Purification and Identification of Potentially Bioactive Peptides from Enzyme-Modified Cheese

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ABSTRACT

Antihypertensive peptides inhibiting angiotensin I-converting enzyme have been isolated from enzymatic hydrolysates of various food materials, but no information is available on the isolation of antihypertensive peptides from enzyme-modified cheese. In this study, several bioactive peptides, mainly potential antihypertensive peptides from enzyme-modified cheese prepared by commercial and *Lactobacillus casei* enzymes, were purified and identified. Enzyme-modified cheese samples were prepared by combination of Neutrase® (1883.0 U/ml), *L. casei* enzymes (amino peptidase activity 86.4 leucine aminopeptidase U/g), and Debitrase™ (22.0 leucine aminopeptidase U/g). The water-soluble fractions of the enzyme-modified cheeses that were prepared by different enzymes were subjected to reverse-phase HPLC on a Delta Pak C_{18} column. Each peak was purified on the same column using a binary gradient. One peak from the Neutrase® digest, five peaks from the Neutrase®-Debitrase™ digest, and two peaks from the Neutrase®-*Lactobacillus* enzyme digest were purified and identified by API mass spectrometry. On the basis of their molecular masses, amino acid sequences of purified peptides were identified. β-Casomorphin with a sequence like that of β-casein (YPFPGPI f 60–66) was found after the Neutrase® digest. All of the peptides purified from the digests with combination of Neutrase® and Debitrase™ or Neutrase® and *L. casei* enzymes contained active sites in their sequences. The presence of sites containing potential antihypertensive peptides suggests that the purified peptides may have antihypertensive properties. Thus, the enzyme-modified cheese process, mainly designed to produce flavor ingredients, may simultaneously produce bioactive peptides, which are considered to be of physiological importance.

(**Key words**: bioactive peptide, antihypertensive peptide, enzyme-modified cheese, *Lactobacillus casei*)

Abbreviation key: **ACE** = Angiotensin-converting enzyme, **BAP** = bioactive peptide, **EMC** = enzyme-modified cheese, **EMC + N8** = EMC treated with Neutrase® only for 8 h, **EMC + ND96** = EMC treated with Neutrase® and Debitrase™ for 96 h, **EMC + NL72** = EMC treated with Neutrase® and *Lactobacillus* enzyme for 72 h, $\mathbf{IC_{50}}$ = the concentration of an ACE inhibitor required to inhibit 50% of ACE activity.

INTRODUCTION

Milk protein is a rich source of bioactive peptides (**BAP**) such as antihypertensive peptides [angiotensin-converting enzyme (**ACE**) inhibitory peptides], opiod peptides, immunostimulating peptides, antimicrobial peptides, and cholesterol-lowering peptides (11). Possible roles of casein hydrolysates as antihypertensive agents, immunostimulants, and exorphins have recently been suggested by several groups (5, 7, 8, 10, 13, 14, 18, 19). β-Casomorphins are particular peptide sequences in β-CN that have received more research attention than other BAP. Various lengths of β-casomorphin have been isolated from enzymatic digests of casein or have been synthesized. The primary sequence of these β-casomorphins corresponds to amino acid residues of β-CN (f 60–70) (16). The N-terminal Tyr residue found in all β-casomorphins is critical to their bioactivity (17). Because of the high content of proline residues, these peptides are apparently resistant to proteolytic attack (6). During digestion of β-CN, these BAP are absorbed intact, and they inhibit gastrointestinal motility and the emptying rate of the stomach by direct interaction with opioid receptors (4).

The ACE is a dipeptidyl carboxypeptidase that catalyzes the production of the vasoconstrictor angiotensin II, and the inactivation of the vasodilator bradykinin plays an important role in blood pressure regulation and hypertension (19). Recently some

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ACE inhibitors were produced by the enzymatic hydrolysis of caseins. Nakamura et al. (14) reported that two peptides with amino acid residues of VPP and IPP, isolated from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, exhibited ACE inhibitory and antihypertensive activities. The concentrations of those peptides required to inhibit 50% of angiotensin I-converting enzyme activity ($\mathbf{IC_{50}}$) were 9 and 5 μM, respectively.

