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ISOLATION AND CHARACTERIZATION OF A TUMOR-DERIVED HUMAN PANCREASTATIN-RELATED PROTEIN

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SUMMARY A protein with pancreastatin-like immunoreactivity has been isolated and purified from liver metastasis of a patient with insulinoma. NH_2-terminal sequence analysis in conjunction with the use of antibodies specific for the C-terminal structure of pancreastatin identified this protein as a 186-amino acid residue protein corresponding to human chromogranin A-116-301. Using a sensitive radioimmunoassay it was found that serum from the patient with insulinoma contains two peptide species; one comigrates with the 186-amino acid residue pancreastatin and the other the 48-residue pancreastatin.

Pancreastatin (PS), a 49-residue peptide amide, was first isolated from porcine pancreas by Tatemoto *et al.* in 1986 (1) using an analytical tool specific for the C-terminal glycine amide structure of the peptide. This peptide has been shown to inhibit glucose-induced insulin release from the isolated perfused pancreas (2). Porcine pancreastatin shows striking sequence homology to a part of bovine chromogranin A (3), a protein known to be present in the secretory granules of endocrine and neuronal cells (4). Recently, Iacangelo *et al.* showed that porcine chromogranin A is the precursor of porcine pancreastatin (5). In 1987, Konecki *et al.* (6) suggested that the amino acid sequence of human pancreastatin (hPS) is a 52-residue peptide amide (hPS-52), since the gene structure of human chromogranin A contained a sequence (positions 250-301) homologous to porcine pancreastatin. Sekiya *et al.*, reported the isolation of a 29-residue peptide (hPS-29) from a pancreatic glucagonoma (7) and showed that this peptide was identical to a portion of the cDNA-

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The abbreviations used are: hCgA, human chromogranin A; hCgA-116-301, human CgA residues 116-301 (other fragments are abbreviated similarly); PS-LI, pancreastatin-like immunoreactivity.

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derived sequence of human chromogranin A. Schmidt *et al.*, reported the isolation and amino acid sequence analysis of tumor-derived peptides related to human pancreastatin and chromogranin A (8). These peptides consisting of 29 and 92 amino acid residues, respectively, are identical to the corresponding part of human chromogranin A.

In this paper, we report the isolation and characterization of a 186-residue polypeptide amide with PS-LI from the tumor of human insulinoma liver metastasis.

MATERIALS AND METHODS

Bioassay for Synthetic Peptides

Human chromogranin A fragments hCgA-273-301 (hPS-29), hCgA-254-301 (hPS-48), hCgA-250-301 (hPS-52) and the other human chromogranin A related peptide fragments were synthesized manually (9) according to the principle of Sheppard *et al.* by using N-fluorenyl-methyloxycarbonyl (Fmoc)-protected amino acids, and purified by gel filtration and HPLC. Rats were prepared with cannulae to drain pure pancreatic juice and bile separately and a duodenal cannula is installed to return pancreatic juice and bile to the intestine and with a jugular vein cannula (10). After 90 min of basal collection of pancreatic juice, cholecystokinin-8 (100 *p*mol/kg/h) was infused for 3 h with or without human pancreastatins (100 *p*mol/kg/h).

Human Pancreastatin Radioimmunoassay

^{125}I-labeled Tyr-hPS-29 was prepared by the chloramine-T method, and purified by Sephadex G-10 chromatography followed by DEAE-ion exchange chromatogphy (eluted with a gradient of 0 to 1 M NaCl in 0.01 M imidazole buffer, pH 7.5). Peptide sample of the standard (hPS-52) or an unknown was incubated with porcine pancreastatin C-terminal-specific rabbit antiserum T-2602 (11) or human pancreastatin C-terminal-specific rabbit antiserum R711 (12) (final dilution, 1 : 330,000) for 48 h at 4°C in a total volume of 500 μl of 0.01 M phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin, 0.025 M EDTA, 0.14 M NaCl, 0.05% (v/v) Tween 20 and 0.01% sodium azide. The tracer (0.1 ml, approximately 5,000 cpm) was then added and the mixture was incubated further for 48 h at 4°C. The bound and unbound peptides were separated by adding goat anti-rabbit IgG.

