In vivo evaluation of free chymotrypsin activity in the lumen using benzoyl-L-tyrosyl-p-aminobenzoic acid in portal cannulated rats

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In vivo chymotrypsin activity in lumen was estimated by portal absorption of p-aminobenzoic acid (PABA) in the portal cannulated rats after feeding of diets containing benzoyl-L-tyrosyl-PABA (BT-PABA), to predict protease activities regarding the negative feedback regulation of exocrine pancreatic secretion. The PABA concentrations of portal plasma depending on hydrolysis of BT-PABA in the lumen was maximum at 20 min after feeding of both an 8% casein and an 8% soybean protein isolate (SPI) diet containing 3% BT-PABA. The concentrations of the casein group were decreased more rapidly, and were significantly lower at 80 and 100 min than that of the SPI group. The concentrations were minimum at 120 min in both the groups, and then increased rapidly in the casein group. Casein, SPI, and their peptic hydrolysates inhibit in vitro chymotrypsin-catalyzed hydrolysis of BT-PABA more than 50%. Thus, the rapid decrease in the portal concentration of PABA may depend on the competition with the dietary proteins or their hydrolysates in the lumen. The inhibition of in vitro hydrolysis by SPI and its hydrolysate were a little stronger than that by casein and its hydrolysate, respectively. The discrepancy between in vivo and in vitro BT-PABA hydrolysis suggests that factors other than the competition also affect the in vivo hydrolysis of BT-PABA. High activities of chymotrypsin in the lumen at 20 min after feeding may be able to inhibit the exocrine pancreatic secretion, and low level of the protease activity in the casein group at 120 min may be able to enhance the secretion by removal of the negative feedback mechanism.

Keywords: chymotrypsin; portal absorption; casein; soybean protein; benzoyl-L-tyrosyl-p-aminobenzoic acid; rats

Introduction

We previously demonstrated the different digestibility of oligo-L-methionine (OM), which is slightly digestible peptide, between the rats fed a low casein and a low soybean protein isolate (SPI) diet.[1,2] The difference of OM digestibility between both the diets may depend on the intraluminal proteolytic activities. A purpose of the present studies is the estimation of in vivo activities of chymotrypsin in the lumen after feeding of both the low protein diets under the same dietary regimens with the OM study. Chymotrypsin is one of the proteases which is able to degrade OM.

The in vivo activity of chymotrypsin in the lumen was evaluated by the portal absorption of p-aminobenzoic acid (PABA) derived from the intraluminal hydrolysis of benzoyl-L-tyrosyl p-aminobenzoic acid (BT-PABA), which is a synthetic substrate of chymotrypsin used for diagnosis of the pancreatic function.[3,4] Liberated PABA by the hydrolysis of BT-PABA in the lumen is absorbed by passive diffusion mechanism, and PABA absorbed is not recycled to the intestinal blood flow because PABA is acetylated simultaneously in the liver. So, the portal concentration of PABA reveals the absorption of PABA, which depends on the hydrolysis of BT-PABA in the small intestinal lumen.

The factors influencing the luminal hydrolysis of

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BT-PABA are the competition with the dietary proteins on the chymotrypsin-catalyzed hydrolysis, the concentration of BT-PABA in the small intestinal lumen mainly depending on the gastric emptying, and the concentration of active chymotrypsin.

The activity of endopeptidases in the lumen regulate the exocrine pancreatic secretion by negative feedback mechanism, which is known as the regulatory mechanism of the pancreatic secretion by dietary protein.[5-10] In vivo free chymotrypsin activity in the lumen as a result of the combination of many factors estimates the protease activities regarding the negative feedback regulation of the exocrine pancreas. This is the second purpose of this study.

We also observed the hydrolysis of BT-PABA by upper small intestinal chyme in vitro to examine the existence of hydrolytic activity of BT-PABA directly, and the competition with the dietary proteins in chymotrypsin-catalyzed hydrolysis of BT-PABA in vitro.

