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STIMULATORY EFFECT OF AN ENDOGENOUS PEPTIDE IN RAT PANCREATIC JUICE ON PANCREATIC ENZYME SECRETION IN THE PRESENCE OF ATROPINE: EVIDENCE FOR DIFFERENT MODE OF ACTION OF STIMULATION FROM EXOGENOUS TRYPSIN INHIBITORS

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SUMMARY: A new factor which activated the secretion of pancreatic enzymes was discovered and purified from rat bile-pancreatic juice. A fraction below M.W.10,000 of rat bile-pancreatic juice enhanced trypsinogen secretion by injection into anesthetized rat duodenum. The factor was purified from this fraction using its biological activity as an index by Sephadex G-50, SP Sephadex C-50 and HPLC. This factor was a peptide of which molecular weight was about 6,000 and had trypsin inhibitory activity. From these and some other findings, it was suggested that the peptide was identical with the "Kazal type" inibitor. In the anesthetized and atropine-treated rat, of which intestinal trypsin was removed by thoroughly washing with saline containing 5 μM soybean trypsin inhibitor (SBTI), pancreatic secretion became basal state, and was not stimulated by injection of SBTI into its duodenum any longer. Under this condition, however, injection of this purified peptide brought about markedly stimulation of pancreatic enzyme secretion. These results suggest that this peptide has a certain function which enhances pancreatic enzyme secretion by the different manner from exogenous trypsin inhibitors such as SBTI.

Pancreatic enzyme secretion is one of the most important step in the digestive process of food protein. It is due to an interplay of neural and hormonal mechanisms(1). However, detailed mechanisms of pancreatic enzyme secretion in the response to chemical properties of ingested food or its components have not been clear. It has been pointed out that intraluminal pancreatic protease (trypsin and/or chymotrypsin) levels may regulate pancreatic enzyme secretion in rat(2-5). This mechanism has been recognized as a feedback inhibition of protease on pancreatic enzyme secretion.

The authors have investigated the response of pancreatic enzyme secretion to ingested meals in the intestinal phase in anesthetized rats, cannulated with tube into the main bile-pancreatic duct. During the course of the studies, the authors found a phenomenon that a fraction of rat bile-pancreatic juice at

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M.W. below 10,000, enhanced considerably the stimulation of pancreatic enzyme secretion by food protein.

This communication deals with the characterization of the stimulatory substance found in rat bile-pancreatic juice and with its mode of action in the pancreatic enzyme secretion system responding to food protein ingestion.

MATERIALS AND METHODS: Animal preparation for bioassay; Male Wistar rats weighing 300-350 g were anesthetized by pentobarbital sodium (20mg/kg/hr, i.p. PITMAN-MOORE) and prepared with bile-pancreatic and duodenal cannula. Materials were injected into the intestine via the duodenal cannula. Bile-pancreatic juice was collected by the bile-pancreatic cannula at 15 minute intervals and continuously returned to intestine(except for 10 μl sample for enzyme assay).

Atropine treatment and intestinal washing; Atropine (100 μg/kg/hr) was injected into the saphenous vein. In this system, the collected bile-pancreatic juice was not returned into the intestine and saline containing 5 μM soybean trypsin inhibitor(SBTI; SIGMA) was infused slowly into the intestine.

Procedure of purification of the stimulatory substance for pancreatic enzyme secretion; Bile-pancreatic juice was fractionated by collodion bag and Amicon membrane filter UM2 into three fractions. Their molecular weight were above 10,000, from 10,000 to 1,000, and below 1,000, respectively. The intermediary fraction was charged directly to a Sephadex G-50(PHARMACIA) column (6 x 120 cm) previously equilibrated with phosphate buffer(pH 7.5, 0.05 M) containing 0.45 M NaCl. The flow rate was 80 ml/hr. The active fraction was concentrated by Sep-pak column (WATERS) equilibrated with phosphate buffer(pH 7.2, 0.05 M) and eluted by acetonitril:0.1% HCl=80:20 (v/v). The concentrate was applied to a SP-sephadex C-50(PHARMACIA) column (2.5 x 50 cm) equilibrated with sodium acetate buffer(pH 4.5, 0.05 M). The active fractions were eluted with a linear NaCl gradient (0 - 60 %), and the main active fraction was concentrated by Sep-pak column as described above.

