Differential Increases in Syntheses of Newly Identified Trypsinogen 2 Isoforms by Dietary Protein in Rat Pancreas

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We have found that dietary protein markedly induced pancreatic serine protease activity via a mechanism independent of luminal trypsin activity in pancreatico-biliary–diverted (PBD) rats. The aim of this study was to examine the effects of dietary protein on the synthesis of trypsinogen isoforms by comparing *in vivo* incorporation of [^{35}S] L-methionine into isoform proteins in PBD and sham-operated rats. A small duodenal segment including the ampulla of Vater was sectioned and transposed to the upper ileum with end-to-side anastomosis (PBD) or duodenal transection was followed by reanastomosis (sham) in male Sprague-Dawley rats. After recovery, PBD and sham rats were fed a 25% or 60% casein-sucrose–based diet (NC or HC) for 14 days. Rats were then intravenously injected with [^{35}S] L-methionine (15 MBq/kg body weight) 30 mins before being sacrificed for analysis of pancreatic enzymes by two-dimensional SDS-polyacrylamide gel electrophoresis. By using electrophoresis with narrow range of isoelectric focusing (pI 4.5–5.5), five trypsinogen 2 (2-x) isoform spots were identified using both [^{35}S] incorporation and Coomassie brilliant blue (CBB) staining in PBD rats, but not in sham rats. N-terminal sequences of these trypsinogen 2-x spots were identical to known rat trypsinogen 2 with the exception that the third valine was changed to isoleucine in one isoform. In PBD rats, feeding of HC specifically increased the [^{35}S] and CBB intensities of these trypsinogen 2-x isoforms and trypsinogen 3. The degree of induction of the five trypsinogen 2-x molecules by HC varied greatly. Trypsinogen 1 and 4, which are the major trypsinogens in normal rats, showed no changes. We conclude that increases in synthesis of a few newly identified trypsinogen 2-x isoforms mainly contribute to the induction of trypsin activity in the pancreas by HC in PBD rats. Exp Biol Med 229:772–780, 2004.

Key words: dietary protein; trypsinogen 2; exocrine pancreas; bile-pancreatic juice-diverted rats

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Pancreatic digestive enzyme activities are known to adapt to ingested dietary components; that is, high-protein intake increases pancreatic protease and lowers amylase levels (1–3). Dietary protein is known to increase pancreatic protease synthesis via cholecystokinin (CCK) secretion (4). We previously found that dietary proteins or peptides are directly sensed by the intestinal mucosal cells and induce CCK release in pancreatico-biliary–diverted (PBD) rats (5–7).

The control mechanism of CCK secretion by dietary protein depends on the masking of luminal protease activities, which is mediated by endogenous CCK-releasing factors (8–10). Pancreatic serine protease activities were found to markedly increase in response to feeding PBD rats a diet with high protein (11). This showed that dietary protein induces pancreatic protease independent of luminal protease activities (12). Several trypsinogen isoforms (trypsinogen 1, 2, 3, 4, and 5) have been identified in the rat, and several isoforms are also present in the human pancreas. It has been reported that anionic (trypsinogen 1 and 2) and cationic (trypsinogen 3 and 4) trypsinogens change differentially with the feeding of a high-protein diet and hyperCCKemia induced by PBD (13–16).

The aims of the present study were to evaluate the relative synthesis rate of each trypsinogen isoform against total pancreatic protein synthesis by feeding a high-protein diet to PBD rats. We found five protein spots newly identified as trypsinogen 2 by using two-dimensional SDS–polyacrylamide gel electrophoresis with narrow-range isoelectric focusing (trypsinogen 2 isoforms were labeled trypsinogen 2-x). Incorporation of [^{35}S]-L-methionine into these trypsinogen 2-x isoforms was greatly influenced by feeding a high-protein diet to PBD rats. These results provide additional insights about the nature of pancreatitis induced by hyperCCKemia.

Materials and Methods

Animals and Diets. This study consisted of two separate experiments using PBD and sham rats. Male Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan) weighing about 220 g were fed a semipurified, sucrose-

based diet containing casein (250 g casein, 50 g corn oil/kg diet) with an AIN-93G mixture of minerals and vitamins (Table 1, Basal diet; Ref. 17) for 7 days. Bile-pancreatic juice (BPJ) was diverted to the ileum by transposition of a duodenal segment including the ampulla of Vater to establish a chronic PBD (18). The acclimated rats underwent the PBD operation in Experiment 1 and a sham operation in Experiment 2 under pentobarbital anesthesia (sodium pentobarbital, 40 mg/kg body weight; Abbott Laboratories, North Chicago, IL) after a 24-hr fast. Briefly, a 2- to 3-cm segment of the duodenum containing the ampulla of Vater was cut off after ligation of the proximal end of the segment. End-to-side anastomosis was carried out between the edge of the distal segment and the lateral opening on the upper ileum (45 cm distal to the ligament of Treitz), and both cut edges of the duodenum were end-to-end anastomosed (PBD rat). The rats in Experiment 2 were subjected to a sham operation in which a 1-cm segment distal to the ampulla of Vater was transected and end-to-end anastomosed (sham rat). Rats did not receive food or water on the day following surgery, but were fed the basal diet during a 14-day recovery period. Rats were then divided into two groups and fed a fat-free diet containing either 25% (NC) or 60% (HC) casein with the difference accounted for by sucrose (Table 1). Fat was eliminated from test diet because it also is an inducer of CCK secretion. The recovery period of the sham rats was shortened so that the sham rats would be of similar body weight to the PBD rats at the initiation of the feeding of test diets. Only PBD and sham rats that gained weight during the recovery phase were included in the experimental phase of the study. Twenty and 16 rats were used for PBD and sham operations, respectively. We used six and seven PBD rats for the NC and HC diet groups, and six and seven sham rats for the NC and HC diet groups, respectively. Rats in all groups had free access to the assigned test diet and water. The experiments were performed in a room controlled at 23° ± 2°C, with a 12:12-hr light:dark cycle (0800–2000 hrs light period). The study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

