Casein Enhances Stability of Peptides in Intestinal Lumen: Role of Digested Products of Casein

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Purpose. To investigate the inhibitory activity of casein on proteases in detail, the effect of digested products of casein itself on trypsin and chymotrypsin in rat small intestine was examined.
Methods. Male Sprague-Dawley rats weighing 200–300 g were used as the animal model. The luminal content of the jejunum was prepared, and the enzymatic activities of trypsin and chymotrypsin were determined using a specific substrate for each protease. Then, the effect of enzymatic digested products of casein on them was examined.
Results. The inhibitory activity of trypsin-digested casein against trypsin decreased as its digestion proceeded, but its inhibitory activity against chymotrypsin came to be more effective. On the other hand, the inhibitory activity of chymotrypsin-digested casein against chymotrypsin decreased with the degree of digestion, but no change in the inhibitory activity against trypsin was observed. Even the completely digested products of casein with trypsin or chymotrypsin showed inhibitory activities against the two proteases.
Conclusions. It was suggested that not only the intact casein but also the products digested with trypsin or chymotrypsin contribute to the inhibitory effect of casein on the proteases in the intestinal lumen.

KEY WORDS: casein; digestion product; enzyme inhibition; protease; small intestine.

INTRODUCTION

To improve the quality of life, oral administration of biologically active peptide drugs would be the most attractive route for patients, but the low drug bioavailability as a result of their nonlipophilicity and instability to proteases in the gastrointestinal tract makes it difficult to develop biologically active peptide drugs as an oral dosage form. We have reported that recombinant human insulin-like growth factor-I (rhIGF-I) can be absorbed after oral administration together with casein or aprotinin in rats (1). Although rhIFG-I was relatively stable in the gastric and large-intestinal contents and the subcellular fraction of the small-intestinal mucosa, it rapidly degraded in the small-intestinal contents. However, casein and aprotinin could protect rhIGF-I from the degradation in the small-intestinal contents. The protective effect of casein on the degradation of IGF-I in the intestinal contents was also confirmed recently (2). Our recent paper clarified that casein has a strong inhibitory activity against trypsin and chymotrypsin in the intestinal lumen. Among the proteases in the intestinal epithelial cells, casein inhibited an endopeptidase, cathepsin B, but not exopeptidases. By kinetic analysis, the type of inhibition on trypsin and chymotrypsin was characterized as competitive (3). Because casein is a major component of milk, it is expected that casein can be a safe and useful adjuvant for oral delivery of peptide drugs. However, because casein itself is a protein and is reported to be degraded in the gastrointestinal tract (4), the inhibitory activity of digested products of casein against proteases remains to be clarified.

In this study, the digestion of casein by trypsin and chymotrypsin and the effects of the resulting digested products on casein's inhibitory activity against proteases in rat small intestine were examined.

MATERIALS AND METHODS

Materials

Casein phosphopeptides (CPP), CPP-I, CPP-II, and CPP-III, were kindly supplied from Meiji Seika Co. (Tokyo, Japan). CPP-I is a spray-dried digested product of casein with trypsin, and the CPP content is 12–15%; CPP-II is a spray-dried product that can be obtained by removing the bitter-taste peptides from CPP-I by aminopeptidase-M, and the CPP content is 12–15%; CPP-III is a spray-dried product of the ethanol precipitate obtained from the calcium-added supernatant after removal of impurities by isoelectric precipitation (pH 4.5) from CPP-I, and the CPP content is 85–90%. Casein dodecapeptide (CDP, Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) was a generous gift of Kanebo Pharmaceutical Co. (Osaka, Japan). Trypsin (from porcine pancreas, type IX), chymotrypsin (from bovine pancreas, type II), 7-amino-4-methyl-coumarin (AMC) (Sigma Chemical Co., St. Louis, MO), *n*-butoxycarbonyl-L-Gln-L-Ala-L-Arg-(4-methylcoumarinyl-7-amine) (MCA), a specific substrate for trypsin (5), and N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-MCA (Peptide Institute, Inc., Minoo, Japan), a specific substrate for chymotrypsin (6), were used as supplied. Other reagents used in this study were analytic grade commercial products.

