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Isolation and characterization of a 60-residue intestinal peptide structurally related to the pancreatic secretory type of trypsin inhibitor: Influence on insulin secretion

(amino acid sequence similarity/disulfide bridges/protease inhibitor)

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ABSTRACT **We have isolated from pig intestine a 60-residue polypeptide initially identified by its inhibition of glucose-induced insulin secretion from perfused pancreas. The amino acid sequence of this porcine polypeptide was determined and found to be markedly similar to that of the pancreatic secretory trypsin inhibitor (41% residue identities). Furthermore, the disulfide arrangements of these two proteins appear identical, suggesting related overall conformations. However, the polypeptide, now named PEC-60 (peptide with N-terminal glutamic acid, C-terminal cysteine, and a total of 60 residues), was found not to inhibit trypsin. The amino acid sequence is also similar to that of a peptide recently isolated from rat bile/pancreatic juice which stimulates the release of cholecystokinin. The biological role of PEC-60 is not known, but the effect on insulin secretion and the homologies observed suggest important biological activities and interesting structural relationships.**

Gastrointestinal peptides exhibit a wide range of biological activities and diverse structural relationships (1). Frequently, the relationships between these peptides are unexpected, as when it was recently reported that an intestinal peptide that inhibits insulin release was identical to the diazepam binding inhibitor (2). Further, a second gastrointestinal peptide was found to be identical to the mitochondrial coupling factor 6 (3). These studies, as well as others on the effect of various polypeptides on the secretion of pancreatic hormones (2, 4), have led us to isolate a 60-residue peptide from pig upper intestinal tissue. This peptide significantly suppressed glucose-induced insulin secretion. We found that the amino acid sequence of this polypeptide is clearly related to that of pig pancreatic secretory trypsin inhibitor (PSTI) (5, 6) and to that of a peptide recently isolated from rat bile/pancreatic juice, which was found to release cholecystokinin on duodenal infusion in rats (7). Combined, these similarities suggest that the 60-residue intestinal polypeptide could have interesting functions.

The homology with the secretory trypsin inhibitors is significant. Two types of such polypeptide inhibitors were characterized earlier from extracts of ox pancreas: the Kunitz type of inhibitor (8) and the Kazal type (9). The latter is secreted in the pancreatic juice and is now often referred to as PSTI (10). This inhibitor has 56 residues and three disulfide bridges (10). It resembles the Kunitz type of inhibitor, with 58 residues and also three intrachain disulfide bridges (11–13). However, the amino acid sequences of the two types of inhibitor appear to be unrelated. Both types have been characterized from many different species. Their activity has been localized to various organs, and both have been shown to be derived from larger proteins (10, 14–19). The Kunitz type of peptide has also been isolated from rat peritoneal mast cells (20) and has been shown to be a part of a human brain amyloid protein (21–23). Additionally, the boar seminal acrosin inhibitor is structurally related to, but not identical with, PSTI (24), as is the cholecystokinin-releasing peptide from rat bile/pancreatic juice (7). The PSTI-like family is now extended further and shown to probably have additional functions.

MATERIALS AND METHODS

Materials. Carboxymethyl cellulose CM-22 was from Whatman, Sephadex G-25 fine from Pharmacia, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin from Worthington, endoproteinase Asp-N from Boehringer Mannheim, iodo[^{14}C]acetic acid from Amersham, and dimethylaminoazobenzene isothiocyanate from Pierce. Other chemicals were of highest commercial grade.

Purification. The peptide fraction now used for the isolation of the hexacontapeptide was prepared from an aqueous solution of a concentrate of thermostable intestinal peptides (CTIP) of pig upper intestine (2, 25). The concentrate was fractionated with 2-propanol, the fraction CTIP-F1 was chromatographed in 0.2 M acetic acid on Sephadex G-25 fine, and the eluate was divided into three fractions as described (3). The third fraction was saturated with NaCl and the precipitate, CTIP-F1-Sx3, was collected by suction filtration. The NaCl was removed by gel chromatography on Sephadex G-25 coarse in 0.2 M acetic acid and the peptide fraction (CTIP-F1-Sx3) was lyophilized.