Isolation of Human CgA-Derived Peptide

Human PS-LI was detected by RIA using porcine pancreastatin C-terminal-specific rabbit antiserum T-2602 in an extract of a liver metastasis derived from an insulinoma patient. Tumor tissue was obtained after autopsy and frozen at -80°C. The frozen tumor (5 g) was dissected, suspended in EGTA 1mM/maleimide 1 mM solution, and homogenized at 0°C for 2 min (twice). The extract was adjusted to 1 N AcOH-20mM HCl and then boiled for 10 min. The extract was homogenized again for 2 min. Then, the extract was centrifuged at 16,000 x g, at 4°C for 20 min. The precipitate was homogenized again in 2 N AcOH 25 ml and centrifuged as described above. Supernatants were combined and concentrated to 20 ml in vacuo. The concentrate was separated into 100 fractions by gel-filtration on a Sephadex G-50 column (3.9 x 42 cm) using 1 N AcOH as eluent (one fraction = 5 ml). Each fraction was lyophilized and assayed for PS-LI. Fractions with PS-LI were combined and subjected to HPLC purification on an Ultrapore RPSC column [Beckman, 1 x 25 cm, flow rate 2 ml/min]. The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Fractions (4 ml each) were collected and lyophilized. The residues were dissolved in phosphate buffered-saline pH 7.2 and assayed for PS-LI. Immunoreactive fractions were collected and rechromatographed on a Cosmosil 5PhT-300 column [Nacalai tesque Co., Ltd, 4.6 x 150 mm, flow rate 1 ml/min] using the same solvent system as described above. Each fraction was lyophilized and assayed for PS-LI. Immunoreactive fractions were combined and subjected to automated Edman degradation on a gas-phase sequencer (Model 470A, Applied Biosystems).

Trypsin Digestion

Tryptic digestion of purified peptide was performed in 0.2 M $AcONH_4$ buffer pH 8.4 at 37°C with enzyme:peptide ratio of 1:100 or 1:1000, wt/wt. After digestion, solution was applied to HPLC and fractions were collected in 1 min interval. Each fraction was assayed for PS-LI and the immunoreactive fractions were combined and subjected to Edman degradation. The C-terminally amidated amino acid residue was identified after tryptic cleavage and HPLC separation according to Schmidt *et al.* (13).

Plasma Extraction

Plasma (1 ml) was mixed with an equal volume of acid solution (formic acid : trifluoroacetic acid : water = 2:2:96) and passed through a Sep-Pak C_{18} cartridge at a flow rate of 0.5 ml/min. PS-LI was eluted with 2.5 ml of a mixture of methanol, TFA and water (80:1:19), and dried under nitrogen gas. The residue was dissolved in 0.5 ml of 1 M acetic acid and then applied on a Sephadex G-50 (fine) column (1.2 x 65.5 cm), equilibrated, and eluted with 1 M acetic acid containing 0.05% gelatin at a flow rate of 5 ml/h. Two-milliliter fractions were collected, lyophilized and redissolved in 0.5 ml assay buffer for RIA. Fractions of the pancreastatin-like immunoreactive peaks from the Sephadex G-50 fractionation of plasma were pooled, lyophilized, dissolved in 0.1% TFA and applied on a HPLC column. The column was eluted as described above and 1 ml fractions were collected. Each fraction was lyophilized and assayed for PS-LI as described above.

RESULTS

Human Pancreastatin Radioimmunoassay

Human pancreastatin C-terminal-specific rabbit antiserum R711 cross-reacted with of hPS-29 (hCgA-273-301), hPS-48 (hCgA-254-301), hPS-52 (hCgA-250-301) and hPS-10 (hCgA-292-301) and also cross-reacted with porcine PS-49 (3.8%) and porcine PS-17 (positions 33-49) (4.9%), but did not cross-react with hCgA-292-300 (des-Gly-NH_2-hPS-10), bovine PS-(32-47), [Arg^8]-vasopressin and gastrin-I (<0.001%) (Fig. 1). The sensitivity of the assay was 1.7 fmol/tube. Dilution curves of human tissue and plasma extracts paralleled with synthetic hPS-52. These results demonstrated that antiserum R711 was specific for the C-terminal decapeptide amide and for the C-terminal glycine amide group.

