Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl

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ABSTRACT

The proteolytic and ACE inhibitory activities of low-moisture Mozzarella cheese (LMMC) as affected by partial substitution of NaCl with KCl were investigated. Experimental LMMC were made and salted with 4 salt mixtures: NaCl only (control), 3NaCl:1KCl, 1NaCl:1KCl, and 1NaCl:3KCl, and then proteolytic activity and angiotensin-converting enzyme inhibitory activity were determined. Salt treatment significantly affected angiotensin-converting enzyme inhibitory activity and phosphotungstic acid-soluble N of LMMC during storage. Water-soluble N, trichloroacetic acid-soluble N, lactic acid bacteria population, and total free amino acids were unaffected during storage. Nonetheless, water-soluble N and trichloroacetic acid-soluble N increased significantly during storage within a salt treatment. Peptide profiles and urea-PAGE gels did not differ between experimental cheeses at the same storage time.

Key words: proteolysis, NaCl substitution, angiotensin-converting enzyme inhibition, low-moisture Mozzarella cheese

INTRODUCTION

Proteolysis is the major biochemical event to occur in cheese, besides glycolysis and lipolysis, that affects textural and functional properties of cheeses (Fox and McSweeney, 1996; Upadhyay et al., 2004). In general, cheeses show similar proteolysis trends; however, differences in cheese nature and manufacturing processes affect the proteolytic pattern. In general, proteolysis in rennet-coagulated cheeses starts with residual rennet activity or plasmin retained in curd after draining of whey and is called primary proteolysis. In this phase, caseins are broken down to large and small peptides. Second, other milk-indigenous enzymes and microbial enzymes (proteinases and peptidases) hydrolyze large peptide units to smaller units. Finally, bacterial en-

zymes from lactic acid bacteria (**LAB**) and nonstarter LAB hydrolyze small peptides into dipeptides and amino acids (Fox and McSweeney, 1996; Sousa et al., 2001; Upadhyay et al., 2004). Several factors have been found to affect proteolysis in low-moisture Mozzarella cheese (**LMMC**) that in turn affect its characteristics, including ripening temperature (Feeney et al., 2001), pH (Yun et al., 1993a,b; Yazici and Akbulut, 2007), starter culture type (Zisu and Shah, 2005, 2006), salt concentration, but not salt type (Rowney et al., 2004; Olson, 2007), manufacturing procedure (Renda et al., 1997; Osaili et al., 2010), and concentration of rennet (Dave et al., 2003). Salt (NaCl) is an important factor that affects the proteolytic pattern of LMMC during storage. Salt reduces the free water available in cheese, which consequently affects enzyme activity and growth of LAB (Guinee, 2004a; Upadhyay et al., 2004). Although NaCl addition is an essential step during LMMC production, it also causes major health hazards. A strong positive correlation has been found between NaCl and hypertension (Kotchen, 2005), osteoporosis (Heaney, 2006), and kidney stones (Massey, 2005). The World Health Organization (2007) recommended that food manufacturers reduce salt in their products. Numerous studies have reported a range of contribution of cheese to daily sodium intake (Guinee, 2004b; Fischer et al., 2009; Meneton et al., 2009; Anderson et al., 2010), which highlights the need to reduce NaCl in major cheeses such as LMMC. Currently, dairy plants produce tonnes of LMMC per day (Kindstedt et al., 2004). Nonetheless, simply reducing NaCl in cheeses can adversely affect cheese quality and safety. Decreasing the NaCl level accelerates proteolysis, produces off-flavors, and allows pathogens to grow (Guinee, 2004b; McMahon, 2010). Therefore, studies have focused on replacing NaCl with KCl, which is the most successful candidate to be used as NaCl replacer (Guinee, 2004a,b). Intake of KCl intake has not been linked to development of hypertension or cardiovascular diseases (Buemi et al., 2002; Geleijnse et al., 2007). Few studies have been carried out to investigate the effect of substitution of NaCl with KCl on proteolysis of Feta cheese (Katsiari et al., 2000), Halloumi cheese (Ayyash and Shah, 2010), and Fynbo cheese (Zorrilla et al.,

