Evaluation of Inhibitory Activity of Casein on Proteases in Rat Intestine

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Received September 17, 2002; accepted January 3, 2003

Purpose. To investigate the possible use of casein as an adjuvant for oral delivery of peptide drugs, the inhibitory activity of casein on proteases in rat small intestine was examined.

Methods. Male Sprague–Dawley rats weighing 200–300 g were used as the animal model. The luminal contents of the small-intestinal tract and mucosal subcellular fractions of the small intestine were prepared; the enzymatic activities of trypsin, chymotrypsin, aminopeptidase-B, leucine aminopeptidase, dipeptidylaminopeptidase-IV, cathepsin B, and dipeptidylaminopeptidase-II were determined using a specific substrate for each protease; and the effect of casein on the protease activity was examined.

Results. Casein strongly inhibited trypsin and chymotrypsin in the concentration-dependent manner. As to the proteases in the intestinal epithelial cells, casein inhibited an endopeptidase, cathepsin B, but not other exopeptidases. The inhibitory activity was independent of the type of casein. The kinetic analysis characterized the type of inhibition on trypsin and chymotrypsin to be competitive.

Conclusions. Casein was shown to have strong inhibitory activity on trypsin and chymotrypsin in the intestinal lumen. Taken into consideration that trypsin and chymotrypsin are endopeptidases, it is suggested that casein has strong inhibitory activity only on endopeptidases.

KEY WORDS: casein; trypsin; chymotrypsin; inhibitory activity; small intestine.

INTRODUCTION

Recent developments in biotechnology have made it possible to supply biologically active peptides. However, there are some problems with the medical use of peptides. The route of administration of peptide drugs is mostly limited to injections because their bioavailability after oral administration is quite low. In addition, their biologic half-lives are so short that frequent administration is needed to maintain their action, resulting in the endurance of pain. To improve the quality of life, the oral administration of peptide drugs is the most attractive for patients, but the low bioavailability because of their nonlipophilicity, relatively high molecular weight, and/or instability to proteases in the gastrointestinal tract makes it difficult. We have reported that recombinant human insulin-like growth factor-I (rhIGF-I) can be absorbed after oral administration together with aprotinin or casein 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, aprotinin and casein could protect rhIGF-I from the degradation in the small-intestinal contents (1). The protective effect of casein on the degradation of IGF-I in the intestinal contents was also confirmed recently (2). However, details of the inhibitory activity of casein on proteases remain to be clarified. In this study, the inhibitory activity of casein on proteases in rat small intestine was examined using a specific substrate for each protease.

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MATERIALS AND METHODS

Materials

Casein (from cow milk, Nacalai Tesque Inc., Kyoto, Japan), α-casein (from bovine milk, Sigma Chemical Co., St. Louis, MO, USA), and β-casein (from bovine milk, Sigma Chemical Co.) were purchased from the sources indicated. Gly-L-Pro-(4-methylcoumarinyl-7-amine; MCA), *n*-Butoxycarbonyl-L-Gln-L-Ala-L-Arg-MCA, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-MCA, benzyloxycarbonyl-L-Arg-L-Arg-MCA, L-Lys-L-Ala-MCA (Peptide Institute, Inc., Minoo, Japan), L-Arg-MCA, L-Leu-MCA (Bachem Ltd., Essex, UK), and 7-amino-4-methyl-coumarin (AMC; Sigma Chemical Co.) were used as supplied. Other reagents used in this study were analytical 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 prior to the experiments. Rats were randomly assigned to each experimental group. Our investigations were performed after the 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 and Mucosal Subcellular Fractions

The luminal contents of the small-intestinal tract were prepared as follows. Briefly, under urethane anesthesia, the loops of jejunum (20 cm lower from the ligament of Treitz) and ileum (20 cm upper from ileocecal junction) were prepared, and 5 mL of isotonic phosphate buffer (pH 7.4) was introduced to each loop. After standing for 5 min, the luminal contents from the jejunum and the ileum were collected by flushing another 5 mL of the same buffer solution. The solution at the protein concentration of 0.005–0.050 mg/mL was used to determine the enzymatic activity.