Several peptides derived from α_{s1}-CN (f 24–31, f 170–199) and β-CN (f 168–175, f 183–190, f 113–127, f 193–210, f 70–97, f 191–210, and f 16–97) by *L. helveticus* CP790 proteinase also exhibited ACE inhibitory and antihypertensive activities after oral administration to spontaneously hypertensive rats (19). Among those peptides, one (f 43–69) derived from β-CN showed the highest ACE inhibitory activity; IC_{50} = 4 μM. A potent antihypertensive heptapeptide (KVLPVPG; f 169–175) from β-CN, also identified in casein hydrolysate, was produced using proteinase from *L. heleveticus*, but this peptide has lower ACE inhibitory activity; IC_{50} = 1000 μM (8). Antihypertensive effect of the peptide was found to be dose dependent in that study. The objectives of the present work were 1) to purify bioactive peptides from enzyme-modified cheese (**EMC**) produced by *Lactobacillus casei* and commercial enzymes and 2) to identify the peptides by comparing their sequences with sequences of known bioactive peptides.

MATERIALS AND METHODS

Strain and Preparation of Enzyme Extracts

Lactobacillus casei isolated from a mild Cheddar cheese was obtained from the Food Research and Development Center, Agriculture and Agri-Food Canada (St. Hyacinthe, QC, Canada). The strain was grown in MRS broth (Difco Laboratories, Detroit, MI) for 18 h at 30°C. The cells were harvested by centrifugation (8000 × *g*, 15 min, 4°C) and washed with 0.05 *M* phosphate buffer (pH 7.0). The harvested cells were then disintegrated at 2-s intervals for 1 min with an Ultrasonic Processor XL (Heat System, New York, NY), and crude extract was obtained after centrifugation (10,000 × *g*, 30 min, 4°C).

Enzyme Purification

Crude extract was precipitated using ammonium sulfate fractionation (45 to 80%). The precipitate was dissolved in a minimum volume of 0.05 *M* phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer using a Spectrapor membrane (30,000 Da molecular mass cutoff; Spectrum Co., Houston, TX). Aminopeptidase was purified using an FPLC® system equipped with an ion-exchange column (Mono Q 16/10; Pharmacia, Montreal, Canada) and a gel-filtration column (Superose, HR 10/30; Pharmacia), according to the methods described by Arora and Lee (1).

Protein and Enzyme Assays

Protein was determined spectrophotometrically at 562 nm using bicinchoninic acid assay reagents supplied with the system (Pierce Chemical Ltd., Rockford, IL). Bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as a standard. The assay mixture containing 180 μl of 0.05 *M* buffer (pH 7.0), 10 μl of substrate, and 10 μl of enzyme fraction was incubated at 32°C for 30 min. Activity was determined spectrophotometrically at 410 nm by measuring the amount of *p*-nitroaniline produced from leucine-p-nitroanilide. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline/min from leucine *p*-nitroanilide. Specific activity was expressed as the enzyme unit per milligram of protein.

Preparation of EMC

Milk Cheddar cheese (Cracker Barrel; Kraft, Don Mills, ON, Canada), manufactured by traditional procedures and containing 38% moisture and 33% fat, was purchased from a local grocer. The EMC was prepared according to the method of Park et al. (15). Shredded milk Cheddar cheese (600 g) was mixed with 2.5% Na_2HPO_4 and 195 ml of distilled water. The mixture was heated at 90°C for 3 min to emulsify the cheese slurry and to inactivate endogenous enzymes and then was cooled to 50°C. Neutrase® [0.5 L (0.05) Anson U/g; Novo Nordisk A/S, Bagsvared, Denmark] a neutral protease produced from *Bacillus subtilis*, was filtered through a 0.45-μm membrane filter and added to the cheese slurry. The slurry was incubated for 8 h under vacuum at 45°C. Portions (50 g) of the resulting cheese slurries were then treated separately with 1) crude extracts of *L. casei* (86.4 leucine aminopeptidase U/g) at 34°C for 72 h and 2) Debitrase™ DBP20 (22.0 leucine aminopeptidase U/g; Imperial Biotech, London, United Kingdom), a peptidase mixture extracted from *Lactococcus lactis* and *Aspergillus oryzae*, at 40°C for 96 h. All EMC samples were incubated under static conditions.

Extraction of Water-Soluble Peptides

The modified method of McGugan et al. (9) was used to extract the water-soluble peptides of the EMC. Portions (5 g) of EMC samples were centrifuged (25,000 × *g*, 50 min, 20°C). The aqueous layer (1 ml) was mixed with 1 ml of methylene chloride and 0.6 ml of water and shaken vigorously. After centrifugation (25,000 × *g* for 30 min), 1 ml of the methanol-water fraction was concentrated by a Speed Vac (Savant Instruments, New York, NY) for 3 h and dissolved with 50 μl of distilled water.