At 0900–1030 hrs on the last day of test feeding, [^{35}S]-L-methionine in saline (15 MBq/kg body weight; ICN Biochemicals Inc., Aurora, OH) was injected into the jugular vein under light anesthesia induced by inhalation of diethyl ether. Thirty minutes after the injection, the rats were sacrificed by withdrawal of aortic blood under pentobarbital anesthesia. The pancreatic tissue was removed, immediately frozen in liquid nitrogen, and freeze-dried.

Two-Dimensional SDS–Polyacrylamide Gel Electrophoresis of the Pancreatic Extracts. Freeze-dried pancreatic tissue was homogenized in a solution containing 8 mol urea/L, 65 mmol CHAPS/L, and 50 mmol dithiothreitol/L with a Polytron homogenizer, and pancreatic proteins were precipitated with choloroform-methanol (1:3). The precipitated proteins were dissolved in the homogenizing solution, and protein content was measured using Bio-Rad protein reagent (the Bradford method; Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard protein. The pancreatic protein extract (300 μg in 300 μl) was applied to immobilized dry strips, pI 3–10 (wide range, IPG Ready Strip; Bio-Rad Laboratories) and pI 4.5–5.5 (narrow range, Immobiline Dry Strip; Amersham International, Little Chalfort, UK) for isoelectric focusing electrophoresis. After soaking for 12 hrs, the first electrophoretic step was performed at 250 V for 0.5 hr, increased linearly to 10,000 V for 5 hrs, and maintained at 10,000 V for an additional 6 hrs. The strips were treated with an equilibrium buffer for the SDS–polyacrylamide gel electrophoresis, then applied on the polyacrylamide gel (2.67%–10%). The second electrophoretic process was performed with 16 mA/gel for 30 mins and 24 mA/gel for 5–5.5 hrs. The gel was stained with Coomassie brilliant blue (CBB) and dried on filter paper. [^{35}S]-L-methionine incorporated into pancreatic protein on the dried gels was measured by a radioactive image analyzer (BAS 1000, Fuji Film, Tokyo, Japan). Quantification of separated spot intensities was performed by an analysis system for two-dimensional electrophoresis.

Identification of each spot was performed by the matrix-assisted desorption ionization time of flight mass spectrometry (MALDI-TOF/MS; Voyager DE-STR/15000, Applied Biosystems, Foster City, CA) after trypsin digestion of proteins extracted from each spot, and the extracted proteins were identified by protein prospect with MS-Fit (University of California, San Francisco, CA). Trypsinogen isoforms were identified by N-terminal amino acid sequence analysis using a protein sequencing system (Procise 492/AS; Applied Biosystems) with the known trypsinogen isoform sequences of rats (19–21).

Other Analyses. Protein concentration in the freeze-dried pancreas was measured by a modified Lowry method (22, 23). The concentration of total RNA was determined colorimetrically by the orcinol method (24) following extraction as described by Fleck and Munro (25).

Trypsinogen and chymotrypsinogen in the freeze-dried pancreas were activated by addition of enterokinase (Sigma) at 30°C for 20 mins in 15 mmol Tris/L buffer (pH 8.1). Trypsin and chymotrypsin activities were estimated photometrically using the synthetic substrates *N*α-*p*-toluenesulfonyl-L-arginine methyl ester (TAME; Ref. 26) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE; Ref. 27), respectively. Pancreatic amylase activity was measured with procion yellow starch as the substrate (28).

Calculations and Statistics. The protein and RNA contents of the pancreas were expressed as milligrams per pancreas (mg/pancreas) in whole pancreas. Activities of the pancreatic enzymes were expressed as units per milligram (U/mg) total pancreatic protein (specific activity). One unit of trypsin or chymotrypsin activity was defined as the

Table 1. Composition of Basal and Test Diets

	Basal[a] and 25% casein diet (NC) (g/kg)	60% casein diets (HC) (g/kg)
Casein[b]	250	600
Corn oil	50 (0 for NC)	0
Mineral mixture[c]	40	40
Vitamin mixture[c]	10	10
Choline bitartrate	4.0	4.0
Sucrose	646 (696 for NC)	346

[a] The basal diet was given during the acclimation and recovery periods.
[b] Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).
[c] AIN-93G formulas.

amount of activity hydrolyzing 1 μmol of substrate per minute at 30°C. Procion yellow starch, the substrate for the amylase assay, was calibrated using purified α-amylase from the porcine pancreas (Type 1A; Sigma) at 37°C. Relative synthesis rate (evaluated by [^{35}S] radioactivity) and relative content (evaluated by CBB staining) of each pancreatic digestive enzyme were evaluated as a percentage of each separated spot intensity against the total intensity of all spots in the two-dimensional electrophoresis with wide-range (pI 3–10) isoelectric focusing. The percentage intensity of non-separated anionic serine protease clusters between pI 4.5–5.5 was divided into separated protease spots by electrophoresis with narrow-range (pI 4.5–5.5) isoelectric focusing.

