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Original article

Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic *Lactobacillus casei* sp.

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Abstract – The aim of the study was to examine the release of ACE-inhibitory peptides in Cheddar cheeses made with starter lactococci and *Lactobacillus casei* 279 or *Lb. casei* LAFTI® L26 during ripening and to isolate, purify and identify such peptides. Addition of probiotic organisms increased the ACE-inhibitory activity of the cheeses during ripening at 4 ºC possibly due to the increased proteolysis. The IC_{50} (concentrations of ACE needed to inhibit 50% of ACE activity) was the lowest after 24 weeks of ripening in the probiotic cheeses (0.23–0.25 mg·mL^{-1}) compared to 36 weeks for cheeses without any probiotic (0.28 mg·mL^{-1}). Water-soluble extracts of each cheese were subjected to several stages of chromatography fractionation. Inhibitory activity found in the crude fractions ranged from 0.1 to 2.0 mg·mL^{-1}. The fraction with the highest activity was purified using a second stage chromatography. Various ACE-inhibitory peptides corresponding to the α_{s1}-casein N-terminal peptides [(f 1–6), (f 1–7), (f 1–9), (f 24–32) and (f 102–110)] and β-casein N-terminal peptides [(f 47–52) and (f 193–209)] were found. Our results suggested that ACE inhibition in Cheddar cheeses was dependent on proteolysis to a certain extent. Probiotic organisms used in this study can be added successfully in Cheddar cheeses in order to provide health benefits while simultaneously producing bioactive peptides for additional health attributes.

angiotensin converting enzyme-inhibitor (ACE-I) / bioactive peptide / Cheddar cheese / probiotic bacteria

摘要 – 添加益生性干酪乳杆菌的契达干酪中血管紧张素转换酶抑制活性。 本文研究了以乳球菌 (lactococci) 和干酪乳杆菌 (*Lactobacillus casei* 279, *Lb. casei* LAFTI® L26) 为发酵剂制造的切达干酪成熟过程中血管紧张素转换酶 (ACE) 抑制肽的释放，并且分离和纯化了这种 ACE 抑制肽。使用了益生菌的契达干酪在 4 °C 糁下成熟后，由于蛋白质的水解量增加使得对 ACE 的抑制活性增加。使用益生菌的干酪成熟 24 周后其 IC_{50} (抑制 50% ACE 活性所需要肽的浓度) 值为 0.23–0.25 mg·mL^{-1}，而未使用益生菌的普通干酪成熟 36 周后其 IC_{50} 值为 0.28 mg·mL^{-1}。干酪的水溶性抽提物经色谱分离后得到多组馏分，粗馏分的 IC_{50} 值在 0.1–2.0 mg·mL^{-1}。将其中 ACE 抑制活性最高的馏分进一步用反相液相色谱纯化。经测定分离、纯化得到的不同 ACE 抑制肽 α_{s1}- 酪蛋白的 N 端肽分别为 (f 1–6), (f 1–7), (f 1–9), (f 24–32) 和 (f 102–110), β- 酪蛋白的 N 端肽为 (f 47–52) 和 (f 193–209)。实验结果证明，契达干酪的 ACE 抑制作用完全取决于蛋白质的水解程度。本研究成功地将益生菌用于契达干酪的生产，即可以提高干酪产品的功能性，同时还提高干酪产品中生物活性肽的产量。

血管紧张素转换酶抑制剂 (ACE-I) / 生物活性肽 / 契达干酪 / 益生菌

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Résumé – Activité inhibitrice de l'enzyme de conversion de l'angiotensine dans les fromages Cheddar fabriqués avec ajout de probiotique *Lactobacillus casei* sp. Cette étude a eu pour but d'examiner le relargage, au cours de l'affinage, de peptides inhibiteurs de l'enzyme de conversion de l'angiotensine (ACE) dans des cheddars fabriqués à l'aide de levains lactocoques et *Lactobacillus casei* 279 ou *Lb casei* LAFTI® L26, et d'isoler, purifier et identifier ces peptides. L'ajout de probiotiques augmentait l'activité inhibitrice de l'ACE dans les fromages au cours de l'affinage à 4 °C, probablement en raison de la protéolyse accrue. La concentration IC_{50} (concentration en ACE nécessaire pour inhiber 50 % de l'activité de l'enzyme) était la plus faible après 24 semaines d'affinage dans les fromages avec probiotique (0,23–0,25 $mg \cdot mL^{-1}$), en comparaison aux 36 semaines d'affinage pour les fromages sans ajout de probiotique (0,28 $mg \cdot mL^{-1}$). Les extraits hydrosolubles de chaque fromage ont été soumis à différentes étapes de fractionnement chromatographique. L'activité inhibitrice retrouvée dans les fractions brutes variait de 0,1 à 2,0 $mg \cdot mL^{-1}$. La fraction possédant l'activité la plus élevée a été purifiée à l'aide d'une seconde étape de chromatographie. Différents peptides inhibiteurs de l'ACE correspondants à la partie N-terminale de la caséine α_{s1} [(f 1–6), (f 1–7), (f 1–9), (f 24–32) et (f 102–110)] et à la partie N-terminale de la caséine β [(f 47–52) et (f 193–209)] ont été retrouvés. Ces résultats suggèrent que l'inhibition de l'ACE dans les cheddars dépendent dans une certaine mesure de la protéolyse. Les probiotiques utilisés dans cette étude ont été ajoutés de façon efficace dans le cheddar, afin de procurer les effets bénéfiques pour la santé en même temps que la production de peptides bioactifs.

enzyme de conversion de l'angiotensine (ACE-I) / peptide bioactif / cheddar / probiotique

Abbreviation key: **ACE** = angiotensin-I-converting enzyme, **CAF** = chemically assisted fractionation, **CN** = casein, **HHL** = hippuryl-histidiyl-leucine, **IC_{50}** = 50% inhibitory concentration, **MALDI-TOF-MS** = matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry, **RP-HPLC** = reverse phase high performance liquid chromatography, **TFA** = trifluoroacetic acid, **WSE** = water-soluble extract.

1. INTRODUCTION

Milk protein is a rich source of biologically active peptides such as antihypertensive, antithrombotic, opioid, immunostimulating, antimicrobial, mineral carrying and cholesterol-lowering peptides [41]. Most of these peptides are hidden in the inactive state in the original parent protein structure and may be released by proteolysis. Protease such as plasmin in milk can hydrolyze milk proteins during cheese ripening. Proteolytic enzymes from bacterial cultures may be responsible for the breakdown of protein into peptides and amino acids. Intracellular peptidases of lactic acid bacteria may also contribute to further degradation after cell lysis.

Angiotensin-I-converting enzyme (**ACE**; peptidyldipeptide 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 [17]. ACE inhibition results in an antihypertensive effect and may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity [25]. The first reported competitive inhibitor of ACE is the naturally occurring peptides in snake venom [32]. Many other ACE inhibitors have been discovered from enzymatic hydrolysis of bovine caseins (**CNs**), plant and other food proteins [31].

Various studies have been reported on ACE-inhibitory peptides found in fermented milk products [30, 40, 44]. Nakamura et al. [30] reported that two peptides with amino acid residues of Val-Pro-Pro and Ile-Pro-Pro, isolated from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, exhibited ACE inhibitory and antihypertensive activities. The concentrations of the peptides required to inhibit 50% of angiotensin-I-converting enzyme activity (**IC_{50}**) were 9 and 5 $\mu mol \cdot L^{-1}$, respectively. Several other ACE inhibitory peptides derived from β-CN including (f 6–14), (f 47–52) and (f 73–82) have been isolated from fermented milk

with *Lb. delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4 [11]. Peptides derived from α_{s1}-CN including (f 24–31) and (f 170–199) and those from -CN such as (f 168–175), (f 183–190), (f 113–127), (f 193–210), (f 70–97), (f 191–210) and (f 16–91) by *Lb. helveticus* CP790 proteinase have shown to exhibit ACE inhibitory activities after oral administration to spontaneously hypertensive rats. Among those peptides, (f 43–69) of the β-CN showed the highest ACE inhibitory activity with IC_{50} of 4 $\mu mol \cdot L^{-1}$ [45].