Further purification on CM-cellulose (2.5 × 32 cm) was carried out in 0.01 M ammonium bicarbonate. Reverse-phase high-performance liquid chromatography (HPLC) utilized a Waters system with detection at 215 nm and a Vydac 218 TP column (15–20 μm; 22 × 250 mm; The Separations Group, Hesperia, CA) or, for final purification, detection at 215 nm plus 280 nm and a TSK ODS 120T column (10 μm; 7.8 × 300 mm; LKB).

Structural Analysis. The intact peptide (250 nmol) was dissolved in 1 ml of 0.4 M Tris·HCl/6 M guanidinium chloride/2 mM EDTA, pH 8.1, reduced with 10 μl of 0.5 M dithioerythritol under nitrogen for 2 hr at 37°C, carboxymethylated with 150 μl of neutralized 0.1 M iodo[^{14}C]acetate under nitrogen for 1.5 hr at 37°C, and desalted on Sephadex G-25 fine (0.6 × 90 cm) in 0.2 M acetic acid. Cleavage with CNBr was carried out in 70% formic acid for 24 hr at room temperature; cleavage with trypsin in 1% ammonium bicarbonate for 4–6 hr at 37°C, with 5 μg of protease per 20 nmol of peptide; and cleavage with endoproteinase Asp-N in 10%

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Abbreviation: PSTI, pancreatic secretory trypsin inhibitor.

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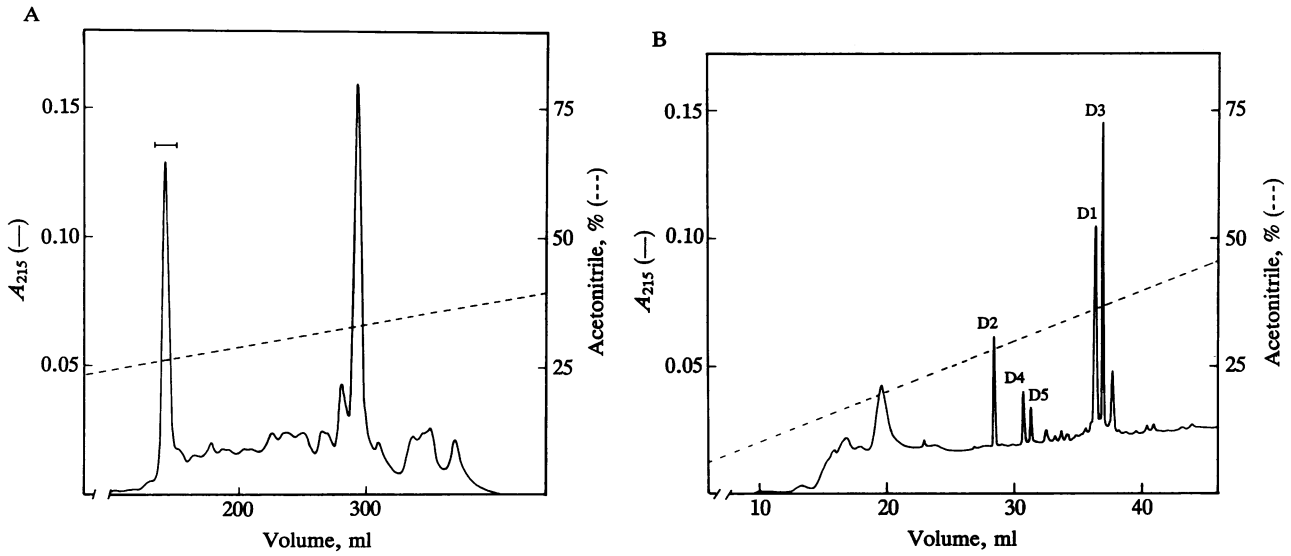

