Changes in Activity, Antigenicity, and Molecular Size of Rice Bran Trypsin Inhibitor by In Vitro Digestion

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Summary Rice bran trypsin inhibitor (RBTI) was digested by pepsin alone or by pepsin and pancreatin with or without bovine serum albumin (BSA) to clarify the changes in trypsin inhibitory activity, apparent antigenicity, and molecular size of RBTI. In vitro pepsin digestion of RBTI in the absence of BSA caused the gradual loss of the trypsin inhibitory activity and antigenicity. This was mostly due to a progressive degradation of the native 14.5-kDa RBTI molecule to small molecular mass products. The presence of BSA in the digestion mixture prevented the RBTI degradation and was accompanied with a considerable protection of the activity and antigenicity. Similar results were also given by in vitro pepsin-pancreatin digestion. These findings suggest that RBTI may be present in its active form in the gastrointestinal tract when fed to animals, especially with a dietary protein.

Key Words in vitro digestion, trypsin inhibitor, pepsin, pancreatin, antigenicity, rice bran

It is well known that the ingestion of plant trypsin inhibitors by rats, mice, chicks and other species stimulates the exocrine pancreas, resulting in increased enzyme production and organ enlargement (1, 2). These effects of the inhibitors may depend mostly on their stability in digestive tracts, since the first step in the stimulation is probably that an ingested inhibitor reaches the small intestine in its active form and combines with trypsin. Therefore, it is extremely important to know the behavior of an inhibitor in the gastrointestinal tract to understand its nutritional significance.

We have previously reported on the purification, characterization, and amino acid sequence of rice bran trypsin inhibitor (RBTI) (3–6). Briefly, this inhibitor is a heat-stable protein with a molecular weight of 14,500 consisting of two tandem, homologous Bowman-Birk type inhibitors and can inhibit trypsins from various species such as bovine, hog, rat, and human. These properties imply that RBTI plays an important role in animal nutrition when ingested. However, the behavior of the RBTI molecule in the digestive tracts is not clear after being fed to animals.
In order to clarify this behavior, we thus constructed in vitro digestion systems of RBTI. In this paper, we describe the changes in trypsin inhibitory activity, apparent antigenicity, and molecular size of RBTI by in vitro pepsin or pepsin-pancreatin digestion.

MATERIALS AND METHODS

**Materials.** RBTI was prepared from the bran of rice (*Nihonbare*) as described previously (3). Bovine trypsin (Type III), porcine pepsin (crystallized and lyophilized), and bovine serum albumin (BSA) (fraction V, powder) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pancreatin (pancreatin N.F.), α-N-benzoyl-D,L-arginine-p-nitroanilide HCl (Bz-DL-Arg-pNA), horseradish peroxidase-labeled goat anti-rabbit IgG, and polyvinylidenfluoride (PVDF) membrane were from Difco Laboratories (Detroit, MI, U.S.A.), Peptide Institute, Inc. (Minoh), Bethy Laboratories, Inc. (Montgomery, AL, U.S.A.), and Atto Corp. (Tokyo), respectively. DEAE-Sepharose CL-6B and CNBr-activated Sepharose 4B were products of Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were obtained from Nacalai Tesque, Inc. (Kyoto) and Wako Pure Chemical Industries (Osaka).

**Trypsin inhibitory assay.** Trypsin inhibitory activity was measured with bovine trypsin as a target enzyme and Bz-DL-Arg-pNA as a trypsin substrate by our method described previously (5).

**Preparation of antiserum against RBTI.** RBTI was emulsified with an equal volume of Freund’s complete adjuvant and injected into a male rabbit (3 kg of body weight) intracutaneously three times at 2-week intervals at a dose of 2 mg/rabbit. The rabbit was bled a week after the last injection to collect serum. The IgG fraction was prepared from the serum by salting-out and the subsequent chromatography on DEAE-Sepharose 4B and polyclonal antibodies against RBTI were purified from the IgG fraction by affinity chromatography on antigen-Sepharose 4B.

