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Producibility and Digestibility of Antihypertensive β-Casein Tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the Gastrointestinal Tract: Analyses Using an *in Vitro* Model of Mammalian Gastrointestinal Digestion

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Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) are antihypertensive tripeptides isolated from milk fermented with *Lactobacillus helveticus* and inhibit angiotensin-converting enzyme (ACE). We investigated whether these peptides were generated from β-casein by digestive enzymes and whether they were resistant to enzymatic hydrolysis, using an *in vitro* model. VPP and IPP were not generated from β-casein by gastrointestinal enzymes; instead, a number of longer peptides including VPP and IPP sequences were detected. The fermentation step would therefore be necessary to produce these antihypertensive tripeptides. VPP and IPP themselves were hardly digested by digestive enzymes, suggesting that orally administered VPP and IPP remain intact in the intestine, retaining their activity until adsorption. The present study also demonstrated that various functional peptide sequences in β-casein were resistant to gastrointestinal enzymes. There may be a strong correlation between the resistance of peptides to gastrointestinal digestion and their real physiological effects after oral administration.

KEYWORDS: Antihypertensive tripeptide; gastrointestinal digestion; casein

INTRODUCTION

A number of physiologically functional peptides, such as opioid (*1, 2*), antibacterial (*3*), immunostimulating (*4*), antioxidant (*4*), and angiotensin-I converting enzyme (kininase II; EC 3.4.15.1) (ACE) inhibitory peptides (*5–7*), have been discovered in the enzymatic hydrolysates of various food proteins, synthetic peptides, and fermented products (*8–10*).

Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) are antihypertensive peptides isolated from milk fermented with *Lactobacillus helveticus* (*6, 7*). They are elicited from β-casein during the fermentation process and showed a potent ACE-inhibitory activity *in vitro*. A blood pressure-lowering effect of this fermented milk or the tripeptides was observed also *in vivo* using spontaneously hypertensive rats (*7*). Furthermore, fermented milks including these peptides revealed antihypertensive effects in Japanese and Finish hypertensive subjects (*11, 12*). On the other hand, casein itself did not show any significant blood pressure-lowering effect (*7*), suggesting that VPP and IPP are not produced by gastrointestinal digestion of casein but would specifically be produced by fermentation with *L. helveticus*. Mizuno et al. (*13*) have recently found that VPP and IPP can be produced by proteolytic enzymes derived from *Aspergillus oryzae* as well. The *in vivo* studies as mentioned above suggest that VPP and IPP, once produced, are resistant to the gastrointestinal hydrolysis. However, no detailed study has yet been performed on the gastrointestinal producibility of VPP and IPP from milk and their digestibility in the gastrointestinal tract.

In vitro hydrolysis of proteins with digestive enzymes such as pepsin, trypsin, and chymotrypsin (named "stage I digestion" in this paper) has been generally attempted to estimate the production of bioactive peptides in the gastrointestinal tract (*14, 15*). However, the peptides produced by stage I digestion will be further hydrolyzed with the brush-border peptidases on intestinal epithelial cells (named "stage II digestion"). To predict whether a functional peptide expresses its biological function *in vivo*, it is important to know whether the peptide is susceptible to the digestive enzymes not only in stage I digestion but also in stage II digestion. Knowing the final structure of peptides after gastrointestinal digestion is also valuable for estimating its biological activity *in vivo*.

The human colonic carcinoma cell line Caco-2 has the ability to differentiate into enterocyte-like cells that express several

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characteristic features of mature small intestinal cells (*16*). Caco-2 cells form monolayers with tight junctions and express brush-border enzymes including peptidases. Howell et al. (*17*) have reported that aminopeptidase N, aminopeptidase P, aminopeptidase W, dipeptidyl peptidase IV, endopeptidase-24.11, γ-glutamyl transpeptidase, microsomal dipeptidase, and peptidyl dipeptidase A (ACE) are expressed in Caco-2 cells. This suggests that Caco-2 cells would provide a good *in vitro* model for the stage II digestion of peptides.