Data were analyzed by two-way analysis of variance (ANOVA; PBD and diet), and the significance of intergroup differences was determined by Duncan's multiple range test in the case that the interaction (PBD × diet) was significant ($P < 0.05$; SAS version 6.07; SAS Institute Inc., Cary, NC). The differences in trypsinogen 2-x between NC and HC groups for PBD rats were analyzed by Student's t test (Tables 2 and 3).

Results

There were no significant differences in food intake over the 2 weeks of feeding among the four groups (18.2 ± 0.29 and 19.3 ± 1.27 g/day for the PBD NC and HC groups, respectively; 18.5 ± 0.12 and 18.1 ± 0.12 g/day for sham NC and HC groups, respectively). Final body weights were similar in all of the groups (314 ± 4.1 and 315 ± 5.7 g for PBD NC and HC groups, respectively; 315 ± 2.1 and 321 ± 2.2 g for sham NC and HC groups, respectively).

Pancreatic dry weight and total protein content were

Table 2. Incorporation of [^{35}S]-L-Methionine into Digestive Enzymes in the Pancreas Separated by Two-Dimensional Gel Electrophoresis in Sham and Bile-Pancreatic Juice-Diverted (PBD) Rats Fed a 25% or 60% (of Total) Casein Diet for Two Weeks[a]

	Sham		PBD		ANOVA		
	25% casein	60% casein	25% casein	60% casein	P	D	P × D
Trypsinogen							
1	14.0 ± 2.13	19.0 ± 2.36	10.9 ± 2.60	9.09 ± 1.71	**		
2-1	—	—	0.78 ± 0.17	2.86 ± 0.59	—	**	—
2-2	—	—	0.91 ± 0.23	1.74 ± 0.18	—	**	—
2-3	—	—	1.77 ± 0.43	7.40 ± 0.99	—	**	—
2-4	—	—	1.36 ± 0.25	2.45 ± 0.29	—	*	—
2-5	—	—	1.33 ± 0.28	1.72 ± 0.31	—		—
3	—	—	2.20 ± 0.48	4.16 ± 0.69		*	
4	10.9 ± 0.51a	13.8 ± 0.40a	8.21 ± 1.28b	6.09 ± 0.58b	**		**
5	—	—	2.41 ± 0.54	2.93 ± 0.41			
Total	25.8 ± 2.59	33.8 ± 2.78	29.9 ± 3.98	38.4 ± 3.10		**	
Chymotrypsinogen							
B-1	3.64 ± 0.86	8.98 ± 0.88	8.53 ± 2.40	14.6 ± 2.10	**	**	
B-2	0.84 ± 0.20c	2.08 ± 0.32b	4.44 ± 0.50a	2.78 ± 0.29b	**		**
Total	4.48 ± 1.06	11.1 ± 1.05	13.0 ± 2.68	17.1 ± 2.34	**	**	
Proelastase 1	2.06 ± 0.40	2.74 ± 0.35	3.51 ± 0.54	3.62 ± 0.37	**		
Procarboxypeptidases	—	—	1.86 ± 0.58	1.08 ± 0.20	—		—
Amylase 1 + 2	25.3 ± 3.17	15.3 ± 1.80	5.77 ± 1.79	2.05 ± 0.28	**	**	
Lipase	2.51 ± 0.14	2.79 ± 0.32	1.27 ± 0.38	1.21 ± 0.20	**		
Digestive enzymes (total)	60.2 ± 3.30	65.7 ± 4.23	55.3 ± 5.79	63.4 ± 4.89			

[a] Values (mean ± SEM) are % intensity of each enzyme spot identified by MALDI-TOF/MS analysis or N-terminal amino acid sequence to the sum of all spots intensity measured by a BAS system. PBD, Pancreatico-biliary–diverted.
*, ** Results of two-way (PBD [P] and diet [D]) or one-way (in the case of no data in more than one group) analysis of variance (ANOVA; $*P < 0.05$, $**P < 0.01$). Dashes indicated no detectable spots. Mean values not sharing a lower case letter are significantly different between groups by post hoc test ($P < 0.05$).

Table 3. Amounts of Digestive Enzymes in the Pancreas as Evaluated by Coomassie Brilliant Blue (CBB) Staining Densities After Separation by Two-Dimensional Gel Electrophoresis in Sham and Bile-Pancreatic Juice-Diverted (PBD) Rats Fed a 25% or 60% (in Total) Casein Diet for Two Weeks[a]