Animals

Male Sprague-Dawley rats weighing 200–300 g (Charles River Japan, Inc., Yokohama, Japan), maintained at 25°C and 55% of humidity, were allowed free access to standard laboratory chow (Clea Japan, Tokyo, Japan) and water before the experiments. Rats were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with "Principles of Laboratory Animal Care" (NIH publication #85-23).

Preparation of Luminal Contents

The luminal contents of the small-intestinal tract were prepared as follows. Under urethane anesthesia, a loop of jejunum (20 cm distal to the ligament of Treitz) was prepared, and 5 ml of isotonic phosphate buffer (pH 7.4) was introduced into the loop. After the solution had remained for 5 min, the luminal content was collected by flushing another 5 ml of the same buffer solution. The solution, with a protein concentration of 0.005–0.050 mg/ml, was used to determine the enzy-

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matic activity. The concentration of protein was determined by the method of Lowry *et al.* (7).

Determination of Enzymatic Activities

To examine the effect of casein on enzymatic activities in the intestinal tract, the specific substrate for each protease, which releases AMC from the N-terminal of each substrate, was selected. The incubation mixture consisted of 250 μl of the luminal contents, 100 μl of a specific substrate, 25 μl of 2.5 M NaCl solution, 25 μl of a catalytic solution, and 100 μl of a corresponding buffer solution. As the catalytic solution, 200 mM $CaCl_2$ was used for trypsin and chymotrypsin (8,9). After 5 min of the reaction time at 37°C, the reaction was stopped by the addition of 2 ml of ice-cold 15% trichloroacetic acid solution. Then, after centrifugation of the reaction mixture at 3000 rpm for 10 min, the fluorescence of AMC released in the supernatant was determined (excitation 380 nm; emission 460 nm), and the enzymatic activity (nmol/min/mg protein) was calculated.

Treatment of Casein with Trypsin or Chymotrypsin

Five grams of casein was dissolved in 100 ml of distilled water adjusted to pH 7.0 by 3 N NaOH. The reaction at 37°C was started by the addition of crystalline trypsin (0.5 mg) or chymotrypsin (12.5 mg), where doses of these enzymes were fixed based on the results of preliminary studies to obtain the significant amount of CCPs and CDP-like fractions. At 0.5, 1, 3, 6, 24, and 48 h after the start of the incubation, 10 ml of the reaction mixture was sampled out and inactivated by heating. The resultant solution was lyophilized to get the powder.

Fractionation of CPP-I

Two grams of CPP-I was dissolved in 100 ml of distilled water adjusted to pH 7.0 with 3 N NaOH, and then the pH was adjusted to 4.5 with 1 N HCl for the isoelectric precipitation of nondigested casein and relatively large peptides. After removal of salts, the obtained precipitate was lyophilized to yield a powder, which is designated "Ppt-1." Calcium chloride was added to the supernatant to a final concentration of 1%, and ethanol was added to 50%. After the centrifugation, the precipitate and the supernatant were named "Ppt-2" and "Sup," respectively.

Gel Filtration

The gel filtration of the sample was performed using Sephadex G200 (Sigma-Aldrich, Milwaukee, WI) column. The column size used was 1.0 × 50 cm, and the bed volume was 40 ml. The sample applied was 0.25 mg/0.5 ml, and the mobile phase was 100 mM phosphate buffer (pH 7.4). The eluted solution was collected every 2 ml, and the digested products were detected spectrophotometrically at 220 nm. As molecular weight markers, fluorescein isothiocyanate-labeled dextran (FD)-4, FD-10, FD-20, and FD-40 were applied and detected spectrofluorometrically at excitation 495 nm and emission 512 nm.

Statistical Analysis

Results are expressed as the mean ± SD of three or more experiments. Analysis of variance (ANOVA) was used to test the statistical significance among groups. Statistical significance of the differences of the means was determined by Dunnet's method.