This study was undertaken to examine whether VPP and IPP can be generated from β-casein by gastrointestinal enzymes including brush-border peptidases and whether these peptides are resistant to gastrointestinal enzymes, by using an *in vitro* digestion model. Generation of other peptides from β-casein by gastrointestinal enzymes was also examined.

MATERIALS AND METHODS

Materials. Bovine β-casein was purchased from Sigma-Aldrich Japan (Tokyo, Japan). VPP and IPP were purchased from Bachem. The Caco-2 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and Hank's balanced salts solution (HBSS) were purchased from Sigma (St. Louis, MO). Penicillin−streptomycin (10000 units mL^{-1} and 10 mg mL^{-1} in 0.9% sodium chloride, respectively) and nonessential amino acids were purchased from Gibco (Gaithersburg, MD). Fetal bovine serum was purchased from Asahi Technoglass (Chiba, Japan). Plastic dishes and plates were purchased from Corning-Coster Japan (Tokyo, Japan). Dulbecco's PBS(−) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Cell matrix type I-C was purchased from Nitta Gelatin Inc. (Osaka, Japan). Millicell-HA with a 0.45 μm cellulose membrane of 12 or 30 mm in diameter was purchased from Millipore (Molsheim, France).

Peptide Syntheses. Tripeptides except VPP and IPP were manually synthesized by the solid-phase Fmoc method, and Fmoc-amino acids were purchased from Noba-Biochem Japan (Tokyo, Japan). The synthesized peptides were cleaved from the resin by a trifluoroacetic acid−phenol−thioanisole−ethanedithiol solution (*18*) and purified by high-performance liquid chromatography (HPLC).

Cell Culture. Caco-2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with a culture medium consisting of DMEM, 10% fetal bovine serum, 1% nonessential amino acids, 200 units mL^{-1} penicillin, and 200 μg mL^{-1} streptomycin. The cells were seeded at 1.4×10^5 cells per well in a 24-well plate that had been precoated with collagen and were cultured for 2 weeks. The Caco-2 cells used in this study were between passages 40 and 70. The activity of aminopeptidase P was measured by detecting free Arg using Arg-Pro-Pro as a substrate. The activity of dipeptidyl peptidase IV and aminopeptidase N was measured by detecting free 7-amino-4-methylcoumarin using Gly-Pro-MCA and Ala-MCA as substrates. Cells for the peptide digestion studies were grown in a Millicell insert with a membrane coated with type I collagen. The cells were seeded at a density of 1.3×10^5 cells/mL of the medium, the medium being changed every 2 or 3 days. The cell layers were used for the experiments after culturing for 14–17 days. The integrity of the cell layer was evaluated beforehand by measuring the transepithelial electrical resistance (TEER).

***In Vitro* Method Estimating Protein and Peptide Digestion with Enzymes (Stage I Digestion).** This procedure was used to mimic the *in vivo* conditions of the gastrointestinal tract described by Robert et al. (*15*). Aqueous solutions of the synthetic peptides of VPP and IPP (1 mg/mL) and β-casein (1 mg/mL) were first hydrolyzed with pepsin (EC 3.4.23.1; 370 units/mg) at pH 2.0 and 37 °C for 120 min. The solution was neutralized to pH 7.0 with 1 N NaOH, and further digestion was carried out for 120 min at 37 °C with pancreatin or trypsin and chymotrypsin. All of the reactions were carried out at an enzyme: substrate ratio of 1:25 (w/w). After the digestion samples were boiled for 5 min to inactivate the enzymes. The samples were analyzed by liquid chromatography–mass spectrometry (LCMS) to determine the amounts of VPP and IPP. The peptide sequences of β-casein digests were analyzed by liquid chromatography ion trap time of flight mass spectrometry (LCMS-IT-TOF).