HPLC system with Model 660 solvent programmer (WATERS) was used with 0.1 % phosphoric acid as starting solution. The concentrate was pumped directly onto a μ-Bondapak C 18 column (WATERS). Elution was carried out by gradient of the eluate (acetonitril:0.1% phosphoric acid=80:20) from 0 to 50% in 10 minute. The flow rate was 2.0 ml/min and effulent was collected in 0.5 ml fraction. The bioactive fraction was re-charged onto the μ-bondapak C 18 column. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn(6) with slight modifications.

Enzyme assay procedure; Trypsin activity was determined using Benzoyl arginine-p-nitroanilide·HCl (BApNA; NAKARAI CHEMICAL Ltd.) as substrate after incubation with enterokinase(SIGMA) at 37° for 40 min.

Other chemicals were of analytical grade and purchased from NAKARAI CHEMICAL Ltd., Japan.

RESULTS AND DISCUSSION: Rat pancreatic juice contains a large quantity of proteases. Thus, injection of whole pancreatic juice into the duodenum markedly lowers pancreatic enzyme secretion(2-4). The pancreatic juice was fractionated by its molecular weight into three fractions, above 10,000, lower 1,000 and intermediary fraction, respectively.

"Above M.W. 10,000 fraction" was abundant of proteases, which decreased pancreatic enzyme secretion as shown in Fig.1. On the contrary, "intermediary

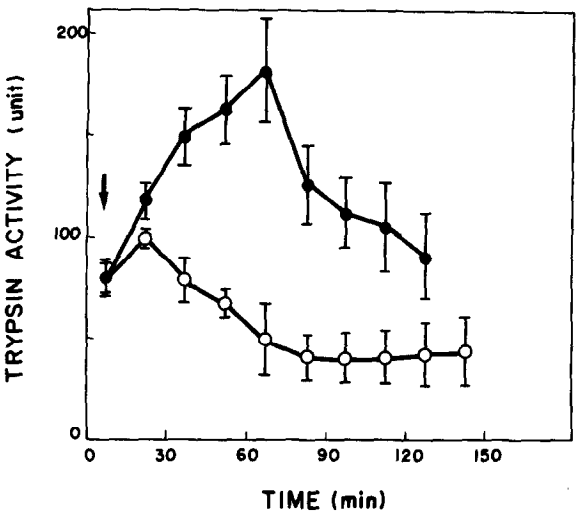
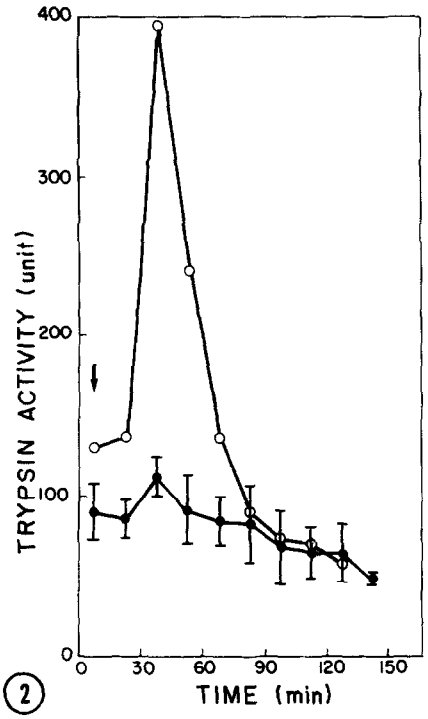