RESULTS

Figure 1 shows the elution profiles of casein and its trypsin-digested products, CPP-I, CPP-II, CPP-III, and CDP, which were commercial products, on Sephadex G200 column chromatography. The gel filtration profile indicated that casein includes relatively larger molecules eluted in the void volume as shown by the sharp peak around 10 ml of elution volume. The treatment of casein with trypsin reduced the molecular weight, but CPP-I and CPP-II still have a peak at the void volume. However, as can be seen in CPP-III, the purification of CPPs removed large molecules eluted at the void volume. CDP, which has a molecular weight of 1322.5, eluted more slowly. Table I shows the comparison of four trypsin-digested products of casein with casein in their inhibitory activity against trypsin and chymotrypsin. Inhibitory activities of the four products against trypsin were significantly lower than that of casein. On the other hand, CPP-I and CPP-II showed significantly higher inhibitory activities against chymotrypsin. It suggests that the removal of bitter-taste peptides from CPP-I does not affect the inhibitory activity because CPP-II is a product obtained by removing the bitter-taste peptides from CPP-I. The inhibitory activity of purified CPP, CPP-III, against both proteases was extremely low. CDP showed lower but significant inhibitory activity against both proteases.

CPP-I was fractionated following a similar process to that for obtaining CPP-III. Figure 2 shows the gel filtration profile of each fraction obtained. The peak around 25 ml of elution volume was relatively reduced, but no predominant qualitative change was observed in the elution profile of Ppt-1. The inhibitory activity of Ppt-1 against trypsin was reduced compared with CPP-I, but its inhibitory activity against chymotrypsin was similar to that of CPP-I (Table II). Figure 2 shows that Ppt-2 gave a similar elution profile to CPP-III (Fig. 1),

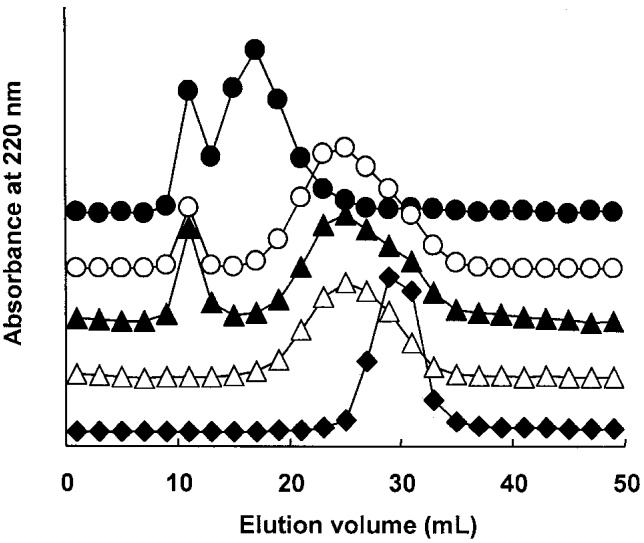

Fig. 1. Typical elution profiles of casein and its trypsin-digested products, CPP-I, CPP-II, CPP-III, and CDP, in Sephadex G-200 chromatography: ●, casein; ○, CPP-I; ▲, CPP-II; △, CPP-III; ◆, CDP.

Table I. Inhibitory Effect of Casein and Its Trypsin-Digested Products, CPP-I, CPP-II, CPP-III, and CDP, on Trypsin and Chymotrypsin Activities in Luminal Contents of Rat Jejunum

	Inhibitory activity (% inhibition)					
	Against trypsin			Against chymotrypsin		
Inhibitor	0.4 mg/mL	2 mg/mL	10 mg/mL	0.4 mg/mL	2 mg/mL	10 mg/mL
Casein	7.4 ± 1.8	30.3 ± 1.3	78.6 ± 1.3	10.7 ± 1.0	41.1 ± 2.9	83.2 ± 3.6
CPP-I	0.0 ± 10.2	20.0 ± 1.4**	62.0 ± 1.4**	30.6 ± 2.4**	77.1 ± 0.6**	90.7 ± 3.9
CPP-II	0.0 ± 5.2	20.7 ± 5.2*	63.4 ± 3.5**	31.0 ± 0.6**	72.3 ± 1.1**	85.3 ± 3.9
CPP-III	4.1 ± 7.4	7.7 ± 3.9**	0.0 ± 0.3**	12.1 ± 9.0	4.2 ± 10.3**	49.7 ± 8.4**
CDP	3.7 ± 1.2	26.6 ± 1.8	73.7 ± 1.1*	8.3 ± 2.3	23.1 ± 2.1**	68.9 ± 1.2*