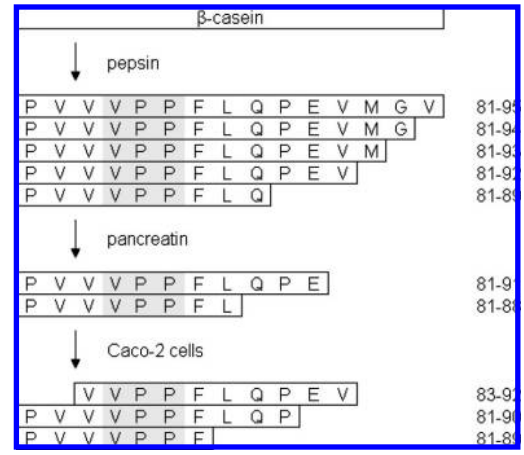

Figure 1. VPP-containing fragments detected after digestion of β-casein with gastrointestinal enzymes. The first, second, and third digestion steps were performed with pepsin, trypsin/chymotrypsin or pancreatin, and Caco-2 enzymes, respectively. The fragments were separated and identified by LCMS-IT-TOF.

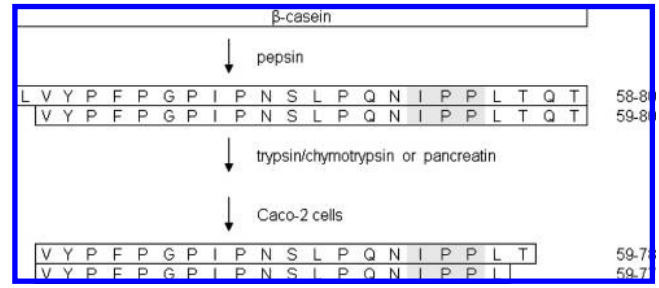

Figure 2. IPP-containing fragments detected after digestion of β-casein with gastrointestinal enzymes. The experimental procedures are shown in **Figure 1**.

***In Vitro* Method Estimating Protein Digestion on Caco-2 Cells (Stage II Digestion).** The cell monolayers grown in 24-well plates (12 mm in diameter) or Millicell insert (12 mm in diameter) were gently rinsed with HBSS (pH adjusted to 7.4 with HEPES). Then, an appropriate volume of HBSS was added to the cells, and the cells were incubated for equilibration at 37 °C for 30 min. HBSS on the cell monolayers was then replaced by a β-casein hydrolysate or a peptide solution. After incubation for the appropriate time, the apical solution was taken. The amount of VPP and IPP was determined by LCMS, and the peptide sequences of β-casein digests were analyzed by LCMS-IT-TOF. The peptide concentration was determined by high-performance liquid chromatography (HPLC).

High-Performance Liquid Chromatography (HPLC). A Trirotar IV liquid chromatography system (Jasco, Tokyo, Japan) equipped with a YMC-Pack ODS-AQ312 column (6 × 150 mm; YMC, Kyoto, Japan) and a UV detector at 210 nm was used. Peptides were applied to the column that had been equilibrated with 0.09% (v/v) trifluoroacetic acid and were eluted with a linear gradient of acetonitrile at a flow rate of 1.0 mL/min. Each peptide was determined by measuring the peak area. Data were statistically analyzed by one-way ANOVA and Tukey test.

Liquid Chromatography–Mass Spectrometry (LCMS). LCMS-2010A (Shimadzu, Kyoto, Japan) equipped with a RP-aqueous column (Nomura Chemical, Aichi, Japan) was used for the analysis. The column was eluted at 50 °C with a linear gradient of solvent B (CH_3CN containing 0.1% HCOOH) in solvent A (H_2O containing 0.1% HCOOH) from 0% to 4% in 24 min. The flow rate was 200 μL/min. The content of peptides was determined by measuring the peak area.

Liquid Chromatography Ion Trap Time of Flight Mass Spectrometry (LCMS-IT-TOF). LCMS-IT-TOF (Shimadzu, Kyoto, Japan) equipped with a RP-aqueous column was used to analyze the peptide