Materials and methods

Diet

The composition of the stock diet (25% casein diet) and 8% protein diet were shown in *Table 1*. The 8% protein diets were prepared containing 8% net protein (Protein = $N \times 6.25$), to which 9.4% of casein material (ALACID; New Zealand Dairy Board, Wellington, NZ) or 9.6% soybean protein isolate (SPI; Fujipro R; Fuji Oil Co., Osaka, Japan) was added. Nitrogen contents of both materials were estimated by the semimicro Kjeldahl method. The diets for the observations of in vivo hydrolysis of benzoyl-L-tyrosyl p-aminobenzoic acid (BT-PABA) were prepared with addition of 3% BT-PABA to both the 8% protein diets, or with the addition of 0.5% or 1% p-aminobenzoic acid (PABA) to 8% casein diet for control study. Benzoyl-L-tyrosyl PABA and PABA were obtained from Eizai Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industry (Osaka, Japan), respectively.

Table 1 Composition of diets

	Stock diet	8% Casein[f] diet	8% SPI[f] diet
	%	%	%
Casein[a]	25.0	9.4	—
SPI[a]	—	—	9.6
Sucrose	63.1	78.5	78.3
Corn oil[b]	5.0	5.0	5.0
Mineral mixture[c]	4.0	4.0	4.0
Vitamin mixture[d]	1.0	1.0	1.0
Granulated vitamin E[e]	0.1	0.1	0.1
Choline chloride	2.0	2.0	2.0

[a] Casein and SPI contained 13.7% and 13.4% nitrogen, respectively.
[b] Retinyl palmitate (6000 IU/kg diet) and ergocalciferol (800 IU/kg diet) were added to the corn oil.
[c] The mineral mixture is identical to the mineral mixture (MM2) described by Ebihara, Imanura, and Kiriyama.[11] It provided (mg/kg diet); Ca, 4491; P, 2997; K, 3746; Mg, 375; Fe, 38.0; I, 0.31; Mn, 81.1; Zn, 25.9; Cu, 15.3; Na, 4342; Cl, 6678; Se, 0.27; Mo, 1.12; Cr, 0.49; B, 0.35; V, 0.22; Sn, 1.05; As, 1.20; Si, 15.7; Ni, 3.00; F, 2.71; Co, 0.20.
[d] The vitamin mixture was prepared in accordance with the AIN-76 mixture[12] except that menadione and L-ascorbic acid were added to make a 1 mg/kg[13] and 50 mg/kg[14] diet, respectively.
[e] Vitamin E (Yuvela, Eizai Co., Tokyo, Japan) supplied 200 mg all-rac-α-tocopheryl acetate in one kg diet.
[f] Three percent of BT-PABA, 0.5% PABA, or 1% PABA added to these basal diets in concurrence with a removal of a corresponding amount of sucrose.

Experiment 1: In vivo hydrolysis of BT-PABA

Male Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan) were housed in individual cages in a temperature-controlled room throughout the experiment. After a 3-day feeding of the stock diet, rats weighing 230–250 g were operated on for implantation of portal cannula under sodium pentobarbital anesthesia (40 mg/kg body weight, Nembutal, Abbott, North Chicago, IL). The catheters (polyethylene tube, sp 28, Natsume Seisakusho, Tokyo, Japan) were directly inserted into the portal vein. The details were described previously.[15] After a 3-day recovery period with the stock diet and a 24-hr fast, 18 operated rats were divided into four groups. The 12 rats in the two groups were fed 2 g of an 8% casein or an 8% SPI diet containing 3% BT-PABA, and six rats of the other two groups were fed 2 g of an 8% casein diet containing 0.5% or 1% PABA for a control study. The portal blood was collected through the implanted cannula with the specified intervals of 20 or 40 min after feeding for 4 hr. Forty microliters of blood were sampled at a time.