Ripened-type cheeses contain numerous peptides that originate mainly from casein released as a result of proteolysis during ripening. Some ACE-inhibitory peptides have been isolated from several Italian Cheeses (Crescenza, Gorgonzola, Mozzarella and Italico) [43] and other cheeses (Camembert, Edam, Gouda, Cheddar, Roquefort, Emmentaler and Parmesan) [26, 31, 37]. The appearance of these bioactive peptides is influenced by proteolysis, but only to a certain degree. An α_{s1}-CN derived antihypertensive peptide isolated from Parmesan cheese at 6 months of ripening could not be found after 15 months [1]. Similarly the antihypertensive activity found in long-ripened Gouda cheese was half as much as that found in its medium-aged counterpart [26]. Consistent with these findings, Gomez-Ruiz et al. [12] who studied the ACE-inhibitory peptides in Manchego cheese found the antihypertensive activity to decrease within the first 4 months, was maximum at 8 months of ripening and decreased again at 12 months. Following isolation from Gouda cheese, Saito et al. [38] analyzed the structure of the antihypertensive peptides and concluded that the strongest depressive effect on the systolic blood pressure and the highest ACE-inhibitory capacity was associated with peptides found in 8 months old cheese. A fermented low-fat hard cheese produced with probiotic lactic acid bacteria was found to produce high amounts of ACE-inhibitory peptides derived from α_{s1}-CN during maturation [37]. The peptides emerged at the age of 3 months and their level remained stable for 6 months.

In our previous study, probiotic organisms including *Lb. casei* 279 or *Lb. casei* LAFTI® L26 were used as adjunct in the development of probiotic Cheddar cheeses. These organisms have been selected based on their acid and bile tolerance, adhesion to intestinal cell line, anticarcinogenic properties, oxygen sensitivity and ability to modify gut microflora of human subjects [21]. Our previous reports show that these strains maintained a high level of viability of > 7.0 $\log_{10}$ $cfu \cdot g^{-1}$ at the end of ripening period of 6 months at 4 ºC with minimal effect on the cheese composition [33, 34]. Addition of the probiotic adjuncts also changed the proteolytic pattern and more peptides were released into probiotic cheeses. The objective of the present report was to study the influence of probiotic adjuncts on the ACE-inhibitory activity of the cheeses during ripening and to isolate, purify and identify the ACE-inhibitory peptides.

2. MATERIALS AND METHODS

2.1. Starter and probiotic organisms

Freeze-dried form of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris,* obtained from DSM Food Specialties Pty. Ltd. (Moorebank, NSW, Australia) were used as the starter culture. The organisms were activated by growing two times at 30 ºC overnight in 12% (wt/vol) sterile reconstituted skim milk (RSM) containing 2% (wt/vol) glucose and 1.2% (wt/vol) yeast extract prior to use as bulk culture (2%, vol/vol).

The probiotic organism, *Lb. casei* 279, was obtained from the Australian Starter Culture Collection Center (ASCC) (Werribee, Vic, Australia), while *Lb. casei* LAFTI® L26 was obtained from DSM Food Specialties Pty. Ltd. Both *Lactobacillus* species were subcultured (1%, vol/vol) two times at 37 °C overnight in 12% (wt/vol) sterile RSM prior to use as a bulk culture (2%, vol/vol).

2.2. Cheddar cheese manufacture

Three batches of Cheddar cheeses were made using 10 L of pasteurized milk in a pair of custom made cheese vats. Batch 1

was produced with starter lactococci alone using 1.5% inoculum of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Batch 2 was produced with starter lactococci and probiotic *Lb. casei* 279 and Batch 3 was produced with starter lactococci and probiotic *Lb. casei* LAFTI® L26. The probiotic bacteria were added as adjunct at 1.2% (vol/vol). Cheeses were manufactured in triplicate according to the standard procedures of Kosikowski [19] as described previously by Ong et al. [33]. Fresh cheeses were removed from the mold, packed in oxygen barrier Cryovac® bags (Cryovac® Pty. Ltd., Fawkner, Vic, Australia), heat-sealed with a Multivac® vacuum packaging equipment (Multivac Sepp Haggenmüller, Wolfertschwenden, Germany) and ripened at 4 °C for 36 weeks.

2.3. Reverse-phase HPLC of cheese water-soluble extract

Cheese samples (10 g) were homogenized in 10 mL of water with an ultraturrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at 10 000× *g* for 2 min. The slurry was centrifuged (Sorvall, Newtown, CT, USA) for 20 min at 4000× *g* and 4 °C. The soluble fraction located between the upper layer (fat) and the precipitate (casein) was filtered through Whatman No. 41 filter paper. The extracts were further centrifuged at 4000× *g* for 20 min at 4 ºC and filtered through glass wool to obtain the clear supernatant (water-soluble extract, **WSE**). The extracts were then concentrated by freeze drying (Dynavac FD300; Airvac Engineering Pty. Ltd., Rowville, Australia) at –20 ºC and –100 kPa for 72 hours. The freeze-dried WSE was then stored in vacuum at –20 ºC for analysis with reverse phase–high performance liquid chromatography (**RP-HPLC**) and for determination of ACE-inhibitory activity.

An aliquot of freeze-dried WSE of 40 mg was dissolved in 1 mL of solvent A (10% of acetonitrile; Merck, South Granville, NSW, Australia) containing 0.05% trifluoroacetic acid (**TFA**) solution (Sigma-Aldrich, St. Louis, MO, USA), centrifuged (14 000× *g*, 10 min) using a bench top centrifuge (Sorvall RT7, Newtown, CT, USA) and filtered through a 0.45-µm filter (Millipore Corp., Bedford, MA, USA). RP-HPLC was performed using Varian HPLC (Mulgrave, Vic, Australia) consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength ultraviolet-visible tunable absorbance detector and a 730 data module. A sample size of 50 µL was injected into the reverse-phase column (C18, 250 mm × 4.6 mm, 5 µm, Grace Vydac, Hesperia, CA, USA) with a guard column and disposable cartridge (10 mm, 12 µm, Grace Vydac). The separation was conducted at room temperature (~22 °C) at a flow rate of 0.75 mL·min^{-1}. The eluent B was 60% acetonitrile containing 0.05% TFA. A linear gradient was applied from 0 to 80% eluent B over 100 min. The detection device was the ultraviolet-visible detector set at 215 nm.

2.4. ACE-inhibitory activity of cheese water-soluble extract

The ACE-inhibitory activity of the freeze-dried WSE was measured using the spectrophotometric assay of Cushman and Cheung [5] and Donkor et al. [6]. The method is based on the liberation of hippuric acid from hippuryl-histidyl-leucine (**HHL**, Sigma) catalysed by ACE. Each assay mixture contained 200 µL HHL solution (3.8 mmol·L^{-1} HHL, 100 mmol·L^{-1} sodium borate buffer, 300 mmol·L^{-1} NaCl, pH 8.3), 2 mU ACE (from rabbit lung; Sigma) and 35 µL sample solution (15 mg freeze-dried WSE in 1 mL of distilled water). After 30 min of incubation at 37 ºC, the hippuric acid was extracted with 1.7 mL ethyl acetate. The mixture was centrifuged and 1.5 mL of the organic phase (ethyl acetate) was transferred to a fresh test tube and evaporated to dryness on a water bath for 15 min at 100 ºC. The residue containing hippuric acid was dissolved in 1 mL deionised water and the solution was measured using Cary IE UV visible spectrophotometer (Varian) at 228 nm against deionised water as a blank. The percent inhibition was calculated as follows: ACE-inhibition (%) = [1– (A–C)/(B–D)] × 100, where A is the absorbance with ACE, HHL and ACE-inhibitory sample, B is the absorbance with

ACE and HHL without ACE-inhibitory sample, C is the absorbance with HHL and ACE-inhibitory sample without ACE and D is the absorbance with HHL without ACE and ACE-inhibitory sample. The inhibition was also expressed as IC_{50} (the concentration of an ACE inhibitor needed to inhibit 50% of ACE activity). All inhibition values reported are the means of two determinations of three replicates (n = 6).

2.5. Protein assays

The protein content in the cheese WSE and in the purified fractions was determined by the Folin-Lowry method [22]. A sample (100 μL) was mixed with an alkaline-copper reagent (3 mL) and folin phenol reagent (0.3 mL; two-fold dilution with distilled water of folin-phenol; Sigma). After the solution was allowed to stand for 30 min, the absorbance was measured at 650 nm using a Pharmacia LKP Novaspek II Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). All determinations were carried out in duplicate.