Substrates used for the measurements of trypsin and chymotrypsin activities were *n*-butoxycarbonyl-Gly-Ala-Arg-4-methylcoumarinyl-7-amine and N-succinyl-Ala-Ala-Pro-Phe-4-methylcoumarinyl-7-amine, respectively. Results are expressed as the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$, compared with the corresponding concentration of casein.

which lacks the peak at the void volume. The inhibitory activity of Ppt-2 fraction against the two proteases was as low as that of CPP-III (Tables I and II). The Sup fraction contains lower-molecular-weight components than CPP-III (Fig. 2) and has an inhibitory activity against trypsin, which is similar to CPP-I (Table II). Its inhibitory activity against chymotrypsin was also significant, although it was lower than that of CPP-I. These results strongly suggest that each component of trypsin-digested casein has an inhibitory activity against trypsin and chymotrypsin.

To investigate the mechanism behind the protease inhibition by casein, the inhibitory activity of the digested products against trypsin and chymotrypsin was examined. The change in the gel filtration profile in Fig. 3 shows that the digestion of casein proceeds as a function of treatment period with trypsin or chymotrypsin. Digested products of casein through a 30-min treatment with trypsin showed a similar profile to CPP-I (Fig. 3A). From the elution profile, treatment for 1–3 h or 6 h gave a gel filtration pattern similar to that of CPP-I or CPP-III, respectively. Furthermore, its digestion for 24 h or more shows the identical filtration profile similar to that of CDP, suggesting the complete digestion of casein. As shown in Table III, the inhibitory activity of trypsin-digested casein against trypsin decreased, but that against chymotrypsin increased, as the trypsin digestion proceeded. Figure 3B shows the gel filtration profiles of chymotrypsin-digested caseins. It seems that treatment for 1–3 h and for more than 6 h gave the intermediate products and the final products by complete digestion, respectively. The inhibitory activity of chymotrypsin-digested casein against trypsin hardly changed (Table III). However, casein digested with chymotrypsin for 3 or 24 h had less inhibitory activity than 0.5-h digested casein against chymotrypsin (Table III).

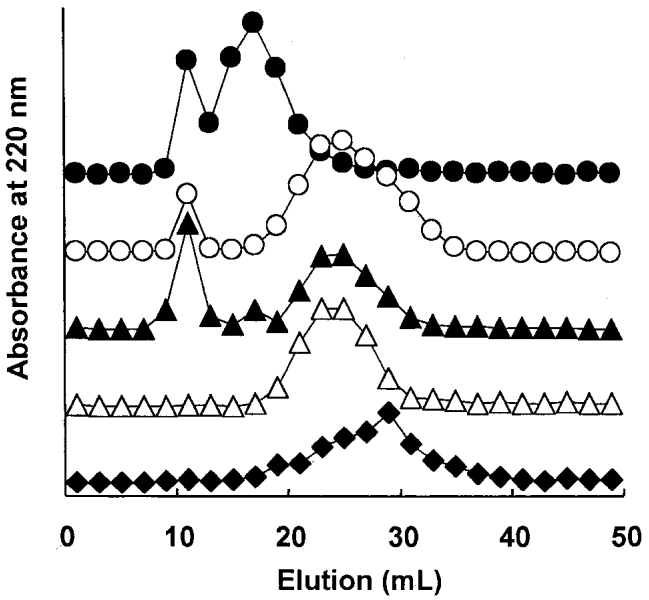

Fig. 2. Typical elution profiles of fractions of CPP-I in Sephadex G-200 chromatography: ●, casein; ○, CPP-I; ▲, Ppt-1, precipitates of isoelectric precipitation (pH 4.5) of CPP-I; △, Ppt-2, precipitates by the addition of ethanol to the above supernatant after the addition of $CaCl_2$; ♦, Sup, the supernatant fraction after the addition of ethanol as above.

