Dietary Protein Peptic Hydrolysates Stimulate Cholecystokinin Release via Direct Sensing by Rat Intestinal Mucosal Cells

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We previously demonstrated that a peptic hydrolysate of guanidinated casein strongly stimulates exocrine pancreatic secretion in chronic bile-pancreatic juice-diverted rats and cholecystokinin (CCK) release from dispersed rat intestinal mucosal cells. These results reveal that the chemically modified protein hydrolysate stimulates CCK secretion and increases pancreatic secretion by a luminal trypsin-independent direct action on the small intestine. In the present study, we examined the direct effect of peptic hydrolysates of naturally occurring dietary proteins, casein, soybean protein isolate (SPI), egg white, and wheat gluten on CCK release under *in vitro* trypsin-independent conditions. All protein hydrolysates significantly stimulated CCK release from dispersed rat intestinal mucosal cells. Among the hydrolysates treated, SPI hydrolysate was the most effective in stimulating CCK release. The potential of SPI hydrolysate to stimulate CCK release was increased by long-term peptic digestion. However, an SPI-like amino acid mixture did not effect CCK release. In conclusion, peptic hydrolysates of commonly ingested dietary proteins stimulate CCK release via trypsin-independent direct sensing by intestinal mucosal cells. [Exp Biol Med Vol. 226(11):1031–1036, 2001]

Key words: cholecystokinin release; dietary protein; mucosal cells

Dietary protein stimulates pancreatic exocrine secretion by a mechanism dependent on trypsin activity in the lumen (1, 2). Its regulation is mediated by cholecystokinin (CCK) (3–5), and the stimulation of CCK release by dietary protein is mediated through endogenous trypsin-sensitive CCK-releasing peptides (6–8). The potential of protein to stimulate CCK release and pancreatic secretion depends on its capacity to mask trypsin in this mechanism. It is likely that a protein hydrolysate does not stimulate pancreatic secretion (9) and CCK release (10) through this mechanism, except where trypsin inhibitory activity is increased by pepsin hydrolysis (11).

Recent studies have shown or suggested that protein hydrolysates are effective in stimulating rat pancreatic exocrine secretion (12, 13) and CCK release (14–16) in a luminal trypsin-independent manner. We previously found that guanidyl residues might be involved in the trypsin-independent stimulatory effects of protein (13, 17); however, in that study, we used a chemically modified protein. Cuber *et al.* (14) and Beucher *et al.* (15), using washed-out isolated duodenojejunal loops, showed that dietary protein stimulates CCK release; however, their neural system may still be functional. Cordier-Busset *et al.* (18) reported that protein hydrolysates stimulate CCK release from cultured STC-1 cells, an enteroendocrine cell line. However, these cells are mutated mucosal cells. The mechanism of trypsin-independent stimulation of CCK release by dietary protein is still unknown, and whether CCK release is directly stimulated by naturally occurring protein in nonmutated intestinal mucosal cells remains to be clarified.

The aims of the present study were to determine whether peptic hydrolysates of naturally occurring dietary proteins stimulate CCK release from isolated rat intestinal mucosal cells, and to examine the effective structure through a comparison of the stimulatory potential of peptic hydrolysates of several dietary proteins.

Materials and Methods

Materials. Materials used were purchased as follows: lactic casein from ALACID (New Zealand Daily Boad, Wellington, New Zealand); soybean protein isolate (SPI, Fujipro R) from Fuji Oil (Osaka, Japan); egg white powder from Taiyo Kagaku Co. (Yokkaichi, Japan); wheat gluten and phenyl isothiocyanate from Wako Pure Chemical Industries (Osaka, Japan); pepsin and ovalbumin hydrolysate from Sigma Chemical (St. Louis, MO); and synthetic CCK

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octapeptide sulfate (CCK-8) from the Peptide Institute (Osaka, Japan). Ovalbumin is the major component of egg white, and ovalbumin hydrolysate contains much smaller peptides, as shown in Table 1. An amino acid mixture simulating SPI, it contains (in millimoles per gram) 1.00 L-aspartic acid, 1.52 L-glutamic acid, 0.53 L-serine, 0.61 L-glycine, 0.14 L-histidine, 0.39 L-arginine, 0.51 L-threonine, 0.54 L-proline, 0.20 L-tyrosine, 0.43 L-valine, 0.04 L-methionine, 0.03 L-cysteine, 0.36 L-isoleucine, 0.65 L-leucine, 0.19 L-phenylalanine, and 0.46 L-lysine, and was prepared based on the analysis of amino acid composition of SPI.

