Intestinal free trypsin concentrations fall only transiently when rats are fed raw soya flour

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SUMMARY Raw soya flour (RSF) is thought to cause pancreatic hypertrophy in rats because trypsin inhibitor in the flour binds trypsin in the gut lumen and this lowering of free trypsin concentrations leads to the release of cholecystokinin (CCK). Intestinal enzyme concentrations were therefore studied in rats fed RSF for from one hour to 400 days to determine whether free trypsin concentrations were depressed during the period of pancreatic growth (up to six to eight weeks after starting RSF). Intestinal levels of enzymes were raised from one hour after starting the diet up to 400 days, indicating that stimulation of pancreatic secretion continued for as long as this diet was fed. Free trypsin concentrations were, however depressed only for the first 12 hours. It seems unlikely, therefore, that lowered free trypsin concentrations alone stimulate the release of CCK in these animals.

When rats are fed raw soya flour (RSF) marked pancreatic growth occurs. This growth is seen initially as hypertrophy, with increases in weight, protein, and RNA within one week of starting the diet, reaching a plateau after four weeks, followed by an increase in total pancreatic DNA, indicating hyperplasia. The increase in DNA does not appear until the second week on the diet and reaches a plateau after six to eight weeks.^{1,2}

A plausible mechanism to explain the effect of RSF on pancreatic growth has been advanced by Green, Lyman and coworkers.³ This theory suggests that the release of the gastrointestinal hormone cholecystokinin (CCK) is normally inhibited by the presence of the pancreatic enzyme trypsin in the gut lumen. Because CCK stimulates pancreatic enzyme secretion (including trypsin), a negative feedback loop is established. Raw soya flour contains high levels of trypsin inhibitor (around 6%), so when this diet is fed intestinal trypsin is bound to the inhibitor, free trypsin concentrations fall and the inhibition of CCK release is lost. Plasma CCK concentrations are, therefore, thought to rise, and, because of the trophic action of CCK on the pancreas, pancreatic growth occurs.

On the basis of this theory it might be predicted that intestinal free trypsin concentrations would be low for several days after starting RSF but that they

would then begin to rise as hypertrophy followed by hyperplasia occurred, to reach normal intestinal levels again when growth plateaued after four to six weeks. Total trypsin concentrations would be expected to be high at all times, with most of the trypsin bound to trypsin inhibitor, while other pancreatic enzyme concentrations should also be raised because of the continued stimulation to secrete from the raised plasma CCK concentrations. In a method developed for the measurement of enterokinase, it was shown recently that the trypsin-inhibitor complex dissociates at low pH, and the released trypsin can be measured if a suitable small molecular weight substrate such as tosyl-L-arginine methyl ester, (TAME) is present when the pH is raised again.⁴ We have used this technique to measure free and total trypsin and the levels of other pancreatic enzymes in the intestinal contents from rats fed RSF for periods from one hour to 400 days. The results show the predicted high output of all pancreatic enzymes at all times in rats fed RSF. The return of free trypsin concentrations to normal values, however, is surprisingly rapid.

Methods

ANIMALS AND DIETS

Male Wistar rats, locally inbred for the past 27 years, were used. The animals were two months old and averaged 200 g in weight at the start of the study. The animals were fed standard rat chow (Milne Feeds,

Welshpool, Western Australia) from weaning. To ensure that new diets were started as soon as the animals were exposed to them, all animals were fasted for 24 hours with free access to water, starting at 9 am. At 9 am on the following day eight rats were killed to provide fasting data and the remainder fed either standard rat chow (47 rats), heated soya flour (HSF, 60 rats) or RSF (79 rats). Soya flour was obtained from Soy Products of Australia, Bayswater, Victoria and was supplemented with vitamins and minerals as recommended by Fölsch and Wormsley.⁵ Chow and HSF contained negligible trypsin inhibitor activity, while various batches of RSF inhibited 33–42 mg trypsin per g flour as measured by the method of Kakade *et al.*⁶ Standard chow contained 52 % carbohydrate, 22 % protein and 5 % fat and soya flour approximately 25 % carbohydrate, 40 % protein, and 20 % fat.

ANALYTICAL TECHNIQUES

At various times after starting the diets groups of four to eight rats were killed by ether anaesthesia and cardiac incision. The gut was removed in two equal segments and each segment washed out twice with 10 ml volumes of ice cold saline. All washings were combined and made up to 50 ml. Washings were either assayed immediately for amylase, lipase, chymotrypsin and free and total trypsin or frozen at -80°C until assay, usually within one week of collection. Intestinal washings were assayed for free trypsin activity (EC 3.4.21.4) directly, using tosyl-L-arginine methyl ester (TAME) as substrate and also after acidification to pH 2.0 to dissociate the trypsin-inhibitor complex for the measurement of total trypsin activity, by the method of Green.⁴ Washings were also assayed for α -amylase activity (EC 3.2.1.1.) by the method of Dahlqvist,⁷ lipase activity (EC

3.1.1.3) by the method of Brockman⁸ and chymotrypsin (EC 3.4.21.1), under the conditions used for trypsin assay, with acetyl-L-tyrosine ethyl ester as substrate.

STATISTIC ANALYSIS

Data was analysed by multifactor analysis of variance.

Results

VALIDATION OF TRYPSIN ASSAY

Studies with purified bovine trypsin (Boehringer Mannheim) and purified soya bean trypsin inhibitor (Sigma) showed that trypsin activity was completely lost in the presence of inhibitor but the initial activity could be measured once again when the trypsin inhibitor complex was assayed after acidification to pH 2 as described above. With a sample of freeze dried rat pancreatic juice activated with enterokinase 95 % of the trypsin activity was blocked by a suspension of RSF but again all the trypsin activity could be measured after acidification. In rat intestinal contents 97 % of the trypsin activity was blocked by RSF and 87 % of the initial activity was measured after acidification (Table).