2.6. Isolation of ACE- inhibitory peptides

For peptide separation, the WSE from 24 weeks old cheese was used. A sample size of 100 μL (80 mg freeze-dried WSE in 1 mL of solvent A) was injected into a reverse-phase column and forty fractions from each batch of cheeses were collected using a fraction collector (Pro Star 704; Varian) on a 2-min collection time basis. This step was repeated 10 times and the 40 fractions from the various chromatographic runs were pooled and concentrated using a vacuum evaporator (SpeedVac SC110 concentrator, Savant Instruments Inc., Farmingdale, NY, USA) to a final volume of ~1 mL and filtered through a low protein binding 0.2 μm filters (Millipore Corp., Bedford, MA, US). An aliquot of the concentrated fractions (50 μL) was used to determine the ACE-inhibitory activity. The fractions with the highest ACE-inhibitory activity were subjected to further separation and purification by RP-HPLC. The chromatographic conditions used were as described in Section 2.3.

2.7. Identification of peptides

Purified peptides were sequenced by an automated Edman degradation method using a protein sequencer (490 Procise, Perkin Elmer Co. Ltd., Applied Biosystem Division, Foster City, CA, USA) with a PTH-C18 column (2.1 × 220 mm; Perkin Elmer Co. Ltd.). Chemicals used in the 140C microgradient delivery system included two types of solvent (Perkin Elmer Co. Ltd.): A3 (3.5% vol/vol aqueous tetrahydrofuran) and B (acetonitrile and isopropanol).

The molecular mass of purified peptides was analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF-MS**) (Bruker Daltonics Inc., Billerica, MA, USA). The matrix used for the analysis was α-cyano-4-hydroxycinnamix acid (Aldrich Chemical Co., Milwaukee, WI, USA). A saturated solution of matrix was prepared in acetonitrile/water (1:1) containing 0.25% (vol/vol) TFA. The crystal matrix-analyte was ionized by a 337 nm nitrogen laser pulse and accelerated under 25 000 V before passing through the time-of-flight mass spectrophotometer. The instrument was set in the positive linear mode.

Peptide sequences were also obtained by using chemically assisted fragmentation (CAF) in conjunction with MALDI-TOF-MS. Ettan™ CAF MALDI sequencing kit (Amersham Biosciences, Uppsala, Sweden) was used to derivatize purified peptides. Peptides were bound to a prepacked reversed-phase C18 matrix in pipette tips (ZipTip™ pipette tips, Millipore Corp.). Acquisition of spectra in reflectron and post source decay (PSD) modes was performed using an Ettan MALDI-TOF-MS. For each analysis, the selected mass of the peptide from the reflectron spectrum was subjected to PSD mode. Mass differences between the fragment ions were calculated to determine the peptide sequence.

2.8. Statistical analysis

Data analysis was carried out with Minitab statistical package. One-way analysis of variance was used to find out differences

between means of ACE-inhibition, IC_{50} and total area of peaks with a significant level at $\alpha = 0.05$.

3. RESULTS

3.1. Reverse-phase HPLC of cheese water-soluble extract

The RP-HPLC peptide profiles of control Cheddar cheeses and probiotic Cheddar cheeses with *Lb. casei* 279 or *Lb. casei* LAFTI® L26 at week 1, 12, 24 and 36 are shown in Figure 1. More peptides were released into the cheeses as the ripening period increased. The number of peaks and total area increased significantly ($P < 0.05$) during the first 12 weeks (data not shown), continued to increase slowly but insignificantly ($P > 0.05$) until 24 weeks and remained constant after that period in all cheeses. The results show that the rate of proteolysis in the cheeses was more extensive at the early stage of ripening. Previously we have reported that cheese with the addition of *Lb. casei* 279 or *Lb. casei* LAFTI® L26 had increased proteolysis with higher concentration of water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) [35]. In the present study, the peptide profiles of the three different batches of cheeses were however very similar (Fig. 1). There are no significant differences ($P > 0.05$) in total area or the number of peaks between the cheeses (data not shown).

3.2. ACE-inhibitory activity of cheese water-soluble extract

The ACE-inhibitory activity of WSE of cheese samples collected at 12 weeks intervals is shown in Figure 2. ACE-inhibitory activity of the cheeses increased significantly ($P < 0.05$) especially during the first 12 weeks of ripening. The inhibitory activity continued to increase in all cheeses for 24 weeks and stabilized after that period. The IC_{50} of control cheese (Batch 1) was the lowest after 36 weeks of ripening at 0.28 $mg \cdot mL^{-1}$ and those of probiotic cheeses were the lowest after 24 weeks of ripening at 0.23 and 0.25 $mg \cdot mL^{-1}$ in cheeses with *Lb. casei* 279 (Batch 2) and *Lb. casei* LAFTI® L26 (Batch 3), respectively. The IC_{50} of the probiotic cheeses (Batches 2 and 3) at 24 weeks of ripening was significantly lower ($P < 0.05$) than that of the control cheese (0.37 $mg \cdot mL^{-1}$, Batch 1). The IC_{50} between the cheeses (Batches 1–3) at 36 weeks of ripening was, however, not significantly different ($P < 0.05$). The result shows that cheeses with the addition of probiotic had higher ACE-inhibitory activity during the first 24 weeks of ripening at 4 °C. There was no significant difference ($P > 0.05$) between the percentage inhibition or the IC_{50} of the cheeses between the ripening period of 24 weeks and 36 weeks. The isolation of ACE-inhibitory peptides in all cheeses was thus performed on 24 weeks old cheeses.

3.3. Isolation of ACE- inhibitory peptides

Forty fractions from each batch of the cheeses were collected by RP-HPLC connected to a fraction collector on a 2-min collection time basis. The ACE-inhibitory activities of the 40 fractions collected from control cheese (Batch 1), probiotic cheese with *Lb. casei* 279 (Batch 2) and probiotic cheese with *Lb. casei* LAFTI® L26 (Batch 3) are shown in Figure 3. Several fractions distributed throughout the acetonitrile gradient consistently showed high ACE-inhibition index. The ACE-inhibition and IC_{50} of fraction 9 were 87% (IC_{50}, 0.20 $mg \cdot mL^{-1}$), 60% (IC_{50}, 0.18 $mg \cdot mL^{-1}$) and 64% (IC_{50}, 0.25 $mg \cdot mL^{-1}$) in Batches 1, 2 and 3, respectively. Fraction 15 consistently showed a high ACE-inhibition and low IC_{50} value, especially in probiotic cheeses. The ACE-inhibition and IC_{50} of fraction 15 were 42% (IC_{50}, 0.16 $mg \cdot mL^{-1}$), 79% (IC_{50}, 0.12 $mg \cdot mL^{-1}$) and 69% (IC_{50}, 0.14 $mg \cdot mL^{-1}$) in Batches 1, 2 and 3, respectively. Fraction 27 showed a consistently high ACE-inhibition and low IC_{50} value in all three batches of cheeses. The ACE-inhibitions and IC_{50} of fraction 27 were 54% (IC_{50}, 0.12 $mg \cdot mL^{-1}$), 61% (IC_{50}, 0.09 $mg \cdot mL^{-1}$) and 63% (IC_{50}, 0.11 $mg \cdot mL^{-1}$) in Batches 1, 2 and 3,