Fig. 1 Change in trypsinogen secretion in rats with bile-pancreatic cannula. One ml of fraction below M.W. 10,000(●) or above M.W. 10,000(○) of bile pancreatic juice was injected(↓) with 20 mg of egg albumin(SIGMA) into the duodenum. Pancreatic juice was collected at 15 min intervals and continuously returned to the intestine except for 10 μl sample for enzyme assay using BApNA as substrate after activation by enterokinase at 37° for 40 min. Trypsin activity is expressed as unit (1 unit=1 μmol p-nitroaniline liberated/min at 37 °) of total pancreatic secretion for 15 min.
Values are given as Mean±SEM of 4 rats.

fraction (M.W.1,000-10,000)" markedly enhanced the enzyme secretion when injected together with 20 mg of ovalbumin. The enhancement was two times higher than that of injection of 20 mg ovalbumin alone. Moreover, ten-fold concentrated fraction increased the response more than four times compared with the ovalbumin alone(Fig. 2). This secretion level was almost maximal for the response of pancreas to intraluminal secretagogues. This intermediary fraction showed rather smaller increase of the secretion in the absence of ovalbumin. Injection of "fraction below M.W. 1,000" had no effect in the presence or absence of ovalbmin. These results suggested that there was an endogenous substance which enhanced pancreatic enzyme secretion and was contained in the fraction of M.W. 1,000 to 10,000 of bile-pancreatic juice.

Purification of the endogenous substance was carried out using its biological activity for the pancreatic enzyme secretion as an index. The final preperation was homogeneous on SDS-polyacrylamide gel electrophoresis(Fig 3) and its molecular weight was approx. 6,000 on Sephadex G-50. The purified substance had potent inhibitory activity to rat trypsin, but weakly inhibited



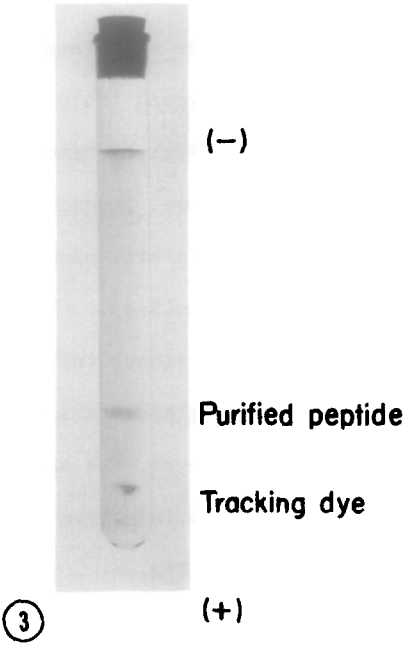


Fig. 2 Change in trypsinogen secretion in rats with bile-pancreatic cannula. One ml of 10-fold concentrate of fraction below M.W. 10,000 was injected(↓) with 20 mg of egg albumin into the duodenum. Sampling and estimation of trypsinogen secretion were the same as described in Fig. 1.
One of typical patterns is given(○).
Twenty mg of egg albumin alone was injected as control(●).
Values are given as Mean±SEM of 5 rats.

Fig. 3 SDS-polyacrylamide gel electrophoresis of the purified peptide from rat pancreatic juice. Six μg of purified peptide was applied to the separating gel containing 15 % acrylamide and 0.1 % SDS in sodium phosphate buffer, pH 7.2. A constant current(8 mA per gel) was applied until tracking dye, bromophenol blue, had moved about 7.5 cm into the gel. Gels were stained with coomassie brilliant blue. The direction of migration is from cathode to anode.

bovine trypsin. The occurrence of a trypsin inhibitor in rat pancreatic juice, so called "Kazal type" inhibitor, or commonly called pancreatic secretory trypsin inhibitor(PSTI) was presented(7), and recently a part of its properties was reported(8). These results described above suggested that the purified substance was probably identical with the Kazal type inhibitor, though further investigation was required.