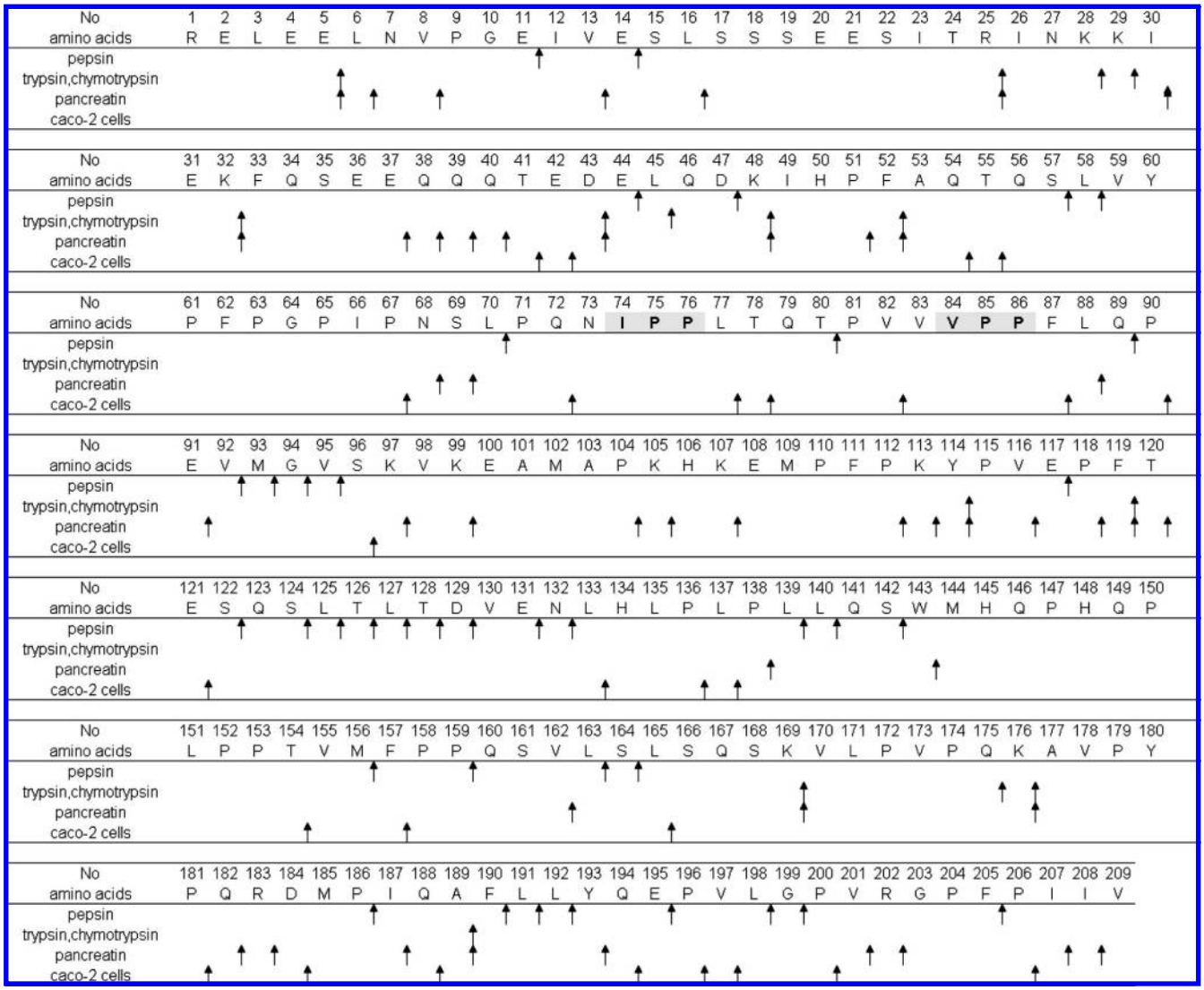

Figure 3. Primary structure of β-casein. The sequences of β-casein hydrolysates were analyzed by LCMS-IT-TOF to define which peptide bonds were cleaved. All of the sites of cleavage by each digestion step are shown by arrows.

sequences. The column was eluted at 40 °C with solvent A (H_2O containing 0.1% HCOOH), and solvent B (CH_3CN containing 0.1% HCOOH) formed the eluent in the following linear gradient steps: from 0% to 30% B over 120 min, then to 90% B over 30 min, and 10 min of isocratic elution at 90% B; and finishing with 10 min of equilibration at 100% A before the next run was started. Total run time was 170 min, and the flow rate was 200 μL/min. The raw data were calculated by Mascot Distiller analyzed by Mascot search (Matrix Science, Tokyo, Japan).