Isolation and Amino Acid Sequence of a Human CgA-Derived Peptide

Gel-filtration of tumor extracts on Sephadex G-50 yielded two fractions with human PS-LI at fraction numbers, 33-44 and 55-61 (Fig. 2). The recoveries were 20.5 nmol for peak I (P-I) and 9.0 nmol for peak II (P-II) as measured by pancreastatin radioimmunoassay using synthetic pancreastatin-52 as a standard. A single immunoreactive peak PH-I (Fig. 3) was obtained upon purification of P-I fraction on HPLC. This fraction was further purified by HPLC on an analytical Cosmosil 5PhT-300 column using the same solvent system. A broad immunoreactive peak was

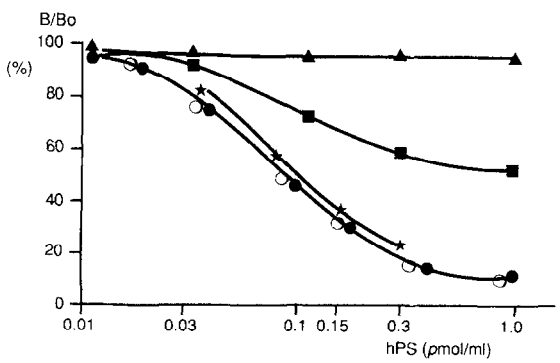

Fig. 1. Displacement curves of ^{125}I-tyrosyl hPS-29 by various molecular forms of pancreastatin using anti-hPS-29 sera (R711). hPS-10 (hCgA-292-301)(■), des-Gly-NH_2-hPS-10 (hCgA-292-300) (▲), hPS-29 (hCgA-273-301) (★), hPS-48 (hCgA-254-301) (○), hPS-52 (hCgA-250-301) (●).

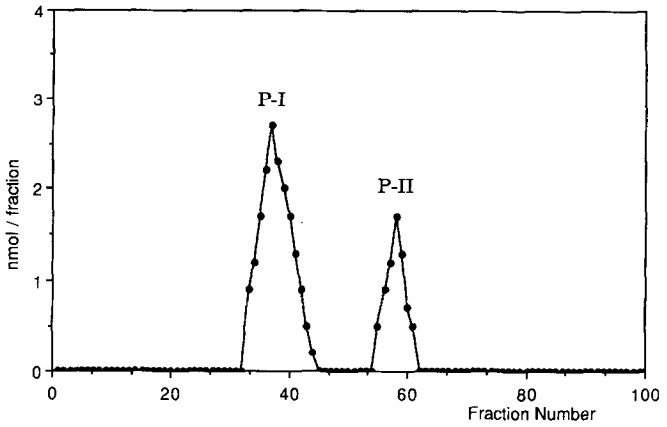

Fig. 2. Gel-filtration profile of PS-LIs in the tumor-extract on Sephadex G-50 (one fraction=5ml).

detected. The main fraction was collected and rechromatographed to yield a single homogeneous peak with PS-LI (Fig. 4). Gas-phase sequencing of this peak revealed the 49-residue sequence from the NH_2-terminus. The sequence obtained was found to be identical with hCgA-116-164 (6). To investigate whether this peptide included the carboxy-terminal glycine amide of hCgA-116-301, approximately 1.5 nmol of the peptide was treated with trypsin (S:E = 100:1, w/w). From the tryptic peptide mixture, glycylamide was identified as its phenylthiocarbamoyl-derivative. The anti-porcine pancreastatin serum T-2602, used in this investigation, possesses specificity for the C-terminal decapeptide of porcine pancreastatin including the glycine amide (10). The use of this antibody in combination with the sequence analysis of tryptic peptides (see below) established that the purified pancreastatin-like immunoreactive peptide was a large chromogranin A-derived peptide (hCgA-116-301) consisted of 186 amino acids.

Tryptic Digestion of Human Pancreastatin-186 (hCgA-116-301)

When the purified human pancreastatin-186 was treated with trypsin at S:E ratio = 100:1 no immunoreactive peptide was isolated, suggesting that the C-terminal glycine amide was released

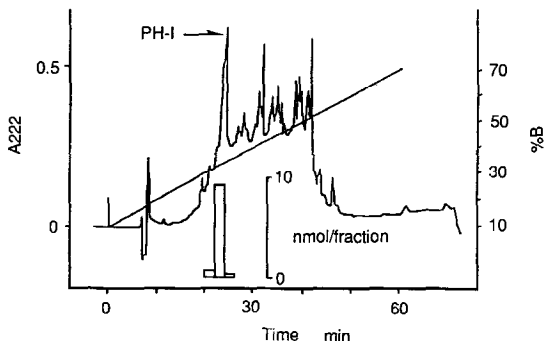

Fig. 3. HPLC elution profile of peak I (P-I) on Ultrapore RPSC column using the 0.1% trifluoroacetic acid aq./acetonitrile solvent system and a linear gradient of 10% to 70% solvent B in 60 min; flow rate was 2ml/min; absorbance was monitored at 222nm. (inner histograms; nmol of PS-LI per fraction).