Experiment 2: Hydrolysis of BT-PABA by the intestinal chyme

After a 7-day feeding of the stock diet, six Sprague-Dawley male rats weighing 260–280 g were divided into two groups and were fed an 8% casein or an 8% SPI diet. Twenty min after feeding of the diets, the rats were sacrificed by decapitation, the abdomens were opened, and the whole quantity of the chyme from the duodenum and upper jejunum was drawn by a syringe with a needle (18G Terumo Co., Tokyo, Japan). The volume of the chyme was measured, and the chyme was frozen quickly by liquid nitrogen. The frozen chyme was preincubated for 1 min to thaw and warm, and concentrated BT-PABA solution (1/10 vol of the collected chyme) was added to the preincubated chyme up to 4.9 mM in the reaction medium. The hydrolysis reaction was performed at 37° C. The chyme volumes were 0.49 ± 0.05 ml in the casein fed group and 0.35 ± 0.03 ml in the SPI fed group.

Experiment 3: In vitro hydrolysis of BT-PABA by chymotrypsin

The hydrolytic rate of BT-PABA (4.9 mM) by chymotrypsin (C-4129, Sigma Chemical Co., St. Louis, MO) under the presence of casein, SPI, or their peptic hy-

drolysate was determined in 80 mM Tris buffer (pH 7.8) at 37° C. We confirmed previously that the velocity of the hydrolysis of BT-PABA is proportional to the concentration of chymotrypsin up to 3.3 U/ml in reaction medium and to the incubation time up to 30 min. We measured the amount of PABA liberated from BT-PABA for 15 and 30 min with casein, SPI, or their hydrolysates on the concentrations of 0.25, 0.5, and 1%, respectively, in the medium containing 3 U/ml chymotrypsin. Tyrosyl and phenylalanyl residues in 1% casein and SPI solution are 8.6 mM/l and 9.8 mM/l, respectively. The concentration of major susceptible sites for chymotrypsin in 0.5% SPI solution is equal to that in 4.9 mM BT-PABA solution.

Analyses

The concentrations of PABA in plasma (experiment 1) and reaction medium (experiments 2 and 3) were measured by HPLC as phenylthiocarbamyl (PTC) derivatives with phenylisothiocyanate (Tokyo Kasei Kogyo, Tokyo, Japan).[16,17] The liquid chromatography system was constructed by Mini-Solvent Delivery System M 600 (Waters Assoc., Milford, MA) and PICO-TAG column (15 × 3.9 mm, Waters Assoc.).

Calculation and statistics

We calculated the percentages of BT-PABA absorbed from the intestine for 240 min to the amount of BT-PABA intake (absorptive efficiency). The changes in PABA concentration of portal plasma after feeding of the diet containing 0.5% or 1% free PABA, and the graph plotting the area under the curves of PABA concentration against PABA% in the diets, are shown in *Figure 1*. The number of PABA residues in 3% BT-PABA diet is equivalent to that in 1% free PABA diet. The absorptive efficiency was evaluated by fitting of the area under the curve of PABA concentration in the portal plasma of the rats fed the BT-PABA diet on the slope of *Figure 1* (lower panel). The absorption of PABA nearly finished in both 0.5% and 1% PABA diet up till 240 min after feeding. Therefore, we used the calibration curve (*Figure 1*, lower) for both casein and SPI test diets. Inhibitory action of the dietary proteins on the hydrolysis of BT-PABA in vitro are presented as percent activities, which are the rate of the amount of released PABA with the various concentration of proteins or hydrolysates to the amount of released PABA without them.

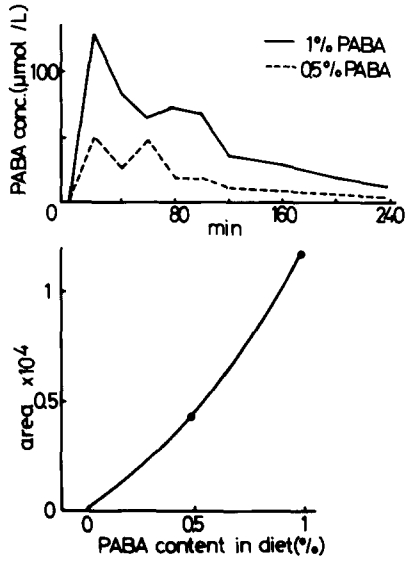

Figure 1 The changes in PABA concentration after feeding of diet containing 0.5% or 1% PABA (upper) and in calibration curve to estimate the absorption efficiency of BT-PABA (lower). The calibration curve is plotted with the values of area under the curves of the upper panel (μmol/l × min) against the content of free PABA in the diet (%).