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1996). However, no information is available on proteolysis or production of angiotensin-converting enzyme (ACE) inhibitory peptides in LMMC during ripening. Angiotensin-I-converting enzyme (peptidyl dipeptide hydrolase, EC 3.4.15.1) increases blood pressure by converting angiotensin-I to angiotensin-II, a potent vasoconstrictor, and by degrading bradykinin, a vasodilatory peptide (Johnston, 1992). Inhibition of ACE leads to a decrease in the level of the vasoconstrictory peptide, and therefore may result in an antihypertensive effect and may influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity (Meisel, 1998; Ong and Shah, 2008). The presence of these ACE inhibitory peptides is influenced directly by proteolysis, but only to a certain degree (Ong et al., 2007). Therefore, and because proteolysis is affected by salt substitution (Ayyash and Shah, 2010), the authors hypothesized that ACE inhibitory activity might be affected by substitution of NaCl with KCl. The objectives of this study were to investigate the effect of partial substitution of NaCl with KCl on proteolysis, production of ACE inhibitory peptides, and LAB growth during ripening.

MATERIALS AND METHODS

Cheese Making

Homogenized and pasteurized full-fat (3% fat) bovine milk was purchased from a local dairy plant (Melbourne, Victoria, Australia) and tempered to 40°C. Experimental LMMC was manufactured according to Feeney et al. (2001) with some modifications. Forty liters of milk was inoculated with TCC-4 direct-in-vat starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* according to manufacturer instructions (Chr. Hansen, Bayswater, Victoria, Australia). After 40 min, diluted single-strength chymosin (1:20) was added according to the manufacturer's instructions (Chr. Hansen) and the milk coagulated in 35 min. Curd was cut into 1-cm cubes and cooked with continuous agitation at 40°C for 15 min. Whey was drained and curd was manually pressed to remove additional whey from the curd. The curd was cheddared and milled when the pH of the slabs reached to 5.3 to 5.2. The milled curd was dry-salted with 1 of 4 NaCl/KCl combinations: only NaCl (A; control), 3NaCl:1KCl (B), 1NaCl:1KCl (C), and 1NaCl:3KCl (D) at a level of 46 g/kg and the curd was allowed to mellow for 20 min. The salted curd was plasticized in 4% brine solutions (A, B, C, and D) at 80°C for 5 min and then manually kneaded. The plasticized curd was molded in circular molds 23 × 8 cm (diameter × height; ~2.5 kg per block). Cheese blocks were kept

at 4°C until the temperature reached 15°C. The 2.5-kg blocks were cut into smaller blocks (500 g per block) and vacuum-packaged into barrier bags using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) and then stored at 4°C for 27 d. Experimental cheeses were made in triplicate. Experimental cheeses were sampled at 0, 9, 18, and 27 d of storage. The cheese samples were shredded and then sub-sampled for the following analyses.

Gross Composition

Moisture was determined by the oven-drying method at 102°C, protein by the Kjeldahl method, fat by the Babcock method, and ash using the muffle furnace method, all according to AOAC (1995). For pH measurement, grated cheese (40 g) was macerated with 40 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MettlerLab, Pacific Laboratory Products, Blackburn, Victoria, Australia).

Enumeration of LAB

Counts of LAB in cheeses were enumerated according to Ayyash and Shah (2010). Grated cheese (10 g) was taken from the shredded cheese block, placed in a stomacher bag, and blended with 90 mL of sterile distilled water using a Stomacher-400 laboratory blender (Seward Medical, London, UK). Appropriate serial dilutions were made using 0.1% peptone, and LAB populations were counted using de Man, Rogosa, and Sharpe agar (Merck Pty. Ltd., Victoria, Australia). Inoculated plates in duplicate were incubated anaerobically at 37°C for 48 h using anaerobic jars. Plates having 25 to 250 colonies were counted and expressed as colony-forming units per gram of sample.