FIG. 1. HPLC purification of the 60-residue peptide (*A*) and its constituent fragments obtained by treatment with endoproteinase Asp-N (*B*). (*A*) The nonadsorbed peptides from CM-cellulose chromatography of the methanol-insoluble portion of fraction CTIP-F1-Sx3 were fractionated on a Vydac 218 TP column (15–20 μm; 22 × 250 mm) in 0.1% trifluoroacetic acid with a gradient of 20–45% acetonitrile in 45 min with a flow of 10 ml/min. (*B*) Peptides from endoproteinase Asp-N digestion were separated on a Vydac 218 TP (5 μm; 4.6 × 250 mm) in 0.1% trifluoroacetic acid with a gradient of 0–60% acetonitrile at 1 ml/min for 60 min.

acetonitrile/0.1 M ammonium bicarbonate for 22 hr at 37°C, with 2 μg of protease per 40 nmol of peptide. All fragments were separated by HPLC on Vydac 218 TP (5 μm; 4.6 × 250 mm). Thin-layer chromatography was carried out on silica gel plates (Riedel-de Haën, Hannover, F.R.G.) in 1-butanol/pyridine/acetic acid/water (15:10:3:12, vol/vol) (26). After migration for 5 hr, material was revealed with ninhydrin.

Total compositions were determined with a Beckman 121 M amino acid analyzer after hydrolysis in evacuated tubes with 6 M HCl/0.5% phenol at 110°C for 22 hr. Peptides were degraded in an Applied Biosystems 470A gas-phase sequencer, and phenylthiohydantoin derivatives were analyzed with HPLC on a Nucleosil C_{18} column with an acetonitrile gradient in sodium acetate as described (27). N-terminal analysis was also carried out with the manual dimethylaminoazobenzene isothiocyanate method (28), utilizing by-products to assist identification (29).

Bioactivity Assays. For tests with isolated perfused pancreas (30), male Sprague–Dawley rats (Alab, Stockholm) of 200–250 g, fed ad libitum, were used. After anesthesia (intraperitoneal injection of pentobarbital, 50 mg/kg), the pancreatic glands were removed and isolated free from adjacent tissues. The basal perfusing medium was Krebs–Ringer bicarbonate solution supplemented with bovine serum albumin (20 g/liter) and 3.3 mM glucose. The medium was infused into the celiac artery and fed into the pancreas by an open circuit in a noncycling perfusion system with a rate of 2.8 ml/min. The pancreas was equilibrated with a basal medium for 30 min before introduction of a higher concentration of glucose (16.7 mM at time zero). The peptide was given from 10 min before zero time to the end of the supplement with high glucose concentration. Samples for determination of insulin and somatostatin in the perfusate were collected in prechilled tubes containing Trasylol (aprotinin, Bayer).

RESULTS

Isolation of the Hexacontapeptide. A peptide fraction from a porcine intestinal extract (CTIP-F1-Sx3, 14.6 g; see *Materials and Methods*) was extracted with 730 ml of methanol for 15 min. The suspension was suction-filtered and the insoluble portion was washed with ether on the filter. After evaporation of the ether, 800 mg of this material was dissolved in water (10 ml) and adjusted to pH 8.0 ± 0.1 with 0.02 M ammonia. After centrifugation, the supernatant was chromatographed on CM-cellulose (2.5 × 32 cm) in 0.01 M ammonium bicarbonate. The nonadsorbed peptides were lyophilized (364 mg) and 100 mg was dissolved in 0.1% trifluoroacetic acid and submitted to reverse-phase HPLC (Fig. 1*A*). The fraction indicated with a bar was lyophilized (yield, 9.75 mg). A 2.6-mg portion of this material was rechromatographed by reverse-phase HPLC (TSK-ODS 120T; 7.8 ×

