Chymotrypsinogen in the Intestine of Rats Fed Soybean Trypsin Inhibitor and Its Inability to Suppress Pancreatic Enzyme Secretions

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ABSTRACT Recent investigations have shown that intestinal trypsin and chymotrypsin exert a negative feedback control on pancreatic enzyme secretion in the rat. The present study was conducted to see if soybean trypsin inhibitor (SBTI) effected changes in the intestinal activities of these enzymes which could provide an explanation for the pancreatic enzyme response evoked when SBTI is fed to rats. Rats were intubated with either a control diet or diet containing SBTI. One hour after feeding, intestinal contents were removed and washed with either saline or saline containing chicken ovomucoid. Trypsin and amylase activities were determined and chymotrypsin activity was measured before and after incubation with trypsin. In control rats, intestinal chymotrypsin activity was not increased by incubation with trypsin. In animals fed SBTI, however, trypsin incubation increased intestinal chymotrypsin activity to 3 to 5 times the pre-incubation levels. Intestinal trypsin activity of rats fed SBTI was reduced to less than 10% of control values. Continuous infusion of purified bovine chymotrypsin or chymotrypsinogen into rats with a bile-pancreatic juice fistula demonstrated that while chymotrypsin suppression pancreatic enzyme output, chymotrypsinogen did not. It was concluded, therefore, that SBTI acts to stimulate pancreatic secretion by binding intestinal trypsin so tightly it cannot fully activate chymotrypsinogen. Since chymotrypsinogen cannot suppress pancreatic secretion, SBTI effectively removes both enzymes from the intestine. An increased pancreatic enzyme secretion is initiated because of loss of the negative feedback regulation normally exerted by the active enzymes. J. Nutr. 104: 105-110, 1974.

INDEXING KEY WORDS trypsin • trypsinogen • chymotrypsin • chymotrypsinogen • soybean trypsin inhibitor • amylase • pancreatic enzymes • pancreatic secretion • feed-back control

Investigations during the past several years have shown that if rats are given a single feeding of a diet containing either unheated soybean meal (1, 2), soybean trypsin inhibitor (SBTI) or chicken ovomucoid (OMTI), (3, 4), pancreatic enzyme secretion may be increased 3 to 5 times that produced by the diet without the inhibitor. In these instances, enzyme secretion has generally been determined indirectly by feeding the inhibitor, then measuring the depletion of enzyme activity in the pancreas relative to that of a control or by an increased enzyme activity in the small intestine. Chickens fed raw soybean meal often showed an initial reduction in the level of intestinal proteolytic enzyme activity that increased to greater than normal values after a period of time. It was suggested that the increased enzyme output may have been a compensatory response to overcome a deficiency of intestinal trypsin induced by the inhibitor (5). However, this explanation seemed not to apply to the rat fed SBTI, since intestinal proteolytic enzyme activity was generally equal to or greater than that of the controls (3, 6, 7).

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1 A preliminary report of this study was presented at the Federation of American Societies for Experimental Biology, Atlantic City, N. J., 1973. Federation Proc. 32, 646 (abstr.).
Recently, Green and Lyman (8) reported that when SBTI was fed to rats and intestinal chymotrypsin and trypsin activities were individually determined, nearly all of the proteolytic enzyme activity in the intestine was due to greatly increased amounts of chymotrypsin activity as compared with control rats. Trypsin activity was depressed below that of control animals. This observation suggested that depressed intestinal trypsin levels might possibly be involved in the increased pancreatic secretory response to SBTI in this species also. Such a possibility was tested directly in anesthetized rats (9) in which bile-pancreatic juice could be collected and its enzymes analyzed while either proteolytic enzymes, SBTI, or bile-pancreatic juice was infused into the intestine. The experiments showed that the intestinal infusion of bile-pancreatic juice produced a constant basal enzyme secretion that increased sharply when the juice was diverted from the intestine. If either trypsin or chymotrypsin was infused in place of the bile-pancreatic juice, a basal level of secretion was maintained. Infusion of SBTI, sufficient to bind the proteolytic enzymes, evoked a sudden output of pancreatic enzymes. It was proposed that trypsin and/or chymotrypsin regulated pancreatic enzyme secretion in the rat by negative feedback control. When active trypsin or chymotrypsin was present in the small intestine, enzyme secretion was suppressed; when the enzymes were removed or bound by SBTI, enzyme secretion rose to a maximum. These experiments have now been extended to conscious, recovered animals (10) with similar results.

If, in fact, SBTI does cause pancreatic secretion by binding intestinal proteases, and explanation must be provided for the high level of chymotrypsin activity in the intestine of rats fed SBTI, since chymotrypsin is as effective as is trypsin for suppressing pancreatic enzyme output in the rat. Green (2) has reported observations which suggest that the high intestinal chymotrypsin activity may be an artifact produced during preparation of the intestinal contents for enzyme analyses and that, in vivo, the enzyme may be present mainly as its unactivated zymogen.