Assessment of Proteolysis

The water-soluble N (WSN) of the cheese samples was evaluated according to Kuchroo and Fox (1982). The nitrogen in the extract was estimated by Kjeldahl method (AOAC, 1995). Twelve percent trichloroacetic acid-soluble N (TCA-SN) and 5% phosphotungstic acid-soluble N (PTA-SN) were determined according to Ayyash and Shah (2010). The TCA-SN was estimated in 9 mL of filtrate obtained after precipitation of filtered water-soluble extract (WSE) of cheese with 24% TCA (Sigma-Aldrich, St. Louis, MO). The extent of secondary proteolysis (PTA-SN) was assayed similarly by using 9 mL of filtrate obtained after precipitation of filtered WSN of cheese with 10% phosphotungstic acid (Sigma-Aldrich).

Urea-PAGE

Preparation of cheese samples and urea-PAGE analysis were carried out according to Ayyash and Shah (2010) with some modifications. In brief, grated (1 g) cheese was homogenized with 10 mL of treatment buffer [6 M urea, 0.1 M β -mercaptoethanol, and 0.5% bromophenol blue (0.05% wt/vol) in 50% ethanol] for 3 min at 10,000 rpm using a tissue homogenizer (Polytron, Kinematica AG, Lucerne, Switzerland). The slurry was held at 40°C for 15 min, and then centrifuged at $3,000 \times g$ for 30 min at 4°C. The upper fat layer was removed and 0.5 mL of the supernatant was mixed with 3.5 mL of treatment buffer. An aliquot (1 mL) from the latest mixture was placed in a 1.5-mL Eppendorf tube and centrifuged at $3,000 \times g$ for 15 min. Eleven microliters of whole casein solution (2 mg/mL; Sigma) was mixed with 40 μ L of treatment buffer. From each sample and prepared whole casein, 11 μ L of sample was loaded onto ready-gel Tris-HCl gel (12% resolving gel, 4% stacking gel, 10-well, 30 μ L, 16 $\times$ 16 cm; Bio-Rad Laboratories Pty. Ltd., Gladesville, New South Wales, Australia). All gels were run in a tank buffer (10 g of Tris and 29.9 g of glycine, wt/vol, in 2 L of distilled water) using a Bio-Rad Protean II xi cell powered by a Power Pac 300 and run for 30 min at 16 mA and then for 3 h 30 mA. The gels were fixed in destaining solution A (40% methanol, 7% acetic acid) for 30 min, and then gels were stained with 0.025% Coomassie Brilliant Blue (ICN Biochemicals Inc., Aurora, OH; 40% methanol, 7% acetic acid) for 4 h. The gels were then destained in destaining solution A for 1 h followed by destaining in destaining solution B (7% acetic acid, 5% methanol) until the background became clear. The gel images were recorded using a Fuji film intelligent dark box II with Fuji film LAS-1000 L V1.3 software (Fujifilm Co., Brookvale, NSW, Australia).

Preparation of WSE for Peptide Profile, ACE Inhibitory Activity, and Total Free Amino Acids

Grated cheese (10 g) was homogenized with 90 mL of MilliQ-water using an Ultraturrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at 20,000 rpm for 3 min to prepare another WSE. The slurry was centrifuged at $4,000 \times g$ for 20 min and the fat layer was removed. A 10-mL aliquot was stored at -20°C for ACE-inhibitory activity, 10 mL was used to assess total free amino acids (TFAA), and then 10 mL of supernatant was freeze-dried (Dynavac FD300 freeze-dryer; Airvac Engineering Pty. Ltd., Rowville, Australia) at -20°C and -100 kPa for 72 h. The freeze-dried lyophilized samples were then stored at -20°C

for analysis with reverse phase (RP)-HPLC as detailed below.