Inhibitory effects of CDP against trypsin and chymotrypsin were kinetically analyzed in luminal contents of rat jejunum, using their specific substrates (Fig. 4). Figure 4A shows the Dixon plot for trypsin in the absence or in the presence of CDP at 0.5, 1, and 2 mg/ml for three substrate concentrations, 5, 10, and 15 μM. The fitting analysis was performed independently for each plot, and the obtained x-intercepts were statistically not different from one another, meaning that the three lines should cross on a single point on the horizontal axis and that the type of inhibition should be noncompetitive. A similar analysis was also performed for chymotrypsin activity in luminal contents of rat jejunum (Fig. 4B), and the inhibition by CDP was examined at concentrations of 0.5, 1, and 2 mg/ml for three substrate concentrations, 50, 100, and 150 μM. Contrary to the case of trypsin, x-intercepts obtained by the independent fitting analysis were statistically different, and the three lines crossed on a single point in the second quadrant, meaning that the type of inhibition should be competitive. K_i values of CDP calculated by the simultaneous fitting of the three lines for trypsin and chymotrypsin were 1.1 ± 0.1 and 2.2 ± 0.1 mg/ml, respectively.

DISCUSSION

In our previous paper, we showed that casein markedly inhibits the degradation of rhIGF-I in rat small-intestinal contents and could enhance the bioavailability after oral administration in adult rats (1). Further study showed that casein

Table II. Effect of Fractions of CPP-I on Trypsin and Chymotrypsin Activities in Luminal Contents of Rat Jejunum

	Inhibitory activity (% inhibition)					
	Against trypsin			Against chymotrypsin		
Fraction	0.4 mg/mL	2 mg/mL	10 mg/mL	0.4 mg/mL	2 mg/mL	10 mg/mL
Casein	7.4 ± 1.8	30.3 ± 1.3	78.6 ± 1.3	10.7 ± 1.0	41.1 ± 2.9	83.2 ± 3.6
CPP-I	0.0 ± 10.2	20.0 ± 1.4*	62.0 ± 1.4**	30.6 ± 2.4**	77.1 ± 0.6**	90.7 ± 3.9
Ppt-1	0.0 ± 3.4*	5.6 ± 5.3**	37.5 ± 3.3**	15.9 ± 3.0*	63.4 ± 1.4**	81.5 ± 1.8
Ppt-2	0.0 ± 2.1**	13. ± 5.2**	14.1 ± 4.1**	0.0 ± 0.4**	2.1 ± 1.5**	32.1 ± 5.6**
Sup	0.0 ± 1.3**	23.0 ± 3.3	68.1 ± 0.4**	0.0 ± 1.4**	13.2 ± 1.8**	63.9 ± 0.7**

Substrates used for the measurements of trypsin and chymotrypsin activities were *n*-butoxycarbonyl-Gly-Ala-Arg-4-methylcoumarinyl-7-amine and N-succinyl-Ala-Ala-Pro-Phe-4-methylcoumarinyl-7-amine, respectively. Results are expressed as the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$, compared with the corresponding concentration of casein.

has a strong inhibitory activity on trypsin and chymotrypsin in the intestinal lumen (3). The kinetic analysis characterized the inhibition of trypsin and chymotrypsin as competitive (3). Because casein itself is a protein and is reported to be degraded in the gastrointestinal tract (4), the inhibitory activity of the digested products from the digestion of casein by trypsin and chymotrypsin on these proteases in rat small intestine was examined.

