Purification and Sequencing of a Trypsin-sensitive Cholecystokinin-releasing Peptide from Rat Pancreatic Juice

ITS HOMOLOGY WITH Pancreatic SECRETORY TRYPSIN INHIBITOR*

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Kazuo Iwai, Shin-Ichi Fukuoka, Tohru Fushiki, Masahiro Tsujikawa, Masaki Hirose, Susumu Tsurasawa, and Fumio Sakiyama

From the Laboratory of Nutritional Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan and the Institute for Protein Research, Osaka University, Osaka 565, Japan

The trypsin-sensitive cholecystokinin-releasing peptide is a peptide purified from rat pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion. We postulate that the peptide acts as a mediator of pancreatic enzyme secretion in response to dietary protein intake and that it (designated as "monitor peptide" from its role in the intestine) could be responsible for the feedback regulation of pancreatic enzyme secretion. About 20 nmol of the highly purified peptide were obtained from 800 ml of rat pancreatic juice by reverse-phase high performance liquid chromatography. It was then sequenced. The peptide comprises 61 amino acid residues (Table I). It has a sequence that closely resembles that of a highly conserved region in pancreatic secretory trypsin inhibitors (PSTIs, Kazal type inhibitor): -Ile-Tyr-Asx-Pro-Val-Cys-Gly-Thr-Asx-Gly-. However, the peptide is less related to other mammalian PSTIs than they are to each other. The additional 5 residues at the NH2 terminus make the peptide larger than the common 56-residue PSTIs. The trypsin-sensitive cholecystokinin-releasing peptide is to be classified as a Kazal-type inhibitor and may be one of the rat PSTIs or a related peptide. The present results and increasing evidence from other laboratories and ours suggest that Kazal-type inhibitors play previously unrecognized multiple physiological roles.

We previously found and purified a peptide, with a molecular weight of about 6500, from rat bile-pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion (1). The peptide was found to exhibit cholecystokinin (CCK)1-releasing activity in the rat intestine (2). We postulate that this peptide acts as a mediator of pancreatic enzyme secretion in response to dietary protein intake (3-5); the peptide is trypsin-sensitive, and when dietary protein, which serves as a substrate for trypsin, enters the intestine, it prevents inactivation of the peptide. The presence of this peptide in the proximal part of the small intestine could stimulate pancreatic enzyme secretion by acting as a CCK releaser. This trypsin-sensitive CCK-releasing peptide could be responsible for the feedback regulation of pancreatic enzyme secretion, as proposed by Green and Lyman (6). We designate this peptide as "monitor peptide" because it was speculated that this peptide "monitors" the amount of food protein intake through the competition between endogenous (this peptide) and exogenous (food protein) substrates for trypsin activity in the small intestine (3-5).

In this communication, we report the purification and results of sequence analysis of this trypsin-sensitive CCK-releasing peptide from rat pancreatic juice.

MATERIALS AND METHODS

Purification—The trypsin-sensitive CCK-releasing peptide was purified from rat pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion. Rat pancreatic juice was collected from anesthetized rats of the Wistar strain (body weight 300-350 g) via a pancreatic cannula inserted with bile duct ligation. The pancreatic juice collected was immediately acidified by the addition of an equal volume of sodium citrate buffer to pH 2.5, and then NaCl was added to a final concentration of 2 M. Heat treatment at 80 °C in a water bath was carried out for 40 min. After insoluble materials had been centrifuged off, the resulting supernatant was desalted and concentrated on a Sep-Pak cartridge (Waters Associates) previously equilibrated with 0.05% trifluoroacetic acid. Eighty percent acetonitrile in 0.05% trifluoroacetic acid was used as eluent. The concentrate was dried in a small amount of distilled water. This sample was subjected to preparative high performance liquid chromatography (HPLC) on a reverse-phase column (BioRad RF 304, 10 x 250 mm). The stimulatory activity toward rat pancreatic enzyme secretion in the effluent fractions was assayed. Peak I, showing the activity, was pooled and further fractionated. The pooled fractions were rechromatographed on the analytical HPLC column, the final preparation being isolated as a single peak. The purity of the peptide preparation was examined by NH2-terminal sequencing.

Bioassay—Male Wistar rats weighing 350 g were anesthetized with pentobarbital. Atropine (100 μg/kg) was injected into a vein, and subsequent injections were given at 1-h intervals (100 μg/kg/h) throughout the assay. Duodenal and bile-pancreatic cannulae, with ligation of the main bile-pancreatic duct at the Vater papilla, were inserted. The procedure is described in detail elsewhere (3,4). One h after the first atropine injection, saline containing soybean trypsin inhibitor (0.1 mg/ml) was infused slowly into the small intestine to get rid of intraluminal proteases, with draining at the distal end of the small intestine. One h after the intestinal washing, the samples to be tested were infused into the intestine via the duodenal cannula. Bile-pancreatic juice was collected via the bile-pancreatic cannula at 15-min intervals for enzyme assay. All the assays were performed under anesthesia with pentobarbital (20 mg/kg/h).