Peptides Profile of WSE by RP-HPLC

Peptide profile of LMMC during storage was examined by HPLC according to Ayyash and Shah (2010) and Ong and Shah (2008) with some modifications. An aliquot of freeze-dried WSE (80 mg) was mixed with 2 mL of solvent A containing 0.1% trifluoroacetic acid (Sigma Aldrich), centrifuged ($3,000 \times g$ for 10 min) using a bench-top centrifuge (Sorvall RT7, Newtown, CT), and filtered through a 0.45- μ m filter (Millipore Corp., Bedford, MA). The RP-HPLC analysis was carried out using HPLC 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., Palo Alto, CA). A sample size of 40 μ L was injected into the reverse-phase column (C18, 250 mm $\times$ 4.6 mm, 5 μ m; Grace Vydac, Hesperia, CA) with a guard column (10 mm, 12 mm, Grace Vydac). Separation was conducted at room temperature ($\sim$ 22°C) at a flow rate of 0.75 mL/min. Eluent B was 60% acetonitrile (Merck, South Granville, New South Wales, Australia) containing 0.05% trifluoroacetic acid. A linear gradient was applied from 0 to 80% eluent B over 100 min. The detection device was an UV-visible detector set at 215 nm, and RP-HPLC chromatograms were visually analyzed.

Measurement of TFAA

Concentrations of TFAA of LMMC were measured by using the Cd-ninhydrin method according to Folkertsma and Fox (1992), and WSE prepared as previously were used in the analysis. An aliquot (100 μ L) was placed in a glass tube and diluted with 1 mL of Milli-Q water (Millipore Corp.). Two milliliters of Cd-ninhydrin reagent [0.8 g of ninhydrin were dissolved in a mixture of 10 mL of glacial acetic acid (100%) and 80 mL ethanol (99.5%), followed by the addition of 1 g of CdCl₂ dissolved in 1 mL of Milli-Q water] was added. The mixture was heated at 84°C for 5 min and cooled to room temperature; then, absorbance at 507 nm was measured. All chemicals in this analysis were purchased from Sigma (St. Louis, MO) and analyses were carried out in duplicates.

ACE Inhibitory Activity in WSE

The ACE inhibitory activity was measured according to Wanasundara et al. (2002) using an HPLC method.

Angiotensin-converting enzyme and hippuryl-histidyl-leucine (**HHL**) were purchased from Sigma and prepared in Tris buffer (50 mM, pH 8.3) containing 300 mM NaCl. The assay consisted of 50 μ L of 3.0 mM HHL, 50 μ L of 1.25 milli-units ACE enzyme (from rabbit lung), and 50 μ L of assay WSE sample. The mixture was placed in a glass tube, incubated for 30 min at 37°C in a water bath without mixing, and then incubated for an additional 30 min after mixing. Glacial acetic acid (150 μ L) was added to stop ACE activity. The reaction mixture was kept at -20°C for further analysis by HPLC. The hippuric acid (**HA**) released from HHL by ACE was determined by HPLC. An external standard curve of HA was prepared to quantify the resultant HA in cheese samples. An aliquot (20 μ L) of the mixture was injected onto the HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV-visible tunable absorbance detector, and a 730 data module. The system was fitted with reverse-phase column (C18, 250 mm $\times$ 4.6 mm, 5 μ m; Grace Vydac) with a guard column (10 mm, 12 mm, Grace Vydac). 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, and the pH was adjusted to 3.0 using glacial acetic acid. The detection device was an UV-visible detector set at 228 nm. The control reaction mixture contained 50 μ L of buffer instead of the assay sample; the control 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

Two-way ANOVA was performed to investigate the significant effect of salt treatment and storage period

interaction on cheeses attributes. One-way ANOVA was performed to investigate the significant difference at a level of 0.05 between experimental cheeses at the same storage time using SAS software (SAS Institute, 2008). Fisher's test was carried out to examine the differences in means between experimental cheeses at same storage period (least significant difference, LSD). The significance of storage period was analyzed at same salt treatment using LSD. Pearson correlations were calculated to investigate correlation between 4 measured minerals (Ca, P, K, and Na) and proteolysis within a salt treatment.