The experiments to be reported, therefore, were designed to test the hypothesis that intestinal trypsin is bound so tightly by the inhibitor it cannot activate chymotrypsinogen, which in its zymogen form cannot suppress pancreatic secretion.

**EXPERIMENTAL METHODS**

**Animals and preparation of intestinal contents.** Male, Sprague-Dawley rats, weighing about 150 g, were maintained on laboratory stock diet (9) prior to an experiment. Animals were fasted overnight (approximately 18 hours), then were fed, by tube, about 2 g of a casein diet (mixed with enough purified trypsin inhibitor to provide 75 mg of SBTI per 100 g body weight. Control animals were fed the same diet, except that additional casein was substituted for the trypsin inhibitor. One hour after feeding, the rats were exsanguinated by decapitation and the entire small intestine from the pylorus to the ileal-cecal junction was quickly removed and chilled in ice. It was then divided into four equal parts and the contents were washed from each segment with two rinsings (2.5 ml each) of cold saline or cold saline containing 100 µg chicken ovomucoid (OMTI)*/ml. (Chicken ovomucoid is an almost pure trypsin inhibitor and contains little or no chymotrypsin inhibitor activity. Its inclusion in the rinse was to prevent activation of chymotrypsinogen by any free trypsin during the preparation of the intestinal contents.) Total contents and washings were brought to 20 ml and homogenized in a glass grinder for 1 minute. Ten milliliters of the homogenate was centrifuged and the supernatant was directly assayed for chymotrypsin and trypsin activity by the method of Hummel (11), using benzoyl-L-tyrosine ethyl ester* (BTEE) as

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*Horton’s Animal Suppliers, Gilroy, Calif.
*Rajston Purina Laboratory Rat Chow, St. Louis, Mo. Protein 22%, fat 4.0%, fiber 5.0%.
*Sucrose 71%, casein 18%, cottonseed oil 5%, fortified oil 1%, salts 4% (Williams, M. A. & Briggs, G. M. 1968) A new mineral mixture for experimental rat diets and evaluation of other mineral mixtures. Federation Proc. 27, 1968). B-vitamin mix 1%, choline chloride 0.2%. Fortified oil provides per 100 g of diet: α-tocopherol 10.0 mg, retinyl palmitate 400 IU, ergocalciferol 200 IU. B-vitamin mix provides (mg/100 g of diet): thiamin-HCl 0.5, riboflavin 0.5, pyridine acid 2.5, Ca pantothenate 2.0, pyridoxine-HCl 0.25, vitamin K (menadione) 0.05, biotin 0.01, folic acid 0.02, vitamin B-12 0.002, inositol 10.0.
*Worthington Biochem., Freehold, N. J.
substrate for the former and tosyl-arginyl methyl ester \(^6\) (TAME) for the latter. Another aliquot of the supernatant was incubated with trypsin (800 \(\mu\)g/ml) at 0°C to activate any chymotrypsinogen and then the total chymotrypsin activity of the intestinal contents was measured. BTEE and TAME units were converted to equivalent milligrams of purified bovine chymotrypsin \(^6\) (EC 3.4.4.5) or trypsin \(^6\) (EC 3.4.4.4) activity in the total intestinal contents (12).

The remaining 10 ml of homogenate was frozen and assayed at a later time for amylase (EC 3.2.1.1) activity by the method of Smith and Roe (13).

Collection of bile-pancreatic juice and enzyme infusions in the conscious rat. These experiments involved use of the conscious rat, surgically prepared so that enzymes could be infused into the intestine at the same time enzyme activity of the bile-pancreatic juice was determined. Briefly, male Wistar rats \(^7\) weighing 300 to 325 g were anesthetized. The duodenum was exposed and the junction at which the common bile-pancreatic duct enters the intestine was located. A cannula was inserted into the common duct near the ampulla, for collection of the bile-pancreatic juice which began flowing immediately. A second cannula was inserted into the intestine near the ampulla for returning the juice to the intestine or for infusing it with enzymes or other substances during an experiment. The abdominal incision was closed and the animals were placed in a restraining cage (Boilman type) and allowed to recover from the operation for about 49 hours. During this period, animals were fed the laboratory stock diet ad libitum. Details of the operative procedure have been reported by Green et al. (10).

Prior to each experiment, the surgically modified rat was fasted for about 8 hours. Bile-pancreatic juice was analyzed for chymotrypsin (11) by activating the zymogen in 25 \(\mu\)l of the juice with 475 \(\mu\)l of a trypsin solution (40 \(\mu\)g trypsin/ml in 0.04 \(M\) Tris-HCl, 0.01 \(M\) CaCl\(_2\) buffer at pH 8.1) and incubating for 15 minutes at 0°C.