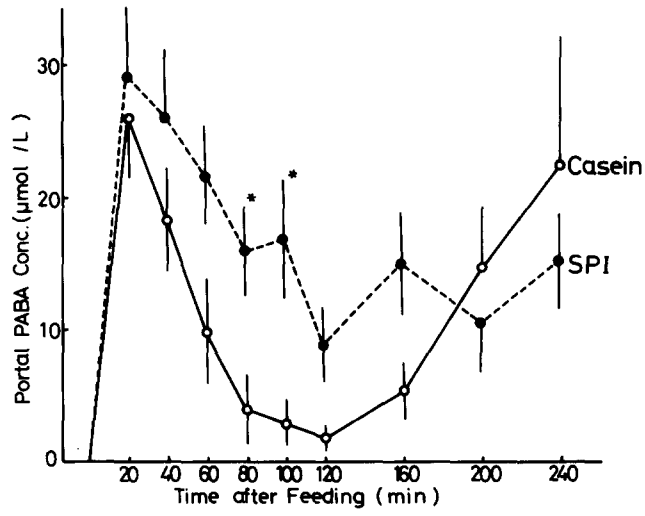

Figure 2 The changes in the PABA concentration of portal plasma after feeding of an 8% casein or an 8% SPI diet containing 3% BT-PABA. From the results of two-way ANOVA, "time" and "diet" were significantly different ($P < 0.05$), and "time × diet" was not significant. Asterisks represent the significant difference between the casein and the SPI group at each time.

Statistical analyses were performed by one-way and two-way ANOVA. Significant differences between the average values of casein and SPI fed groups or among the different times were determined by the LSD method. The results are presented in the figure legends.

Results

In *Figure 2*, the changes in PABA concentrations of portal plasma after feeding of an 8% casein or an 8% SPI diet containing 3% BT-PABA are shown. The concentrations in both the groups were maximum at 20 min and minimum at 120 min after feeding. The level of PABA in the casein group was decreased more rapidly after 20 min, and was significantly lower at 80 and 100 min than that in the SPI group. The value at 120 min was reduced to 7.8% of the value at 20 min and then increased rapidly in the casein-fed group. *Table 2* shows the area under the curves of PABA concentrations of portal plasma after feeding of the casein-based or the SPI-based diet. The value of 60–120 min in the SPI group was 5-fold higher than

Table 2 Area under the curve of portal PABA concentration after feeding of the test diets containing 3% BT-PABA and absorptive efficiency of BT-PABA

Min	0–60	60–120	0–120	0–240	Absorptive efficiency
		× 10^3 (μmol/l × min)			%
Casein	0.98 ± 0.17	0.12 ± 0.07	1.10 ± 0.28	2.17 ± 0.36	28.6
SPI	1.32 ± 0.19	0.58 ± 0.14*	0.90 ± 0.30	2.92 ± 0.48	37.7

Note: Absorptive efficiency is evaluated by fitting the values of the area under the curves of PABA concentration from 0 to 240 min (*Figure 2*) on the curve of *Figure 1* (lower). Values are mean ± SEM, (n = 6).
* Significant difference between the casein and the SPI group ($P < 0.02$).

that in the casein group. The percentages of BT-PABA absorbed from the intestine to the intake of BT-PABA, which was evaluated by using the slope of *Figure 1*, were 28.6% and 37.7% until 240 min after feeding in the casein and SPI groups, respectively.

As shown in *Figure 3*, in vitro hydrolysis of BT-PABA by the chyme of the upper small intestine at 20 min after feeding of an 8% casein or an 8% SPI diet was very high, because the time course of the increment of liberated PABA was not linear from the early stage of the reaction. The hydrolysis for 60 min incubation tended to be higher in the chyme of the rats fed the SPI diet than that of the rats fed the casein diet, but the values corrected by chyme volumes were similar between both the groups.