RESULTS AND DISCUSSION

Gross Composition

Gross composition of LMMC salted with 1 of 4 ratios of NaCl and KCl mixture stored at 4°C at 0 d is presented in Table 1. Moisture, protein, fat, and ash contents did not differ ($P > 0.05$) between experimental LMMC at 0 d of storage. This suggests that variations in the LMMC measured in this study were mainly related to salt treatment. These results are in agreement with those of Ayyash and Shah (2010), Fitzgerald and Buckley (1985), and Katsiari et al. (2000) who reported similar results in Halloumi, Cheddar, and Feta cheeses, respectively. The pH values were significantly ($P < 0.05$) higher in experimental cheeses salted with NaCl/KCl mixtures (B, C, and D) compared with the control (A). This may be attributed to higher pH of KCl (by ~0.3 pH units) compared with NaCl. This is in accordance with pH results of Ayyash and Shah (2010), who reported a slight increase in pH values of Halloumi cheese kept in NaCl/KCl mixture compared with the control.

Proteolysis and LAB Growth

The WSN, TCA-SN, and PTA-SN of experimental cheeses are presented in Table 2. Growth of LAB in experimental LMMC during storage period is shown

Table 1. Moisture, protein, fat, and ash contents and pH values of low-moisture Mozzarella cheese made with 4 different salt treatments and stored at 4°C at 0 d of storage¹

Salt treatment ²	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
A	50.99 $\pm$ 1.59 ^a	26.81 $\pm$ 0.84 ^a	21.70 $\pm$ 0.99 ^a	3.10 $\pm$ 0.14 ^a	5.14 $\pm$ 0.02 ^b
B	51.13 $\pm$ 0.60 ^a	26.53 $\pm$ 1.43 ^a	22.20 $\pm$ 1.19 ^a	3.10 $\pm$ 0.15 ^a	5.18 $\pm$ 0.04 ^{ab}
C	50.53 $\pm$ 0.86 ^a	27.77 $\pm$ 0.49 ^a	21.20 $\pm$ 0.44 ^a	3.03 $\pm$ 0.07 ^a	5.18 $\pm$ 0.03 ^{ab}
D	50.80 $\pm$ 0.84 ^a	27.93 $\pm$ 0.13 ^a	21.73 $\pm$ 0.82 ^a	3.07 $\pm$ 0.16 ^a	5.24 $\pm$ 0.02 ^a

^{a,b}Means in each column with same letter do not differ significantly ($P > 0.05$).

¹Mean values $\pm$ SE of 3 trials.

²Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

in Figure 1. Concentrations of WSN and TCA-SN did not differ ($P > 0.05$) between experimental LMMC at the same storage time. However, WSN and TCA-SN increased significantly ($P < 0.05$) during storage within a salt treatment. This increase was due to the increase in proteolytic activity by coagulant enzyme residues, indigenous milk enzymes, and bacterial peptidases. These enzymes are reported to be responsible for the increase in proteolysis during cheese ripening (Fox and McSweeney, 1996; Upadhyay et al., 2004). These findings are in agreement with those of Ayyash and Shah (2010) and Katsiari et al. (2000, 2001), who reported similar results in Halloumi, Feta, and Kefalograviera cheeses during storage, respectively. A significant ($P < 0.05$) difference was observed in PTA-SN (Table 2) and LAB growth (Figure 1) between experimental cheeses at the same storage time. Analysis of variance showed that PTA-SN increased ($P < 0.05$) during storage within a salt treatment. However, LAB growth was insignificant during storage within a salt treatment (Figure 1). The observed differences in PTA-SN suggested that replacement of NaCl with KCl affected proteolytic enzymes secreted by LAB, but not logarithm growth of LAB. Ayyash and Shah (2010) reported similar observations in PTA-SN of Halloumi cheese. Armenteros et al. (2009) reported that some of the proteolytic enzymes were activated when the level of KCl was increased in a meat product, whereas other enzymes were inhibited. This supports our hypothesis that the presence of higher concentrations of KCl in cheeses stimulated