Preparation of Protein Peptic Hydrolysates. Hydrolysis of protein by pepsin was performed as previously described with slight modification (19). Briefly, Casein, SPI, egg white, and wheat gluten were hydrolyzed by pepsin at pH 1.8 and 37°C for 10 min. A second SPI peptic hydrolysate was also prepared by treatment with pepsin for 60 min. These hydrolysates were then neutralized, desalted, and lyophilized. Molecular weight distribution was analyzed in all hydrolysates by gel permentation chromatography using high-performance liquid chromatography (HPLC) with two connected Protein Pak 60 columns (7.8 × 300 mm; Waters, Milford, MA). These data are shown in Table 1. To estimate arginine content, all proteins were hydrolyzed by 6N HCl (110°, 24 hr) and amino acid compositions were analyzed by HPLC as phenyl thiocarbamyl (PTC) derivatives with phenyl isothiocyanate (20). The HPLC system comprised a mini-solvent delivery system, M-600 (Waters), and a Wakopak WS-PTC column (4.0 × 200 mm; Wako Pure Chemical Industries). The *N*-acetylneuraminic acid (NANA) content in those proteins was measured by thiobarbituric acid assay method (21). Hexosamines in those proteins were extracted through the Dowex 50 (H^+) resin column after acid hydrolysis with 4N HCl at 100°C for 8 hr, and then the hexosamine content was measured by Elson-Morgan reaction (22). NANA and hexosamine content for each hydrolysate are also shown in Table 1.

Cell Preparation. Dispersed intestinal mucosal cells were prepared as previously described (17). Briefly, male Sprague-Dawley rats, weighing 250–350 g (Japan SLC Inc., Hamamatsu, Japan) were deprived of food overnight and were sacrificed under sodium pentobarbital anesthesia. Twenty centimeters of the proximal small intestine, beginning at the ampulla of Vater, was resected, the lumen was washed with saline, and the specimen was everted. Then, the intestinal specimen was incubated for 5 min in 15 ml of oxygenated (with 95% O_2:5% CO_2) calcium-free, modified Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 2.5 mM EDTA at 37°C. After incubation, the mucosa was immediately scraped from the intestinal specimen, mixed with the buffer, and mechanical dispersion was performed using an autopipette. The suspension was centrifuged for 3 min at 500 rpm to eliminate mucus, and pellets (mucosal cells) were resuspended in oxygenated HEPES buffer (pH 7.4). Viability of the cells was confirmed with 0.5% trypan blue solution. The cells from three rats were pooled and incubated twice in the HEPES buffer for 30 min at 37°C with slight shaking for cell equilibration. The cells were resuspended in fresh HEPES buffer at a volume 200 times that of the packed cell volume and incubated at 37°C with slow stirring.

Experimental Procedure. The mucosal cell suspension (1 ml) was added to a plastic vial containing each test hydrolysate and was incubated for 30 min at 37°C. The suspension was centrifuged for 7 sec at 10,000 rpm after incubation, and 0.5 ml of supernatant was collected. CCK in the supernatant was extracted and concentrated with a C18 Sep-pak cartridge to extract CCK as follows. The supernatant was passed through a cartridge pretreated with 10 ml of acetonitrile, 10 ml of methanol, and 20 ml of distilled water.