RESULTS AND DISCUSSION

Hydrolysis of proteins with pepsin, trypsin, chymotrypsin, and pancreatin is usually used to simulate digestion in the gastrointestinal tract (*19*). The digestibility of bovine β-casein has also been previously analyzed by using pepsin and trypsin (*14, 20*). In addition to digestion with these enzymes (stage I digestion), further digestion with brush-border enzymes (stage II digestion) proceeds in the intestinal tract, although an appropriate model for the later process was not available. Here we introduced Caco-2 cells as a model for stage II digestion. As had been reported by Howell et al. (*17*), Caco-2 cells expressed various brush-border peptidases. Our Caco-2 cells also expressed such peptidases as aminopeptidases N and P and dipeptidylpeptidase IV, the activities (milliunit: $n = 3$, means $\pm$ SD) being 3.24 $\pm$ 0.14, 2.12 $\pm$ 0.07, and 3.51 $\pm$ 0.73, respectively. This suggests the Caco-2 cells be used as a model of digestion by brush-border enzymes. The present study was the first approach to simulate gastrointestinal digestion of bovine β-casein by both secretory enzymes and brush-border enzymes to analyze the production of peptides including VPP and IPP in the gastrointestinal tract.

Table 1. Residual Ratio (%) of Val-Pro-Pro and Ile-Pro-Pro to Initial Peptide Concentrations after Sequential Digestion with Pepsin, Trypsin, Chymotrypsin, and Pancreatin

	residual ratio[a]	
	Val-Pro-Pro	Ile-Pro-Pro
pepsin	101 $\pm$ 2	99 $\pm$ 4
trypsin	100 $\pm$ 1	95 $\pm$ 3
chymotrypsin	97 $\pm$ 1	98 $\pm$ 3
pancreatin	97 $\pm$ 3	99 $\pm$ 2

[a] Results are expressed as means $\pm$ SD.

VPP and IPP Are Not Generated from β-Casein by Gastrointestinal Enzymes. Bovine β-casein has the sequence of both VPP (fragment 84–86) and IPP (f74–76). The producibility of these tripeptides by pepsin, trypsin, chymotrypsin, and pancreatin (stage I digestion) was examined by analyzing the

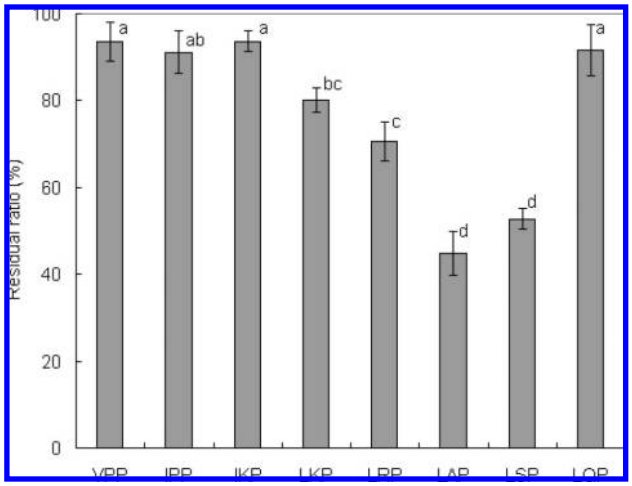

Figure 4. Residual ratio (%) of each antihypertensive tripeptide in the apical solution. The amount of tripeptides in the apical chamber was measured by HPLC after incubation for 60 min. Each value is the mean ± SD ($n = 3$). Data were statistically analyzed by one-way ANOVA and Tukey test. Values not sharing common letters are significantly different at $P < 0.05$.

Table 2. IC_{50} and Source Proteins of Antihypertensive Tripeptides[a]

	IC_{50} (μmol/L)	source protein
VPP	9	bovine milk/β-casein
IPP	5	bovine milk/β-casein, human milk/κ-casein
IKP	1.6	fish/dried bonito
LKP	0.3	chicken/chicken muscle, plant/pea vicilin
LRP	1.0	fish/bonito bowels
	0.3	plant/α-zein, maize endosperm
LAP	3.5	chicken/chicken muscle
LSP	1.7	plant/α-zein, maize endosperm
LQP	1.9	plant/α-zein, maize endosperm

[a] See ref *10*.