LAB to produce proteolytic enzymes different from those in cheeses brined in NaCl, or activated particular enzymes and inhibited others different from those in cheeses brined in NaCl. However, more studies need to be conducted to investigate the effect of NaCl/KCl mixtures on proteolytic enzymes from LAB. In addition, the initial differences in pH may also have contributed to differences in PTA-SN (Upadhyay et al., 2004). The lower pH in treatment A (control) compared with other treatments may have been caused by increased chymosin retention in cheese and improved chymosin activity. A higher pH value in cheese is not favorable for chymosin activity (Feeney et al., 2002). Thus, the increase in chymosin activity enhanced proteolytic activity of LAB enzymes, which, in turn, increased PTA-SN in control cheese. The initial difference in pH may also be affected by metabolism of LAB, which affected the enzymes produced by LAB. Hence, this caused the significant differences in PTA-SN between the experimental cheeses (Feeney et al., 2002).

Urea-PAGE and TFAA

The proteolytic pattern of the experimental LMMC salted with 4 different NaCl/KCl mixtures are shown in Figure 2. Salt treatment had no significant effect on LMMC at the same storage time. Moreover, α - and β -caseins showed a slight hydrolysis during storage within a salt treatment. This suggests that partial substitution of NaCl with KCl had similar effects on

Table 2. Water-soluble N (WSN), 12% trichloroacetic acid-soluble N (TCA-SN), and 5% phosphotungstic acid-soluble N (PTA-SN) of low-moisture Mozzarella cheese salted with 4 different salt levels of NaCl and KCl during storage at 4°C for 27 d¹

Storage time (d)	Salt treatment ²	% of total N		
		WSN	TCA-SN	PTA-SN
0	A	3.79 ± 0.26 ^a	2.95 ± 0.26 ^a	0.73 ± 0.06 ^a
	B	3.94 ± 0.16 ^a	2.61 ± 0.04 ^a	0.65 ± 0.04 ^{ab}
	C	3.33 ± 0.27 ^a	2.58 ± 0.13 ^a	0.55 ± 0.03 ^{ab}
	D	3.78 ± 0.18 ^a	2.45 ± 0.14 ^a	0.53 ± 0.03 ^b
9	A	5.34 ± 0.38 ^a	4.03 ± 0.25 ^a	0.97 ± 0.04 ^a
	B	5.60 ± 0.74 ^a	3.93 ± 0.51 ^a	0.78 ± 0.07 ^{ab}
	C	4.69 ± 0.33 ^a	3.46 ± 0.43 ^a	0.62 ± 0.09 ^{ab}
	D	4.53 ± 0.40 ^a	4.01 ± 0.22 ^a	0.40 ± 0.20 ^b
18	A	5.08 ± 0.12 ^a	4.69 ± 0.17 ^a	1.03 ± 0.06 ^a
	B	3.85 ± 0.14 ^b	3.05 ± 0.18 ^b	0.78 ± 0.01 ^{ab}
	C	4.07 ± 0.28 ^b	3.48 ± 0.81 ^{ab}	0.67 ± 0.06 ^{bc}
	D	3.97 ± 0.30 ^b	4.21 ± 0.38 ^{ab}	0.62 ± 0.03 ^c
27	A	8.55 ± 0.19 ^a	6.56 ± 0.42 ^a	1.07 ± 0.08 ^a
	B	8.42 ± 0.60 ^a	7.36 ± 0.83 ^a	0.98 ± 0.07 ^a
	C	7.09 ± 0.49 ^a	6.35 ± 0.68 ^a	0.80 ± 0.08 ^{ab}
	D	7.19 ± 0.40 ^a	5.20 ± 0.85 ^a	0.68 ± 0.11 ^b

^{a-c}Means in each column and at the same storage time with same letter do not differ significantly ($P > 0.05$).

¹Mean values ± SE of 3 trials.

²Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

Figure 1. Urea-PAGE electropherograms of low-moisture Mozzarella cheese salted with A = only NaCl (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); and D = 1NaCl:3KCl (wt/wt) and stored at 4°C for 0, 9, 18, and 27 d.

LMMC compared with control. This finding agrees with those of Ayyash and Shah (2010) and Katsiari et al. (2000, 2001).