Table I. Proportions of Peptides in Each Molecular Weight Range, L-Arginine and Sugar Content in Peptic Hydrolysates of Casein, Soybean Protein Isolate (SPI), Egg White, Wheat Gluten, and an Enzymatic Hydrolysate of Ovalbumin

Molecular weight	Hydrolysate (%)				
	Casein	SPI	Egg white	Wheat gluten	Ovalbumin
>20,000	19	14	29	23	0
20,000–10,000	11	15	12	9	0
10,000–5,000	9	14	12	17	7
5,000–1,000	31	31	28	25	16
<1,000	30	26	19	26	77
L-arginine (mmol/g protein)	0.256	0.394	0.360	0.230	
NANA[a] (μmol/g protein)	0.157	0.283	0.362	0.277	
Hexosamine[b] (μmol/g protein)	5.26	7.02	8.77	8.60	

Note. Casein, SPI, egg white, and wheat gluten were hydrolyzed with pepsin for 10 min by the authors, whereas the ovalbumin had already been hydrolyzed by digestive enzymes. The molecular weight distribution of each hydrolysate was determined by high-performance liquid chromatography with two Protein Pak 60 columns (7.8 × 300 mm, Waters). L-arginine content was analyzed by high-performance liquid chromatography with a Wakopak WS-PTC column (4.0 × 200 mm, Wako Pure Chemical Industries) after hydrolysis by 6 N HCl at 110°C for 24 hr. Details are described in "Materials and Methods."

[a] N-acetylneuraminic acid.

[b] galactosamine and glucosamine.

After washing in the cartridge with 20 ml of distilled water, CCK was extracted by 1 ml of 50% acetonitrile and was stored at −80°C until assay. CCK concentration was measured using a bioassay as previously described (23, 24). We previously showed that stimulation of amylase secretion from pancreatic acini by Sep-pak extract of effluent of isolated intestinal mucosal cells was completely abolished by a specific CCK-A receptor antagonist, FK480 (data not shown).

Calculation and Statistical Analysis. The amount of released CCK was estimated in 1 ml of the suspension as a CCK-8 equivalent. All results were expressed as mean ± SEM. Data were analyzed by Duncan's multiple range test. Differences of $P < 0.05$ were considered significant.

Results and Discussion

Figure 1 shows the amounts of CCK released in a 30-min period by dispersed rat intestinal mucosal cells with various protein hydrolysates. The amount of released CCK by the hydrolysate-free control medium was 12.7 ± 2.2 fmol. CCK release from intestinal cells in the presence of any of the hydrolysates was significantly greater than that of the control. SPI hydrolysate had a greater effect than did the hydrolysates of casein, egg white, and wheat gluten (44.2 ± 5.6, 32.0 ± 4.7, 28.4 ± 2.2, and 28.0 ± 4.4 fmol, respectively). The results demonstrate that isolated intestinal mucosal cells directly recognized these protein hydrolysates.

It had been shown that a well-digested (for 60 min) peptic hydrolysate of casein was not effective in stimulating pancreatic secretion or CCK release via a trypsin-independent mechanism both *in vivo* (25) and *in vitro* (17). In contrast, casein hydrolysate digested with pepsin for a short time stimulated CCK release from the jejunal mucosal cells in the present study. On the basis of these results, we speculate that larger peptides in the casein hydrolysate may be involved in the direct stimulation of CCK release. Gastric emptying of one-half of the ingested casein diet was performed within 20 min (26). We prepared protein hydrolysates by peptic digestion for 10 min in the present study, as a well-digested casein hydrolysate may not be representative of those produced under physiological conditions. Beucher *et al.* (15), using isolated vascularly perfused rat duodenojejunum, have shown that a hydrolysate produced by pepsin hydrolysis for a short time can stimulate CCK release, but this effect disappeared upon treatment with pepsin for a longer time. Their observations support our hy-

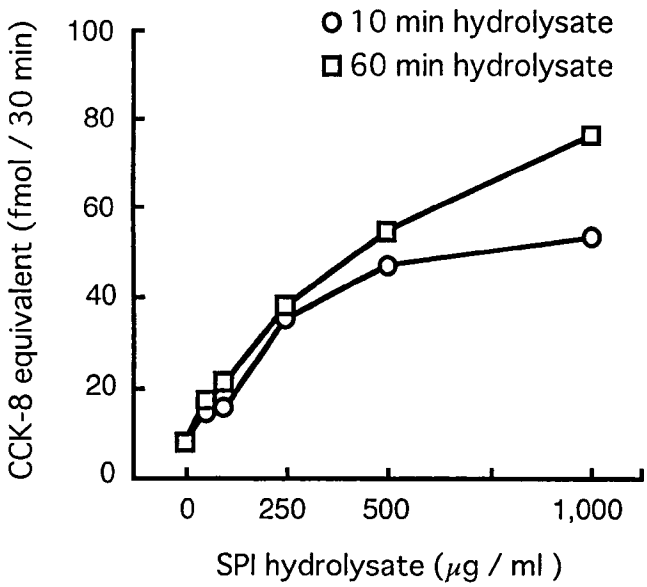

Figure 2. Dose responses of CCK release from dispersed rat intestinal mucosal cells to stimulation with 10 and 60 min of peptic hydrolysate of SPI. Values are mean of two to three experiments.