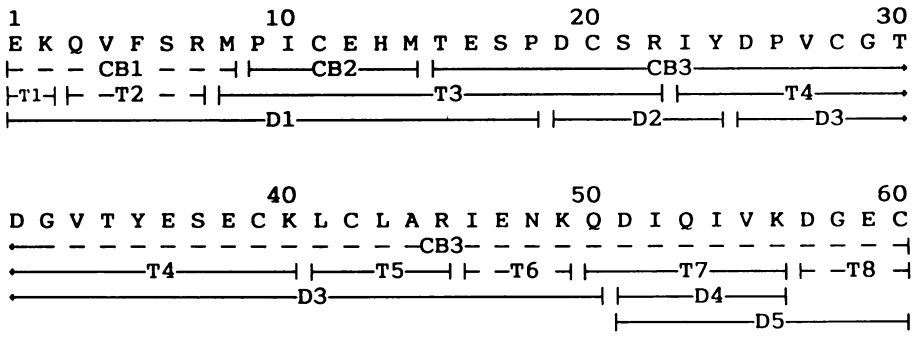

FIG. 2. Amino acid sequence (one-letter symbols) of the hexacontapeptide. All peptides analyzed are indicated (CB, T, and D indicate peptides generated by cleavage with CNBr, trypsin, and Asp-specific protease, respectively), with solid lines to show regions analyzed by sequencer degradations and with dashed lines for remaining regions proven by total compositions.

Table 1. Composition of PEC-60

Amino acid	Residues, mol/mol*	Amino acid	Residues, mol/mol*
Cys†	5.2 (6)	Val	4.0 (4)
Asp	6.3 (5)	Met	1.4 (2)
Asn	(1)	Ile	4.5 (5)
Thr	2.9 (3)	Leu	2.2 (2)
Ser	3.8 (4)	Tyr	1.7 (2)
Glu	10.9 (7)	Phe	0.9 (1)
Gln	(3)	Lys	4.2 (4)
Pro	3.2 (3)	His	1.4 (1)
Gly	3.7 (3)	Arg	3.5 (3)
Ala	1.6 (1)		

*Values shown are molar ratios from acid hydrolysis and (within parentheses) from sequence analysis.
†Analyzed as Cys(Cm) after reduction and carboxymethylation.

300 mm) and eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. The yield was 1.73 mg after lyophilization.

The peptide obtained was found to be essentially pure. It gave only one component on thin-layer silica gel chromatography and only one N terminus.

Structural Determination. The N terminus was found to be glutamic acid. The total composition of the intact peptide indicated that it contained cysteine/half-cystine. It was therefore reduced with dithioerythritol and carboxymethylated with neutralized iodo[^{14}C]acetate. The peptide was carboxymethylated at six positions, and since analysis by the Ellman method (31) showed that free thiol groups were not present, the peptide was concluded to contain 6 half-cystine residues. Gas-phase sequencer degradation of the carboxymethylated peptide revealed the sequence of the first 29 residues.

Proteolytic fragments were generated and the entire primary structure was determined by analysis of three sets of peptides obtained by treatments with CNBr, trypsin, and endoproteinase Asp-N. Peptide purification is shown in Fig. 1*B* and the primary structure in Fig. 2. The sum of the sequence analysis is in excellent agreement with the total composition from acid hydrolysis (Table 1). Since the sequence begins with glutamic acid (E) and ends with half-cystine (C), we have named the 60-residue peptide PEC-60.

The disulfide bridge pattern was estimated by analysis of separate digests with trypsin and pepsin of the intact polypeptide. The products of both digests were purified by HPLC and all peptides containing half-cystine were analyzed by sequencer degradation. Peptic digestion indicated that the fragment at positions 23–29 was associated with that at 57–60, suggesting that Cys-28 and Cys-60 are joined by a disulfide bridge as they are in PSTI (10). The other two disulfide bridges are probably also identical to those in PSTI. Analysis of an additional peptic fraction showed an association between residues 8–14 with 35–44, indicating that Cys-11 is joined with Cys-39 or Cys-42, and Cys-20 with the remaining Cys of the latter two.