Mucosal subcellular fractions of the small intestine were prepared according to the methods of Maunsbach (3) and Bai (4,5), with slight modification. The intestinal mucosa of each segment was scraped off, suspended in 2 mM Tris-HCl buffer (pH 7.5) containing 50 mM mannitol and then homogenized using a blender homogenizer (Nihon Seiki Ltd., Tokyo). The homogenate was centrifuged at 700×g for 10 min at 4°C to remove nuclear fraction. The supernatant was centrifuged at 9,000×g for 20 min at 4°C, and the precipitate was used as the lysosomal fraction. The purity of the fraction was estimated to determine the activity of acid phosphatase, a lysosomal marker (3,6). The enrichment obtained was 11.6 ± 0.8, show-

0724-8741/03/0400-0611/0 © 2003 Plenum Publishing Corporation

ing the purification of lysosomal fraction. The supernatant was added with 1 M $CaCl_2$, of which the final concentration is 10 mM, and was centrifuged at 27,000×g for 30 min at 4°C. The precipitate was used as the brush border membrane (BBM) fraction. Activity alkaline phosphatase, a marker enzyme for BBM (7,8), was determined to assess the purity of BBM. On average, BBM was 35.5 ± 2.2 times enriched. Further, the supernatant was centrifuged at 100,000×g for 1 h at 4°C, and the resulting supernatant was used as the cytosolic fraction. By determining the lactate dehydrogenase activity, a cytosolic marker (4), the purification of cytosol was assessed and 85.1 ± 4.5% of the activity in homogenate was accounted for by the cytosol purified in the present study. The lysosomal fraction was resuspended with 1 M acetate-NaOH buffer (pH 4.5) and the BBM fraction was with 100 mM Tris-HCl buffer (pH 7.5). They were used to determine the enzyme activity at the protein concentrations of 0.200–0.500 mg/mL and 0.020–0.100 mg/mL, respectively. The cytosolic fraction was diluted with 100 mM Tris-HCl buffer (pH 7.5) to be 0.100–0.600 mg protein/mL. The concentration of protein was determined by the method of Lowry *et al.* (9).

Determination of Enzymatic Activities

To examine the effect of casein on enzymatic activities in the intestinal tract, the specific substrate for each protease (10–16), which releases AMC from the N-terminal of each substrate, was selected. They are listed in Table I together with the reaction time at 37°C. The incubation mixture consisted of 250 μL of each protease solution, 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; 2 M KCl was for aminopeptidase-B (AP-B); 40 mM dithiothreitol, 40 mM EDTA, and 10 mg/mL soybean trypsin inhibitor were for cathepsin B; and distilled water was for the other proteases (4,5). After each appropriate reaction time, the reaction was stopped by the addition of 2 mL of ice-cold 15% trichloroacetic acid solution. Then, after the centrifugation of the reaction mixture at 1,700×g for 10 min, the fluorescence of AMC released in the supernatant was determined (ex. 380 nm; em. 460 nm), and the enzymatic activity (nmol/min/mg protein) was calculated. In some experiments, casein, aprotinin, sodium glycocholate or bovine serum albumin (BSA; as a reference protein) was added to examine its inhibitory activity.

Kinetic Analysis of Inhibitory Activity of Casein

Estimation of K_i and the type of inhibition by casein was performed based on Lineweaver–Burk plots using the nonlinear least-square regression program MULTI (17).

Statistical Analysis

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

RESULTS

The activities of trypsin and chymotrypsin in luminal contents and the effect of casein, a crude acid casein, on their activities were examined for rat jejunum and ileum (Table II). The activities of trypsin and chymotrypsin were significantly higher in the jejunal contents than those in the ileal ones. Two mg/mL casein significantly inhibited the activities of both enzymes and 10 mg/mL casein extensively decreased both enzyme activities down to about 20% of control. BSA, a reference protein, also inhibited the enzyme activities, but the inhibitory effect was not so large, comparing with that by casein. Although the regional differences in trypsin and chymotrypsin activities between the jejunum and the ileum were observed, there was no obvious difference in the inhibitory activities of casein between the two sites. Figure 1 shows that casein inhibited trypsin and chymotrypsin activities in luminal contents of rat jejunum with increasing the concentration. Although BSA exerted a similar inhibitory effect up to 2 mg/mL, the inhibitory effect was found to be saturated and much lower than that by casein in the range of higher concentrations.