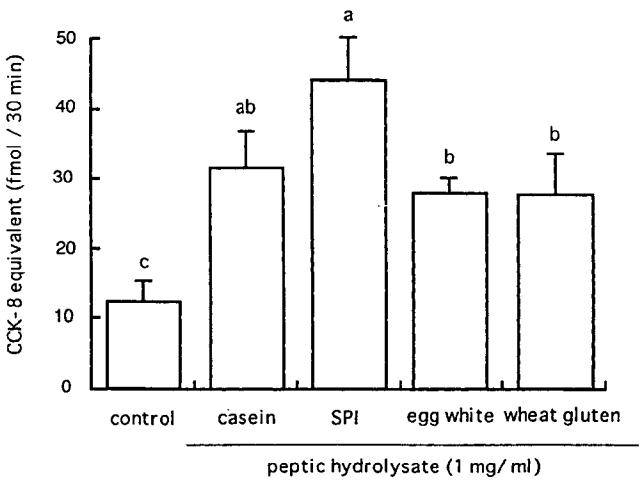

Figure 1. CCK release from dispersed rat intestinal mucosal cells in response to a 1-mg dose of several dietary protein peptic hydrolysates in 1 ml of incubation medium. Values are mean ± SEM of five experiments. Columns not sharing a common letter are significantly different ($P < 0.05$).

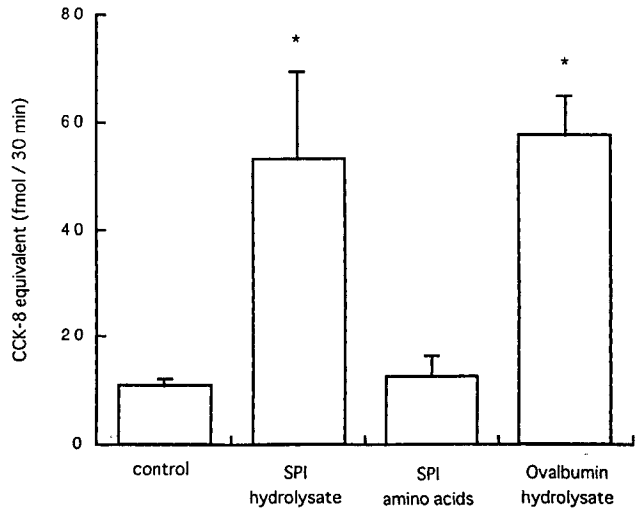

Figure 3. Effect of 1 mg/ml of the SPI-like amino acid mixture and ovalbumin hydrolysate on CCK release from dispersed rat intestinal mucosal cells. Values are mean ± SEM of five to eight experiments. The asterisk represents a significant difference to the control value ($P < 0.05$).

pothesis regarding the involvement of large peptides in CCK release, mentioned above.

Hydrolysates of SPI, egg white, and wheat gluten also stimulated CCK release (Fig. 1). In particular, the SPI hydrolysate was the most potent in stimulating CCK release among the hydrolysates tested. In Figure 2, both 10- and 60-min SPI hydrolysates stimulated CCK release from dispersed intestinal mucosal cells in a dose-dependent manner. We confirmed that the 60-min peptic hydrolysate of SPI contained more small peptides than did the 10-min hydrolysate by measurement of amino terminal content using trinitrobenzene sulfonic acid (27) (amino term contents of SPI, SPI 10-min hydrolysate, and SPI 60-min hydrolysate were 0.303, 4.132, and 4.639 mmol/g material, respectively). The dose-response curve of 60-min SPI hydrolysate continued to stimulate ever greater CCK release up to 1,000 μg/ml, whereas 10-min SPI hydrolysate-stimulated CCK release reached a plateau at 500 μg/ml. Additionally, the amount of CCK released by the stimulation of 1,000 μg/ml of 60-min SPI hydrolysate being significantly higher than that of 10-min SPI hydrolysate; this is a result other than that shown in Figure 2, which was obtained with too low number of ex-