**Immunoassay.** Double immunodiffusion was carried out in agarose gel according to the method of Ouchterlony (7), and showed that the antiserum against RBTI gave a single fused precipitin line without any spurs as shown in Fig. 1. Single radial immunodiffusion (SRID) (8) was employed for the estimation of apparent antigenicity of enzymic digests using the antiserum against RBTI. Briefly, 10 ml of prewarmed 1.1% agarose in 10 mM Na-phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.1% (w/v) NaN₃ were mixed with 0.2 ml of the antiserum and poured onto a glass plate (8.5 × 11 cm). After the formation of a gel, 2-mm-wide holes were punched in the gel on the glass plate and filled with 3 μl of the antigen solutions. The gel plate was incubated for 72 h under the condition of high humidity and each area of precipitin circles formed was determined by diameter measurement as a function of the antigen concentration. Calibration curves were made with the purified RBTI at concentrations from 0 to 0.2 mg/ml.

Fig. 1. Immunodiffusion analysis with rabbit antiserum against RBTI. A, antiserum; B, purified RBTI; C, crude RBTI obtained from rice bran extract by heat treatment and salting-out with 90% ammonium sulfate saturation.

**SDS-PAGE and immunoblot analysis.** SDS-PAGE in the presence of a reducing agent was done in 15% slab gels by the method of Laemmli (9). After electrophoresis, proteins in a slab gel were electrophoretically transferred to a sheet of PVDF membrane as described by Towbin et al. (10). Proteins with antigenicity were then stained by an enzyme immunoassay technique (11) with the purified rabbit anti-RBTI IgG, horseradish peroxidase-labeled goat anti-rabbit IgG, 4-chloro-1-naphthol, and hydrogen peroxide.

**HPLC.** Molecular sieve HPLC was carried out using a TSK-SW 2000 column (0.75×60 cm) equilibrated with 0.2 M K-phosphate buffer (pH 6.5).

**In vitro digestion.** In vitro pepsin digestion was done by incubating RBTI (1.2 mg) with pepsin (0.12 mg) in 1 ml of 0.1 M NaCl-HCl (pH 1.8) at 37°C in the absence or presence of BSA (6 mg). After appropriate incubations, aliquots (0.1 ml) were withdrawn and neutralized by adding 0.2 ml of 0.1 M Na-phosphate buffer (pH 7.0), and the mixtures were used for SDS-PAGE and HPLC analyses in addition to the determinations of trypsin inhibitory activity and apparent antigenicity. In vitro pepsin-pancreatin digestion was carried out by mixing 1 ml of the above 30-min pepsin digest with 2 ml of pancreatin solution (0.3 mg/ml in 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM CaCl₂). The addition of the pancreatin solution made the pH of the reaction mixture 8.0. Aliquots (0.3 ml) were taken out at appropriate intervals, heated at 100°C for 5 min, and then used for analysis. This heating did not yield any precipitable materials even in the case of the digestion with BSA, because BSA was already degraded by pepsin. The effect of the heat
treatment on the inhibitory activity and antigenicity of the digestes was considered to be negligible.

RESULTS

In vitro pepsin digestion of RBTI

RBTI was digested by pepsin at 37°C and pH 1.8 using the enzyme-to-RBTI ratio of 1:10 (w/w) in the absence or presence of BSA. Figure 2 shows the changes in trypsin inhibitory activity and antigenicity of RBTI during pepsin digestions. The trypsin inhibitory activity and apparent antigenicity of intact RBTI were taken as 100%. As shown in Fig. 2, in vitro pepsin digestion of RBTI without BSA resulted in a 30% loss of the original trypsin inhibitory activity after 30 min of incubation and a 70% loss after 24 h, while the presence of BSA in the digestion mixture significantly prevented the activity loss. That is to say, only a 5% loss of the inhibitory activity was shown by a 30-min digestion and a 20% loss by a 24-h digestion. The apparent antigenicity of RBTI determined by SRID was also decreased by the pepsin digestion in the absence of BSA, though the antigenicity loss was lower than the activity loss. For example, no detectable loss of the antigenicity was shown by a 30-min digestion and only a 40% loss by a 24-h digestion. Furthermore, the addition of BSA to the digestion mixture completely protected the antigenicity.