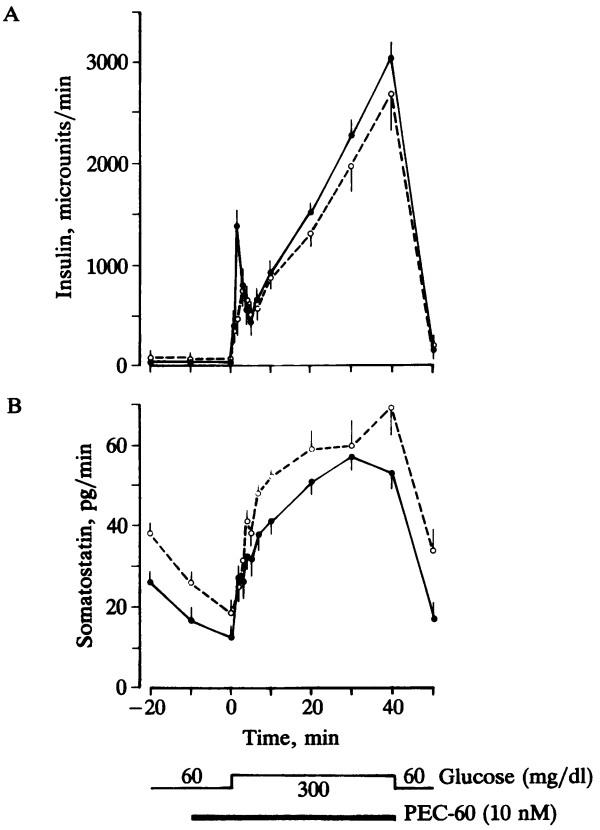

FIG. 3. Effects of PEC-60 on insulin release (*A*) and somatostatin release (*B*) from isolated perfused rat pancreas. Pancreata were perfused with glucose as indicated, in the absence (●; $n = 8$) or presence (○; $n = 8$) of PEC-60 from time −10 to +40 min. Insulin and somatostatin in the perfusion effluent were measured by radioimmunoassays.

Biological Activity. The effects of the 60-residue peptide on glucose-induced hormone release were tested. In control experiments, 16.7 mM glucose induced biphasic responses of both insulin and somatostatin release (Table 2, Fig. 3). When PEC-60 (10 nM) was given 10 min prior to, and simultaneously with, the high glucose concentration, the first phase of the glucose-induced insulin response was suppressed by 38% ($P < 0.05$). The second phase, however, was not significantly influenced. Glucose-induced somatostatin release was not significantly affected by PEC-60.

DISCUSSION

We have isolated a 60-residue intestinal polypeptide and determined its amino acid sequence. Since the polypeptide has N-terminal glutamic acid (E) and C-terminal half-cystine (C) and is 60 residues long, we designate it PEC-60. It

Table 2. Effects of PEC-60 on glucose-induced insulin and somatostatin responses of isolated perfused rat pancreas

	Hormone secretion		
Hormone response	First phase (0–5 min)	Second phase (5–40 min)	Total (0–40 min)
Insulin (microunits)			
Control	3340 ± 330	60,900 ± 2800	64,200 ± 3000
PEC-60 (10 nM)	2080 ± 250*	53,500 ± 5800	55,600 ± 5900
Somatostatin (pg)			
Control	63 ± 12	1,280 ± 85	1,340 ± 90
PEC-60 (10 nM)	44 ± 10	1,360 ± 180	1,400 ± 180

Values given are mean ± SEM ($n = 8$ perfusions).
*$P < 0.05$ vs. control.