The competitive action of casein, SPI, and their peptic hydrolysates on the chymotrypsin-catalyzed hydrolysis of BT-PABA in vitro are shown in *Figure 4*. The decreasing curves of percent activities with the increasing concentration of the pepsin hydrolysates were similar to the curves of the intact proteins for 15 min incubation (left panel) or for 30 min incubation (right panel). The inhibitory action of SPI and its hydrolysate in the hydrolysis of BT-PABA was a little stronger than that of casein and its hydrolysate, respectively. The percent activity with 0.5% level of protein or hydrolysates was about 30–60%, which represents that the affinity of chymotrypsin for these proteins is comparable to that for BT-PABA because the number of the sensitive sites for chymotrypsin in 0.5% solution of these materials is almost the same as that in 4.9 mM BT-PABA.

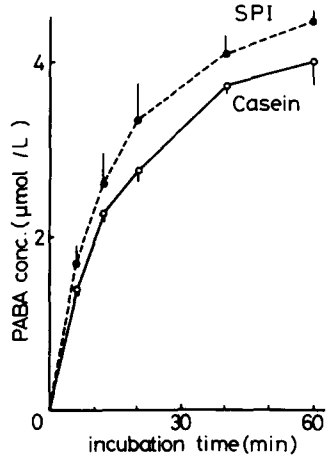

Figure 3 The hydrolytic activities of BT-PABA in the chyme of upper small intestine at 20 min after feeding of an 8% casein or an 8% SPI diet. The values represent the PABA concentration liberated from BT-PABA in the reaction mixture. Vertical bars represent the SEM (n = 3).

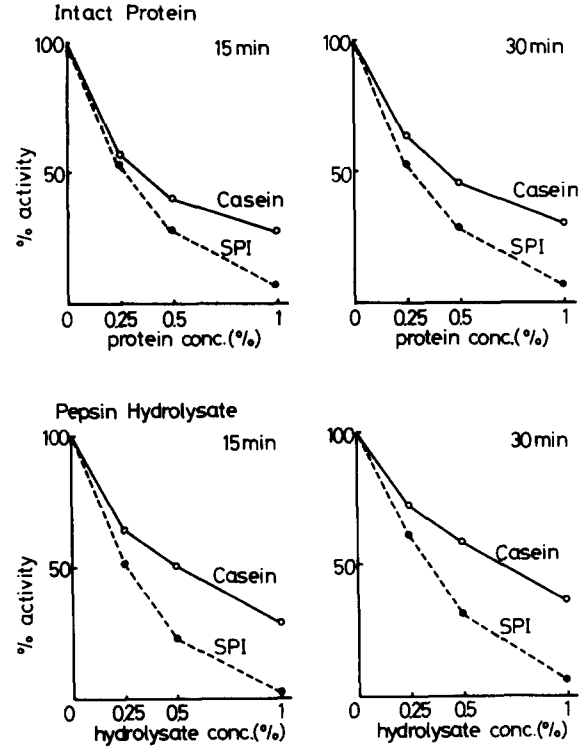

Figure 4 The effects of casein, SPI, and their peptic hydrolysates on in vitro hydrolysis of BT-PABA (4.9 mM) by chymotrypsin (3 U/ml). The hydrolytic reaction was performed in Tris buffer (pH 7.8) at 37° C, and the results showed the percentages of liberated PABA under 0.25–1.00% protein concentrations to the values without protein (% activity) for 15 min and 30 min. The values are the average of two assays.

Discussion

We observed that the absorption of PABA derived from the hydrolysis of BT-PABA, which represents the in vivo activity of chymotrypsin in the lumen, was decreased abruptly from the maximum level at 20 min

after feeding of the casein diet, and then became significantly lower at 60–80 min than that after feeding of the SPI diet. Berger and Schneeman[18] reported that intraluminal activities of carboxypeptidase A and B of SPI-fed rats are higher than that of casein-fed rats 1 hr after feeding, which agrees with our results of chymotrypsin activity in the lumen.They describe that the difference between casein and SPI depends on the pancreatic secretion, which is enhanced by the trypsin inhibitor contained in each material.