![Fig. 2. Effect of in vitro pepsin digestion on the trypsin inhibitory activity and antigenicity of RBTI. RBTI was digested by pepsin at 37°C and pH 1.8 in the absence (○) or presence (●) of BSA. ---, trypsin inhibitory activity; ----, antigenicity determined by SRID.](J. Nutr. Sci. Vitaminol.)
Figure 3 shows SDS-PAGE and immunoblot patterns of the pepsin digests. It was clearly seen that pepsin progressively degraded the native 14.5-kDa RBTI molecule to small molecular mass products in the absence of BSA. The degradation products detected by SDS-PAGE with a reducing agent were mainly 7.5-kDa and 8.5-kDa species within 2 h of digestion, and those with less molecular mass species over 2 h of digestion. It also turned out that intact RBTI completely disappeared over 3 h of digestion. Among the degradation products, the 8.5-kDa species was found to be most immunoreactive when judged by immunoblot patterns. On the other hand, pepsin scarcely degraded the RBTI molecule when BSA was added to the reaction mixture. This resistance to digestion by pepsin was still observed even after the original BSA molecule completely disappeared.

The change in the molecular size of RBTI during the pepsin digestion was also examined by HPLC, in which a successive diminution in the original RBTI peak was shown in the absence of BSA and the nearly complete maintenance of the peak in the presence of BSA, as shown in Fig. 4. These findings are in good agreement with the results obtained from SDS-PAGE.

**In vitro pepsin-pancreatin digestion of RBTI**

RBTI was digested by pepsin for 30 min under the same conditions as above, and subsequently by pancreatin for 24 h at pH 8.0. In the digestion, the pancreatin...
Fig. 4. HPLC patterns of pepsin digests of RBTI. A sample solution (50 μl) of the digests was applied to a TSK-SW 2000 column (0.75 × 60 cm) equilibrated with 0.2 M K-phosphate buffer (pH 6.5). Elution was done with the phosphate buffer at a flow rate of 1.0 ml/min and monitored at 280 nm. Arrowheads indicate the elution position of native RBTI. A, pepsin digestion in the absence of BSA; B, pepsin digestion in the presence of BSA.

used included the respective activities corresponding to 0.018 mg of trypsin and 0.012 mg of α-chymotrypsin. Figure 5 shows the changes in trypsin inhibitory activity and antigenicity of RBTI caused by this in vitro digestion. Results shown in Fig. 5 were very similar to those obtained in the above in vitro pepsin digestion. That is to say, the trypsin inhibitory activity and antigenicity of RBTI were gradually decreased by the pepsin-pancreatin digestion in the absence of BSA, whereas the addition of BSA to the digestion mixture significantly prevented the activity loss and completely protected the antigenicity. A slight difference between in vitro pepsin and pepsin-pancreatin digestions was seen only in the degree of activity and antigenicity losses.
Fig. 5. Effects of *in vitro* pepsin-pancreatin digestion on the trypsin inhibitory activity and antigenicity of RBTI. RBTI was digested by pepsin for 30 min at 37°C, pH 1.8 and subsequently by pancreatin for 24 h at pH 8.0 in the absence (○) or presence (●) of BSA. ---, trypsin inhibitory activity; ----, antigenicity determined by SRID.

Fig. 6. SDS-PAGE and immunoblot of the pepsin-pancreatin digests of RBTI. The upper half of the pictures shows SDS-patterns and the lower half immunoblot patterns. A, pepsin-pancreatin digestion in the absence of BSA; B, pepsin-pancreatin digestion in the presence of BSA.
The profiles of SDS-PAGE and immunoblot for the pepsin-pancreatin digests are presented in Fig. 6. SDS-PAGE of the digests without BSA clearly revealed that RBTI was resistant against pancreatin attack, since the density of the original RBTI band was not diminished by pancreatin treatment. It was also indicated that the band of 8.5-kDa degradation product formed by pepsin digestion was not reduced in its density by digestion with pancreatin and only the band of 7.5-kDa degradation product with a little immunoreactivity disappeared after 3 h of digestion by pancreatin. These phenomena were caused essentially by several proteinases including α-chymotrypsin except for trypsin in the pancreatin preparation, since an excess amount of RBTI remaining must inactivate trypsin.

