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RESEARCH NOTE

Occurrence in Leguminous Seeds, Resistance to Protease Digestion and Antigenicity of an α-Amylase Inhibitor

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A rabbit was immunized with the purified α-amylase inhibitor from cranberry beans to obtain a specific antiserum. With respect to antigenicity, the inhibitor was examined for its distribution in leguminous seeds, and was proved to occur exclusively in cultivars of the same kind (*Phaseolus vulgaris*) but not in those of other kinds. In addition, its in-vitro digestibility was investigated by the use of porcine and bovine enzymes. Consequently it was revealed that the inhibitor underwent a gradual inactivation by chymotrypsin digestion (but not by pepsin and trypsin digestions) and that its antigenicity was removed more slowly than its inhibitor activity.

INTRODUCTION

In previous papers (Kotaru *et al.*, 1987*a*,*b*), we described the purification of α-amylase inhibitor from cranberry bean (*Phaseolus vulgaris*) and some physicochemical properties. The inhibitor was a glycoprotein with molecular weight of 45 000, which specifically acted on mammalian pancreatic α-amylases. Such a proteinaceous inhibitor has been found in a few cultivars of kidney beans (Powers & Whitaker, 1977; Pick & Wöber, 1978*a*; Lajolo & Filho, 1985). There is, however, little information on its distribution in legumes from the viewpoint of antigenicity. The immunochemical approach is useful in investigating to what extent the inhibitor is susceptible to digestion by proteases in the mimetic digestive tract.

This preliminary report deals with the inhibitor distribution in various bean cultivars as well as variation in antigenicity during the in-vitro protease digestion using the rabbit antiserum against α-amylase inhibitor purified from cranberry beans.

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MATERIALS AND METHODS

Materials

Leguminous seeds used in this experiment are as follows; seven cultivars of *Phaseolus vulgaris* (great northern beans, small white kidney beans, light red kidney beans, kintoki beans, tora beans, uzura beans, and cranberry beans), two cultivars of *Phaseolus lunatus* (butter beans and baby lima beans), two cultivars of *Vigna angularis* (bamboo beans and adzuki beans), and cultivars of *Phaseolus coccineus* (runner beans) and *Psophocarpus tetragonolobus* (winged beans). Kintoki, tora, uzura, and adzuki beans were purchased from a grocery in Kyoto and the others from a seed importer in Kobe. Porcine pancreatic α-amylase, bovine pancreatic trypsin

and chymotrypsin, and porcine pepsin were obtained from the Sigma Chemical Co., St Louis, MO, USA. All other chemicals are commercially available and were of analytical grade and were used without further purification.

Water extraction of pulverized beans

Flour ground from air-dried beans was passed through a 60-mesh screen and stirred for 2 h at room temperature with five volumes of distilled water, followed by centrifugation at 15 000*g* for 60 min. The resulting supernatant was heated at 70°C for 15 min, centrifuged at the same speed as above, and subjected to immunoassay in the manner described below.

Purification of α-amylase inhibitor and its activity measurement

A highly purified preparation of α-amylase inhibitor was obtained from pulverized cranberry beans by means of water extraction (step 1), heat treatment (step 2), ethanol precipitation (step 3), ion-exchange chromatography with DEAE-cellulose (step 4) and gel filtration with Sephacryl S-200 (step 5) as previously described (Kotaru et al., 1987*a*). The final preparation was homogeneous in polyacrylamide gel electrophoresis, and is hereinafter referred to as 'CBAI'. The amylase activity was measured according to the iodine–starch method (Kotaru *et al.*, 1987*b*), and its degree of inhibition, i.e. inhibitor activity, was expressed as a percentage of the difference in absorbance at 660 nm between the absence and presence of CBAI under the routine assay system.

Immunization of a rabbit with CBAI and immunoassay

A New Zealand male rabbit weighing about 3 kg was immunized by subcutaneous injection with a water-in-oil emulsion of CBAI (5 mg), pH 7·2 phosphate buffer (1 ml) and Freund's complete adjuvant (1 ml). A booster shot of 2 mg of CBAI was given two weeks after the first injection, and a week later, the animal was bled to collect the serum. Double simple immunodiffusion for qualitative analysis was carried out in flat agarose gel according to the method of Ouchterlony (1958; 1962). Single radial immunodiffusion technique was employed for quantification of the antigen (Mancini *et al.*, 1965).

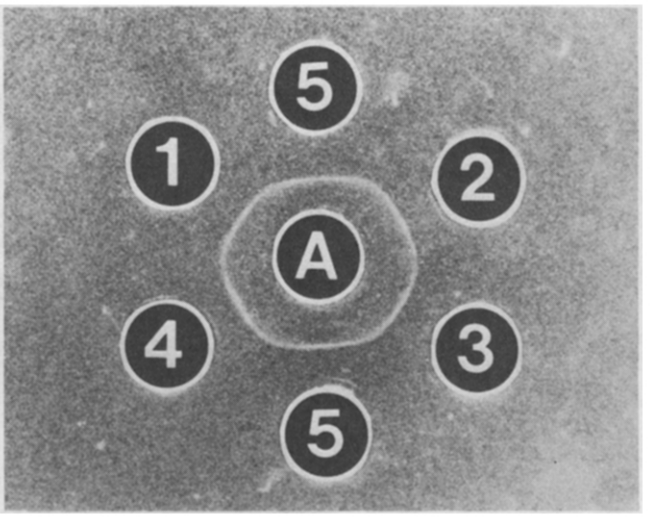

Fig. 1. Immunoprecipitin reaction in agarose gel by double simple diffusion between preparation at each purification and rabbit anti-CBAI serum; (A) antiserum, (1–5) the corresponding preparations at steps which were mentioned in the Materials and Methods section.