Because casein is a mixture of several components, such as α-casein, β-casein, and κ-casein, the effect of major components of casein, α-casein, and β-casein on the activities of trypsin and chymotrypsin in luminal contents of rat jejunum was examined. As shown in Fig. 2, both α-casein and β-casein strongly inhibited trypsin and chymotrypsin, but their inhibitory activities were not so different from that of crude casein. Therefore, further study was performed using less-expensive crude casein.

In both BBM and cytosolic fractions, AP-B, leucine aminopeptidase (Leu-AP), and dipeptidylaminopeptidase-IV (DPP-IV) were examined. The activities of AP-B, Leu-AP, and DPP-IV were significantly higher in the BBM fraction

Table I. Intestinal Proteases and Their Specific Substrates Used in This Study

Site/protease	Specific substrate	Reference	Final concentration	Reaction time
Lumen				
Trypsin	*n*-Butyloxycarbonyl-L-Gln-L-Ala-L-Arg-MCA	(10)	60 μM	5–10 min
Chymotrypsin	*N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-MCA	(11)	60 μM	5–15 min
Brush border membrane and cytosol				
AP-B	L-Arg-MCA	(12)	0.46 mM	30, 60 min
Leu-AP	L-Leu-MCA	(13)	0.71 mM	30, 60 min
DPP-IV	Gly-L-Pro-MCA	(14)	0.45 mM	20, 60 min
Lysosome				
Cathepsin B	Benzyloxycarbonyl-L-Arg-L-Arg-MCA	(15)	40 μM	60 min
DPP-II	L-Lys-L-Ala-MCA	(16)	40 μM	60 min

Note: AP, DPP, and MCA mean aminopeptidase, dipeptidylpeptidase and 4-methylcoumarinyl-7-amine, respectively.

Table II. Protease Activities in Fractions of Rat Jejunum and Ileum with or without Casein or BSA

Fraction (site) protease	Protease activity (nmol/min/mg protein)				
	Control	Plus casein (2 mg/mL)	Plus casein (10 mg/mL)	Plus BSA (2 mg/mL)	Plus BSA (10 mg/mL)
Contents (Jejunum)					
Trypsin	3585.0 (106.1)	2497.8 (45.0)[d]	768.3 (47.6)[d]	3003.2 (117.5)[d]	2502.7 (109.1)[d]
Chymotrypsin	455.6 (48.9)	294.6 (14.3)[d]	83.8 (18.1)[d]	377.7 (8.4)[c]	372.7 (12.0)[d]
Contents (Ileum)					
Trypsin	2657.2 (65.9)[a]	2348.1 (42.4)[d]	783.7 (26.1)[d]	n.e.	n.e.
Chymotrypsin	47.9 (4.1)[a]	31.0 (3.5)[d]	13.4 (2.2)[d]	n.e.	n.e.
BBM (Jejunum)					
AP-B	197.0 (0.1)[b]	196.8 (0.5)	196.8 (0.2)	197.5 (0.4)	198.5 (0.1)[d]
Leu-AP	308.5 (0.2)[b]	308.6 (0.2)	307.9 (0.4)	309.7 (0.1)[d]	310.0 (0.3)[d]
DPP-IV	197.6 (0.3)[b]	196.7 (0.2)[c]	195.2 (0.3)[d]	198.2 (0.4)	198.8 (0.1)[d]
BBM (Ileum)					
AP-B	129.4 (0.0)[a,b]	129.5 (0.0)	129.6 (0.1)[d]	129.4 (0.1)	129.3 (0.0)
Leu-AP	200.2 (0.1)[a,b]	200.5 (0.2)	200.2 (0.2)	200.8 (0.1)[d]	200.4 (0.2)
DPP-IV	127.3 (0.1)[a,b]	127.4 (0.0)	126.1 (0.1)[d]	127.2 (0.1)	127.2 (0.0)
Cytosol (Jejunum)					
AP-B	41.3 (0.0)	41.2 (0.1)	41.3 (0.0)	41.2 (0.0)[c]	41.2 (0.0)
Leu-AP	62.3 (0.0)	62.0 (0.0)[d]	61.8 (0.1)[d]	62.3 (0.0)	62.3 (0.0)
DPP-IV	39.1 (0.0)	39.0 (0.0)[d]	38.9 (0.0)[d]	39.1 (0.0)	39.2 (0.0)[d]
Cytosol (Ileum)					
AP-B	37.4 (0.0)[a]	37.4 (0.0)	37.6 (0.1)[d]	37.5 (0.0)	37.5 (0.0)[d]
Leu-AP	57.0 (0.0)[a]	56.9 (0.0)[d]	56.7 (0.0)[d]	57.1 (0.0)[d]	57.2 (0.0)[d]
DPP-IV	35.9 (0.0)[a]	35.9 (0.0)	35.8 (0.0)[d]	36.0 (0.0)	36.0 (0.0)[d]
Lysosome (Jejunum)					
DPP-II	0.185 (0.002)	0.289 (0.018)[d]	0.310 (0.015)[d]	n.e.	n.e.
Cathepsin B	0.268 (0.003)	0.232 (0.011)[d]	0.136 (0.011)[d]	n.e.	n.e.
Lysosome (Ileum)					
DPP-II	0.280 (0.004)[a]	0.328 (0.032)	0.268 (0.029)	n.e.	n.e.
Cathepsin B	0.259 (0.006)	0.249 (0.032)	0.207 (0.023)	n.e.	n.e.