**DISCUSSION**

Rice bran is a valuable byproduct as a potential source of food protein, because it contains a large amount of proteins with high nutritional quality. However, we have indicated that rice bran also contains a significant amount of a proteinaceous trypsin inhibitor (RBTI) (3) regarded as an antinutritional factor. Therefore, in order to utilize rice bran proteins as a food, it is necessary to clarify the physiological effect of RBTI on animals and man. From this viewpoint, we constructed *in vitro* digestion systems of RBTI and examined the changes in the inhibitory activity, antigenicity, and molecular size of RBTI during digestion.

*In vitro* pepsin digestion in the absence of BSA clearly showed that RBTI gradually lost its inhibitory activity during the digestion. This was mainly due to a progressive degradation of native RBTI to small molecular mass products. However, the residual inhibitory activity did not necessarily correspond to the amount of intact RBTI molecule remaining. In particular, it should be noted that about 30% of the original activity still remained even after the intact RBTI molecule completely disappeared. The above fact demonstrates that one or more degradation products derived from the RBTI molecule have some trypsin inhibitory activity. These products with the inhibitory activity seem to be the 7.5-kDa and 8.5-kDa species that are probably derivatives of the constituent domains of RBTI, since RBTI consists of two tandem, homologous Bowman-Birk type inhibitors with a molecular weight of about 8,000 (6). The idea is also supported by the result of pepsin-pancreatin digestion without BSA, because the activity loss caused by pancreatin is judged to be attributed to the degradation of the 7.5-kDa product.

The inhibitory activity loss caused by pepsin digestion was strongly prevented by the addition of BSA to the digestion mixture. This was clearly due to the protection effect of BSA to the RBTI degradation, as indicated by SDS-PAGE and HPLC. The effect of BSA is believed to be based upon its susceptibility to pepsin. BSA possibly competes with RBTI for pepsin. It is also probable that peptides derived from the BSA molecule by pepsin digestion compete with RBTI for pepsin, because RBTI fairly maintained its molecular size during pepsin digestion even after the original BSA molecule completely disappeared. In any case, a dietary
protein ingested together with RBTI probably works as a protector of RBTI unless the protein is indigestible to pepsin. Therefore, RBTI is thought to be highly active in the stomach after it was ingested as a part of food constituents by animals. In addition, it is likely that RBTI exhibits substantial trypsin inhibitory activity even in the small intestine, since this inhibitor was indicated to be resistant to pancreatin.

The apparent antigenicity of RBTI determined by SRID was likewise reduced by pepsin digestion in the absence of BSA. Like the above activity loss, this antigenicity loss was not consistent with the amount of intact RBTI molecule disappearing. Furthermore, the degree of the residual antigenicity of a digest was generally estimated to be higher than that of the residual inhibitory activity, indicating that a certain degradation product produced by the digestion of RBTI still retains a high level of antigenicity compared to the inhibitory activity. A candidate for the product is the 8.5-kDa degradation product, since the immunoblot patterns of the digests showed that it was highly immunoreactive. The product is also anticipated to combine with the antiserum to form a larger precipitin circle in SRID than intact RBTI, since it seems that the 8.5-kDa species diffuses more rapidly in an agarose gel than RBTI because of its smaller molecular size. This may be the reason why the apparent antigenicity of a digest did not necessarily correspond to the inhibitory activity. However, it is suggested that the antigenicity determined by SRID is a more useful indicator than the inhibitory activity for evaluating the unabsorbed amounts of RBTI and its degradation products remaining in actual digestive tracts.

REFERENCES