Enzyme Assay—Pancreatic enzyme secretion was monitored by estimating trypsin activity in the collected bile-pancreatic juice samples (4,5). Trypsin activity was determined with benzoyl arginine p-nitroanilide (7) after activation with enterokinase (Sigma) at 37 °C for 40 min. Crystalline porcine trypsin was used as a standard.

1 The abbreviations used are: CCK, cholecystokinin; HPLC, high performance liquid chromatography; PSTI, pancreatic secretory trypsin inhibitor; EGF, epidermal growth factor.

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Trypsin Inhibition Assay—Trypsin inhibitory activity was determined from the residual trypsin activity after mixing bovine trypsin (Sigma, Type III) with the purified peptide, using benzoxyarginine p-nitroanilide as a substrate.

Amino Acid Analysis—Amino acid analysis was performed with a Hitachi 835-S amino acid analyzer after hydrolysis with 6 N HCl at 110 °C for 24 h.

Sequence Analysis—Prior to sequencing and protease digestion, the native peptide was oxidized with performic acid (8) to cleave disulfide bonds. Digestion of the oxidized peptide with Staphylococcus aureus V8 protease (Miles) was performed in 1% (NH4)2HCO3 at 37 °C for 17 h at an enzyme-to-substrate ratio of 1:100 (mol/mol). Digestion with Achromobacter lyticus lysylendopeptidase (Wako Pure Chemicals) was carried out in 50 nM Tris-HCl buffer (pH 9.0) at 37 °C for 6 h at an enzyme-to-substrate ratio of 1:500 (mol/mol) after the peptide had been aminoethylated as described (9). The lyophilized digests were fractionated by HPLC. Acetylation of the peptide and subsequent BrCN cleavage were performed as described (10). The resulting sample was used for sequencing without further purification. The sequences of the native peptide, BrCN-treated peptide, and peptide fragments obtained on protease digestion were determined from the residual trypsin activity after mixing bovine trypsin with Achromobacter lyticus lysylendopeptidase (Wako Pure Chemicals) was carried out in 50 nM Tris-HCl buffer (pH 9.0) at 37 °C for 6 h at an enzyme-to-substrate ratio of 1:500 (mol/mol) after the peptide had been aminoethylated as described (9). The lyophilized digests were fractionated by HPLC. Acetylation of the peptide and subsequent BrCN cleavage were performed as described (10). The resulting sample was used for sequencing without further purification.

RESULTS AND DISCUSSION

The trypsin-sensitive CCK-releasing peptide was first purified from rat bile-pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion (1). Using this preparation, preliminary experiments, with the eventual aim of a large scale purification, on the heat stability and behavior on HPLC of the peptide were performed. Based on the results, we could purify the peptide by means of a simple procedure from rat pancreatic juice, since it was shown that the peptide is localized in the zymogen granules of acinar cells in the pancreas and is secreted into the pancreatic juice (12). The peptide was found to be basic (pI = ~9.0), acid-stable, and heat-resistant, but, on the other hand, trypsin, which inactivates the peptide, was heat-labile (12). The heat treatment successfully eliminated endogenous trypsinogen activity without loss of the stimulatory activity of the peptide. When the crude preparation after the heat treatment was subjected to preparative reverse-phase HPLC, the stimulatory activity toward pancreatic enzyme secretion was separated into four peaks with close retention times (Fig. 1A). The activity was detected as a single peak on Sephadex G-50 corresponding to a molecular weight of about 6000 (1). Peak I (the predominant fraction) had the shortest retention time and was further purified. About 20 nmol of the highly purified peptide were obtained from 800 ml of rat pancreatic juice (Fig. 1B).

Amino acid analysis indicated that the peptide comprises 61 amino acid residues (Table I). On direct sequencing of the peptide after performic acid oxidation amino acids were successfully identified up to the 29th step. Only one methionine was detected on amino acid analysis. BrCN treatment was carried out after blocking the NH2 terminus by acetylation, and then the resulting sample was sequenced. Amino acids were determined from residue 19, methionine, up to residue 39. Other parts of the peptide were identified using sequence data obtained from fragments obtained on protease digestion, which are summarized in Fig. 2A, and then the entire amino acid sequence of the peptide could be determined. Residues 38, 41, and 44 were not clearly identified and were deduced from the results of amino acid analysis for each peptide fragment.