Reverse-Phase HPLC Analysis

The Waters HLPC system (600 E system controller, U6K Injector, 486 Tunable Absorbance Detector, and a Millenium 2010 Chromatography Manager; Millipore, Milford, MA) was used to separate peptides. Portions (25 μl) of the water-soluble fractions were injected on a Delta Pak C18 column (100 Å, 30 × 150 mm). The peptides were eluted at a flow rate of 0.5 ml/min with a binary gradient from 20 to 40% of solvent B (0.08% trifluoroacetic acid in a mixture of acetonitrile and water at a 40:60 ratio) for 15 min, from 40 to 60% for 15 min, from 60 to 100% for 5 min, and from 100 to 20% for 35 min using a system controller. Solvent A was 0.1% trifluoroacetic acid in deionized water. The elution was monitored at an absorbance of 214 nm. All samples were filtered through a 0.45-μm high syringe filter before injection.

Identification of Peptides

To identify bioactive peptides from EMC samples, selected peaks were collected and purified (three times) by reverse-phase HPLC on the same column with the same gradient. Collected samples were then analyzed by API-mass spectrometry to determine their molecular masses and their amino acid sequences. Identification of peptides from enzymatic digests was based on the amino acid composition; the nearest integer was determined by the molar ratios of amino acids of the peaks. That of Neutrase® was determined by the ion mass of individual peptides within the range of ±0.5 to 1.0 atomic mass unit (amu) to the actual mass of the peptide with 100 or at least 75% relative intensity.

Mass Spectrometry

Mass spectra were obtained in the positive mode on a triple stage mass spectrometer (Model API III; Scix, Toronto, Canada). Samples were dissolved in 10% acetic acid and infused through a stainless steel capillary (100 μm i.d.). A stream of air (pneumatic nebulization) was introduced to assist the formation of submicron droplets (3). These droplets were evaporated at the interface by nitrogen gas producing highly charged ions, which were detected by the analyzer. The calibration of the system was performed using ammonium adduct ions of polypropylene glycol with a known mass to charge (m/z) ratio throughout the range of 0 to 2470 amu. Mass charges were used throughout the range of the instrument (0 to 2470 amu). Simple algorithms were used to correlate the charges produced by these compounds to their molecular masses. After determining the molecular mass of different fragments, sequences of amino acids for peptides 1, 2, 3, 4, 5, 6, 7, and 8 were proposed.

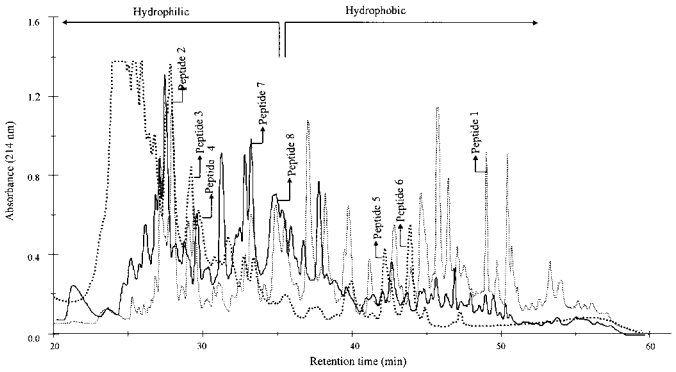

Figure 1. Peptide profiles of enzyme-modified cheese prepared with Neutrase® (Novo Nordisk, A/S, Bagsvared, Denmark) (· · · · ·), Neutrase® plus Debitrase™ (Imperial Biotech, London, United Kingdom) (·····), and Neutrase® plus *Lactobacillus casei* enzymes (—).

RESULTS

Figure 1 shows the peptide profiles of the Neutrase® digest (**EMC + N8**), Neutrase® and *L. casei* enzyme digest (**EMC + NL72**), and Neutrase® and Debitrase™ digest (**EMC + NC96**). Most of the peptides from EMC + N8 were eluted in a wide range of acetonitrile concentrations (from 100% to 20), and more peptides appeared in the hydrophobic region (the second half). In contrast, peptides from EMC + NL72 and EMC + ND96 appeared in the hydrophilic region (the first half). This elution profile indicates that hydrophobic peptides eluted late that were produced by Neutrase were hydrolyzed by peptidases from *L. casei* and commercial enzymes. This finding is