	Sham		PBD		ANOVA		
	25% casein	60% casein	25% casein	60% casein	P	D	P × D
Trypsinogen							
1	9.36 ± 1.09	13.1 ± 1.52	8.54 ± 2.59	6.57 ± 1.36	*		
2-1	—	—	0.53 ± 0.13	2.35 ± 0.34	—	**	—
2-2	0.25 ± 0.05	0.52 ± 0.11	0.72 ± 0.15	1.30 ± 0.21	**	*	
2-3	—	—	1.24 ± 0.33	5.82 ± 0.83	—	**	—
2-4	—	—	1.04 ± 0.37	1.63 ± 0.29	—		—
2-5	0.21 ± 0.04	0.57 ± 0.09	1.16 ± 0.20	1.37 ± 0.34	**		
3	—	—	1.08 ± 0.24	2.32 ± 0.37		*	
4	8.54 ± 0.73a	7.90 ±1.05a	4.94 ± 0.87b	4.09 ± 0.38b	**		**
5	0.30 ± 0.09	0.39 ± 0.08	1.70 ± 0.48	1.73 ± 0.13			
Total	18.8 ± 1.52	22.9 ± 2.40	21.0 ± 4.17	27.2 ± 2.87			
Chymotrypsinogen							
B-1	6.62 ± 0.85	9.85 ± 0.63	4.92 ± 1.08	7.94 ± 1.61		*	
B-2	1.17 ± 0.30b	2.38 ± 0.36a	2.16 ± 0.47a	1.35 ± 0.18b			**
Total	7.79 ± 1.09	12.2 ± 0.81	7.08 ± 1.39	9.29 ± 1.75		*	
Proelastase 1	2.26 ± 0.29	2.53 ± 0.17	3.22 ± 0.48	3.09 ± 0.38	*		
Procarboxypeptidases	1.32 ± 0.24	1.14 ± 0.12	1.93 ± 0.39	1.47 ± 0.16	*		
Amylase 1 + 2	13.8 ± 1.94	11.2 ± 1.46	5.10 ± 1.58	1.55 ± 0.31	**	*	
Lipase	1.72 ± 0.08	1.76 ± 0.20	1.18 ± 0.29	1.04 ± 0.12	**		
Digestive enzymes (total)	45.5 ± 2.54	51.8 ± 4.37	39.5 ± 4.89	43.6 ± 4.80			

[a] Values (mean ± SEM) are % intensity of each enzyme spot identified by MALDI-TOF/MS analysis or N-terminal amino acid sequence to the sum of all spots intensity measured by a BAS system. PBD, Pancreatico-biliary–diverted.
*, ** Results of two-way (PBD [P] and diet [D]) or one-way (in the case of no data in more than one group) analysis of variance (ANOVA; $*P < 0.05$, $**P < 0.01$). Dashes indicated no detectable spots. Mean values not sharing a subscript letter are significantly different between groups by post hoc test ($P < 0.05$).

higher in groups fed HC compared with NC diets for both sham and PBD rats (Fig. 1). The increases induced by feeding the HC diet were much higher in PBD rats than in sham rats. Changes in RNA content were similar to those for weight and protein content in the pancreas, although there were no significant differences between the NC and HC groups in sham rats. Two-way ANOVA showed significant interaction between PBD and diet for each pancreatic marker.

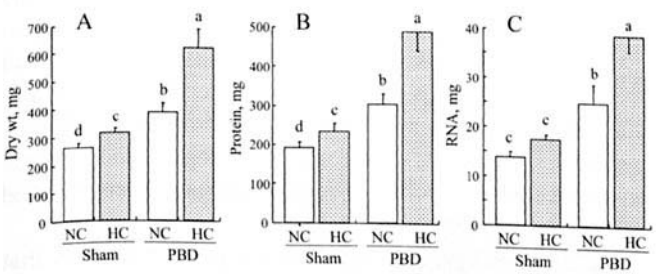


Figure 1. Dry weight (A), protein content (B), and RNA content (C) of pancreas in sham and pancreatico-biliary–diverted (PBD) rats after feeding a low (NC 250 g casein/kg diet) or high-protein diet (HC, 600 g casein/kg diet) for 2 weeks. Values are mean ± SE (six rats in the NC groups and seven rats in the HC groups of both sham and PBD rats). *P* values estimated by two-way analysis of variance (ANOVA) were <0.01 for PBD and diet and <0.05 for PBD × diet in all three variables. Mean values not sharing a letter are significantly different between groups by post hoc test ($P < 0.05$).

Specific activities of trypsin and chymotrypsin (U/mg pancreatic protein) in sham and PBD rats fed HC diet were higher than in both groups of rats fed the NC diet (Fig. 2). Amylase-specific activities changed inversely with serine proteases. Chymotrypsin activities were higher and amylase activities were lower in PBD rats than in sham rats.

Anionic serine protease spots for two-dimensional SDS-PAGE with narrow-range isoelectric focusing (pI 4.5–5.5) detected by CBB staining and [^{35}S] radioactivity are shown in Figure 3A (PBD rats) and B (sham rats). The two upper panels in Figure 3A and B show representative spots detected by CBB staining of pancreatic extract from PBD and sham rats fed NC and HC diets. The lower two panels show spots detected by [^{35}S] radioactivity. CBB staining (upper two panels) and [^{35}S] radioactivity imaging (lower two panels) revealed five trypsinogen 2-x spots in PBD rats, but not in sham rats (Fig. 3A). Trypsinogen 2-x isoforms are numbered from 2-1 (the lowest pI) to 2-5 (the highest pI). The spot densities of trypsinogen 2-1, 2-2, 2-3, but not trypsinogen 2-5, were clearly greater in the HC group compared with those in the NC group. There were no changes in the spots for trypsinogen 1.