CPP is a general name for hydrolytic products of casein with trypsin and contains four to five phosphate groups in a molecule with an average molecular weight of around 3000 (10,11). CPP increases the solubility of calcium by forming water-soluble complexes with calcium in the gastrointestinal tract and, therefore, can enhance the absorption of calcium (12). Thus, CPP-I, CPP-II, and CPP-III are on the market and are used as adjuvants to enhance calcium absorption in foods and drinks. CPP-I is a digested product of casein with trypsin, and the CPP content is 12–15%. It contains bitter-taste hydrophobic peptides and is used in veterinary area. CPP-II is a spray-dried product that is left after removal of the bitter-taste peptides from CPP-I by aminopeptidase-M. CPP-II contains CPP content at 12–15% and is used as an adjuvant in confectionery and foods. CPP-III is a spray-dried product of the ethanol precipitate from the calcium-added supernatant after impurities have been removed from CPP-I by isoelectric precipitation (pH 4.5). CPP-III, used as an adjuvant in drinks, contains CPP content of 85–90%. As shown in Fig. 1, the treatment of casein with trypsin produces molecules with smaller molecular weights, whereas CPP-I and CPP-II still have large molecules eluted at the void volume. Although the average molecular weight of casein is considered to be 23,000, this peak in the void volume seems to be derived from casein micelles (4) and was removed by the purification of CPP because CPP-III does not have that peak in the void volume.

CPP-I showed a lower inhibitory activity against trypsin compared with casein, but the removal of bitter-taste peptides to get CPP-II caused no big change in the inhibitory activity

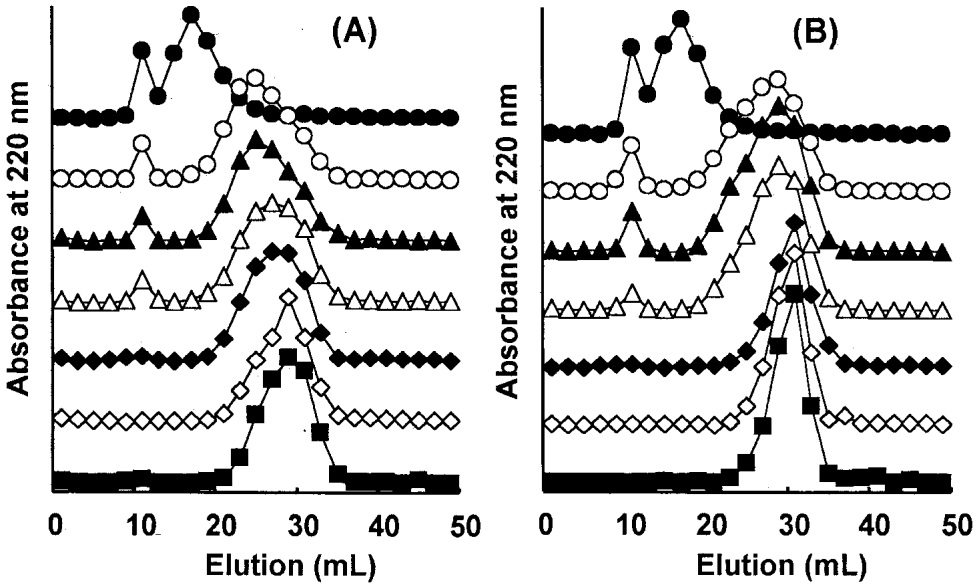

Fig. 3. Changes in elution profiles of casein by digestion with trypsin (A) or chymotrypsin (B) for various reaction periods in Sephadex G-200 chromatography. Trypsin (from porcine pancreas) and chymotrypsin (from bovine pancreas) were used for digestion of casein (5 g) at 0.5 mg and 12.5 mg, respectively. ●, nondigested casein; ○, 30 min; ▲, 1 h; △, 3 h; ♦, 6 h; ◇, 24 h; ■, 48 h.