The in vivo activity of chymotrypsin evaluated by hydrolysis of BT-PABA in the intestinal lumen is affected by many factors including the pancreatic secretion. The major factors other than the pancreatic secretion are the BT-PABA concentrations in the lumen resulting from the movement of BT-PABA along the gastrointestinal tract and the competition with the dietary protein on chymotrypsin-catalyzed hydrolysis. We observed previously that the gastric emptying rate of free PABA was not slowed markedly 20 min after feeding of a low casein or a low SPI diet containing 1% free PABA and was slightly faster in the casein group under the same feeding conditions with the present study.* The gastric emptying of BT-PABA may be similar to free PABA. This reveals that the rapid decrease after 20 min and the difference between the casein and SPI fed group in the in vivo hydrolysis of BT-PABA did not depend on the gastric emptying of BT-PABA. To confirm this phenomenon, the study of the direct challenge of BT-PABA into the small intestine is necessary.

As shown in *Figure 4*, the dietary proteins and their hydrolysates compete with BT-PABA on the chymotrypsin hydrolysis, which can explain the decrease of the hydrolysis of BT-PABA in vivo in the lumen nearing 120 min after feeding, in part. But, the more rapid decrease in the casein-fed group than in the SPI-fed group is not explained by the competition with the proteins because the inhibition of SPI or its peptic hydrolysate on the hydrolysis of BT-PABA was moderately stronger than that of casein or its hydrolysate, respectively. The discrepancy between in vivo and in vitro effect of dietary proteins on BT-PABA hydrolysis may be related to other factors affecting the free protease activity in the lumen, which are chymotrypsin secretion, chymotrypsinogen activation, and chymotrypsin inactivation in the lumen. Green and Nasset[19] described that bile relates to protease inactivation. Intestinal transit, intraluminal pH, or rate of protein hydrolysis may also affect the in vivo BT-PABA hydrolysis.

Richter and Schneeman reported that SPI material used in their experiment has about 4.5-fold stronger trypsin inhibitor activity than casein material.[20] But, our SPI material does not have as strong inhibitor activity for chymotrypsin in comparison with the casein material. The difference of the inhibitory activity on the hydrolysis of BT-PABA between casein and SPI or between casein and SPI hydrolysates may be due to the nature of these proteins or peptides, not due to the existence of protease inhibitors.

The activities of the hydrolysis of BT-PABA in vitro by the chyme of upper small intestine at 20 min after feeding of both the casein and the SPI diet were very high, which was coincident with the in vivo hydrolysis of BT-PABA shown by the portal PABA absorption. These results in vivo and in vitro represent high levels of free chymotrypsin activity in the early stage of feeding the test meal. If the behavior of the activity of free trypsin is similar to that of chymotrypsin, and it may be so, then the high protease activity in the lumen may inhibit the exocrine pancreatic secretion mediated by the inhibition of cholecystokinin (CCK) secretion.[21,22] The decrease in chymotrypsin activity in the lumen was observed 20 min after feeding in our study.

Miyasaka and Green[23] evaluate that the negative feedback mechanism is exerted by disappearance of 90% protease activity from the intestinal lumen. The activity of free chymotrypsin at 120 min was reduced to 7.8% of the value at 20 min in the casein fed group, which can remove the negative feedback inhibition of the CCK secretion. The reduction of the chymotrypsin activity of the SPI group was less. These results may explain the rapid increase in chymotrypsin activity after 120 min in the casein group, but not in the SPI group.

Recently, several authors reported that BT-PABA also hydrolyzed by the brush border membrane peptidases in rats, but the contribution of the hydrolysis of BT-PABA by the peptidases is small.[24] If the activities of the peptidases are significant, the effects of those are the same between the casein and the SPI group in our study.

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