We identified nine spots (Fig. 4) by N-terminal amino acid sequence analysis as trypsinogens and chymotrypsinogens. We detected five separate trypsinogen 2-x spots (from 2-1 to 2-5), one spot each of trypsinogen 1 and 5, and

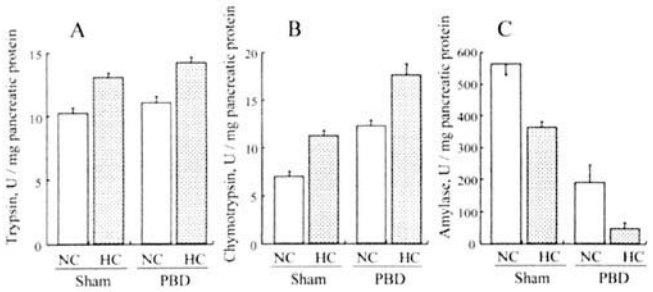


Figure 2. Trypsin (A), chymotrypsin (B), and amylase (C) activities in the pancreas of sham and pancreatico-biliary–diverted (PBD) rats after feeding a low- (NC 250 g casein/kg diet) or high-protein diet (HC, 600 g casein/kg diet) for 2 weeks. Values are mean ± SE (six rats in the NC groups and seven rats in the HC groups of both sham and PBD rats). *P* values estimated by two-way analysis of variance (ANOVA) were <0.05 for PBD and <0.01 for diet in trypsin (A), and <0.01 for PBD and diet in chymotrypsin (B) and amylase (C). All *P* values for PBD × Diet were >0.05 in all three variables.

two chymotrypsin B spots. The N-terminal sequences of the five trypsinogen 2-x spots were identical except for one spot numbered trypsinogen 2-4. In the trypsinogen 2-4 molecules, the third valine from the N-terminal was replaced by isoleucine in the known amino acid sequence of rat trypsinogen 2-x (29).

Relative synthetic rates of digestive enzymes were evaluated by percentages of [^{35}S]-L-methionine radioactivities incorporated into each enzyme spot against total spot activity (Table 2). The sum of the identified digestive enzyme spot radioactivity was 55%–65% of the total radioactivity (total pancreatic protein synthesis). We identified nine trypsinogen isoform spots including five trypsinogen 2-x spots by radioactivity in PBD rats as shown in Figure 4 (labeled from trypsinogen 2-1 to 2-5 or as trypsinogen 2-x); however, we found trypsinogen 1 and 4, but not trypsinogen 2-x or 3, in sham rats. The trypsinogen 2-x and 3 spots in the PBD rats fed an HC diet were greater than in those fed an NC diet except for the trypsinogen 2-5 spot. In contrast, there were no differences in the large trypsinogen 1 and 4 spots. BPD, but not diet, influenced these two trypsinogen isoforms found in both sham and PBD rats.

Two chymotrypsinogen B spots were identified, and radioactivity of the larger (lower pI) chymotrypsinogen B spot and sum of these two spots were influenced by both PBD and diet. Radioactivity of the amylase spots was also influenced by both PBD and diet, and changes were reciprocal to those of the serine proteases. Proelastase and lipase were affected by PBD alone.

Changes in the CBB spot densities in two-dimensional SDS-PAGE were similar to those in spots detected by [^{35}S] radioactivity, except for the trypsinogen 2-2 and 2-5 spots (Table 3). These two trypsinogen 2-x isoforms were also detected in sham rats by CBB staining. The sum of identified digestive enzyme spots stained by CBB was 40%–50% of the total spot density, which was lower than that evaluated by [^{35}S] radioactivity. The results of two-way ANOVA show that the trypsinogen 2-x isoforms (2-2 and 2-5) were influenced by PBD. The large spots of trypsinogen isoforms 1 and 4 were also influenced by PBD, but not by diet. Diet influenced trypsinogen 2-2 and trypsinogen 3 among trypsinogen isoforms, as well as chymotrypsinogen and amylase spot densities.

The ratios of HC to NC dietary groups for each enzyme spot for both [^{35}S] radioactivity and CBB density are summarized in Table 4 as an indicator of induction by feeding a high-protein diet. The ratio of each enzyme for CBB density was similar to that for [^{35}S] radioactivity. Regarding the [^{35}S] radioactivity of the five trypsinogen 2-x molecules in PBD rats, the degrees of induction of trypsinogen 2-1 and 2-3 were highest, those of trypsinogen 2-2 and 2-4 were intermediate, and that of trypsinogen 2-5 was lowest.

Discussion

The present study shows that pancreatic dry weight, protein, and RNA content were increased in PBD rats, and that these changes were further enhanced with the feeding of a high-protein diet. Changes in pancreatic proteases, especially chymotrypsin activity, were similar to those for dry weight, protein, and RNA content. These results agree with our previous studies (30). We have already shown that these increases are dependent on CCK action (12). The pancreatic growth and protease induction in PBD rats show dietary protein-mediated CCK release from the small intestine is responsible for the observed pancreatic adaptations.

In this paper we identified five evident spots as trypsinogen 2-x isoforms in PBD rats fed a 60% casein diet by two-dimensional SDS-PAGE with narrow-range isoelectric focusing. The spots were detected by both CBB staining and [^{35}S]methionine incorporation. The trypsinogen 2-x and 3 spots, but not the trypsinogen 1 and 4 spots, were increased by feeding the high-protein diet to PBD rats. The latter two isoforms are the major trypsinogen molecules in sham and PBD rats fed a 25% casein diet. The minor isoform, trypsinogen 2-x, became the major isoform in PBD rats fed a high-protein diet. The sum of the five trypsinogen 2-x spots accounts for nearly one-half of the total trypsinogens in terms of pancreatic content (CBB staining) and relative rate of synthesis ([^{35}S] radioactivity). Differences in the changes in anionic trypsinogens and cationic trypsinogens in response to stimulation have been reported previously (13–16).

