{bc}	48.35 ± 0.94^{a}	38.22 ± 4.94^{a}	48.42 ± 0.39^{a}
	3:1	91.21 ± 0.34^{b}	55.85 ± 0.96^{b}	63.26 ± 0.27^{a}	47.55 ± 0.64^{a}	40.59 ± 4.21^{a}	49.70 ± 0.85^{a}
	KCl	93.20 ± 0.10^{a}	51.11 ± 0.88^{c}	60.69 ± 0.46^{ab}	41.15 ± 3.07^{b}	39.20 ± 4.99^{a}	46.53 ± 0.45^{b}
	NaCl	54.81 ± 0.32^{e}	54.00 ± 1.21^{b}	54.81 ± 0.32^{c}	49.21 ± 0.18^{a}	46.69 ± 0.54^{a}	49.21 ± 0.18^{a}
5.5	Control	55.05 ± 0.37^{c}	56.67 ± 0.76^{a}	58.29 ± 1.62^{bc}	43.85 ± 0.31^{c}	48.77 ± 0.36^{a}	47.56 ± 0.20^{ab}
	1:1	53.40 ± 0.32^{c}	51.24 ± 1.05^{c}	57.38 ± 2.87^{c}	46.86 ± 0.64^{b}	34.70 ± 3.64^{b}	45.81 ± 1.45^{b}
	3:1	85.29 ± 2.12^{b}	56.66 ± 0.61^{a}	63.43 ± 0.48^{a}	48.52 ± 0.52^{a}	38.97 ± 4.65^{ab}	49.57 ± 0.54^{a}
	KCl	92.58 ± 0.04^{a}	53.69 ± 0.24^{b}	62.17 ± 0.38^{ab}	47.43 ± 0.38^{ab}	40.04 ± 3.86^{ab}	48.68 ± 0.34^{a}
	NaCl	55.16 ± 0.48^{c}	55.16 ± 0.48^{ab}	55.16 ± 0.48^{c}	47.81 ± 0.51^{ab}	46.00 ± 0.51^{a}	47.81 ± 0.51^{ab}
6.0	Control	54.61 ± 1.61^{d}	72.05 ± 2.13^{a}	52.22 ± 0.82^{c}	42.80 ± 0.83^{b}	46.97 ± 0.15^{a}	47.96 ± 0.26^{a}
	1:1	56.76 ± 0.49^{d}	54.32 ± 0.25^{b}	49.32 ± 0.63^{d}	44.15 ± 0.11^{b}	46.11 ± 1.33^{a}	46.48 ± 0.27^{a}
	3:1	80.92 ± 0.26^{b}	54.73 ± 0.36^{b}	63.45 ± 0.36^{b}	48.19 ± 0.34^{a}	38.74 ± 4.95^{a}	49.23 ± 0.22^{a}
	KCl	91.90 ± 0.23^{a}	55.01 ± 0.05^{b}	62.16 ± 0.10^{b}	47.72 ± 0.21^{a}	40.74 ± 4.26^{a}	49.19 ± 0.26^{a}
	NaCl	70.01 ± 0.46^{c}	70.03 ± 0.48^{a}	70.03 ± 0.48^{a}	48.22 ± 1.90^{a}	44.51 ± 1.03^{a}	48.22 ± 1.90^{a}

[a–e]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

tion and pH level (Tables 11 and 12). These differences were found to be inconsistent when pH level changed, suggesting that pH level affected the proteinase activities of CFE and CFS significantly. In conclusion, substitution of NaCl with KCl was found to significantly affect the proteinase activities of starter culture strains *L. acidophilus* and *L. casei*. Further investigation is required to explore the effect of KCl on proteinase activity and production during fermentation or product storage. The salt treatment effect was highly dependent on pH.

CONCLUSIONS

Complete or partial replacement of NaCl with KCl significantly affected the proteinase activities of CFE and CFS from probiotic strains *L. acidophilus* and *L. casei*. The proteolytic activity following incubation with individual milk caseins showed that the presence of KCl in the bacterial culture medium had a significant effect on the proteinases. Salt treatment also significantly affected ACE-inhibitory activity. The effect of KCl on the bacterial proteinases depended on pH, casein type,