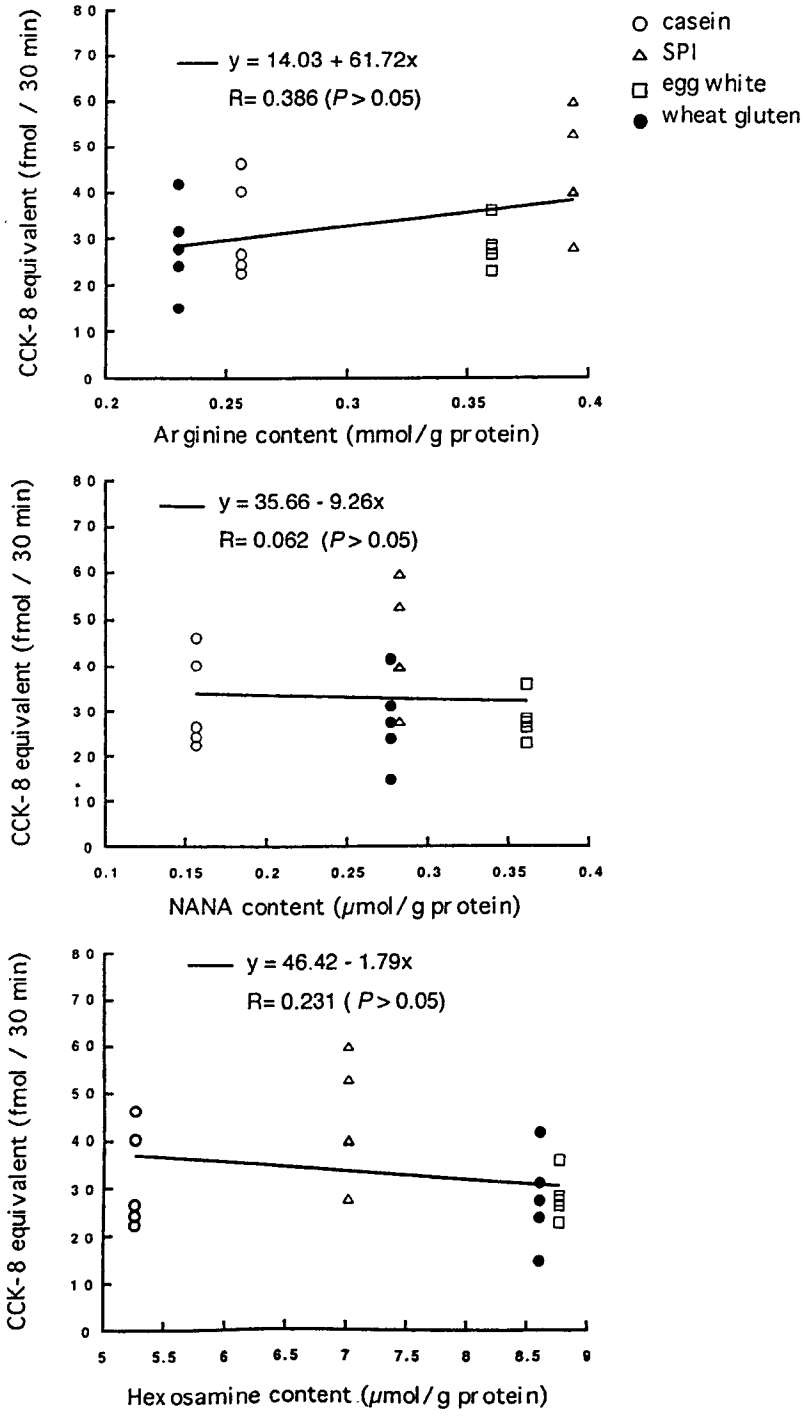

Figure 4. Correlations between the amount of released CCK from dispersed rat intestinal mucosal cells in response to a 1-mg dose of several dietary protein peptic hydrolysates and the content of L-arginine, NANA, and hexosamine in 1 g of those hydrolysates (n = 20).