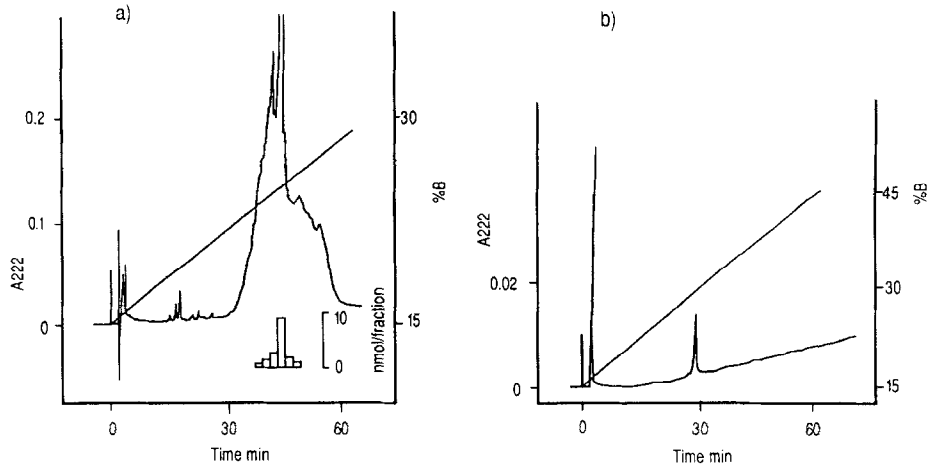

Fig. 4. (a) HPLC profile of PH-1 on a Cosmosil 5PhT-300 column using the same solvent system as in Fig. 3 and a liner gradient: 15% to 30% solvent B in 60min; flow rate was 1ml/min.; absorbance was monitored at 222nm. Each fraction was concentrated and assayed for PS-LI; (b) HPLC profile of the immunoreactive peak shown in Fig.4-a (retention time 42-44min.) on a Cosmosil 5PhT-300 column using the same solvent system as in Fig. 3 and a liner gradient: 15% to 45% solvent B in 60min; flow rate was 1ml/min.; absorbance was monitored at 222nm.

during the tryptic digestion (Fig. 5-a). Treatment of the 186-residue peptide with trypsin at S:E ratio = 1000:1 at 37°C for 5 h yielded three immunoreactive peptide, P-5, P-5' and P-6. The immunoreactivity of peptides P-5, P-5' and P-6 toward antiserum R711 together with partial sequence analysis indicated that these three peptides corresponded positions 116-301 for P-5, positions 124-301 for P-5' and positions 254-301 (hPS-48) for P-6 within the structure of human chromogranin A (Fig. 5-b).