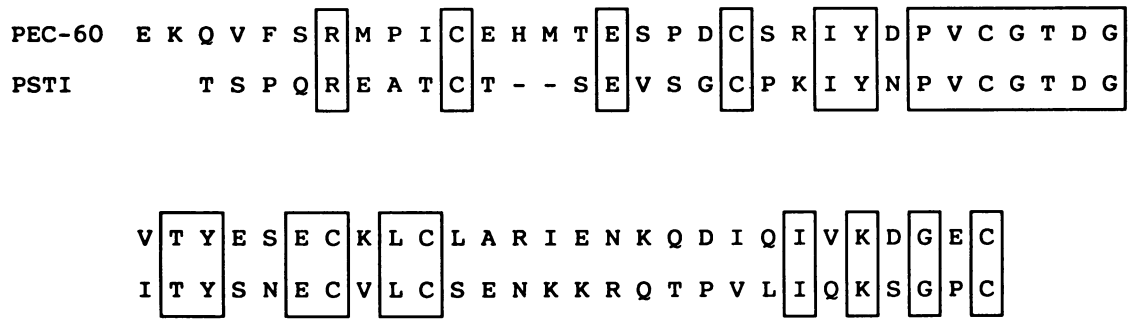

FIG. 4. Comparison of the structures of porcine PEC-60 and PSTI. Boxed letters indicate residue identities. The structure of the trypsin inhibitor is from ref. 6.

contains three disulfide bridges. The primary structure obtained is similar to that of the Kazal type of trypsin inhibitor from pancreas (Fig. 4). The structural similarities between PEC-60 and these antiproteases are interesting.

In the pancreas, two types of antiproteases exist; the Kunitz type (the basic trypsin inhibitor) and the Kazal type (the acidic, secretory trypsin inhibitor). Both are related to peptides involved in other biological functions (7, 20), suggesting a possible general regulatory role of antiprotease-related structures. In view of the significant sequence homology, PEC-60 could be a member of this group of antiproteases. Interestingly, PEC-60 was initially identified by monitoring the effects on insulin release. Although PEC-60 inhibited insulin release in our experimental system (Fig. 3), it is not clear whether PEC-60 plays a physiological role in the regulation of hormonal secretion from the pancreas. A separate, structurally related peptide affecting hormone release has been isolated from rat bile/pancreatic juice (7). This supports the idea that there is a relationship between the effects on hormone release and the structural characteristics of these protease inhibitors.

Extracts of bovine pituitary glands have been reported to contain a survival factor for adult rat hepatocytes in primary culture (32). This factor is identical to the bovine pancreatic trypsin inhibitor of the Kunitz type. Furthermore, the precursor of an amyloid protein from neuritic plaques of patients with Alzheimer disease contains a 56-residue peptide similar to that of the human form of the Kunitz type of pancreatic inhibitor. This suggests that protease inhibitors might be present in these amyloid deposits (21–23).

The Kazal type of pancreatic secretory inhibitor is known not to be confined to pancreas. Recently, a new function has been suggested for a member of this family in the rat, which is to monitor the release of cholecystokinin from the lumen of the intestine (7). The exact relationship of this monitor peptide to the secretory trypsin inhibitor is not yet ascertainable, because the structure of rat PSTI is not known. Further, it is not possible to assess the structural relationship, if any, between the monitor peptide and peptides from the small intestine that have been found to exhibit cholecystokinin-releasing activity (33, 34). However, rat PSTI differs from other mammalian forms since it contains histidine (35), as does the monitor peptide. We now show that PEC-60 also belongs to this family since it contains histidine as well (Table 1). The overall sequence similarity between porcine PEC-60 and porcine PSTI is about 40%, and in the region corresponding to the active site of PSTI (10), heptapeptide sequences are identical.

However, PEC-60 does not seem to inhibit trypsin activity when measured (36, 37) with benzoyl-L-arginine ethyl ester as a substrate. Consequently, PEC-60 has a different activity than PSTI, despite a clear structural similarity. It is, of course, not improbable that PEC-60 inhibits some other proteolytic enzyme with trypsin-like specificity. It may be noted that PEC-60, in addition to its similarity to pig PSTI, boar acrosin inhibitor, and the rat monitor peptide, is also similar to secretin and epidermal growth factor/urogastrone (38). The true biological function of PEC-60 is yet unknown. Nevertheless, its effect on insulin release and its structural similarities with a cholecystokinin release factor and the secretory protease inhibitors suggest important connections between protease inhibitors and hormone release mechanisms. It appears possible that antiproteases regulate important mechanisms of hormone release. In addition, it is probable that PEC-60, and the structural similarities now characterized, may provide further insights into the structures required for antiprotease activity.

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