hydrolysates with LCMS. VPP and IPP were not detected in any of these hydrolysates. To determine the sequences of the peptides produced in the hydrolysates, the peptide fragments containing either VPP or IPP sequence were analyzed by LCMS-IT-TOF. VPP-containing peptides that consisted of 8−15 amino acid residues were identified. The hydrolysates were then incubated with Caco-2 cells for stage II digestion, and the peptides in the hydrolysates were analyzed. Peptides with 7–10 amino acid residues were newly detected (**Figure 1**). IPP-containing peptides that consisted of 22 to 23 amino acid residues were identified after stage I digestion and 19 to 20 after stage II digestion (**Figure 2**), although VPP and IPP were not detected. The shortest VPP-containing peptide was PVVVPPF, which was produced by digesting β-casein sequentially with pepsin, trypsin/chymotrypsin, and Caco-2 cells. The shortest IPP-containing peptide, VYPFPGPIPNSLPQNIPPL, was also produced by the same digestion process. Thus, it was suggested that VPP and IPP were produced from β-casein only when the milk is fermented with bacteria such as *L. helveticus* (*6, 7*) or is digested with enzymes derived from fungi such as *A. oryzae* (*13*).

Generation of Other Antihypertensive Peptides from β-Casein by Gastrointestinal Enzymes. The sequences of β-casein hydrolysates were analyzed with LCMS-IT-TOF. The sequence coverage in this experiment was 85–95%. Each sequence coverage was 85% for pepsin hydrolysate, 92% for pepsin−trypsin−chymotrypsin hydrolysate, 95% for pepsin−pancreatin hydrolysate, and 95% for pepsin−trypsin/chymotrypsin (or pancreatin)−brush-border enzyme hydrolysate. All of the cleavage sites of β-casein after stage I digestion and stage II digestion are shown in **Figure 3**. VYP (f59–61), VYPFPG (f59–64), and TPVVVPPFLQP (f80–90) have been reported as ACE-inhibitory peptides derived from bovine β-casein (*10*). VYP and VYPFPG showed antihypertensive effect both in *in vitro* and *in vivo* (*21*), although TPVVVPPFLQP did not show an antihypertensive effect *in vivo*. In our present study, the sequence of VYP and VYPFPG is resistant to gastrointestinal digestion. On the other hand, TPVVVPPFLQP was readily digested, the peptide bonds of T−P (f80–81), F−L (f87–88), L−Q (f88–89), and Q−P (f89–90) being hydrolyzed by gastrointestinal digestion. Maruyama et al. (*22*) found an ACE-inhibitory peptide, AVPYPQR (f177–183), which showed an antihypertensive effect *in vivo* using SHR (*23*). Our present paper shows that the sequence AVPYP (f177–181) is resistant to gastrointestinal digestion (**Figure 3**). The findings suggest that the resistance of peptides to the digestion relates to their antihypertensive effects *in vivo*.

Generation of Other Functional Peptides from β-Casein by Gastrointestinal Enzymes. YPFPGPI (f60–66), YPFPGP (f60–65), YPFPG (f60–64), and YPFP (f60–63) are β-casomorphins that form a family of opioid peptides derived from β-casein. β-Casomorphins possess the μ-receptor agonist activity (*1, 2*). They are found in the small intestine of adult humans and in the plasma of newborn calves after the ingestion of bovine milk and are resistant to the actions of gastrointestinal enzymes due to a high content of proline residues (*24*). Our present study also shows that the sequence including that of β-casomorphins is highly resistant to gastrointestinal digestion.

SSSEESITR (f17–25), the sequence for phosphorylation sites and also the core region of casein phosphopeptides (CPPs), which have mineral absorption enhancing activity, was also shown to be resistant to the digestive enzymes, supporting the previous findings of Miquel et al. (*25*).

These results suggest that there is a strong correlation between the resistance of peptides to gastrointestinal digestion and their real effects after oral administration. In addition to the sequences described above, the present study demonstrates other sequences (longer than five residues) that would be resistant to gastrointestinal digestion, namely, RELEE (f1–5), FQSEE (f33–37), EAMAP (f100–104), EMPFP (f108–112), MHQPHQPLPPT (f144–154), and VLPVPQ (f170–175). It may be interesting to search for physiological functions in these peptide regions.