Table III. Effect of Casein Digested with Trypsin or Chymotrypsin on Trypsin and Chymotrypsin Activities in Luminal Contents of Rat Jejunum

Digestion	Period of digestion (h)	Inhibitory activity (% inhibition)					
		Against trypsin			Against chymotrypsin		
		0.4 mg/mL[a]	2 mg/mL[a]	10 mg/mL[a]	0.4 mg/mL[a]	2 mg/mL[a]	10 mg/mL[a]
No enzyme	0	7.4 ± 1.8	30.3 ± 1.3	78.6 ± 1.3	10.7 ± 1.0	41.1 ± 2.9	83.2 ± 3.6
With trypsin	0.5	0.0 ± 2.5**	28.6 ± 1.0	69.2 ± 1.5*	19.6 ± 2.9**	67.3 ± 0.9**	83.7 ± 2.5
	3	0.0 ± 1.6**	0.0 ± 2.7**	43.4 ± 2.9**	24.7 ± 4.1**	72.0 ± 0.6**	91.3 ± 0.5**
	24	0.0 ± 2.7**	8.7 ± 2.7**	42.7 ± 5.5**	22.8 ± 0.4**	73.1 ± 0.6**	91.7 ± 1.0**
With chymotrypsin	0.5	8.4 ± 2.8	33.0 ± 4.4	62.7 ± 4.4**	4.0 ± 0.9**	24.4 ± 4.9**	62.8 ± 2.7**
	3	5.5 ± 4.2	27.6 ± 3.6	63.7 ± 3.3**	3.3 ± 3.2**	10.8 ± 1.7**	54.5 ± 1.7**
	24	0.0 ± 2.8*	31.8 ± 1.9	70.2 ± 1.0*	0.0 ± 0.5**	18.0 ± 0.7**	58.1 ± 2.2**

Trypsin (from porcine pancreas) and chymotrypsin (from bovine pancreas) were used for digestion.

[a] Concentrations of nondigested casein or its digested products. Substrates used for the measurements of trypsin and chymotrypsin activities in luminal contents of rat jejunum were *n*-butoxycarbonyl-Gly-Ala-Arg-4-methylcoumarinyl-7-amine and N-succinyl-Ala-Ala-Pro-Phe-4-methylcoumarinyl-7-amine, respectively. Results are expressed as the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$, compared with the corresponding concentration of nondigested casein.

against the protease (Table I). On the other hand, the inhibitory activity of CPP-I and CPP-II against chymotrypsin was significantly larger than that of casein, but again no significant difference was observed between CPP-I and CPP-II, suggesting that the bitter-taste hydrophobic peptides hardly have anything to do with the inhibitory activity against both proteases. The inhibitory activity of CPP-III was markedly low against the two proteases. This purification of CPP-I seemed to remove the components having the inhibitory activity against trypsin almost completely, whereas CPP-III still has a lower but significant inhibitory activity against chymotrypsin.

Another product from trypsin-digested casein, CDP, is a polypeptide consisting of 12 amino acids and has an inhibitory activity against angiotensin-converting enzyme (13). CDP showed similar inhibitory activities against trypsin and chymotrypsin, but they are slightly lower in comparison with casein (Table I). CDP has no bond to be hydrolyzed by trypsin and thus is considered not to be a substrate of trypsin. On the other hand, this peptide has three bonds to be attacked by chymotrypsin. Therefore, the type of CDP inhibition against trypsin could be different from that against chymotrypsin. To solve this question, a kinetic analysis was performed for the inhibitory effect of CDP on trypsin and chymotrypsin activities in luminal contents of rat jejunum. The results of the Dixon-plot analyses showed that the types of inhibition by CDP against trypsin and chymotrypsin are different from each other and are noncompetitive and competitive, respectively (Fig. 4).

CPP-I seems to contain not only various trypsin-digested products but also components of nondigested casein. Ppt-1, a