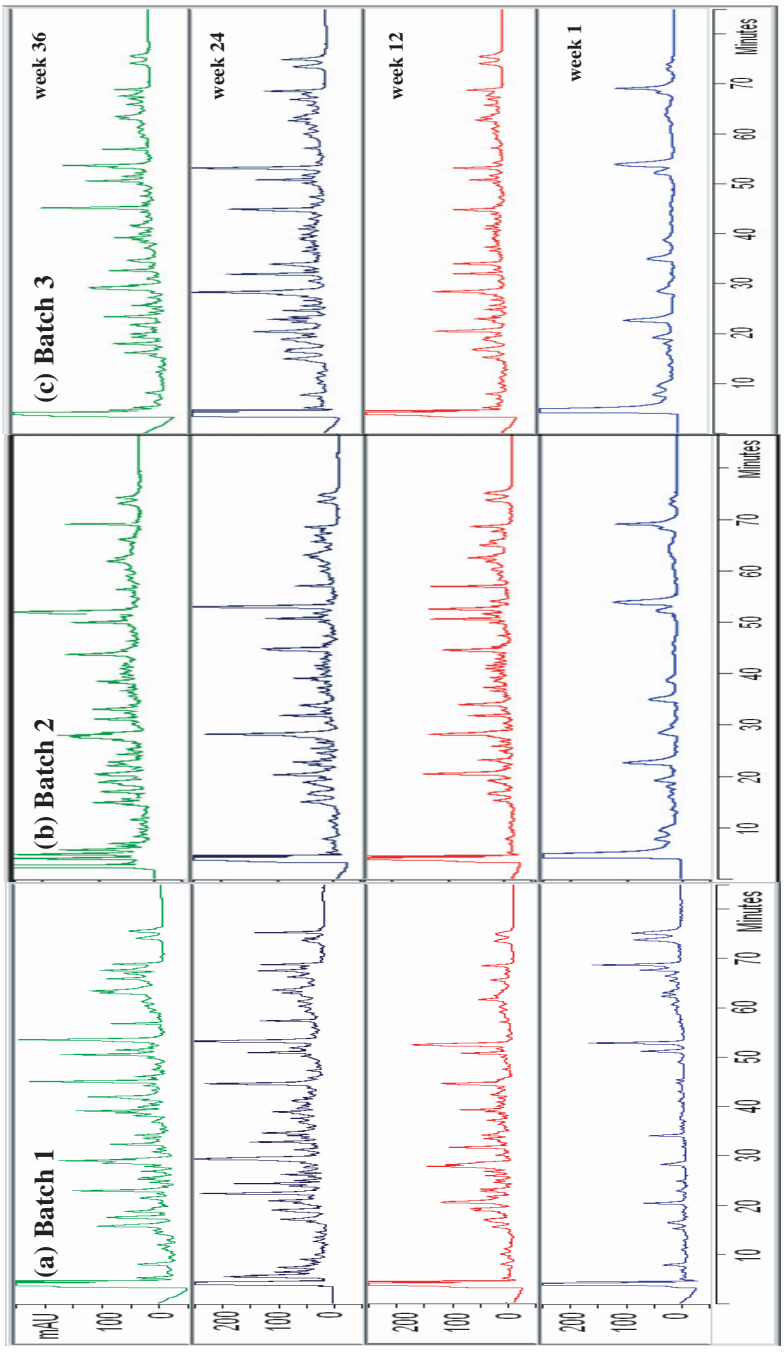


Figure 1. Peptide profile of water-soluble extract (WSE) of (a) control Cheddar cheese (Batch 1), (b) probiotic Cheddar cheeses with *Lb. casei* 279 (Batch 2) and (c) probiotic Cheddar cheese with *Lb. casei* LAFTI® L26 (Batch 3) at weeks 1, 12, 24 and 36. Eluent A was 10% acetonitrile containing 0.05% TFA solution. Eluent B was 60% acetonitrile containing 0.05% TFA. Gradient: 0 to 100 min, 0 to 80% eluent B; 100 to 105 min, 80 to 100% eluent B; 105 to 110 min, 100 to 0% eluent B. Detection was at 215 nm.

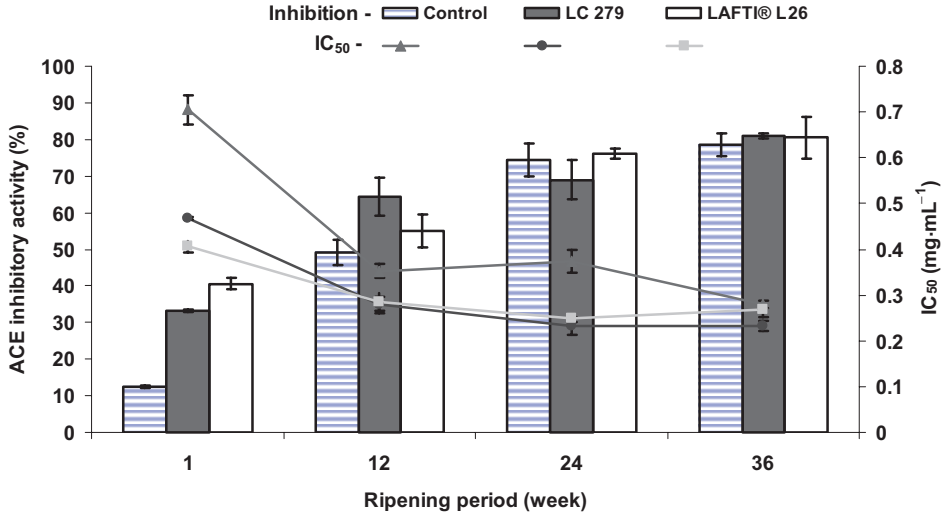


Figure 2. The ACE-inhibitory activity of water-soluble extract (WSE) of control Cheddar cheese (Batch 1) and probiotic Cheddar cheeses with *Lb. casei* 279 (Batch 2) or *Lb. casei* LAFTI® L26 (Batch 3) at weeks 1, 12, 24 and 36. IC_{50} is concentration of ACE inhibitor needed to inhibit 50% of ACE activity. Results were expressed as mean ± SE (n = 6).

respectively. Fraction 28 was collected from a higher concentration of acetonitrile gradient. The ACE-inhibition and IC_{50} of fraction 28 were 57% (IC_{50}, 0.14 mg·mL^{-1}), 50% (IC_{50}, 0.17 mg·mL^{-1}) and 46% (IC_{50}, 0.14 mg·mL^{-1}) for Batches 1, 2 and 3, respectively.

Fractions 9, 15, 27, 28 obtained from the three different batches of the cheeses were re-injected into RP-HPLC and the profiles of the fractions were found to be very similar. Only fractions obtained from *Lb. casei* LAFTI® L26 (Batch 3) were subsequently purified by further RP-HPLC with different gradient or isocratic run to obtain pure peptides for identification purpose.

3.4. Identification of peptides

N-terminal sequencing of the peptides was obtained by using an automated Edman degradation technique on a protein sequencer and the molecular weights of the peptides were determined by MALDI-TOF-MS. The details of the fractions are shown in Table I. Most of the fractions collected contained a mixture of several peptides. The first five residues of fraction 9 obtained from the N-terminal sequencing were Arg-Pro-Lys-His-Pro. Following sequence interpretation and molecular weight determination, the peptides in fraction 9 were identified as α_{s1}-CN (f 1–9), α_{s1}-CN (f 1–7) and α_{s1}-CN (f 1–6) (Tab. I). The first five residues obtained from the N-terminal sequencing of fraction 15 were Asp and Glu in the first, Lys and Val in the second, Ile in the third, His and Glu in the fourth, and Pro in the fifth residue. The mass spectra of fraction 15 only corresponded to sequence Asp-Lys-Ile-His-Pro-Phe with molecular molecular weight of 755.4 g·mol^{-1}. Thus only one peptide derived from β-CN (f 47–52) was identified from fraction 15 (Tab. I). The first five residues of fraction 27 obtained from the N-terminal sequencing were Phe and Lys, Val and Lys, Ala and Tyr, Pro and Lys, Phe and Val in the first, second, third, fourth and fifth residues, respectively. The sequence interpretation and the mass spectrums (1053.3 and 1132.4 g·mol^{-1}) showed that fraction 27 had peptides derived from α_{s1}-CN (f 102–110) and α_{s1}-CN (f 24–32) (Tab. I).