The more important result in the present study is that the degree of change induced by feeding a high-protein diet varied largely among the five trypsinogen 2-x molecules. We calculated the intensity ratios of the 60% casein to the 25% casein groups (HC/NC) for each trypsinogen 2-x spot (Table 4). The lowest ratio for [^{35}S] radioactivity was 1.3 (trypsinogen 2-5), and the highest ratio was 4.8 (trypsinogen 2-3). The intensity ratios of the CBB-stained spots were

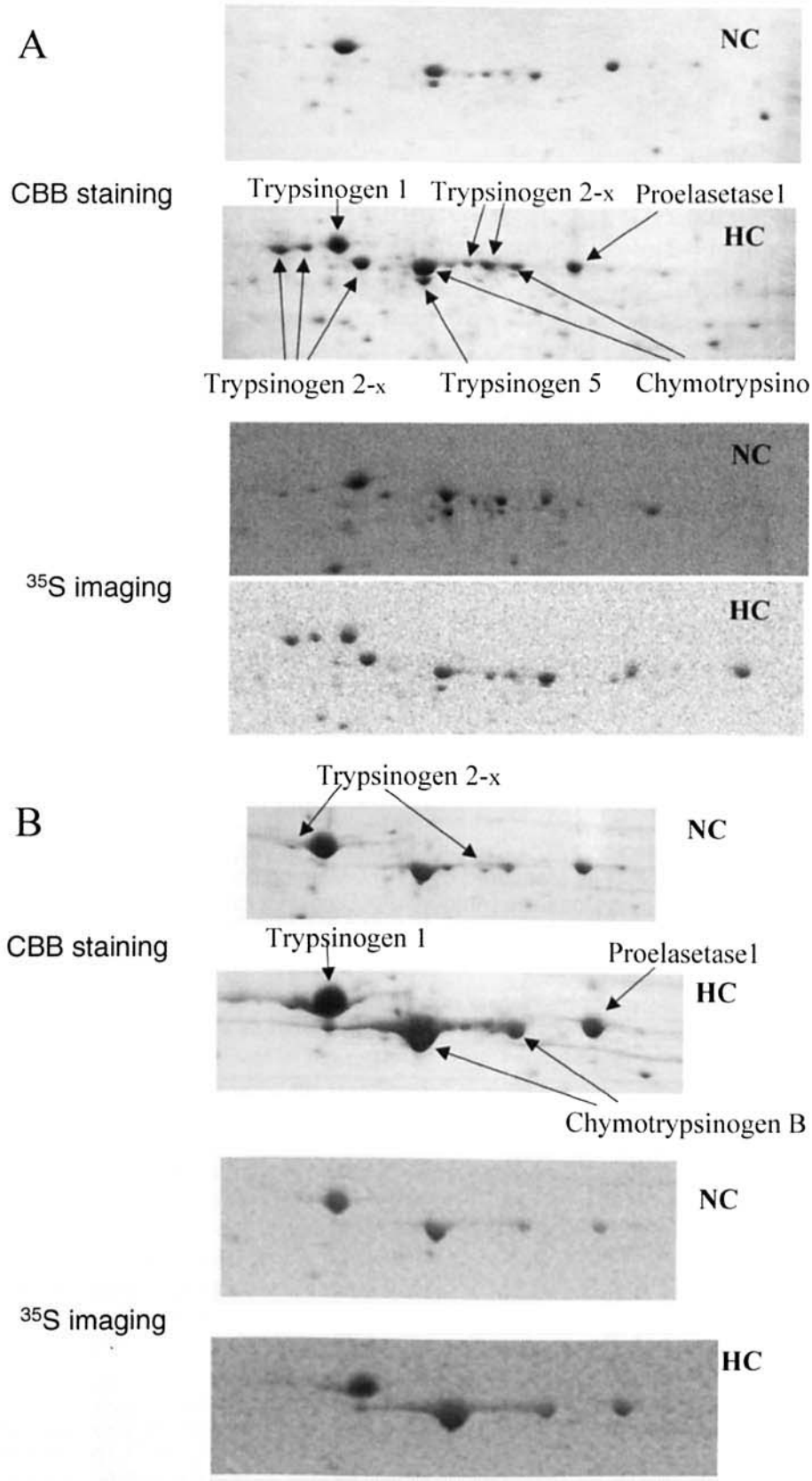
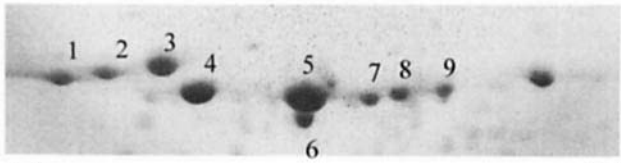


Figure 3. Representative images of two-dimensional SDS-polyacrylamide electrophoresis with narrow-range isoelectric focusing (pI 4.4–5.5) of extracts from the pancreas of PBD (A) and sham (B) rats fed a low- (NC, 250 g casein/kg diet) or high-protein diet (HC, 600 g casein/kg diet) for 2 weeks. The upper two panels show images obtained by CBB staining, and the lower two panels show images obtained by [^{35}S] radioactivity.