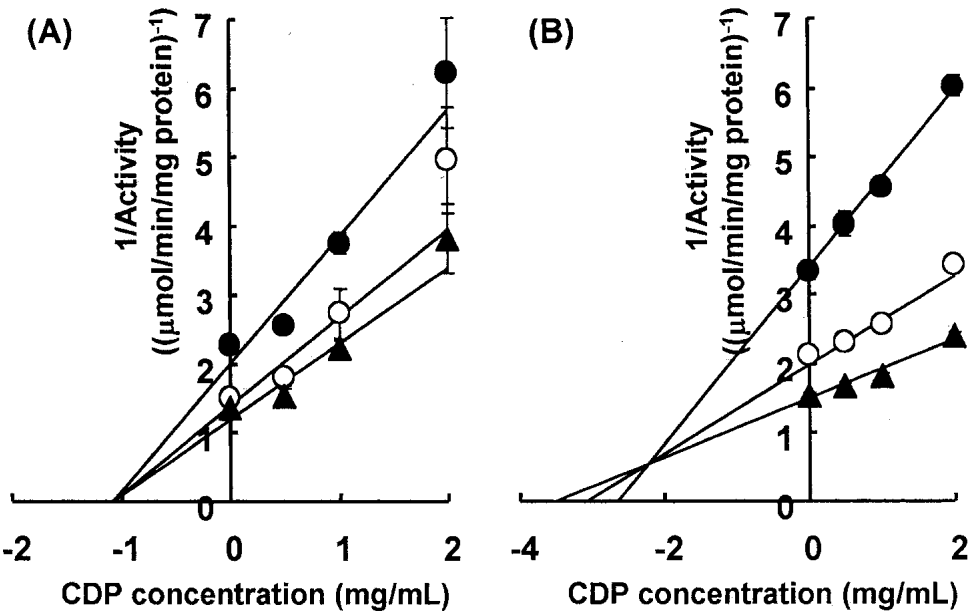

Fig. 4. Dixon-plot analyses for inhibitory effect of CDP on trypsin (A) and chymotrypsin (B) activities in luminal contents of rat jejunum Substrates concentrations were 5(●), 10(○), and 15(▲) μM in A and 50(●), 100(○), and 150 (▲) μM in B. Results are expressed as the mean ± SD of three experiments. Solid lines were obtained by the simultaneous fitting study.

precipitate from CPP-I at pH 4.5, also contains those components of nondigested casein (Fig. 2). The inhibitory activity of Ppt-1 against trypsin was lower than that of CPP-I, but the activity against chymotrypsin was similar to that of CPP-I (Table II). The gel filtration profile of Ppt-2 was similar to that of CPP-III (Figs. 1 and 2), and both Ppt-2 and CPP-III had low inhibitory activities against trypsin. However, chymotrypsin was significantly inhibited by these fractions (Tables I and II). This suggests that CPP-III hardly inhibits trypsin but slightly inhibits chymotrypsin. On the other hand, Sup contains smaller products in comparison with CPPs (Figs. 1 and 2) and has strong inhibitory activities against both trypsin and chymotrypsin (Table II), suggesting that the components with smaller molecular weights would largely contribute to the inhibitory activity of CPP-I and CPP-II.

The treatment of casein with trypsin for 30 min gave a gel filtration profile quite similar to that of CPP-I (Figs. 2 and 3A), of which the inhibitory activity was also similar to that of CPP-I (Tables II and III). Considering the gel filtration profiles (Fig. 3A), casein itself could be almost completely degraded by treatment with trypsin for 24 h or more via the formation of the intermediate products by 1–3 h of treatment. The final products showed a lower but significant inhibitory activity against trypsin, and the inhibition of chymotrypsin was higher than that by casein (Table III). Similarly, chymotrypsin-digested products, even the final products, maintained an inhibitory activity against trypsin, similar to casein itself, even though its inhibition of chymotrypsin was lower than that by casein (Table III). These results clearly show that digested products of casein keep a significant inhibitory activity against trypsin and chymotrypsin. Therefore, the oral administration of casein would make it possible to inhibit the degradation of peptides by trypsin and chymotrypsin as long as the digested products of casein exist in the gastrointestinal tract, even though casein is completely digested by trypsin and chymotrypsin.

In conclusion, not only the digested intermediate products but also the final products can inhibit trypsin and chymotrypsin in the intestinal lumen, although casein itself is digested during the transit in the small intestine. Therefore, using casein as a protease inhibitor could maintain the inhibition of proteases such as trypsin and chymotrypsin during its passage through the gastrointestinal tract.

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