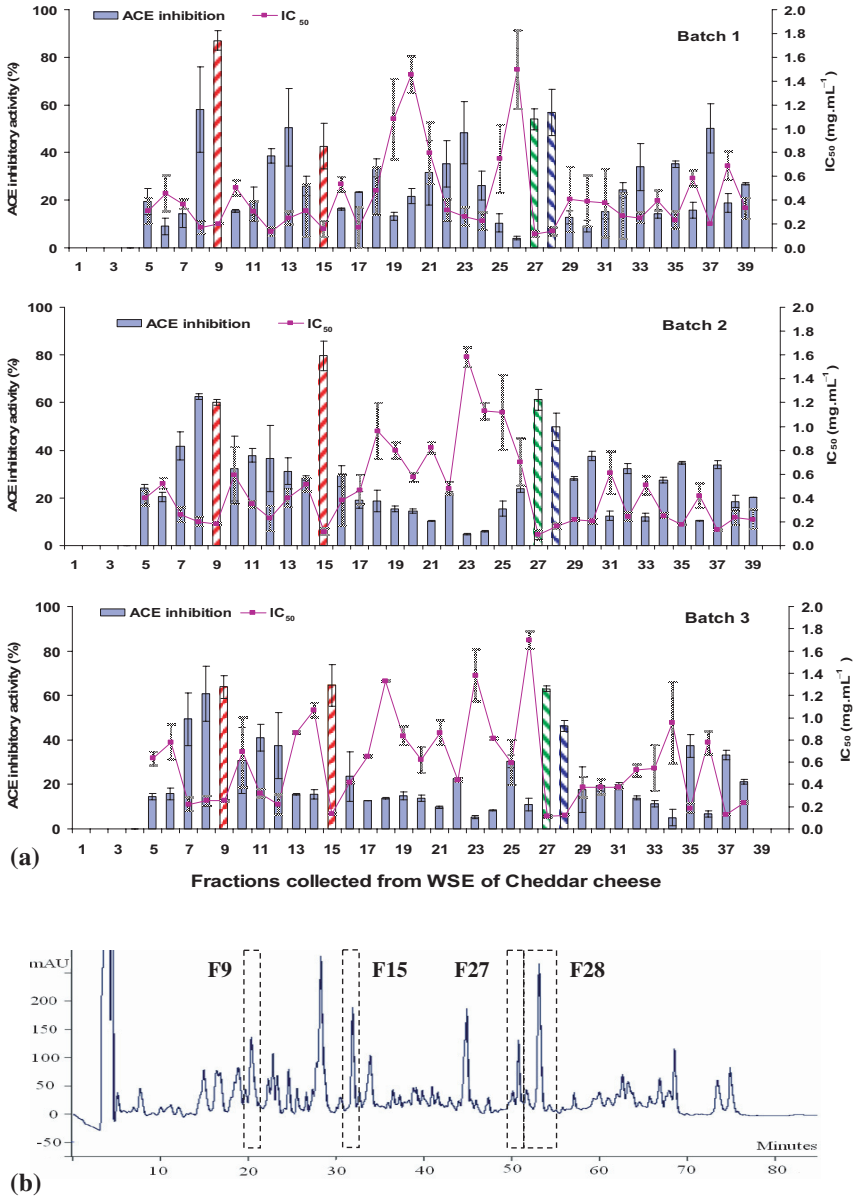
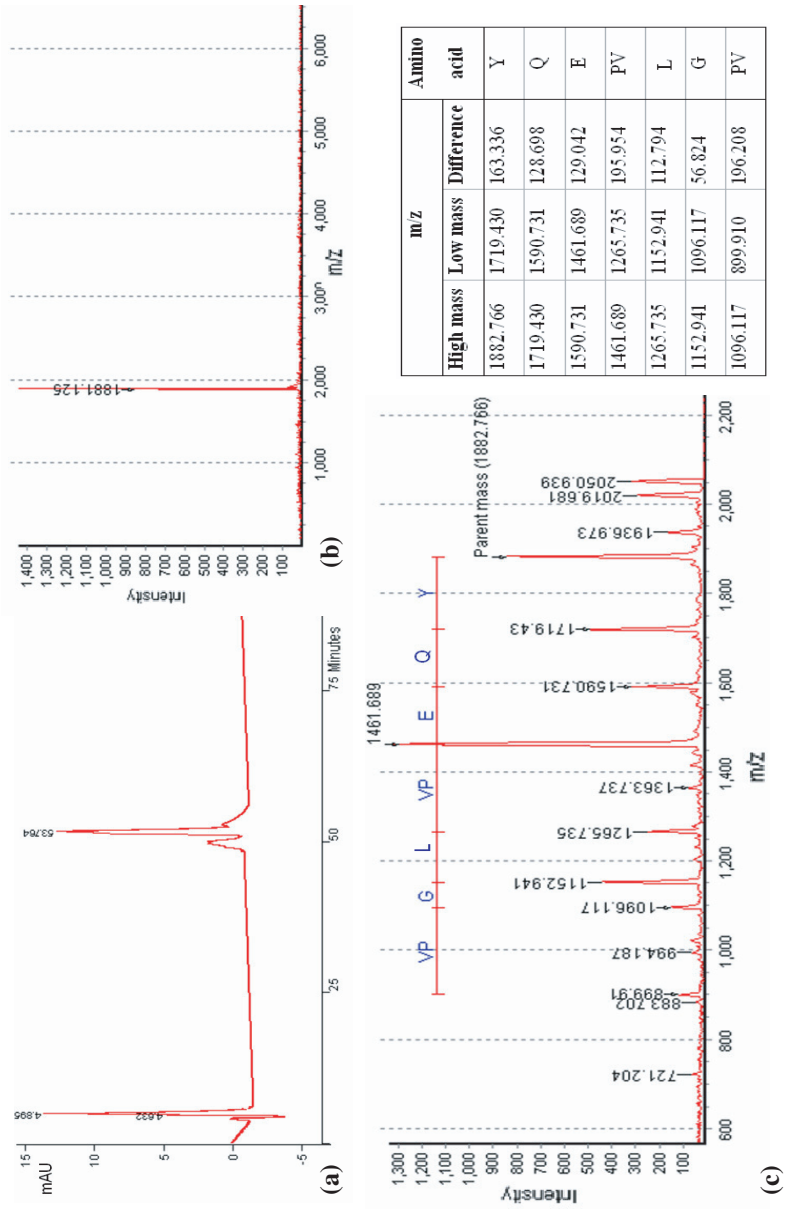


Figure 3. (a) The ACE-inhibitory activity of 40 fractions collected from water-soluble extract (WSE) of control Cheddar cheese (Batch 1), probiotic Cheddar cheeses with *Lb. casei* 279 (Batch 2) and probiotic Cheddar cheese with *Lb. casei* LAFTI® L26 (Batch 3). IC_{50} is concentration of ACE inhibitor needed to inhibit 50% of ACE activity. Results were expressed as mean ± SE (n = 6). Fractions without results mean that no inhibitory activity was obtained. (b) RP-HPLC profiles of fractions with the highest ACE-inhibitory activity. Fractions 9, 15, 27 and 28 were further collected and re-injected into RP-HPLC for further purification (RP-HPLC condition as described in Sects. 2.3 and 2.6).

Table I. Identified peptides in the fractions obtained from water-soluble extract of Cheddar cheese produced with *Lb. casei* LAFTI® L26.

Fraction	Sequences	Origin	Experimental molecular mass ($g \cdot mol^{-1}$)	Theoretical molecular mass ($g \cdot mol^{-1}$)
9	Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln (RPKHPIKHQ)	α_{s1}-CN (f 1–9)	1140.7	1140.4
9	Arg-Pro-Lys-His-Pro-Ile-Lys (RPKHPIK)	α_{s1}-CN (f 1–7)	877.0	875.1
9	Arg-Pro-Lys-His-Pro-Ile (RPKHPI)	α_{s1}-CN (f 1–6)	745.4	746.9
15	Asp-Lys-Ile-His-Pro-Phe (DKIHPF)	β-CN (f 47–52)	755.4	755.5
27	Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe (FVAPFPEVF)	α_{s1}-CN (f 24–32)	1053.3	1052.2
27	Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu (KKYKVPQLE)	α_{s1}-CN (f 102–110)	1132.4	1134.4
28	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (YQEPVLGPVRGPFPIIV)	β-CN (f 193–209)	1881.1	1881.3



m/z			Amino
High mass	Low mass	Difference	acid
1882.766	1719.430	163.336	Y
1719.430	1590.731	128.698	Q
1590.731	1461.689	129.042	E
1461.689	1265.735	195.954	PV
1265.735	1152.941	112.794	L
1152.941	1096.117	56.824	G
1096.117	899.910	196.208	PV

Figure 4. (a) RP-HPLC profiles of fraction 28 of Cheddar cheeses produced with the addition of probiotic *Lb. casei* LAFTI® L26 (condition as described in Sect. 2.3). (b) Molecular weight of purified peptide from fraction 28 obtained with MALDI-TOF-MS (condition as described in Sect. 2.7). (c) Chemically assisted fragmentation (CAF) of purified peptide from fraction 28. The first nine amino acids of the N-terminal was identified as YQEPVLGPV (CAF condition as described in Sect. 2.7). Following sequence interpretation and molecular weight determination, the peptide was identified as β-CN (f 193–209).