INTESTINAL TRYPSIN CONTENT

In rats fed HSF and chow the total trypsin activity in washings assayed as collected (free trypsin) and in the same washings assayed after acidification to pH 2 (total trypsin) were very similar, whereas in rats fed RSF total trypsin activity was about three times the free values (Fig. 1).

In fasted rats intestinal trypsin content was low but within one hour of feeding chow or HSF the trypsin

Table Trypsin activity (U/ml) in samples before and after addition of trypsin inhibitor and after acidification of the trypsin-inhibitor complex

	Without inhibitor	With inhibitor*	With inhibitor after acidification
Bovine trypsin 0.4 mg/ml (n = 3)	67.1 $\pm$ 2.2	0	66.7 $\pm$ 6.4
Freeze dried rat pancreatic juice 2.0 mg/ml (n = 5)	23.9 $\pm$ 0.7	1.3 $\pm$ 0.1	24.1 $\pm$ 1.3
Rat intestinal contents (n = 5)	35.7 $\pm$ 0.6	0.9 $\pm$ 0.06	31.0 $\pm$ 1.5

* An equal volume of purified trypsin inhibitor, 0.4 mg/ml was used added to the bovine trypsin and an equal volume of a 10 % suspension of delipidated RSF in saline to the remaining two samples. Values are mean $\pm$ SEM.

Fig. 1 Comparison of free and total trypsin content in intestinal washings from rats fed RSF, chow or HSF. $\square$ Rats fed RSF $\blacksquare$ Rats fed Chow or HSF

Fig. 2 Trypsin activity in intestinal washings from rats fed RSF, chow or HSF for times varying from 1 hr to 400 days. Free and total trypsin did not differ significantly in rats fed chow or HSF. The graph on the left shows trypsin activity for the first 24 hours, on a linear time scale, the graph on the right activity over the whole test period, on a logarithmic time scale. F→fasting free and total trypsin $\diamond$ Rats fed RSF, free trypsin $\blacksquare$ Rats fed RSF, total trypsin $\square$ Rats fed chow, total trypsin $\blacklozenge$ Rats fed HSF, total trypsin. (Mean $\pm$ SEM).

content had doubled and trypsin content remained at this level for the remainder of the study (Fig. 2).

In rats fed RSF free trypsin was low for the first six hours but within 12 hours of starting on RSF the free trypsin content had risen to the level seen in animals fed diets devoid of trypsin inhibitor (chow and HSF). From this time onwards in rats fed RSF the free trypsin content of the gut was not significantly different from that seen in control animals. In animals fed RSF the total trypsin content was always significantly higher than the free content, and from day 1 onwards also significantly higher than the total content seen in control animals (Fig. 2).

OTHER ENZYMES

Fasting levels of the other enzymes measured were low but within one hour of feeding these increased several fold.

Amylase content fell from the one hour value in chow fed rats to a minimum after six hours and then slowly rose over the remainder of the study. In HSF-fed rats amylase fell to low values after three days on the diet and remained constant from this time onwards. Animals fed RSF showed a similar pattern to those fed HSF but amylase content was significantly greater than that seen in HSF fed rats at all intervals studied (Fig. 3).

Lipase activity was constant throughout the study and not significantly different in rats fed either chow or HSF. In rats fed RSF lipase content was significantly greater than in the other dietary groups at all times except 60 and 180 days after starting the diets (Fig. 3).

Chymotrypsin content was constant throughout the study in rats fed HSF and chow. In rats fed RSF chymotrypsin was initially low but it rose slowly and after three days on the diet was significantly greater than in rats fed the other diets. It remained high in these animals for the remainder of the study (Fig. 3).

Discussion

The validation studies showed that RSF blocks rat trypsin in intestinal contents but most of this activity can be measured in the gut washings after acidification. The failure to recover all the trypsin activity in these studies may reflect incomplete dissociation of the trypsin soya bean trypsin inhibitor complex, as suggested by Green.⁴

Within one hour of starting on a diet of RSF intestinal free trypsin content was very low. Stimulation of CCK secretion by the mechanism suggested by Green and Lyman,² and consequent stimulation of pancreatic secretion, should therefore occur. The high levels of amylase, lipase and total

Fig 3. Amylase, lipase and chymotrypsin activity in intestinal washings from rats fed RSF, chow or HSF for times varying from 1 hr to 400 days. F→fasting values ■ Rats fed RSF □ Rats fed chow ◆ Rats fed HSF. (Mean ± SEM)

trypsin in the gut contents at this time indicates that this does indeed take place. Free trypsin concentrations rose rapidly, however, after the first hour, and from 12 hours onwards were not significantly different from that seen in animals fed chow or HSF. The stimulation of CCK release by the loss of inhibition by trypsin should therefore cease, but the raised intestinal enzyme concentrations indicate that pancreatic hypersecretion is maintained for as long as the RSF diet is fed, presumably as a result of sustained high CCK secretion. It seems unlikely therefore, that lowered free trypsin alone controls CCK release in these animals and that some other component in RSF also stimulates CCK release. Such a possibility was suggested several years ago to explain pancreatic growth seen in chicks fed soya flour preparations free of inhibitor activity⁹ and pancreatic growth greater than could be accounted for by inhibitor content in rats.¹⁰

In rats fed soya flour amylase concentrations were lower than in rats fed chow from day 3 onwards. This almost certainly reflects adaptation in pancreatic enzyme composition to dietary composition^{11,12} as soya flour contains less carbohydrate than rat chow. Within the soya flour fed group, however, amylase output was still three times greater in animals fed RSF than in animals fed HSF, because of the pancreatic hypersecretion.

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