		5 10 15 20 25
1	Trypsinogen 2-1	FPVDDDDKIVGGYTXQENSVPYQVSLN
2	Trypsinogen 2-2	FPVDDDDKIVGGYTXQE
3	Trypsinogen 1	FPLEDDDKIV
4	Trypsinogen 2-3	FPVDDDDKIVGGYTXQENSVPYQVSLN
5	Chymotrypsinogen B-1	XGVPTIQPVLT
6	Trypsinogen 5	FPTEDNDDRI
7	Trypsinogen 2-4	FPIDDDDKIVGGYTXQENSVPYQV
8	Trypsinogen 2-5	FPVDDDDKIVGGYTXQEN
9	Chymotrypsinogen B-2	XGVPTIQPVLT

Figure 4. N-terminal amino acid sequences of anionic trypsinogens and chymotrypsinogens separated by two-dimensional SDS-polyacrylamide electrophoresis with narrow-range isoelectric focusing (pI 4.4–5.5) of pancreatic extracts.

similar to those for [^{35}S] radioactivity. We have not identified mRNA corresponding to five trypsinogen 2-x isoforms. Identification and quantification of these mRNAs will reveal whether transcriptional and/or translational control is involved in the various changes in trypsinogen 2-x synthesis induced by feeding HC.

Post-translational modification, such as glycosylation, is possibly involved in the separation of the five trypsinogen 2-x spots. However, we did find an amino acid replacement in trypsinogen 2-4 from valine to isoleucine in the third position from the N-terminal. We also have observed the same five spots of trypsinogen 2-x in Wistar PBD rats fed a

Table 4. Degree of Induction by Feeding a 60% Casein Diet (Ratios of Values in the 60% Casein Groups [HC] to Those in the 25% Casein Group [NC]) as Measured by [^{35}S]-L-Methionine Incorporation (Synthesis Rate) and Coomassie Brilliant Blue (CBB) Staining Densities (Amounts) of Digestive Enzymes in the Pancreas of Sham and Bile-Pancreatic Juice-Diverted (PBD) Rats[a]

	[^{35}S]-L-methionine incorporation		CBB staining density	
	Sham HC/NC	PBD HC/NC	Sham HC/NC	PBD HC/NC
Trypsinogen				
1	1.36	0.83	1.40	0.77
2-1	—	3.67	—	4.43
2-2	—	1.91	2.08	1.81
2-3	—	4.18	—	4.69
2-4	—	1.80	—	1.57
2-5	—	1.29	2.71	1.18
3	—	1.89	—	2.15
4	1.26	0.74	0.93	0.83
5	—	1.22	1.30	1.02
Total	1.31	1.29	1.22	1.30
Chymotrypsinogen				
B-1	2.47	1.71	1.49	1.61
B-2	2.48	0.63	2.03	0.63
Total	2.47	1.34	1.57	1.31
Proelastase 1	1.33	1.03	1.12	0.96
Procarboxypeptidases	—	0.58	0.86	0.76
Amylase 1 + 2	0.60	0.36	0.81	0.30
Lipase	1.11	0.95	1.02	0.88

[a] Values are the ratio of the means of HC to NC.

high-protein diet (data not shown). These results strongly suggest the possible existence of genes or at least mRNAs corresponding to the five trypsinogen 2-x spots.

Identification of several different trypsinogen 2-x molecules and the different expression of these trypsinogen isoforms were achieved by using very narrow-range (pI 4.5–5.5) isoelectric focusing. Borgström *et al.* (13) reported that PBD increased the ratio of anionic to cationic trypsinogen. Our results demonstrated that specific increases in several trypsinogen 2-x molecules are responsible for the increment of anionic trypsinogen. Schick *et al.* (14) examined the induction of trypsinogen 1, 2-x, and 3 isoforms by feeding graded levels of protein in normal rats. They showed that the trypsinogen 2-x level was very low, and trypsinogen 1, but not trypsinogen 4 (named trypsinogen 3 in that report), was changed by increases in dietary protein. These results agree with our observations in normal rats (Table 3). This agreement with the previous report indicates the reliability of our observations by using two-dimensional electrophoresis with a combination of narrow- and wide-range isoelectric focusing.

Increases in the sum of all trypsinogen isoforms in CBB staining by feeding a high-protein diet (Table 3) should correspond to increases in trypsin activity (Fig. 2). In sham rats, the sum of the spot densities of the trypsinogen isoforms was increased 22% and trypsin activity was increased 27% by feeding a high-protein diet. In PBD rats, the increment was 30% for spot density and 28% for activity. Changes in chymotrypsinogen and amylase showed a similar correspondence. These results also indicate the reliability of the results obtained by two-dimensional electrophoresis.

The degree of increase or decrease in each enzyme spot by feeding a high-protein diet was similar for CBB and [^{35}S] radioactivity, especially in PBD rats (Table 4). This important result shows that changes in digestive enzyme content in the pancreas is coincident with those in enzyme synthesis and reveals that enzyme content in the pancreas reflects their rate of synthesis, at least under the conditions tested in the present study.

In conclusion, five different protein molecules of trypsinogen 2 were found in PBD rats by using two-dimensional SDS-PAGE with very narrow-range isoelectric focusing, and feeding a high-protein diet variously induced these trypsinogen 2-x proteins. These results concerning differences in the induction of trypsinogen isoforms in PBD rats contribute to a better understanding of pancreatitis induced by hyperCCKemia.