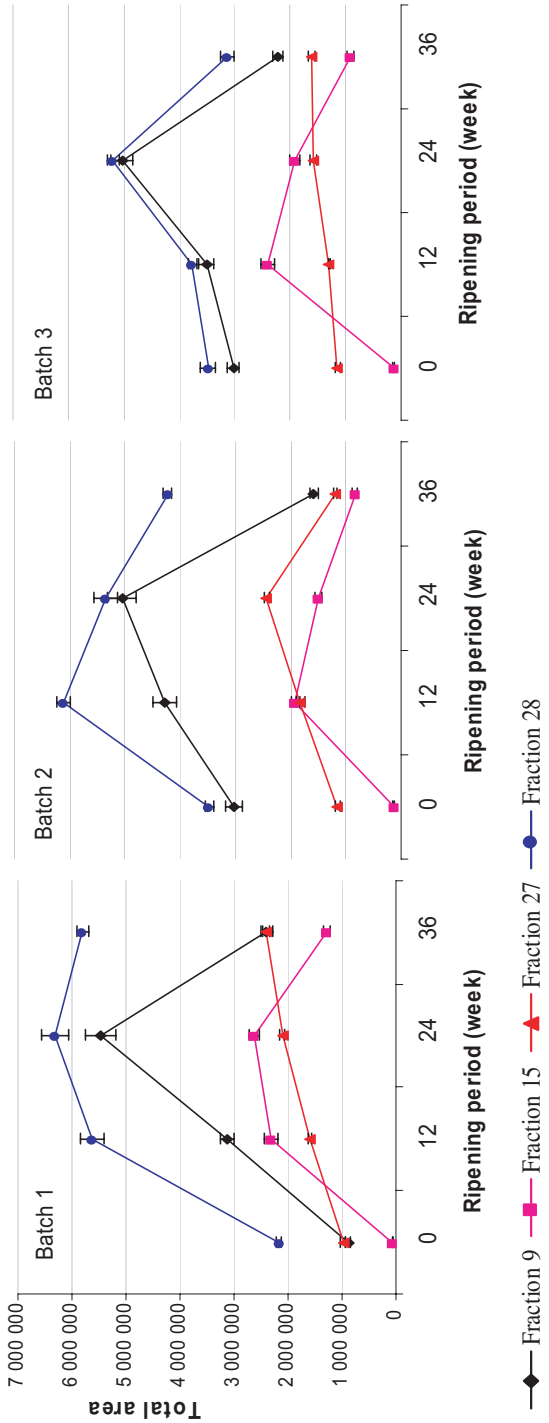


Figure 5. Total area of ACE-inhibitory fractions obtained from water soluble extract of control Cheddar cheese (Batch 1) and probiotic cheeses made with *Lb. casei* 279 (Batch 2) or *Lb. casei* LAFTI® L26 (Batch 3) during ripening at 4 °C for 36 weeks. Total area is the area of the peak (ACE-inhibitory fractions highlighted in Fig. 3b) obtained from HPLC-RP. Results were expressed as total area ± standard error (n = 6).

Sequences and molecular weight of fraction 28 corresponded to peptide derived from β-CN (f 193–209).

The identification method was also confirmed by using chemically assisted fragmentation (CAF) chemistry followed by MALDI-TOF-MS determination (Fig. 4). Purified peaks were derivatized using the Ettan™ CAF-MALDI sequencing kit. CAF-MALDI chemistry is based on the introduction of a negatively charged group to the N-terminus of peptides generated by tryptic digestion. Only y-ions, which retain a net positive charge, are separated and detected while N-terminal fragments are neutral and not detectable. With the generation of only y-ion series fragments, the mass between two peaks on the spectrum could be corresponded to the mass of individual amino acids. The first 9 residues obtained by using CAF-MALDI of fraction 28 were identified as Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val (Fig. 4). Thus the peptide was confirmed as β-CN (f 193–209). Peptides in fractions 9, 15 and 27 were also confirmed using CAF-MALDI (data not shown).

3.5. Formation of peptides during ripening

After 24 weeks of ripening, control cheese had more of the fraction 9 as compared to the probiotic cheeses. Figure 5 shows that the total area of fraction 9 was higher in control cheese (Batch 1) after 24 weeks, which possibly explained the high ACE-inhibitory index in fraction 9 of control cheese (Fig. 3). The total area of fraction 9 reduced after 24 weeks in all cheeses (Fig. 5). Further ripening, possibly degraded these peptides to smaller peptides which may have changed their bioactivity. The peak area of fraction 27 increased significantly ($P < 0.05$) as the ripening time increased to 24 weeks in all cheeses (Batches 1–3) and decreased significantly ($P < 0.05$) in cheese with *Lb. casei* 279 (Batch 2) and remained relatively constant in cheese with *Lb. casei* LAFTI® L26 (Batch 3) after 24 weeks of ripening (Fig. 5). Total area of fraction 27 in control cheese continued to increase even after 24 weeks of ripening (Fig. 5). The peak area of fraction 28 increased during ripening especially during the first 3 months in Batches 1–3, but decreased after further ripening (Fig. 5).

4. DISCUSSION

It has been reported that proteinases of lactic acid bacteria can hydrolyze more than 40% of the peptide bonds of β-CN resulting in the formation of more than 100 different oligopeptides, which are in turn actively degraded by the complex peptidase system. The same pattern is reported for α_{s1}-CN. Consequently lactic acid bacteria could potentially generate a large variety of peptides including bioactive peptides [20]. Rokka et al. [36] have reported the release of a variety of bioactive peptides by enzymatic proteolysis of UHT milk fermented with probiotic *Lb. casei* ssp. *rhamnosus* strain. The type of dairy product, the technology adapted, and the selection of strain based on the specificity of proteolysis are factors that influence the proteolytic activation of bioactive peptides. ACE-inhibitory peptides have been found in several types of cheeses, which differ with respect to the type of starter and the ripening condition used [14, 15, 37, 38].

In our study, *Lb. casei* 279 or *Lb. casei* LAFTI® L26 was added as an adjunct in Cheddar cheeses production and the ACE-inhibitory activity was monitored during a ripening period of 36 weeks at 4 °C. The rate of increase of the ACE-inhibitory activity during ripening was very similar to the rate of proteolysis of cheeses with *Lb. casei* 279 or *Lb. casei* LAFTI® L26 reported previously [34]. Proteolysis was reported to be more extensive in probiotic cheeses with *Lb. casei* 279 or *Lb. casei* LAFTI® L26 after ripening for 24 weeks at 4 °C [34]. The IC_{50} of the probiotic cheeses (Batches 2 and 3) at 24 weeks of ripening was significantly lower ($P < 0.05$) than the control cheese (Batch 1). The data thus show that ACE-inhibitory activity was higher in cheeses received a higher degree of proteolysis. The IC_{50} between the cheeses (Batches 1–3) at 36 weeks of ripening was, however, not significantly different ($P < 0.05$). ACE-inhibition is thus dependent on the extent of

proteolysis but only to a certain extent. Our results support the previous findings of Addeo et al. [1], Gomez-Ruiz et al. [12], Ryahanen et al. [37], and Saito et al. [38].

ACE is predominantly an ectoenzyme with two catalytic sites, one on each lobe of the extracellular portion. Structure activity correlations among different peptides inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tri-peptide sequence of the substrate [24]. ACE appears to prefer substrates or inhibitors that contain mainly hydrophobic (aromatic or branched side chains) amino acid residues at the three C-terminal positions. The structure-activity relationship of ACE-inhibitory peptides has not yet been established and different antihypertensive sequences have been derived from a large number of food proteins. Likewise, most of the potential ACE-inhibitory peptides identified in this study have different structure and sequences.