Note: BBM, brush border membrane; AP-B, aminopeptidase B; Leu-AP, leucine-aminopeptidase; DPP-IV, dipeptidylaminopeptidase IV; DPP-II, dipeptidylaminopeptidase II; BSA, bovine serum albumin. Results are expressed as the means with SD in parentheses of three experiments. n.e., not examined.

[a] $p < 0.001$, compared with the corresponding value in jejunum.

[b] $p < 0.001$, compared with the corresponding value in cytosol.

[c] $p < 0.05$; [d] $p < 0.01$, compared with each control value.

than those in the cytosol, and their activities in both fractions were slightly but significantly higher in the jejunum than those in the ileum (Table II). In contrast with the proteases in the luminal contents, the effect of casein on each protease was negligible. In the lysosomal fraction, cathepsin B activity was not different between the jejunum and the ileum, but cathepsin B in lysosomal fractions of both jejunum and ileum was inhibited by casein. However, dipeptidylaminopeptidase-II (DPP-II) activity in the lysosomal fraction was higher in the ileum than in the jejunum, but the activity was enhanced by casein (Table II).

Inhibitory effects of casein on trypsin and chymotrypsin were kinetically analyzed in luminal contents of rat jejunum, using their specific substrates shown in Table I (Fig. 3). Figure 3(A) shows the Lineweaver–Burk plots in the absence or in the presence of casein at 1 and 2 mg/mL. The fitting analysis was performed independently for each plot and the obtained y-intercepts were statistically not different one another, meaning that the three lines should cross on a single point on the vertical axis and that the type of inhibition should be competitive. Therefore, the simultaneous fitting of the three lines were performed to calculate K_m, V_{max}, and K_i values, resulting in 18.3 ± 4.0 μM, 1.97 ± 0.26 μmol/min/mg protein, and 0.34 ± 0.01 mg/mL, respectively. The similar analysis was also performed for chymotrypsin activity in luminal contents of rat jejunum (Fig. 3B), and the study on the inhibition by casein were examined at the concentrations of 1 and 2 mg/mL for four substrate concentrations, 50, 100, 150, and 200 μM. As shown in Fig. 3(B), the type of inhibition was competitive and the K_m, V_{max}, and K_i values were calculated to be 106.0 ± 26.4 μM, 0.54 ± 0.07 μmol/min/mg protein, and 0.84 ± 0.15 mg/mL, respectively.

Figure 4 shows the comparison of casein with aprotinin and sodium glycocholate in the inhibitory activity on trypsin and chymotrypsin. Here, the concentration of casein was expressed in μM by assuming that the molecular weight of casein is 23,000. As is evident from the figure, the inhibitory activity of aprotinin was stronger than casein especially against trypsin, while the inhibitory activity of sodium glycocholate against the two enzymes was quite weak in the concentration range examined.