Table 11. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.05 ± 1.29^{d}	57.12 ± 0.79^{ab}	58.19 ± 2.01^{a}	44.56 ± 0.67^{a}	46.65 ± 0.53^{a}	46.85 ± 1.26^{a}
	1:1	75.11 ± 0.58^{a}	62.25 ± 0.82^{a}	49.39 ± 1.47^{bc}	43.51 ± 0.07^{a}	45.22 ± 0.61^{a}	46.34 ± 1.34^{a}
	3:1	65.41 ± 1.77^{b}	50.98 ± 6.83^{b}	46.15 ± 0.98^{c}	43.28 ± 1.31^{a}	47.65 ± 1.81^{a}	43.28 ± 1.20^{a}
	KCl	60.43 ± 0.41^{c}	54.51 ± 2.08^{ab}	51.50 ± 1.45^{b}	45.55 ± 0.55^{a}	47.53 ± 0.79^{a}	46.24 ± 0.74^{a}
	NaCl	59.58 ± 0.58^{c}	51.03 ± 1.75^{b}	48.90 ± 2.07^{bc}	43.55 ± 0.83^{a}	40.49 ± 1.98^{b}	46.33 ± 1.05^{a}
5.5	Control	53.42 ± 2.55^{c}	56.38 ± 1.66^{b}	59.34 ± 0.89^{a}	42.98 ± 0.84^{ab}	44.58 ± 1.22^{bc}	46.13 ± 0.36^{a}
	1:1	77.61 ± 0.38^{a}	63.98 ± 1.09^{a}	50.35 ± 2.25^{b}	41.24 ± 2.15^{ab}	46.59 ± 0.97^{ab}	46.75 ± 0.64^{a}
	3:1	73.74 ± 0.94^{a}	61.36 ± 0.50^{a}	48.99 ± 1.07^{b}	41.05 ± 0.44^{b}	42.15 ± 0.38^{c}	40.78 ± 0.03^{b}
	KCl	60.10 ± 0.92^{b}	53.10 ± 1.90^{b}	48.83 ± 0.90^{b}	44.66 ± 1.09^{ab}	48.54 ± 0.85^{a}	44.65 ± 1.02^{a}
	NaCl	60.59 ± 0.45^{b}	52.82 ± 0.56^{b}	49.27 ± 1.63^{b}	45.01 ± 0.91^{a}	43.69 ± 1.35^{bc}	46.08 ± 1.39^{a}
6.0	Control	51.36 ± 3.14^{d}	56.87 ± 6.95^{a}	53.59 ± 2.53^{a}	40.13 ± 0.90^{b}	44.84 ± 0.91^{a}	44.89 ± 0.83^{ab}
	1:1	64.71 ± 4.40^{bc}	57.42 ± 0.90^{a}	50.13 ± 2.62^{ab}	44.85 ± 1.53^{a}	47.65 ± 0.78^{a}	46.63 ± 0.81^{a}
	3:1	73.31 ± 0.43^{a}	61.91 ± 1.26^{a}	50.50 ± 2.51^{ab}	40.18 ± 0.96^{b}	44.52 ± 1.01^{a}	43.26 ± 1.41^{b}
	KCl	71.73 ± 1.54^{ab}	51.77 ± 2.89^{a}	46.46 ± 0.90^{b}	43.95 ± 1.13^{a}	47.66 ± 1.84^{a}	44.83 ± 1.00^{ab}
	NaCl	61.88 ± 0.60^{c}	54.70 ± 2.20^{a}	49.00 ± 1.93^{ab}	45.92 ± 0.70^{a}	44.84 ± 1.96^{a}	45.86 ± 0.96^{ab}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 12. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.05 ± 1.29^{c}	57.12 ± 0.79^{a}	58.19 ± 2.01^{abc}	44.56 ± 0.67^{cd}	46.65 ± 0.53^{a}	46.85 ± 1.26^{b}
	1:1	78.39 ± 0.06^{b}	52.78 ± 0.17^{bc}	56.45 ± 3.94^{bc}	45.74 ± 0.59^{c}	18.73 ± 2.04^{b}	49.08 ± 1.02^{b}
	3:1	91.12 ± 0.07^{a}	54.85 ± 1.55^{ab}	63.44 ± 0.38^{a}	47.28 ± 0.13^{b}	22.38 ± 1.90^{b}	52.18 ± 0.41^{a}
	KCl	92.91 ± 0.02^{a}	48.37 ± 1.29^{d}	60.69 ± 0.57^{ab}	44.17 ± 0.44^{d}	19.11 ± 3.92^{b}	49.05 ± 0.76^{b}
	NaCl	54.07 ± 0.26^{d}	50.30 ± 0.65^{cd}	54.07 ± 0.26^{c}	49.09 ± 0.07^{a}	43.59 ± 0.25^{a}	49.09 ± 0.07^{b}
5.5	Control	53.42 ± 2.55^{c}	56.38 ± 1.66^{a}	59.34 ± 0.89^{bc}	42.98 ± 0.84^{b}	44.58 ± 1.22^{a}	46.13 ± 0.36^{bc}
	1:1	66.52 ± 3.64^{b}	51.97 ± 2.09^{ab}	57.44 ± 1.90^{cd}	43.35 ± 0.88^{b}	17.26 ± 2.26^{b}	46.02 ± 0.82^{bc}
	3:1	89.98 ± 0.05^{a}	52.76 ± 1.12^{a}	62.68 ± 0.47^{a}	46.98 ± 0.71^{a}	18.78 ± 1.71^{b}	50.68 ± 1.10^{a}
	KCl	92.98 ± 0.09^{a}	47.73 ± 1.41^{b}	61.76 ± 0.62^{ab}	45.85 ± 0.69^{ab}	20.63 ± 2.67^{b}	48.84 ± 0.49^{ab}
	NaCl	54.56 ± 0.42^{c}	54.56 ± 0.42^{a}	54.55 ± 0.41^{d}	45.56 ± 1.43^{ab}	43.80 ± 0.64^{a}	45.56 ± 1.43^{c}
6.0	Control	51.36 ± 3.14^{c}	56.87 ± 6.95^{a}	53.59 ± 2.53^{b}	40.13 ± 0.90^{c}	44.84 ± 0.91^{a}	44.89 ± 0.83^{c}
	1:1	55.39 ± 0.42^{c}	51.26 ± 1.32^{a}	47.31 ± 0.10^{c}	43.13 ± 0.59^{b}	43.16 ± 0.63^{a}	45.24 ± 0.37^{bc}
	3:1	80.76 ± 0.45^{b}	53.40 ± 0.55^{a}	63.38 ± 0.75^{a}	46.98 ± 0.34^{a}	17.99 ± 2.37^{b}	50.14 ± 0.47^{a}
	KCl	92.08 ± 0.12^{a}	55.18 ± 0.86^{a}	62.47 ± 0.46^{a}	47.24 ± 0.48^{a}	20.22 ± 4.09^{b}	47.53 ± 1.13^{b}
	NaCl	54.08 ± 0.57^{c}	54.08 ± 0.57^{a}	54.08 ± 0.57^{b}	44.24 ± 0.72^{b}	41.24 ± 0.97^{a}	44.24 ± 0.72^{c}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