In cheeses, bovine α_{s1}-CN can be rapidly hydrolyzed by chymosin at Phe^{23}-Phe^{24} to yield α_{s1}-CN (f 1–23) and α_{s1}-CN (f 24–199) [29]. α_{s1}-CN (f 1–23) is hydrolyzed rapidly in cheese by lactococcal cell envelope proteinases to several small peptides resulting in the formation and accumulation of peptides α_{s1}-CN (f 1–9) and α_{s1}-CN (f 1–13) in Cheddar during ripening [42]. In the present study, three peptides from the same N-terminal region of α_{s1}-CN (f 1–23) were isolated, which corresponded to α_{s1}-CN (f 1–9), (f 1–7) and α_{s1}-CN (f 1–6) (Tab. I). The IC_{50} of the peptides was not determined. These peptides were previously isolated from a fermented low-fat hard cheese produced with probiotic bacteria and have shown to have ACE-inhibitory properties [37]. Peptide α_{s1}-CN (f 1–9) isolated from Gouda cheese gave very low IC_{50} value of 13.4 $\mu mol \cdot L^{-1}$ and antihypertensive effect of –9.3 ± 4.8 mm Hg on spontaneously hypertensive rats (SHR) [38]. The peptide derived from α_{s1}-CN (f 1–6) had been isolated from sheep sodium caseinate hydrolysate and was shown to have ACE-inhibitory activity with an IC_{50} of 30.1 $\mu g \cdot mL^{-1}$ [28], probably due to the presence of hydrophobic Pro-Ile residues at the C-terminal end. Synthesized peptide α_{s1}-CN (f 1–6) treated with trypsin and chymotrypsin was also resistant to hydrolysis [28].

The hexapeptide Asp-Lys-Ile-His-Pro-Phe that originates from β-CN (f 47–52) was also isolated in our study (Tab. I). This peptide contained only hydrophobic amino acid. It is interesting to note that this peptide had the first N-terminal amino acid and the last four C-terminal amino acids in common with the octapeptide angiotensin-II generated by ACE hydrolysis of the decapeptide angiotensin-I. Commercial drugs used in antihypertension therapy are based on compounds which may compete for the receptor sites of the vasoconstrictor angiotensin-II due to their partial homology with the product of ACE activity (angiotensin-II). Angiotensin-II receptor antagonists (such as losartan) competitively block angiotensin-II-induced vascular contraction [16]. A similar peptide was found in milk fermented by *L. lactis* subsp. *cremoris* FT4 [11]. In that study, this peptide was chemically synthesized and the ACE-inhibitory activity was confirmed (IC_{50} = 193.9 $mg \cdot L^{-1}$). The chemically synthesized peptide was also resistant to hydrolysis by trypsin and chymotrypsin [11]. Biochemical properties and cleavage site of proteinases and peptidases of *L. lactis* strain have been studied in detail [18]. The cleavage sites of the peptide bonds residue 46–47 and 52–53 of β-CN are hydrolyzed by all of the lactococcal proteinases studied. The ACE inhibitory index of fraction 15 was higher in probiotic cheeses (Fig. 3). The proteolytic enzymes from the probiotic adjuncts could possibly hydrolyze residue 46–47 and 52–53 of β-CN resulting in the liberation of more of this peptide in probiotic cheeses.

The fraction 27 contained a mixture of two peptides derived from α_{s1}-CN (f 102–110) and α_{s1}-CN (f 24–32) (Tab. I). The peptide Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu derived from α_{s1}-CN (f 102–110) had within its sequence the hexapeptide Tyr-Lys-Val-Pro-Gln-Leu of α_{s1}-CN (f 104–109), which has been proven to have in-vitro ACE-inhibitory activity (IC_{50} = 22 $\mu mol \cdot L^{-1}$) [23]. This peptide, however, did not show a major antihypertensive effect after oral administration to spontaneously hypertensive rats [23]. A similar

peptide derived from α_{s1}-CN (f 101–107) of ovine cheese was isolated from Manchego cheese and had a potent ACE-inhibitory activity with an IC_{50} of 77.1 μmol·L^{-1} [12]. This peptide has a high homology with the peptide α_{s1}-CN (f 102–110) found in our study. The synthesized peptide of fragment α_{s1}-CN (f 101–107) was, however, not resistant to hydrolysis with a pancreatic extract and the ACE-inhibitory activity decreased after digestion [13].

The peptide corresponding to α_{s1}-CN (f 24–32) probably originated from α_{s1}-CN (f 24–199) via hydrolysis by chymosin (cleavage sites Phe^{23}-Phe^{24} and Phe^{32}-Gly^{33}). Peptides from within this sequence, α_{s1}-CN (f 24–27), (f 25–27), (f 27–30) have been produced from bovine casein of enzymatic hydrolysis and have shown bradykinin-potentiating activity on the uteri and ilea of rats [8, 24]. The IC_{50} of some of the above peptides varied largely from 2 to > 1000 μmol·L^{-1}.

According to Fox et al. [9], about 3–6% of the coagulant (chymosin) added to cheese milk is retained in the curd. After cheese processing, peptide corresponding to α_{s1}-CN (f 24–32) was possibly released as the action of the remaining coagulant to a certain extent. However, the decrease in the area of the peak corresponding to α_{s1}-CN (f 24–32) in probiotic cheeses (Batches 2 and 3) indicated that this peptide was hydrolyzed, possibly by the proteolytic enzyme produced by the probiotic adjunct. Carboxypeptidase activity in lactobacilli [2] and in nonstarter lactic acid bacteria (NSLAB) in Feta cheese [27] has been reported to hydrolyze α_{s1}-CN (f 24–32) to α_{s1}-CN (f 24–30) and smaller fragments, which further supported the possibility that the proteolytic enzymes from the adjunct probiotic were responsible for the decrease in total area of fraction 27. Peptides derived from β-CN (f 193–209) was isolated from the fractions eluted with a higher concentration of acetonitrile gradient (Tab. I). Leu^{190}-Tyr^{191} and Leu^{192}-Tyr^{193} were known chymosin cleavage sites in solution [7]. Cell-wall-associated proteinases also appear to cleave Leu^{192}-Tyr^{193} in solution [10]. Both peptides were thus most probably the product of hydrolysis by chymosin and cell-wall-associated proteinases from starter lactococci and/or from probiotic adjunct. Peptides derived from β-CN (f 193–209) has previously been isolated from casein by extracellular proteinase from *Lb. helveticus* CP790 and have shown to have ACE-inhibitory activity with IC_{50} of 101 μg·mL^{-1} [45]. Other studies also reported the various bioactivities of these peptides such as immunomodulating properties [4] and antimicrobial activity [39]. Peptide β-CN (f 193–209) and α_{s1}-CN (f 1–9) have been reported to cause bitterness in cheese [3]. Sensory evaluation of Cheddar cheeses made with *Lb. casei* 279 showed bitterness as the major defect [35], but a correlation between ACE-inhibitory peptides derived from β-CN (f 193–209) and α_{s1}-CN (f 1–9) to the production of bitterness in Cheddar cheese needs to be investigated.

5. CONCLUSION

Addition of probiotic organisms increased the ACE-inhibitory activity of the cheeses after ripening for 24 weeks at 4 °C. This may suggest that the proteolytic enzyme of the probiotic organisms could possibly play a role in increasing the production of ACE-inhibitory peptides in Cheddar cheeses. Some of the peptides isolated were not resistant to further proteolysis and bioactivity may have been enhanced only after subsequent proteolysis. These peptides may become potential substrates for the production of other ACE-inhibitory peptides by the proteolytic enzyme of the probiotic organism. Further study on the individual ACE-inhibitory peptides isolated in this study is needed to better understand the correlation between the proteolytic enzyme produced by the probiotic organisms on the generation of these peptides and other ACE-inhibitory peptides generated by subsequent proteolysis of these peptides.

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REFERENCES

[1] Addeo F., Chianes L., Salzano A., Sacchi R., Cappucio U., Ferranti P., Malroni A., Characterization of the 12% trichloroacetic acid-insoluble oligopeptides of Parmigiano-Reggiano cheese, J. Dairy Res. 59 (1992) 401–411.

[2] Atlan D., Gilbert C., Blank B., Frot-Coutaz J., Portalier R., Proteolytic system of lactobacilli, in: Foo E.L., Griffin H.G., Molby R., Heden C.G. (Eds.), Lactic Acid Bacteria, Horizon Sci. Press, Norfolk, UK, 1993, pp. 31–38.