During a typical experiment, purified bovine chymotrypsin (10 mg/ml in 0.05 \(N\) NaHCO\(_3\)) plus OMTI (1 mg/ml) was infused into the small intestine at a rate of 3 ml/hour, for a period of at least 2.5 hours. At half-hour intervals during this basal period a sample of the collected juice was analyzed for chymotrypsin activity. At the completion of the basal period, either chymotrypsin was deleted from the bicarbonate solution or else chymotrypsinogen \(^6\) (10 mg/ml) was substituted and changes in enzyme activity of the secreted juice determined. Chymotrypsinogen, when fully activated, had nearly the same specific enzyme activity as did the chymotrypsin, so both were infused at similar rates and concentrations. We found it necessary to add a small amount of OMTI (1 mg/ml) to the infusate to prevent activation of the chymotrypsinogen by traces of active trypsin which might be remaining in the intestinal tract and was also present in most of the chymotrypsinogen preparations. Consequently, we included an equal amount of OMTI in all experiments. Upon completion of an experiment, the bile-pancreatic duct cannula was reconnected to the intestinal cannula, so that a normal enterohepatic circulation of bile and pancreatic juice was maintained. Statistical comparisons were made using the \(t\) test described by Snedecor (14). Differences in mean values yielding \(P < 0.05\) were considered significant.

RESULTS

Table 1 shows intestinal chymotrypsin activity, before and after incubation with trypsin, 1 hour after a single feeding of diet or of diet and SBTI. In control animals (group 1) incubation of the intestinal contents with trypsin had no effect on intestinal chymotrypsin activity. However, in the SBTI-fed animals (groups 2 and 3) incubation of the contents with trypsin increased the chymotrypsin activity nearly fivefold. Washing the contents with saline plus OMTI decreased the initial chymotrypsin activity even further below that of the group fed SBTI, but washed only with saline. After trypsin incubation, the chymotrypsin activity in the intestinal contents of SBTI-fed animals was significantly higher (\(P < 0.01\)) than the activities seen in the controls.

The amount of active trypsin detected in the contents of animals fed SBTI was only

\(^*\) Hilltop Lab Animals, Chatsworth, Calif.
TABLE 1

Pancreatic enzyme activity in intestinal contents 1 hour after feeding protein or SBTI to rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chymotrypsin</th>
<th>Trypsin</th>
<th>Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before trypsin activation</td>
<td>After trypsin activation</td>
<td>mg chymotrypsin</td>
</tr>
<tr>
<td>1. Control (basal diet +75 mg prot./100 g BW)</td>
<td>2.41±0.27</td>
<td>2.59±0.31</td>
<td>0.80±0.14</td>
</tr>
<tr>
<td>2. Basal diet +75 mg SBTI/100 g BW (contents washed with cold saline)</td>
<td>1.60±0.49</td>
<td>4.89±1.06</td>
<td>0.09±0.03</td>
</tr>
<tr>
<td>3. Basal diet +75 mg SBTI/100 g BW (contents washed with cold saline + OMTI)</td>
<td>0.92±0.49</td>
<td>4.91±0.71</td>
<td>0.04±0.02</td>
</tr>
</tbody>
</table>

SBTI = soybean trypsin inhibitor; OMTI = chicken ovomucoid. Enzyme activities expressed as milligrams purified bovine chymotrypsin or trypsin/total intestinal contents/100 g body weight; amylase activity is in milligrams per milliliters of four animals per group. Statistical comparisons: Chymotrypsin activity of groups 2 and 3 significantly higher (P < 0.02 and P < 0.01, respectively) than control activity after incubation with trypsin. Chymotrypsin activity of groups 2 and 3 before incubation significantly lower (P < 0.01) than activity after incubation with trypsin. Trypsin activity of groups 2 and 3 significantly lower (P < 0.01) than control activity. Amylase activity of groups 2 and 3 significantly higher (P < 0.01) than control activity.

about one-tenth that of the control rats, indicating that the intestinal enzyme was nearly completely bound by the inhibitor. Intestinal amylase activity was greatly stimulated also by feeding SBTI. This observation has been reported by others previously (2-4).

These results show that when SBTI is fed to rats, most of the chymotrypsin in the small intestine is present as chymotrypsinogen, apparently because insufficient free trypsin is present to catalyze the activation of the zymogen. Since greater than normal amounts of chymotrypsinogen would be present in the intestine of rats fed SBTI, our next efforts were directed towards establishing whether chymotrypsinogen would be unable to suppress pancreatic enzyme secretion. In order to do this, we used the conscious rat, prepared so that either chymotrypsin or chymotrypsinogen could be infused into the intestine while pancreatic enzyme output was being determined in the bile-pancreatic juice.