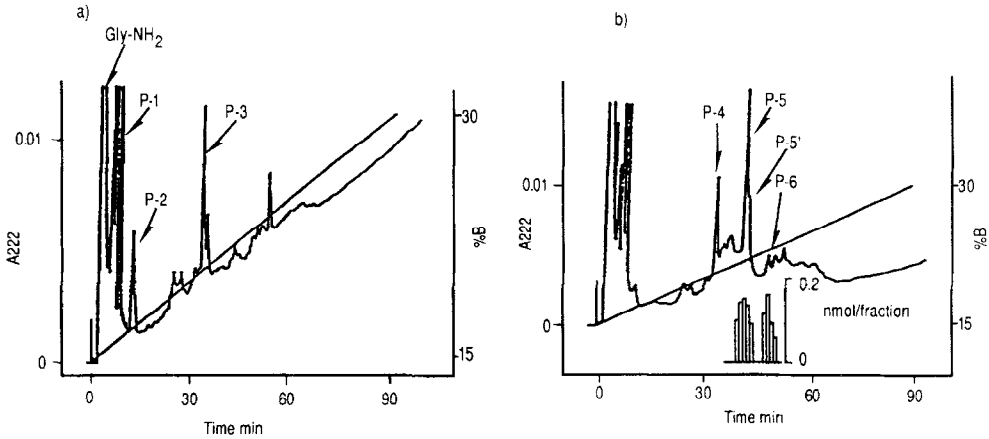

Fig. 5. HPLC elution profile of the tryptic peptides of pancreastatin-186 on a Cosmosil 5PhT-300 column using the same solvent systems as in Fig. 3; tryptic cleavage conditions [S:E ratio=100:1(a), 1000:1(b), reaction time: 2hr (a), 5hr (b)]; flow rate was 1ml/min.; absorbance was monitored at 222nm, a liner gradient: 15% to 30% solvent B in 90min. Each fraction was concentrated and assayed for PS-LI using R711. In the tryptic cleavage [S:E ratio=100:1(a)] no immunoreactive peptide was detected (Fig-5-a). The fraction at retention time; 0-3min containd glycine amide which was identified by amide assay. P-1, P-2, and P-3 were identified as hCgA-198-209, hCgA-176-197, and hCgA-145-175, respectively. In the tryptic cleavage (S:E=1000:1) two main peaks (P-5, P-6) and a small peak (P-5') with immunoreactivities and a non-immunoreactive peak (P-4) were detected (Fig-5-b).

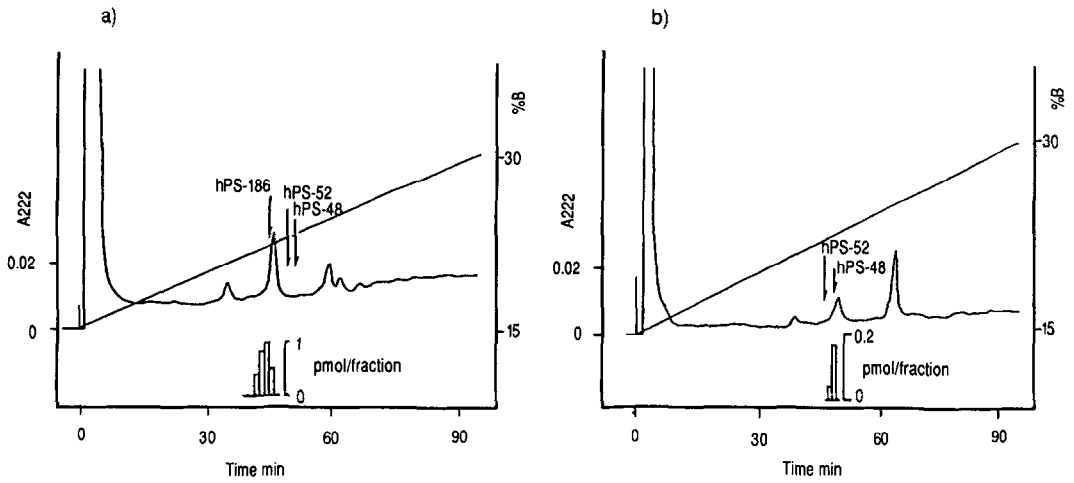

Fig. 6. HPLC elution profile of plasma extracts on a Cosmosil 5PhT-300 column using the same solvent system as in Fig. 3; flow rate was 1ml/min; absorbance was monitored at 222nm; PS-LI was eluted with a liner gradient: 15% to 30% solvent B in 90min. Fractions (1ml each) were collected and assayed for pancreastatin-like immunoreactivity. Prior to HPLC chromatography, plasma extracts were fractionated on Sephadex G-50 column as shown in Fig. 2 to yield two immunoreactive fractions; a high molecular weight fraction and a low molecular weight fraction. (a) HPLC profile of the high molecular wight fraction, (b) HPLC profile of the low molecular weight fraction. Arrows indicate elution positions of synthetic human pancreastatin-52, -48, and the isolated human pancreastatin-186 respectively. (inner histograms; pmol of PS-LI per fraction)

Characterization of Plasma Extract

On gel-chromatography on Sephadex G-50 the C-terminally amidated synthetic peptide, hPS-48 (hCgA-254-301), coeluted with the PS-LI of the tumor extract (Fig. 2, P-II). The plasma of the patient with insulinoma contains an immunoreactive peptide(s) that co-chromatographed with the synthetic peptide hPS-48 upon gel-filtration on Sephadex G-50 in the peak-II position (data not shown). This suggests the possibility of the existence of the molecular form similar or identical to hPS-48 in human plasma and in pancreas. The synthetic hPS-48 co-eluted with the tryptic fragment hPS-48 generated by tryptic digestion of hPS-186 isolated from tumor extract and also with the smaller molecula form of human plasma PS-LI on HPLC (Fig. 6).