Concentrations of TFAA of experimental LMMC during storage are shown in Figure 3. Analysis of variance showed no significant ($P > 0.05$) difference in TFAA between experimental cheeses at the same storage time. Katsiari et al. (2000, 2001) reported similar results in Feta and Kefalograviera cheeses, respectively. Total free AA increased ($P > 0.05$) slightly during storage for all experimental cheeses. These concentrations of TFAA were lower compared with those of Katsiari et al. (2000, 2001) in Feta and Kefalograviera cheeses, respectively. This may be attributed to slower proteolytic activity in LMMC during storage regardless of the salt treatment. Cooking temperature and stretching treatment during the cheese making process may inactivate chymosin residues in LMMC. It has been reported that inactivation of coagulant residues in cheese delays the proteolytic

process in cheese during storage (Fox, 1989; Fox and McSweeney, 1996; Upadhyay et al., 2004).

ACE Inhibitory Activity

The ACE inhibitory activity of LMMC salted with 4 different NaCl/KCl mixtures is shown in Figure 4; activity differed significantly ($P < 0.05$) between salt treatments at the same storage time, except at the end of storage (d 27). During storage of LMMC, ACE inhibitory activity was significantly ($P < 0.05$) higher in D cheeses compared with the other treatments. This suggests that the presence of higher concentrations of KCl increased the ACE inhibitory activity of LAB. In addition, we presumed that KCl affected LAB enzymes in a way that increased ACE activity compared with NaCl. Analysis of variance showed that the ACE inhibi-

Figure 2. Lactic acid bacteria growth (LAB, cfu/g) of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

Figure 3. Total free amino acids as absorbance of water-soluble extracts of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

Figure 4. Angiotensin-converting enzyme (ACE) inhibitory percentage of water-soluble extracts of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

tory activity of LAB in LMMC increased significantly ($P < 0.05$) during storage within a salt treatment.

RP-HPLC Peptide Profile

The RP-HPLC peptide profiles of experimental LMMC during storage period are shown in Figure 5. In general, RP-HPLC chromatograms showed no sig-

nificant differences in peptide profiles of experimental cheeses at the same storage time. Nonetheless, slight differences were observed at the end of storage between experimental LMMC. Peptide profiles varied slightly with LMMC ripening for all salt treatments, which may be due to slow proteolytic activity of enzymes in LMMC.

Correlations

Contents of Ca, P, K, and Na and percentage of soluble Ca are presented in the companion article (Ayyash and Shah, 2011). The Pearson correlations were calculated to investigate any correlation between Ca, P, K and Na. Our results showed negative correlations between total Ca and WSN, TCA-SN, PTA-SN, TFAA, and ACE inhibitory activity, in contrast to a positive correlation between percentage of soluble Ca and the same variables. This indicated that the conversion of insoluble Ca to soluble Ca during storage improved the proteolytic activity of LMMC in all salt treatments (data not shown). It has been reported that Ca in cheeses acts as cross-linkages for casein networks. Hence, the release of Ca from the casein network to the aqueous phase in cheeses (i.e., from insoluble Ca to soluble Ca) increased the susceptibility of caseins to hydrolysis (Feeney et al., 2002; Guinee et al., 2002).

Figure 5. Reversed-phase HPLC profiles of water-soluble extracts of low-moisture Mozzarella cheese (at d 0, 9, 18, and 27) salted with A (red) = only NaCl (control); B (green) = 3NaCl:1KCl (wt/wt); C (blue) = 1NaCl:1KCl (wt/wt); D (yellow) = 1NaCl:3KCl (wt/wt) and stored at 4°C.

CONCLUSIONS

From this study, we concluded that partial substitution of NaCl with KCl did not significantly affect proteolysis of LMMC. A significant effect of partial substitution of NaCl with KCl on ACE inhibitory activity was observed; however, further investigation is needed in this area. In general, proteolysis variables and ACE inhibitory activity correlated positively with percentage of soluble Ca and negatively with Ca in LMMC within a salt treatment.

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