DISCUSSION

It has been suggested that IGF-I must be absorbed from gastrointestinal tract of the suckling newborn, since IGF-I in

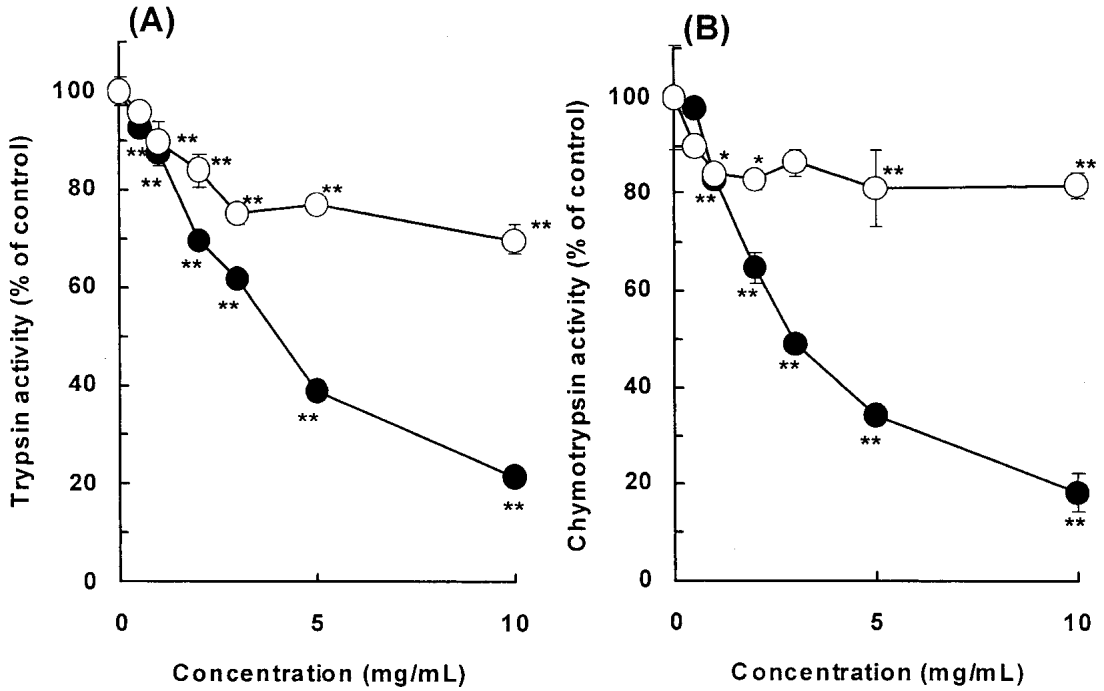

Fig. 1. Effect of casein and bovine serum albumin on trypsin (A) and chymotrypsin (B) activities in luminal contents of rat jejunum. ●, casein; ○, bovine serum albumin. Results are expressed as the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$ compared with the control value.

the maternal milk may play an important role in regulating its postnatal development (18). Then, Vacher et al. (19) and Xu and Wang (20) reported the absorption of IGF-I from gastrointestinal tract of neonatal calves and neonatal pigs, respectively. The presence of protease inhibitors in the milk (21) and the enhancement of the gastrointestinal absorption of somatostatin by the addition of rat milk due to the inhibition of its intestinal degradation (22) have been also reported.