and the concentration of each salt. Further investigation is needed to study the effect of KCl on bacterial proteinases in food (especially cheese).

REFERENCES

Alderman, M. H. 2006. Evidence relating dietary sodium to cardiovascular disease. J. Am. Coll. Nutr. 25(Suppl.):256S–261S.

Armenteros, M., M. C. Aristoy, J. M. Barat, and F. Toldrá. 2009. Biochemical changes in dry-cured loins salted with partial replacements of NaCl by KCl. Food Chem. 117:627–633.

Ash, A., and A. Wilbey. 2010. The nutritional significance of cheese in the UK diet. Int. J. Dairy Technol. 63:305–319.

Ayyash, M. M., and N. P. Shah. 2010. Effect of partial substitution of NaCl with KCl on Halloumi cheese during storage: Chemical composition, lactic bacterial count, and organic acids production. J. Food Sci. 75:C525–C529.

Ayyash, M. M., and N. P. Shah. 2011a. Effect of partial substitution of NaCl with KCl on proteolysis of Halloumi cheese. J. Food Sci. 76:C31–C37.

Ayyash, M. M., and N. P. Shah. 2011b. The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile. J. Dairy Sci. 94:2741–2751.

Ayyash, M. M., and N. P. Shah. 2011c. The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese. J. Dairy Sci. 94:3761–3768.

Ayyash, M. M., and N. P. Shah. 2011d. Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl. J. Dairy Sci. 94:3769–3777.

Ayyash, M. M., F. Sherkat, and N. P. Shah. 2012. The impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. J. Food Sci. 77:M490–498.

Church, F. C., H. E. Swaisgood, D. H. Porter, and G. L. Catignani. 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66:1219–1227.

Fox, P. F. 2003. Biochemistry of cheese ripening. Pages 320–326 in Encyclopedia of Dairy Sciences. Vol. 1. 1st ed. H. Roginski, J. W. Fuquay, and P. F. Fox, ed. Elsevier Academic Press, London, UK.

Heaney, R. P. 2006. Role of dietary sodium in osteoporosis. J. Am. Coll. Nutr. 25:271S–276S.

Kotchen, T. A. 2005. Contributions of sodium and chloride to NaCl-induced hypertension. Hypertension 45:849–850.

Massey, L. K. 2005. Effect of dietary salt intake on circadian calcium metabolism, bone turnover, and calcium oxalate kidney stone risk in postmenopausal women. Nutr. Res. 25:891–903.

McSweeney, P. L. H. 2004. Biochemistry of cheese ripening. Int. J. Dairy Technol. 57:127–144.

Meneton, P., L. Lafay, A. Tard, A. Dufour, J. Ireland, J. Ménard, and J. L. Volatier. 2009. Dietary sources and correlates of sodium and potassium intakes in the French general population. Eur. J. Clin. Nutr. 63:1169–1175.

National Health and Medical Research Council. 2003. Dietary guidelines for children and adolescents in Australia. Accessed Apr. 10, 2003. http://www.nhmrc.gov.au/_files_nhmrc/file/publications/synopses/n34.pdf.

Reddy, K. A., and E. H. Marth. 1991. Reducing the sodium content of foods: A review. J. Food Prot. 54:138–150.

Shin, J. Y., W. M. Jeon, G. B. Kim, and B. H. Lee. 2004. Purification and characterization of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG. J. Dairy Sci. 87:4097–4103.

Sigma-Aldrich. 2011. Bradford reagent technical bulletin. Sigma-Aldrich Inc., St. Louis, MO. http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/b6916bul.Par.0001.File.tmp/b6916bul.pdf.

World Health Organization. 2007. Reducing salt intake in populations. Accessed Sep. 25, 2012. http://www.who.int/dietphysicalactivity/reducingsaltintake_EN.pdf.