[3] Broadbent J.R., Barnes M., Brennand C., Strickland M., Houck K., Johnson M.E., Steele J.L., Contribution of *Lactococcus lactis* cell envelope proteinase specificity to peptide accumulation and bitterness in reduced-fat Cheddar cheese, Appl. Environ. Microbiol. 68 (2002) 1778–1785.

[4] Coste M., Rochet V., Leonil J., Molle D., Bouhallab S., Tomme D., Identification of C-terminal peptides from bovine β-casein that enhance proliferation of rat lymphocytes, Immunol. Letters 33 (1992) 41–46.

[5] Cushman D.W., Cheung H.S, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637–1648.

[6] Donkor O.N., Anders H., Todor V., Shah N.P., Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yoghurt, J. Food Sci. 70 (2005) 375–381.

[7] Exterkate F.A., Lagerwerf F.M., Haverkamp J., Vanschalkwijk S., The selectivity of chymosin action on α_{s1}- and β-caseins in solution is modulated in cheese, Int. Dairy J. 7 (1997) 47–54.

[8] FitzGerald R.J., Meisel H., Milk protein-derived inhibitors of angiotensin-I-converting enzyme, Br. J. Nutr. 1 (2000) S33–S37.

[9] Fox P.F., Law J., McSweeney P.L.H., Wallace J., Biochemistry of Cheese Ripening, in: Fox P.F. (Ed.), Cheese: Chemistry, Physics and Microbiology, 2nd edn., Chapman and Hall, London, UK, 1993, pp. 389–438.

[10] Fox P.F., Singh T.K., McSweeney P.L.H., Proteolysis in cheese during ripening, in: Andrews A.T., Varley J. (Eds.), Biochemistry of milk products, Royal Soc. Chem., Cambridge, UK, 1994, pp. 1–31.

[11] Gobbetti M., Ferranti P., Smacchi E., Goffredi F., Addeo F., Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus latis* subsp. *cremoris* FT4, Appl. Environ. Microbiol. 66 (2000) 3898–3904.

[12] Gomez-Ruiz J.A., Ramos M., Recio I., Angiotensin-I-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures, Int. Dairy J. 12 (2002) 697–706.

[13] Gomez-Ruiz J.A., Ramos M., Recio I., Angiotensin converting enzyme-inhibitory activity of peptides isolated from Manchego cheese: Stability under simulated gastrointestinal digestion, Int. Dairy J. 14 (2004) 1075–1080.

[14] Gouldsworthty A.M., Leaver J., Banks J.M., Application of a mass spectrometry sequencing technique for identifying peptides present in Cheddar cheese, Int. Dairy J. 6 (1996) 781–790.

[15] Haileselassie S.S., Lee B.H., Gibbs B.F., Purification and identification of potentially bioactive peptides from enzyme-modified cheese, J. Dairy Sci. 82 (1998) 1612–1617.

[16] Jagadesh G., Angiotensin II receptors – antagonists, molecular biology and signal transduction, Indian J. Exp. Biol. 36 (1998) 1171–1194.

[17] Johnston C.I., Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control, J. Hypertension 10 (1992) S13–S26.

[18] Juillard V., Laan H., Kunji E.R.S., Jeronimus-Stratingh C.M., Bruins A.P., Konings W.N., The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyse β-casein into more than one hundred different oligopeptides, J. Bacteriol. 177 (1995) 3472–3478.

[19] Kosikowski F., Cheese and fermented milk foods, Edward Brothers Inc, Ann Arbor, MI, USA, 1977.

[20] Kunji E.R.S., Mierau I., Hanting A., Poolman B., Konings W.N., The proteolytic systems of lactic acid bacteria, Antonie van Leeuwenhoek 70 (1996) 187–221.

[21] Lankaputhra W.E.V., Shah N.P., Adherence of probiotic bacteria to human colonic cells, Biosci. Microflora 17 (1998) 105–113.

[22] Lowry O.H., Resebrough N.J., Farr A.L., Randall R.J., Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[23] Maeno M., Yamamoto N., Takano T., Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790, J. Dairy Sci. 79 (1996) 1316–1321.

[24] Maruyama S.K., Mitachi H., Tanaka H., Tomizuka N., Suzuki H., Studies on the active site and antihypertensive activity of angiotensin-I-converting enzyme derived from casein, Agric. Biol. Chem. 51 (1987) 1581–1586.

[25] Meisel H., Overview on milk protein-derived peptides, Int. Dairy J. 8 (1998) 363–373.

[26] Meisel H., Goepfert A., Gunther S., ACE-inhibitory activities in milk products, Milchwissenschaft 52 (1997) 307–311.

[27] Michaelidou A., Alichanidis E., Urlaub H., Polychroniadou A., Zerfiridis G.K., Isolation and identification of some major water-soluble peptides in Feta cheese, J. Dairy Sci. 81 (1998) 3109–3116.

[28] Minervini F., Algaron F., Rizello G., Fox P.F., Monnet F., Gobbetti M., Angiotensin-I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species, Appl. Environ. Microbiol. 9 (2003) 5297–5305.

[29] Minkiewicz P., Slangen C.J., Dziuba J., Visser S., Mioduszewska H., Identification of peptides obtained via hydrolysis of bovine casein by chymosin using HPLC and mass spectrometer, Milchwissenschaft 55 (2000) 14–17.

[30] Nakamura Y., Yamamoto H., Sakai K., Okubo A., Yamazaki S., Takano T., Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk, J. Dairy Sci. 78 (1995) 777–783.

[31] Okamoto A., Hanagata H., Matsumoto E., Kawamura Y., Koizumi Y., Yanagida F., Angiotensin I converting enzyme inhibitory activities of various fermented foods, Biosci. Biotech. Biochem. 59 (1995) 1147–1149.

[32] Ondetti M.A., Williams N.J., Sabo E.F., Pluvec J., Weaver E.R., Kocy O., Angiotensin converting enzyme inhibitors form the venom of *Bothrops jararaca*, isolation, elucidation of structure and synthesis, Biochemistry 10 (1971) 4033–4039.

[33] Ong L., Henriksson A., Shah N.P., Development of probiotic Cheddar cheese containing *Lb. acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid, Int. Dairy J. 16 (2006) 446–456.

[34] Ong L., Henriksson A., Shah N.P., Proteolytic pattern and production of organic acid of Cheddar cheese produced with probiotic *Lb. acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* spp., Int. Dairy J. 17 (2007) 67–78.

[35] Ong L., Henriksson A., Shah N.P., Chemical analysis and sensory evaluation of Cheddar cheese produced with *Lb. acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* sp., Int. Dairy J. (2007) in press.

[36] Rokka T., Syvaoja E.L., Tuominen J., Korhonen H., Release of bioactive peptides by enzymatic proteolysis of *Lactobacillus GG* fermented UHT milk, Milchwissenschaft 52 (1997) 675–678.

[37] Ryahanen E., Pihlanto-Leppala A., Pahkala E., A new type of ripened, low-fat cheese with bioactive peptides, Int. Dairy J. 11 (2001) 441–447.

[38] Saito T., Nakamura T., Kitazawa H., Kawai Y., Itoh T., Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese, J. Dairy Sci. 83 (2000) 1434–1440.

[39] Sandre C., Gleizes A., Forestier F., Gorges-Kergot R., Chilmonczyk S., Leonil J., Moreau M.C., Labarre C., A peptide derived from bovine β-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice, J. Nutr. 131 (2001) 2936–2942.

[40] Seppo L., Jauhiainen T., Poussa T., Korpela R., A fermented milk high in bioactive peptides has a blood-pressure lowering effect in hypertensive subjects, Am. J. Clin. Nutr. 77 (2003) 326–330.

[41] Shah N.P., Effects of milk-derived bioactives: an overview, Br. J. Nutr. 1 (2000) S3–S10.

[42] Singh T.J., Fox P.F., Healy A., Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese, J. Dairy Res. 64 (1997) 433–443.

[43] Smacchi E., Gobetti M., Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin I-converting enzyme, Enzyme Microb. Technol. 22 (1998) 687–694.

[44] Takano T., Milk derived peptides and hypertension reduction, Int. Dairy. J. 8 (1998) 375–381.

[45] Yamamoto N., Akino A., Takano T., Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790, J. Dairy Sci. 77 (1994) 917–922.