Treatment of the intermediary fraction with rat trypsin decreased its stimulatory activity. Therefore, the substance was a trypsin-sensitive peptide. Melmed and Bouchier(9) speculated that PSTI may stimulate pancreatic enzyme secretion such as other trypsin inhibitor as SBTI, but Forell el al.(10) showed

that PSTI was destroyed rapidly in the duodenum. Thus, it is still unclear the fate of PSTI during the digestive process of food. Then, it was investigated whether the purified peptide stimulated pancreatic enzyme secretion by the same manner as the exogenous trypsin inhibitor, SBTI, which was considered to give rise to the stimulatory effect by removal of the feedback inhibition by trypsin in the intestine(3-5). Some investigators observed that pancreatic enzyme secretion was increased when bile-pancreatic juice was kept diverting and was not put back to the duodenum(3,4). Thus, it is difficult to remove trypsin from the intestinal lumen without influence on the pancreatic secretion. This hypersecretion by diverting pancreatic juice was explained by removal of the feedback inhibition. However, the author found that it might be due to in part the neural mechanism, since injection of atropine was able to depress this hypersecretion as shown in Fig. 4. Therefore, trypsin-free system was prepared in anesthetized and atropine-treated rats as follows: Pancreatic juice was diverted by main bile-pancreatic duct cannulation, and residual intraluminal

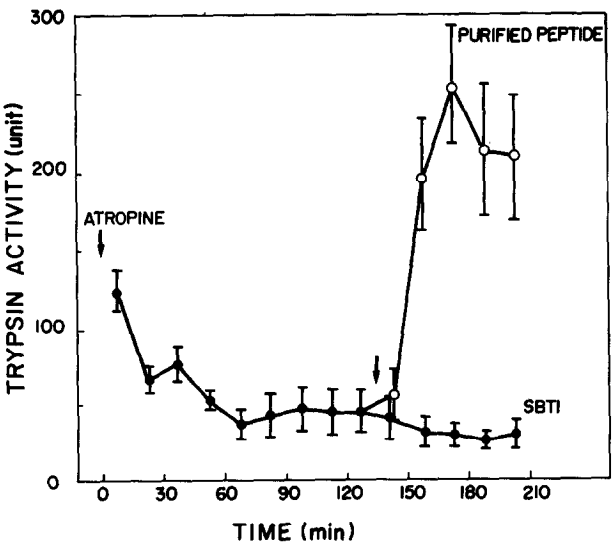


Fig. 4 Effect of the purified peptide(O) from rat bile-pancreatic juice or soybean trypsin inhibitor(SBTI)(●) on trypsinogen secretion in atropine treated rats with bile-pancreatic cannula. Atropine(100 μg/kg/hr) was injected into the saphenous vein. Pancreatic juice was collected at 15 min intervals for analysis and was not returned into the intestine. Residual intraluminal trypsin was washed out by infusing saline containing 5 μM SBTI. After this treatment, the purified peptide (10 μg) or SBTI (5 mg) was injected(↓) into the duodenum. Estimation of trypsinogen secretion was the same as described in Fig. 1. Values are given as Mean±SEM of 8 rats.

trypsin was washing out by infusion of saline with 5 μM SBTI. After this treatment, no trypsin activity was detected in the intestine and pancreatic enzyme secretion became basal level within 2 hours.

Under the conditions, the purified peptide indicated clear action as a secretagogue through different manner from exogenous inhibitors such as SBTI. The additional administration of SBTI to the duodenum did not stimulate pancreatic enzyme secretion. On the other hand, injection of the purified peptide (about 10 μg) brought about the remarkable stimulation of pancreatic enzyme secretion(Fig.4).

The Kazal type inhibitor has been reported earlier, but its physiological function remains unclear. Some investigators believe that the inhibitor acts as a local inhibitor within the pancreas and protects against the deleterious effect of prematurely activated trypsin. The authors suggest, from the present investigations, a new physiological function of the inhibitor as one of the mediators of pancreatic enzyme secretion responding to food intake.

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