Stability of VPP and IPP at Stage I Digestion. VPP and IPP chemically synthesized were incubated with pepsin, trypsin, chymotrypsin, and pancreatin in this order, and the amounts of these peptides after each digestion step were measured by LCMS. As shown in **Table 1**, these tripeptides were highly resistant to the proteases secreted in the gastrointestinal tract. VPP and IPP were not susceptible to pepsin, trypsin, chymotrypsin, and pancreatin, suggesting that they were not digested by secretory enzymes in the gastrointestinal tract.

Stability of VPP and IPP at Stage II Digestion. The digestion rate of VPP and IPP on the brush-border side was estimated by determining the intact tripeptides remaining in the apical chamber after incubation for 60 min. The digestion rate of other proline-containing antihypertensive tripeptides (*10*) was also determined and compared with that of VPP and IPP. **Figure 4** shows the residual ratio of the tripeptides. Leu-Ala-Pro (LAP) and Leu-Ser-Pro (LSP) were quickly digested by the brush-border peptidases, whereas VPP, IPP, Ile-Lys-Pro (IKP), and Leu-Gln-Pro (LQP) were fairly resistant to them. Leu-Lys-Pro (LKP) and Leu-Arg-Pro (LRP) were to some extent resistant to the Caco-2 peptidases. The present results clearly demonstrate

the very low susceptibility of VPP and IPP to the peptidases expressed on the Caco-2 cell surface (**Figure 4**). Among the peptides tested, VPP, IPP, and IKP are reported to be antihypertensive by orally administrating at a relatively low dose (*7, 26*), although their IC_{50} values to ACE were not low (**Table 2**). The high *in vivo* activity of these peptides may be, at least partly, due to their low susceptibility to the brush-border peptidases. The sequence X-Pro-Pro is likely to be particularly stable against peptidases. Masuda et al. (*27*) have reported that VPP and IPP were detectable in the abdominal aorta of SHR 6 h after administrating sour milk containing these peptides, indicating the stable nature of these tripeptides. In a previous paper, we showed that VPP could be transported across the Caco-2 cell monolayers in an intact form via paracellular diffusion (*28*). These X-Pro-Pro tripeptides might therefore be absorbed intact into the portal vein after ingestion, thereby lowering the blood pressure through inhibiting ACE in the blood vessel.

In conclusion, our *in vitro* model for mammalian gastrointestinal digestion supports our hypotheses that VPP and IPP would reach the small intestine as the intact form retaining their ACE-inhibitory activity. This study also shows the particular importance of the fermentation process in producing VPP and IPP, since mammalian gastrointestinal proteolytic enzymes would not participate in the production of these antihypertensive tripeptides from β-casein.

LITERATURE CITED

(1) Meisel, H.; FitzGerald, R. J. Opioid peptides encrypted in intact milk protein sequences. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S27–S31.

(2) Teschemacher, H.; Kock, G.; Brantl, V. Milk protein-derived opioid receptor ligands. *Biopolymers* **1997**, *43*, 99–117.

(3) Hayes, M.; Ross, R. P.; Fitzgerald, G. F.; Hill, C.; Stanton, C. Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Appl. Environ. Microbiol.* **2006**, *72*, 2260–2264.

(4) Lahov, E.; Regelson, W. Antibacterial and immunostimulating casein-derived substances from milk: casecidein, isracidin peptides. *Food Chem. Toxicol.* **1996**, *34*, 131–145.

(5) FitzGerald, R. J.; Meisel, H. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S33–S37.

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

(7) Nakamura, Y.; Yamamoto, N.; Sakai, K.; Takano, T. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* **1995**, *78*, 1253–1257.

(8) Yamamoto, N.; Ejiri, M.; Mizuno, S. Biogenic peptides and their potential use. *Curr. Pharm. Des.* **2003**, *9*, 1345–1355.

(9) Meisel, H.; Bochkelmann, W. Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie van Leeuwenhoek* **1999**, *76*, 207–215.