periments for statistical analysis. These results indicate that well-digested SPI is more effective than less-digested one on CCK release, in contrast with the effect of casein. Hydrolysate of egg white showed moderate stimulation of CCK release (Fig. 1); however, peptic ovalbumin hydrolysate stimulated CCK release much more strongly (Fig. 3). Ovalbumin is the major component of egg white and, as shown in Table 1, there were a lot of small molecular-weight peptides in this hydrolysate. In the cases of SPI and egg white, small peptides are more effective in stimulating CCK release than are large peptides. Some dietary peptones could stimulate CCK release from vascularly perfused rat duodenojejunum (14) and STC-1 cell (28). In these reports, peptones, both casein and ovalbumin, stimulated CCK release significantly despite the distributions of peptide molecular weight of their peptides were largely different. These findings show that the size of the peptides in the hydrolysates is not a crucial determinant in CCK-releasing activity, although it may be a factor. The peptide length that gives a maximal effect on CCK release seems be different among each dietary protein.

In Figure 3, the SPI-like amino acid mixture failed to stimulate CCK release, nevertheless SPI hydrolysate was effective in releasing CCK from mucosal cells. Moreover, we previously observed that a guanidinated casein hydrolysate strongly stimulated CCK release, but an amino acid mixture simulating guanidinated casein did not stimulate CCK release in the rat mucosal cells perifusion system (17). These results indicate that the structure of secretagogue, which acts to the mucosal cells directly on CCK release, is peptide, not the amino acid itself.

Characteristics of the active peptide(s) in the dietary protein hydrolysates, which stimulate CCK release, are still unknown. We have previously observed that guanidinated casein hydrolysate and protamine, both of which have many guanidyl residues, strongly stimulate pancreatic secretion and CCK release (13, 17, 25). In the case of an endogenous CCK-releasing peptide, monitor peptide, has an arginine residue in the site involved in binding to the intestinal cells (29). It is possible that arginine-containing peptides derived from dietary protein stimulate CCK release through a common sensing mechanism for the endogenous peptide. These observations suggest that arginine residues in the peptides partly participate in the direct stimulatory effect of the hydrolysate on CCK release from dispersed intestinal cells. Accordingly, we evaluated the amount of arginine content of each of the hydrolysates tested in the present study. As shown in Table 1, wheat gluten contained the least amount of arginine of all test proteins. Casein, SPI, and egg white contained 1.1, 1.6, and 1.7 times the arginine contained in wheat gluten, respectively. However, in Figure 4, the statistical correlation between the amount of released CCK in response to each hydrolysate and the content of L-arginine in each hydrolysate was not significant. This result suggests that effective arginine residues to stimulate CCK release are not proportional numbers of arginine in hydrolysates, or another factor on CCK release also exist in those peptides. Possibly, appearance or disappearance of the available arginine residues with degrees of hydrolysis is different among dietary protein sources.

Another defined molecular structure associated with CCK release is the oligosaccharide moiety on glycopeptides (15, 30, 31). Therefore, we assayed the NANA and hexosamine content of each of the dietary proteins tested. NANA is one of the components of glycopeptides and one of the molecules thought to be involved in the CCK-releasing activity of casein hydrolysate (15). Therefore, the variety of sugar in the glycopeptide seems to participate in CCK-releasing activity of casein hydrolysate mentioned above. Jordinson *et al.* (30) demonstrated that lectins binding different types of sugar chain showed different effects on CCK release from dispersed rat intestinal cells and hexosamine-binding lectins had a positive effect on CCK release. However, as shown in Figure 4, there was no correlation between the NANA or hexosamine content and the CCK-releasing activity of the hydrolysates tested in the present study. These observations show that the CCK-releasing activity in dietary protein hydrolysate does not seem to be determined by the amount of glycopeptide.

In conclusion, peptic hydrolysates of commonly ingested naturally occurring proteins are effective in stimulating CCK release from dispersed rat intestinal mucosal cells under trypsin-independent conditions. Our findings indicate that the protein hydrolysates act directly on the intestinal mucosa to stimulate CCK release. Total CCK-releasing activity in each protein hydrolysate seems to define the synergism of several established and unknown factors. The structure of the peptides seems to be important to the activity in particularly.

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