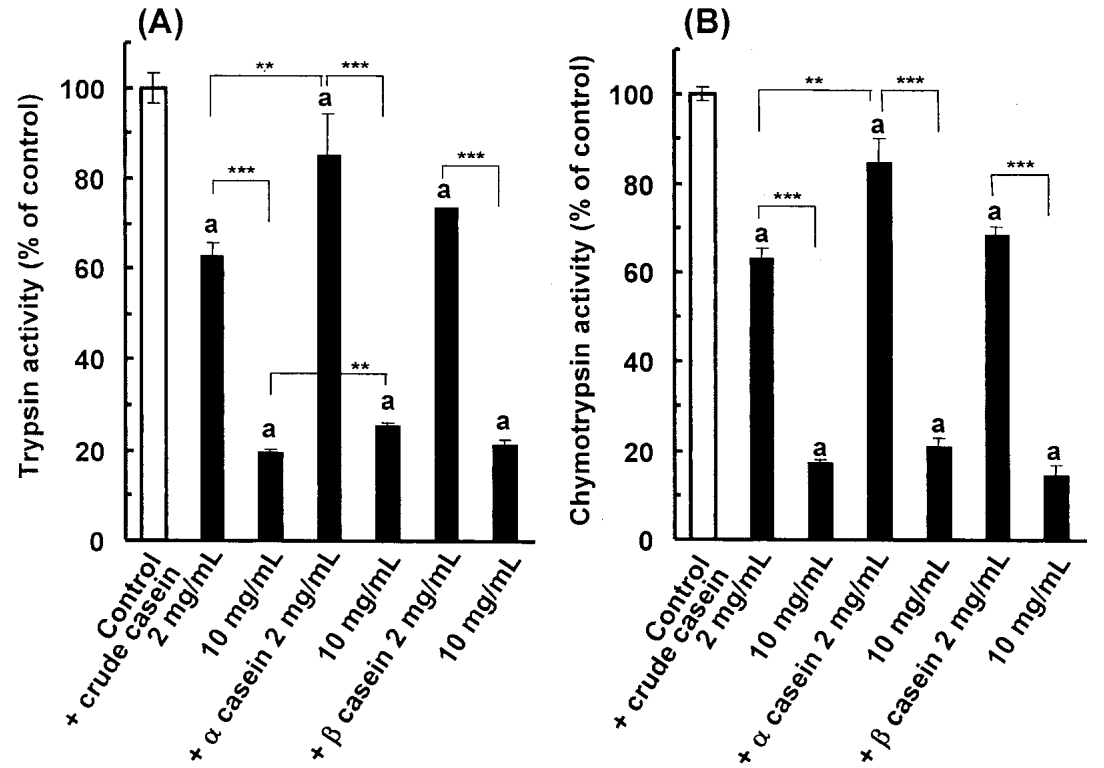

Fig. 2. Effect of caseins on trypsin (A) and chymotrypsin (B) activities in luminal contents of rat jejunum. Results are expressed as the mean ± SD of three experiments. a, $p < 0.001$, compared with the control value. **$p < 0.01$; ***$p < 0.001$.