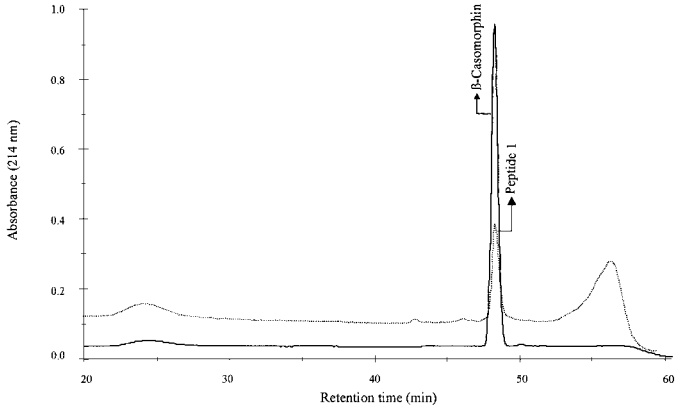

Figure 2. Standard β-casomorphin at 1 μg/ml (—) and β-casomorphin purified from enzyme-modified cheese prepared with Neutrase® (Novo Nordisk, A/S, Bagsvared, Denmark) only (· · · · ·).

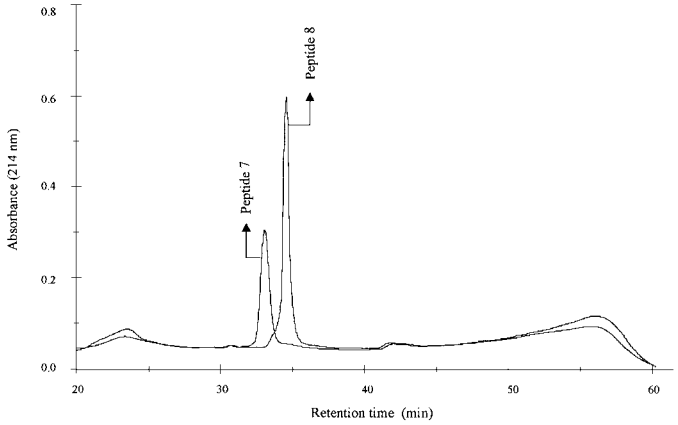

Figure 4. Potential bioactive peptides purified from enzyme-modified cheese prepared with Neutrase® (Novo Nordisk, A/S, Bagsvared, Denmark) + *Lactobacillus casei* enzymes.

similar to those of Minagawa et al. (12), Cliffe and Law (2), and Park et al. (15).

Because of the similarity of the retention time (48 min) to the standard β-casomorphin, one peak that was eluted from EMC + N8 at 48 min was purified and analyzed by API-mass spectrometry. Figure 2 shows the purified peptide from EMC prepared by Neutrase only (peptide 1) and the standard β-casomorphin. The purified peptide was identified as β-casomorphin and had the same sequence (YPFPGPI, f 60–66) of β-CN as did standard β-casomorphin. A total of 5 peaks from EMC + ND96 (Figure 3) were collected and analyzed by API-mass spectrometry: peaks 2, 3, 4, 5, and 6. Two peaks (Figure 4), 7 and 8, from EMC + NL72 were also purified and analyzed by API-mass spectrometry. All of the purified peptides were identified to be potential antihypertensive peptides.

Table 1 shows the sequence of amino acids for the fragments obtained by mass spectrometry. All sequences obtained after fragmentation by API mass spectrometry were derived from the precursor β-CN. A search of other milk proteins confirmed that they were not precursors of the peptides formed. In Table 1 the sequence of amino acids for peaks 1, 2, 3, 4, 5, 6, 7, and 8 were identified as follows: YPFPGPI for peak 1, LTLTDVE for peak 2, YPQRDMPIQAFLLYQEPV for peak 3, EMPFPKYPVEPFTESQSLTL for peak 4, SLVYPFPGPIPNSLPQNIPPLT for peak 5, LVYPF PGPIPNSLPQNIPPLT for peak 6, PGPIP for peak 7, and PKHKEMPFPKYPVEPFT for peak 8.