Results from a series of experiments conducted with individual rats are shown in figure 1. Figure 1a shows that when a solution of sodium bicarbonate and bovine chymotrypsin was infused at a rate of 30 mg/hour, enzyme secretion was maintained at a relatively constant rate. This rate of enzyme output was similar to what we had reported (10) previously when bile-pancreatic juice was returned to the intestine. When the chymotrypsin infusion was discontinued (fig. 1b), enzyme output increased immediately as it also did when the chymotrypsin was replaced with chymotrypsinogen (fig. 1c). Chymotrypsinogen, therefore, does not suppress pancreatic enzyme secretion in the rat as does active chymotrypsin.

DISCUSSION

The results of these experiments provide further evidence that active chymotrypsin, in the small intestine, exerts a regulatory influence on pancreatic enzyme secretion by a mechanism of negative feedback control. When the enzyme is infused into the intestine, pancreatic enzyme secretion was suppressed; when it is removed, enzyme output increased nearly threefold (fig. 1a and b). In addition, the results indicate that disruption of this feedback regulation is the likely mechanism by which SBTI, when fed to rats, evokes a copious outpouring of enzymes even though greater than normal amounts of chymotrypsin activity appear to be present in the small intestine.
When rats are fed a single meal containing SBTI, it tightly binds the secreted trypsin in the upper small intestine as rapidly as it becomes activated. In the absence of trypsin, chymotrypsinogen is not activated. Since the inactive zymogen does not exert feedback control on enzyme secretion (fig. 1c) the effect is the same as removing both enzymes from the intestine, and the pancreas responds by uninhibited secretion of its enzymes. The considerable amount of active chymotrypsin detected in the small intestine of rats 2 or 3 hours after a single feeding of SBTI (8) probably arises from its activation by traces of trypsin during preparation of the intestinal contents for analysis. The evidence for this happening would be the lower chymotrypsin activity detected prior to incubation with trypsin when the intestinal contents were washed with the OMTI solution than when washed only with cold saline. Results recently obtained (unpublished) have also indicated that the proteolytic enzymes do not exert their suppression of pancreatic enzyme secretion when introduced into the lower half of the small intestine. Quite possibly some activation of thezymogen could occur during transport through the gut, but too late to be effective for suppression of enzyme secretion.

Lepkovsky et al. (15) have questioned whether raw soybean meal or trypsin inhibitors really stimulate pancreatic enzyme secretion in rats, since, at the time of their studies, evidence for such a stimulation had been obtained only by indirect methods involving changes in pancreatic and intestinal enzyme activities. In their experiments, they succeeded in collecting pancreatic juice from four rats given, successively, heated soybean and raw soybean diets over a period of 9 days. When these diets were given in reversed order, they found no appreciable differences in enzyme content of the secreted juice. Our investigations in the rat with a bile-pancreatic fistula (9, 10) have clearly shown that when pancreatic juice (bile-pancreatic juice) is returned, a single feeding of diet and SBTI produced a four- to fivefold increase in pancreatic enzyme output. Complete withdrawal of the juice or the proteolytic enzymes from the intestine evoked a similar discharge of enzymes initially, but after several hours enzyme secretion declined to levels approximately 2 times higher than the basal secretions (10). Administration of SBTI during the time the pancreatic enzymes were diverted failed to evoke any further response. Inasmuch as the animals in the experiment by Lepkovsky et al. had their pancreatic secretions diverted from the intestine, little additional influence of the raw soybean meal (trypsin inhibitor) would be expected. We feel, therefore, that previous results (1-4, 6-8) showing changes
in pancreatic and intestinal enzyme activities of rats fed SBTI or raw soybean meal do reflect, reasonably accurately, the influence of the trypsin inhibitor on pancreatic enzyme output.

The results of this and the previous studies (9, 10) indicate that SBTI acts to evoke a pancreatic enzyme response by effectively removing active trypsin and chymotrypsin from the intestine, thereby preventing their normal feedback regulation of secretion. Little is known as to how the enzymes accomplish this intraintestinal control. A mechanism discussed previously (9) proposed that trypsin or chymotrypsin in the intestine interfered with the release of a humoral factor similar to or identical with cholecystokinin–pancreozymin, a known pancreatic enzyme stimulant. Removal of the enzymes from the intestine would result in the release of the humoral factor which then stimulates pancreatic enzyme secretion. Evidence for the existence of such a factor in the blood of rats fed SBTI has been reported (16). However, the nature of the factor and how its release is mediated by removal of trypsin or chymotrypsin from the intestine is not known. Investigations designed to answer these questions are now in progress.

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LITERATURE CITED