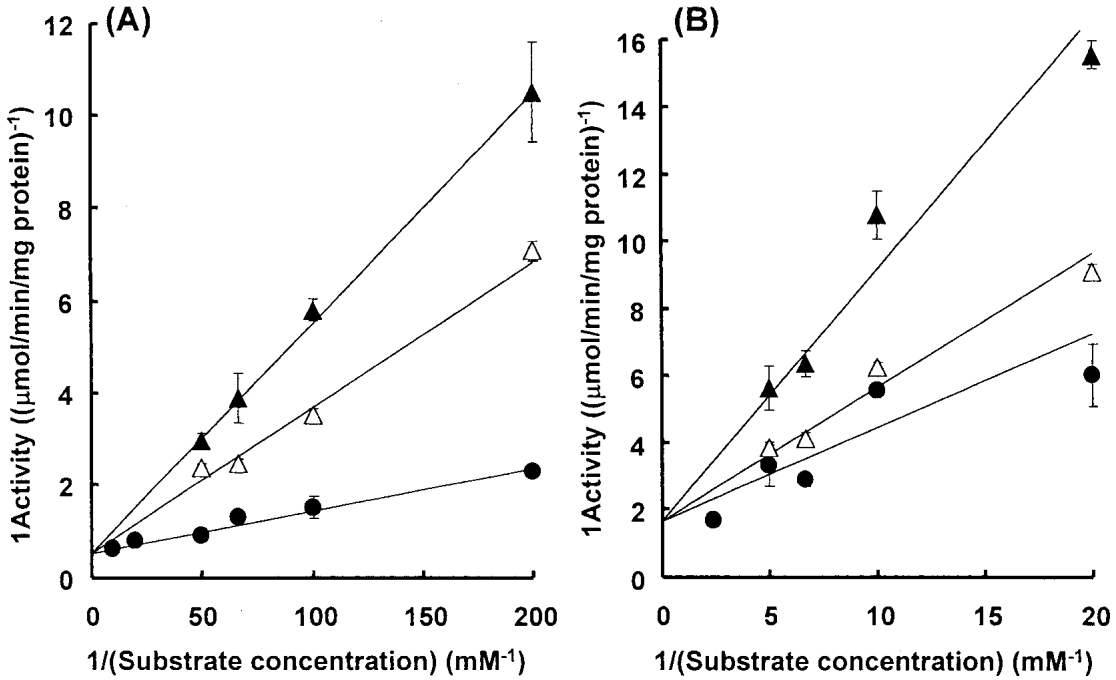

Fig. 3. Lineweaver–Burk-type plots for activities of trypsin (A) and chymotrypsin (B) in luminal contents of rat jejunum vs. initial concentration of each substrate in the presence or absence of casein. ●, without casein; △, with 1 mg/mL casein; and ▲, with 2 mg/mL casein. Results are expressed as the mean ± SD of three experiments. Solid lines were obtained by the simultaneous fitting study.

From these backgrounds, focusing on casein, a major component of the milk, we showed that casein markedly inhibits the degradation of rhIGF-I in rat small-intestinal contents and can enhance the bioavailability after oral administration in adult rats (1). The present study was performed to clarify the characteristics of the inhibitory activity of casein on proteases in the small intestine for developing the oral drug delivery system of peptide drugs using casein as a protease inhibitor.

Casein is a major acidic protein in the milk and can be easily obtained by the isoelectric precipitation of the milk at pH 4.6. This casein is called the acid casein and can be separated by electrophoresis to α-, β-, and κ-casein, which have no

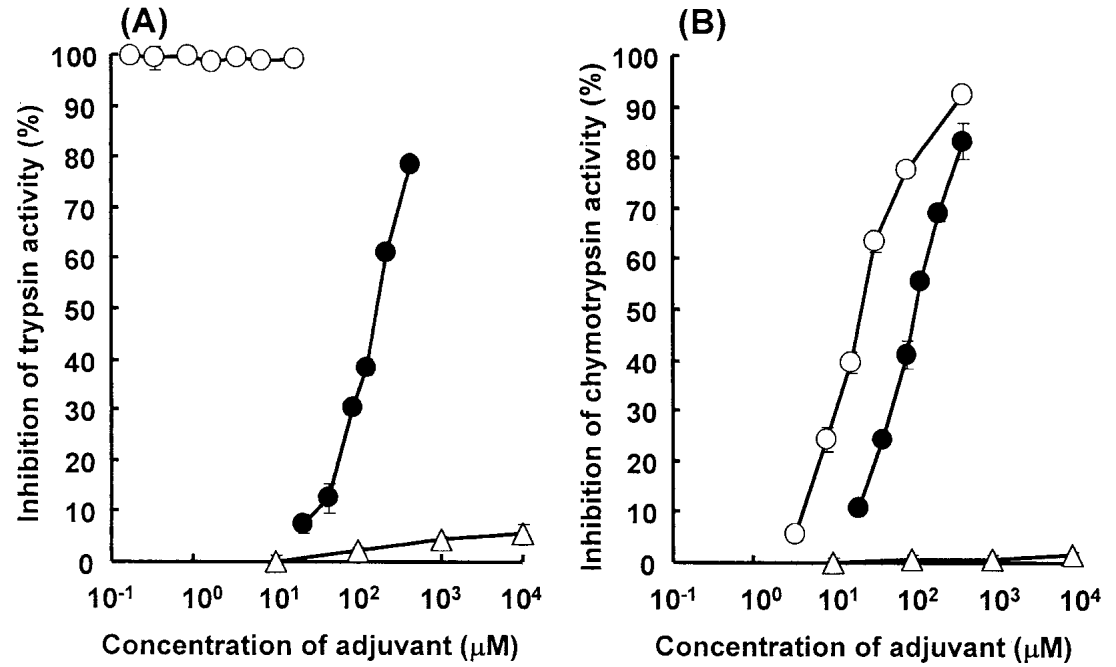

Fig. 4. Comparison of inhibitory effects of casein on trypsin (A) and chymotrypsin (B) activities in luminal contents of rat jejunum with those of aprotinin and sodium glycocholate. ●, casein; ○, aprotinin; △, sodium glycocholate. Results are expressed as the mean ± SD of three experiments.