(10) Meisel, H. ; Walsh, D. J.; Murray, B.; FitzGerald, R. J. ACE inhibitory peptides. In *Nutraceutical Proteins and Peptides in Health and Disease*; Mine, Y., Shahidi, F., Eds.; CRC Press Taylor & Francis Group: Boca Raton, London, and New York, 2006; pp 269–305.

(11) Hata, Y.; Yamamoto, M.; Ohni, M.; Nakajima, K.; Nakamura, Y.; Takano, T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **1996**, *64*, 767–771.

(12) Seppo, L.; Kerojoki, O.; Suomalainen, T.; Korpela, R. The effect of a *Lactobacillus helveticus* LBK-16 H fermented milk on hypertension—A pilot study on humans. *Milchwissenschaft* **2002**, *57*, 124–127.

(13) Mizuno, S.; Nishimura, S.; Matsuura, K.; Gotou, T.; Yamamoto, N. Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. *J. Dairy Sci.* **2004**, *87*, 3183–3188.

(14) Schmelzer, C. E.; Schöps, R.; Ulbrich-Hofmann, R.; Neubert, R. H.; Raith, K. Mass spectrometric characterization of peptides derived by peptic cleavage of bovine β-casein. *J. Chromatogr. A* **2004**, *1005*, 87–92.

(15) Robert, M. C.; Razaname, A.; Mutter, M.; Juillerat, M. A. Identification of Angiotensin-I-Converting Enzyme Inhibitory Peptides Derived from Sodium Caseinate Hydrolysates Produced by *Lactobacillus helveticus* NCC 2765. *J. Agric. Food Chem.* **2004**, *52*, 6923–6931.

(16) Hidalgo, I. J.; Raub, T. J.; Borchardt, R. T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **1989**, *96*, 736–749.

(17) Howell, S.; Kenny, A. J.; Turner, A. J. A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. *Biochem. J.* **1992**, *284*, 595–601.

(18) King, D. S.; Fields, C. B.; Fields, G. B. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Pept. Protein Res.* **1990**, *36*, 255–266.

(19) Vermeirssen, V.; Camp, J. V.; Decroos, K.; Wijmelbeke, L. V.; Verstraete, W. The impact of fermentation and *in vitro* digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and whey protein. *J. Dairy Sci.* **2003**, *86*, 429–438.

(20) Rival, S. G.; Fornaroli, S. G.; Boeriu, C.; Wichers, H. J. Caseins and casein hydrolysates. 1. lipoxygenase inhibitory properties. *J. Agric. Food Chem.* **2001**, *49*, 287–294.

(21) Abubakar, A.; Saito, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* **1998**, *81*, 3131–3138.

(22) Maruyama, S.; Nakagomi, K.; Tomizuka, N.; Suzuki, H. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. *Agric. Biol. Chem.* **1985**, *49*, 1405–1409.

(23) Karaki, H.; Doi, K.; Sugano, S.; Uchiwa, H.; Sugai, R.; Murakami, U.; Takemoto, S. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol.* **1990**, *96*, 367–371.

(24) Umbach, M.; Teschemacher, H.; Praetorius, K.; Hirschhauser, R.; Bostedt, H. Demonstration of a beta-casomorphin immunoreactive material in the plasma of newborn calves after milk intake. *Regul. Pept.* **1985**, *12*, 223–230.

(25) Miquel, E.; Gomez, J. A.; Alegria, A.; Barbera, R.; Farre, R.; Recio, I. Identification of casein phosphopeptides released after simulated digestion of milk-based infant formulas. *J. Agric. Food Chem.* **2005**, *53*, 3426–3433.

(26) Yoshikawa, M.; Fujita, H. Studies on the optimum conditions to utilize biologically active peptides derived from food proteins. In *Developments in Food Engineering*; Yano, T., Matsuno, R., Nakamura, K., Eds.; Blackie Academic & Professional: London, U.K., 1994; pp 1053–1055.

(27) Masuda, O.; Nakamura, Y.; Takano, T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* **1996**, *126*, 3063–3068.

(28) Satake, M.; Enjoh, M.; Nakamura, Y.; Takano, T.; Kawamura, Y.; Arai, S.; Shimizu, M. Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 378–384.

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