DISCUSSION

Using C-terminal specific porcine pancreastatin antibody as a guide, we isolated a C-terminal α-amidated peptide related to human pancreastatin and chromogranin A from liver metastasis of a patient with insulinoma. The established NH_2-terminal 49 amino acid sequence of the isolated protein hPS-186, was identical to the corresponding region (position 116-301) of its cDNA-derived sequence of human chromogranin A (6). Using our newly developed human pancreastatin radioimmunoassay, we identified and isolated three peptides containing the C-terminal glycine amide of hCgA from the tryptic digestion of the 186 residue polypeptide.

The isolation and characterization of pancreastatin-186 suggests that the biosynthesis of various sizes of human pancreastatin from hCgA-1-439 could be initiated with the synthesis of hPS-186 (Fig. 7). This peptide undergoes proteolytic processing to yield betagranin (hCgA-1-115) (14) and hPS fragments of various sizes that retain biological activities.

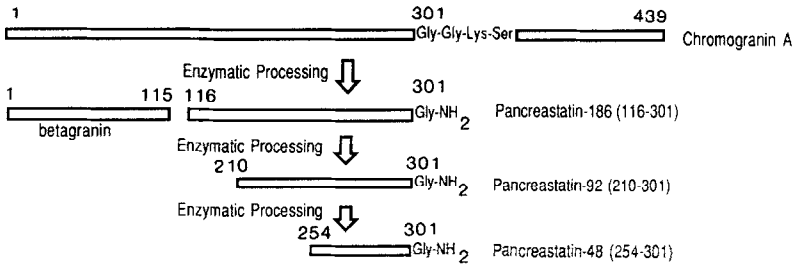

Fig. 7. Schematic representation of the processing of human chromogranin A related peptides.

While hPS-186 was identified as the major form of pancreastatin in a liver metastasis from a patient with insulinoma (4.2 nmol/g wet cell, 67% of the total activity), it is not clear that hPS-186 is the major active form of human pancreastatin in circulation. Analysis of human sera derived from a patient with insulinoma indicated the presence of an immunoreactive polypeptide(s) eluted at or near hPS-186 (Fig. 6-a) and a peptide(s) that co-eluted with a synthetic 48-residue peptide amide hPS-48 (Fig. 6-b). The corresponding major molecular species of pancreastatin in porcine and bovine are 49-residue and 47-residue peptide amide, respectively (1, 15). Therefore, hPS-48 might be a candidate for a naturally occurring pancreastatin in circulation.

Biological studies on synthetic peptides, hPS-29, -48 and -52, indicated that, the potency of hPSs in inhibiting cholecystokinin-stimulated pancreatic protein secretion is similar to that of porcine PS-49 (16), which has a 70% sequence identity with hPS (Table 1). The potent effect of the C-terminal fragment of pancreastatin on pancreatic secretion suggested that this portion of the molecule is important for the biological activity (2).

Table 1. Increments of fluid, bicarbonate and protein outputs stimulated by CCK-8 with human pancreastatin-29, -48, -52 (100 pmol/kg/h) and without pancreastatin[a]

	Fluid output (ml/3h)	HCO_3^- output (mEq/3h)	Protein output[b] (mg/3h)
Without	0.37 ± 0.12 (n=5)	3.7 ± 2.3 (n=5)	47.1 ± 6.3 (n=5)
pancreastatin-29	0.10 ± 0.09* (n=10)	5.6 ± 4.1 (n=10)	19.7 ± 3.9* (n=10)
pancreastatin-48	0.18 ± 0.04 (n=6)	4.2 ± 0.39 (n=6)	23.2 ± 3.9* (n=6)
pancreastatin-52	0.15 ± 0.13 (n=10)	3.8 ± 3.2 (n=10)	21.8 ± 8.7* (n=10)

a: Values were calculated as follows; the sum of values during 3h CCK-8 infusion minus the sum of values 1-h basal secretion (before the infusion of CCK-8) X 3, and expressed Mean ± SE.
b: Significantly different by ANOVA.
Fluid output: $F(3,21) = 2.01$ $P>0.05$
HCO_3^- output: $F(3,21) = 1.78$ $P>0.05$
Protein output: $F(3,21) = 9.65$ $P<0.01$
* Significantly lower than the values without pancreastatin by Newman Keul's multiple comparison test.
Numbers in parentheses indicate the number of rats examined.

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