common amino acid sequences one another (23). In general, casein in cow milk consists of 52, 33, and 15% of α-, β-, and κ-casein, respectively (24). Figure 2 shows the effect of types of casein on trypsin and chymotrypsin activities in luminal contents of rat jejunum. As is evident from the figure, both α-casein and β-casein strongly inhibited both proteases, and their inhibitory activities were not so different from that of crude casein, suggesting that α-casein and β-casein, major components of casein, should mainly be responsible for the inhibitory activity of crude casein.

The specific activities of trypsin and chymotrypsin were much higher in the jejunum than those in the ileum (Table II). The two proteases in the jejunum must be more active than those in the ileum, since the amounts of protein in the luminal contents were not so different in the two regions (results not shown). However, there was not any obvious difference in the inhibitory effect of casein on these enzyme activities between the jejunum and the ileum (Table II). As shown in Fig. 1, casein strongly inhibited trypsin and chymotrypsin in the concentration-dependent manner. Approximately 80% of both enzyme activities were inhibited by the presence of casein at 10 mg/mL. However, BSA, a reference protein, did not show such a marked inhibitory activity, although the significant inhibition was observed. The inhibition by BSA might be due to its interaction with substrates and/or enzymes that could hinder the access of substrates to the enzymes.

In the mucosal cells, the activities of three exopeptidases, AP-B, Leu-AP, and DPP-IV, were higher in the BBM fraction than those in the cytosol, and the enzyme activities in both fractions were slightly higher in the jejunum than those in the ileum (Table II). In contrast with proteases in the luminal contents, the effect of casein on each protease was negligible. Cathepsin B activity in the lysosomal fraction was not different between the jejunum and the ileum, and this endopeptidase was inhibited by casein. On the other hand, DPP-II activity in the lysosomal fraction, which was higher in the ileum than in the jejunum, was enhanced by casein (Table II), but the reason remains to be clarified. Considering the results obtained, it seems that casein has a strong inhibitory activity against endopeptidases, i.e., trypsin and chymotrypsin in the intestinal lumen, cathepsin B in lysosome.

The kinetic analysis for the inhibitory activity of casein on trypsin and chymotrypsin showed that the types of inhibition on both proteases are competitive (Fig. 3). However, because casein itself is a protein and is reported to be degraded in the gastrointestinal tract (25), further study is needed to clarify the mechanism of protease inhibition in more detail. Studies on the characterization of degradation products of casein by trypsin and chymotrypsin and their effects on the two proteases are in progress.

The inhibitory activities of casein on trypsin and chymotrypsin were compared with those of aprotinin and sodium glycocholate (Fig. 4). The inhibitory activity of aprotinin was stronger than casein especially against trypsin, whereas the inhibitory activity of sodium glycocholate was weak in the concentration range examined. Although aprotinin has strong inhibitory activity on proteases, this protease inhibitor has the permeability enhancing property and caused some mucosal damages, which allowed Evans blue systemically injected to leak into intestinal lumen (26). We also confirmed that aprotinin enhanced the intestinal absorption of fluorescein isothiocyanate-conjugated dextran with average molecular weight of 44,000 (FD-4) in rat jejunum (1). However, casein did not enhance the absorption of FD-4 (1), suggesting that casein is a safer adjuvant that can suppress the degradation of peptide drugs.

In conclusion, casein inhibits trypsin and chymotrypsin activities in the intestinal lumen and cathepsin B activity in lysosome. The type of inhibition against trypsin and chymotrypsin is competitive, meaning that the inhibitory effect by casein must be reversible. Furthermore, because casein is a major component of the milk, it is expected that casein can be safe and cannot affect the food digestion so much. Therefore, the use of casein would be promising for developing an oral drug delivery system of peptide drugs.

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