The peptide has a sequence (residues 24-33) that closely resembles that of a highly conserved region in pancreatic secretory trypsin inhibitors (PSTIs, Kazal type inhibitor), residues 19-28, -Ile-Tyr-Asx-Pro-Val-Cys-Gly-Thr-Asx-Gly-.  

![Fig. 1. A, reverse-phase HPLC of the crude preparation from rat pancreatic juice. Acid- and heat-treated rat pancreatic juice was concentrated on a Sep-Pak column, and the concentrate was applied to a preparative HPLC column (Bio-Rad RP-304, 10 x 250 mm) in a system equipped with a model 600 programmer and a model 510 pump (Waters). The column, previously equilibrated with 16% acetonitrile in 0.06% trifluoroacetic acid, was eluted with a linear gradient (16-24% acetonitrile in 0.06% trifluoroacetic acid) over 30 min at a flow rate of 4.0 ml/min. The stimulatory activity toward rat pancreatic enzyme secretion in the eluents was assayed. The active fraction peaks are shadowed. B, final purification of the trypsin-sensitive CCK-releasing peptide by reverse-phase HPLC. The peak I material from the previous chromatography (A) was rechromatographed twice on an analytical column (Bio-Rad RP-304, 4.6 x 250 mm) in the same HPLC system as in A at a flow rate of 1.0 ml/min. The effluent pattern from the final purification is presented.](image-url)
Sequence of a CCK-releasing Peptide

It should be noted that the peptide has an additional 5 residues at the NH₂ terminus compared to PSTIs reported in other mammals. When the COOH terminus of the peptide is aligned with those of other mammalian PSTIs, the sites of the 6 cysteine residues are identical. However, the peptide is less related to other mammalian PSTIs than they are to each other. The purified peptide inhibited bovine trypsin (Fig. 3). Taking these results together with several other lines of evidence (1, 6), the trypsin-sensitive CCK-releasing peptide is to be classified as a Kazal-type inhibitor and may be one of the rat PSTIs or a related peptide. This is the first report of a "large form" 61-residue Kazal-type inhibitor. However, the amino acid composition of the peptide is inconsistent with the results reported by Marks and Ohlsson (13), in that they determined that their purified rat PSTI comprised 56 residues, even after the NH₂-terminal 5 residues are subtracted from our data. This discrepancy cannot be explained well at this time because Marks and Ohlsson have not presented any sequence data for their preparation. It might be possible to attribute the discrepancy to multiplicities of PSTIs, as previously demonstrated in several mammals (14–17) and in the rat (Ref. 13 and our data). Another Kazal-type inhibitor may be present in rat pancreatic juice that is more closely related to PSTIs than the CCK-releasing peptide we described here.

The Kazal-type inhibitor (PSTI) is believed to act as a local inhibitor within the pancreas and to prevent the deleterious effects of autoactivated trypsin. However, nothing as to the physiological function of PSTI as a trypsin inhibitor has been demonstrated yet. Increasing amounts of evidence have led to reconsideration of the physiological roles of Kazal-type inhibitors or PSTI. We demonstrated that the "Kazal-type" peptide is responsible for pancreatic enzyme secretion in response to food protein intake through its CCK-releasing effect (3–5). In addition to its CCK-releasing activity, Ogawa et al. (18) demonstrated stimulation of DNA synthesis by human PSTI, and elevated plasma PSTI levels were detected in various malignant diseases (19). A PSTI-like tumor-associated peptide was detected in the plasma, urine, and tumor tissue of cancer patients (20, 21). Recently McKeehan et al. (22) found endothelial growth factors derived from hepatomas and demonstrated that one of them is identical to human PSTI. These observations suggest that PSTI plays a role as a growth factor.

We demonstrated that the trypsin-sensitive CCK-releasing peptide also exhibits a growth-stimulating activity toward Swiss 3T3 fibroblasts, showing almost the same potency as epidermal growth factor (EGF) (23). We postulate that the growth-stimulating activity of the peptide is involved in proliferation of the intestinal epithelial cells, since about 10 µg/ml of the peptide was estimated to be present in rat pancreatic juice (12, 23). Accordingly, when residues 20–33 of the peptide are compared with residues 5–18 of mouse epidermal growth factor (EGF) (24), 8 of 14 amino acids can be seen to be identical (Fig. 2B). This partial homology between the
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peptide and EGF might explain the EGF-like growth-stimulating activity of the peptide we demonstrated previously (23). It has been pointed out that PSTIs of other animals share common sequences with EGF at both the amino acid (25, 26) and DNA levels (27).

This information and the observations we have reported here strongly imply that Kazal-type inhibitors play previously unrecognized multiple physiological roles.

Note Added in Proof—We have just completed sequencing of the peptide, and residues 38, 39, 41, and 44 were confirmed to be Ser, Glu, Ser, and Phe, respectively. The details will be published elsewhere.

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