1. Grossman MI, Greengard H, Ivy AC. The effect of dietary composition on pancreatic enzymes. Am J Physiol 138:676–682, 1942.
2. Dagorn JC, Lahaie RG. Dietary regulation of pancreatic protein synthesis. I. Rapid and specific modulation of enzyme synthesis by changes in dietary composition. Biochim Biophys Acta 654:111–118, 1981.
3. Giorgi D, Renaud W, Bernard JP, Dagorn JC. Regulation of proteolytic enzyme activities and mRNA concentrations in rat pancreas by food content. Biochem Biophys Res Commun 127:937–942, 1985.
4. Green GM, Jurkowska G, Berube FL, Rivard N, Guan D, Morisset J. Role of cholecystokinin in induction and maintenance of dietary protein-stimulated pancreatic growth. Am J Physiol 262:G740–G746, 1992.
5. Hara H, Narakino H, Kiriyama S. Enhancement of pancreatic secretion by dietary protein in rats with chronic diversion of bile-pancreatic juice from the proximal small intestine. Pancreas 9:275–279, 1994.
6. Hara H, Narakino H, Kiriyama S. Gastric acid-independent enhancement of exocrine pancreatic secretion by dietary protein in chronic bile-pancreatic juice diverted rats. Pancreas 11:173-178, 1995.
7. Nishi T, Hara H, Hira T, Tomita F. Dietary protein peptic hydrolysates stimulate cholecystokinin release via direct sensing by rat intestinal mucosa NC cells. Exp Biol Med 226:1031–1036, 2001.
8. Fushiki T, Kajiura H, Fukuoka S-I, Kido K, Semba T, Iwai K. Evidence for an intraluminal mediator in rat pancreatic enzyme secretion. Reconstitution of the pancreatic response with dietary protein, trypsin and the monitor peptide. J Nutr 119:622–627, 1989.
9. Li L, Louie D, Owyang CA. Cholecystokinin releasing peptide mediates feedback regulation of pancreatic secretion. Am J Physiol 256:G430–G435, 1989.
10. Miyasaka K, Guan D, Liddle R, Green GM. Feedback regulation by trypsin, evidence for intraluminal CCK-releasing peptide. Am J Physiol 257:G175–G181, 1989.
11. Hara H, Ohyama S, Hira T. Luminal dietary protein, not amino acids, induces pancreatic protease via CCK in pancreaticobiliary-diverted rats. Am J Physiol 278:G937–G945, 2000.
12. Nishi T, Hara H, Aoyama Y. Guanidinated casein hydrolysate stimulation of cholecystokinin release *via* pancreatic enzyme- and cholinergic-independent mechanisms in rats. Biosci Biotech Biochem 63:1070–1074, 1999.
13. Borgström A, Axelson J, Ihse I, Rehfeld JF. The ratio between anionic and cationic trypsin in rat pancreas varies with CCK stimulation. Pancreas 11:179–184, 1995.
14. Schick J, Kern H, Scheele G. Hormonal stimulation in the exocrine pancreas results in coordinate and anticoordinate regulation of protein synthesis. J Cell Biol 99:1569–1574, 1984.
15. Schick J, Verspohl R, Kern H, Scheele G. Two distinct adaptive responses in the synthesis of exocrine pancreatic enzymes to inverse changes in protein and carbohydrate in the diet. Am J Physiol 247:G611–G616, 1984.
16. Steinhilber W, Poensgen J, Rausch U, Kern HF, Scheele GA. Translational control of anionic trypsinogen and amylase synthesis in rat pancreas in response to cerulein stimulation. Proc Natl Assoc Sci U S A 85:6597–6601, 1988.
17. Reeves PG, Nielsen FH, Fahey GJ. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951, 1993.
18. Williamson RC, Bauer FL, Ross JS, Watkins JB, Malt RA. Enhanced colonic carcinogenesis with azoxymethane in rats after pancreaticobiliary diversion to mid small bowel. Gastroenterology 76:1386–1392, 1979.
19. MacDonald RJ, Stary SJ, Swift GH. Two similar but nonallelic rat pancreatic trypsinogens. Nucleotide sequences of the cloned cDNAs. J Biol Chem 257:9724–9732, 1982.
20. Lutcke H, Rausch U, Vasiloudes P, Scheele GA, Ker HF. A fourth trypsinogen (p23) in the rat pancreas induced by CCK. Nucl Acids Res 17:6736, 1989.
21. Fletcher TS, Alhadeff MA, Craik CS, Largman C. Isolation and characterization of a cDNA encoding rat cationic trypsinogen. Biochemistry 26:3081–3086, 1987.
22. Lowry OH, Rosebrough HJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. J Biol Chem 193:265–275, 1951.

23. Sugawara K. Influence of triton X-100 on protein determination by Lowry procedure. Agric Biol Chem 93:2429–2430, 1975.
24. Kerr SE, Seraidarian K. The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J Biol Chem 161:293–303, 1945.
25. Fleck A, Munro HN. The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. Biochim Biophys Acta 55:571–583, 1962.
26. Rick W. Trypsin. In: Bergmeyer HU, Ed. Methods of Enzymatic Analysis, Vol. 2, 2nd ed. New York: Academic Press, pp1013–1024, 1976.
27. Rick W. Chymotrypsin. In: Bergmeyer HU, Ed. Methods of Enzymatic Analysis, Vol. 2, 2nd ed. New York: Academic Press, pp1006–1012, 1976.
28. Jung DH. Preparation and application of procion yellow starch for amylase assay. Clin Chim Acta 100:7–11, 1980.
29. Craik CS, Choo QL, Swift GH, Quinto C, MacDonald RJ, Rutter WJ. Structure of two related rat pancreatic trypsin genes. J Biol Chem 259:14255–14264, 1984.
30. Hara H, Ochi Y, Kasai T. Bile-pancreatic juice-independent increases in pancreatic proteases and intestinal cholecystokinin by dietary protein in rats. Proc Soc Exp Biol Med 217:173–179, 1998.