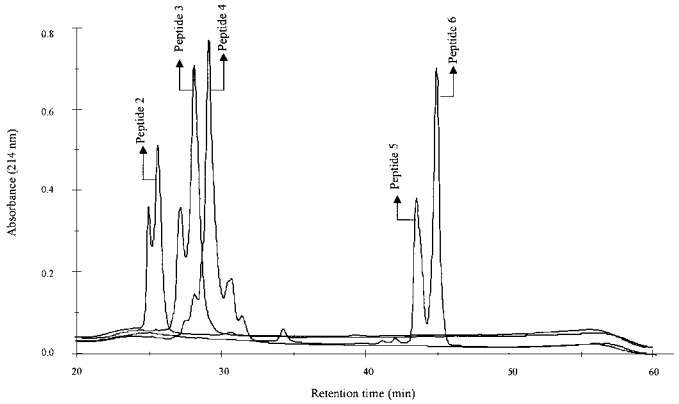

Figure 3. Potential bioactive peptides purified from enzyme-modified cheese prepared with Neutrase® (Novo Nordisk, A/S, Bagsvared, Denmark) plus Debitrase™ (Imperial Biotech, London, United Kingdom).

DISCUSSION

In this study, β-casomorphin with a sequence of YPFPGPI (f 60–66 of β-CN) was detected in the EMC digest with Neutrase® only (EMC + N8). Because rennet and other endogenous enzymes were inactivated prior to incubation, the cleavages found were clearly derived from the protease used in this study. The sequence of cleavage leading to β-casomorphin, however, is not known, and the opioid activity was not determined. β-Casomorphin was absent in EMC + NL72 and EMC + ND96. The absence of β-casomorphin could be the result of two factors. First, β-casomorphin was not resistant to proteolysis and might have been degraded by the proteolytic and peptidolytic system of *Lactobacillus* and the commercial enzymes. Second, β-casomorphin might be

TABLE 1. Sequences of amino acids for the peptides derived from enzyme-modified cheese by Neutrase®, Neutrase® plus *Lactobacillus*, and Neutrase® plus Debitrase™ after fragmentation by mass spectrometry.[1]

Treatment[2]	Peak No.	Ion mass of actual peak (amu)	Fragment	Peptides sequence	Peptide molecular mass (amu)
EMC + N8	1	790.94	(f 60–66)	**YPFPGPI**	791.00
EMC + ND96	2	790.89	(f 125–131)	**LTLTDVE**	791.00
	3	2209.50	(f 180–197)	**YPQRDMPIQ AFLLYQEPV**	2211.00
	4	2341.68	(f 108–127)	**EMPFPKYPVE PFTESQSLTL**	2343.00
	5	2362.77	(f 57–58)	**SL*V*YPFPGPIP NSLPQNIPPLT**	2263.00
	6	2275.50	(f 58–78)	***LV*YPFPGPIP N SLPQNIPPLT**	2275.70
EMC + NL72	7	480.58	(f 63–67)	**PGPIP**	482.00
	8	2073.46	(f 104–120)	**PKHKEMPFP PKYPVEPFT**	2074.00

[1]Fragments of main peptides are in boldface, bioactive sites are underlined, and regions in the main peptides that contain overlapping bioactive sites are italicized.

[2]EMC + N8 = enzyme-modified cheese treated with Neutrase® only for 8 h, EMC + ND96 = enzyme-modified cheese treated with Neutrase® and Debitrase™ for 96 h, and EMC + NL72 = enzyme-modified cheese treated with Neutrase® and *Lactobacillus* enzymes for 72 h.

present in a concentration below the detection threshold value of HPLC. A more sensitive analytical method such as radioimmunoassay might be appropriate to detect lower concentrations. However, if no physiological benefits could be gained by trace amounts, further analysis of lower concentrations might not be necessary. In addition to loss of β-casomorphin, we also observed increase in the peak of the hydrophilic region.

Potential antihypertensive peptides were detected for EMC + ND96 and EMC + NL72. These results suggest that β-casomorphin, or other hydrophobic peptides released by Neutrase®, might serve as a precursor for antihypertensive peptides. The formation of peptides containing active sites suggests that EMC production may be an alternative process for the production of antihypertensive agents. However, it is important to determine the physiological benefits and application of these peptides by measuring their ACE inhibitory activity (IC_{50}) in vitro and in vivo.

CONCLUSIONS

The present study demonstrates that the EMC process simultaneously produces bioactive peptides that may provide savory, nutritional, medical, and health benefits. The stability of β-casomorphin may be dependent on the type of enzyme present in the system. As β-casomorphin can be hydrolyzed by proteolytic enzymes in the living body, fragments with potent inhibitory activity in vivo might be produced. However, further studies are necessary to elucidate the formation mechanism and physiological significance of biologically active peptides.

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