RESULTS AND DISCUSSION

Inspection of antiserum specificity

The rabbit antiserum raised against CBAI was tested

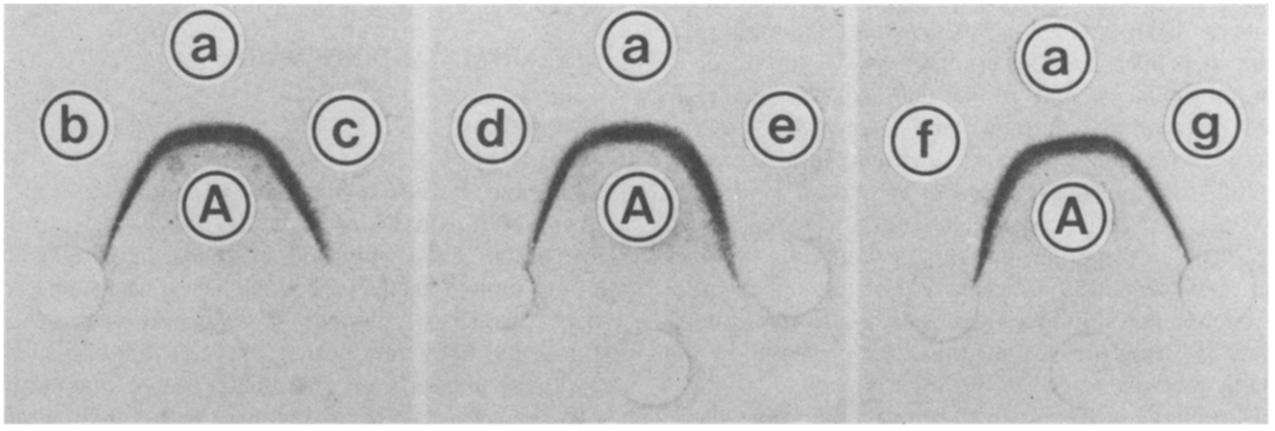

Fig. 2. Immunoprecipitin reaction in agarose gel by double simple diffusion between rabbit anti-CBAI serum and aqueous extracts from cultivars of *Phaseolus vulgaris*: (A) antiserum, (a) cranberry beans, (b) great northern beans, (c) small white kidney beans, (d) light red kidney beans, (e) kintoki beans, (f) tora beans, (g) uzura beans. Precipitin lines were stained with a Coomassie brilliant blue.

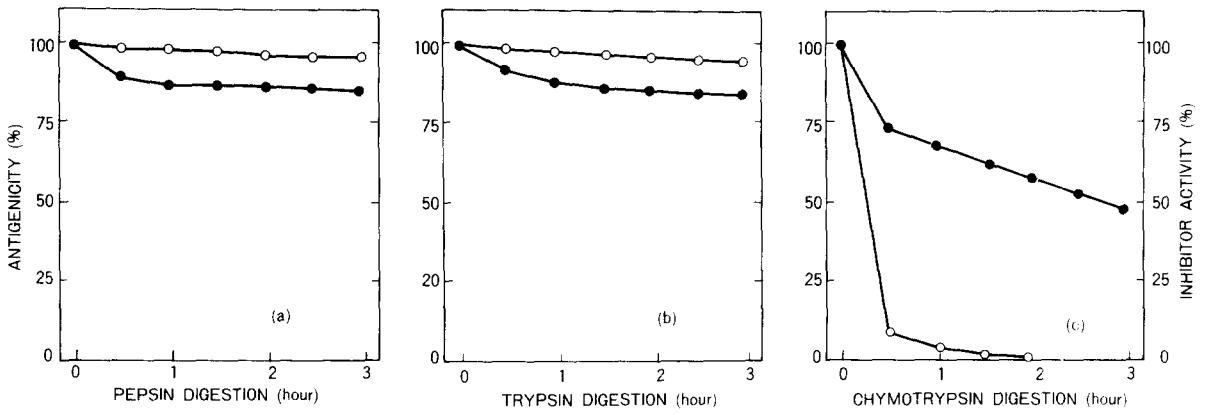

Fig. 3. Effects of digestion by (a) pepsin, (b) trypsin, and (c) chymotrypsin on both inhibitor activity and antigenicity of CBAI. A definite concentration of CBAI ($2{\cdot}2 \times 10^{-5}$ M) was treated at 37°C with fixed concentrations of pepsin (pH 2·2, $2{\cdot}2 \times 10^{-4}$ M), trypsin (pH 7·5, $2{\cdot}2 \times 10^{-3}$ M) and chymotrypsin (pH 7·5, $2{\cdot}2 \times 10^{-4}$ M), respectively. Aliquots (50 μl) were taken out at 30 min intervals. Then, pepsin was inactivated by neutralization with PIPES buffer, while trypsin and chymotrypsin were inactivated by heating at 70°C for 10 min. (○) residual inhibitor activity; (●) residual antigenicity.

for its immuno-reactivity with respective preparations at five purification steps. Immunoprecipitin reaction in agarose gel resulted in a single fused precipitin line without any spur as shown in Fig. 1. This implies that the antiserum contains no antibody against isoinhibitors, if any, nor against contaminants possibly arisen from modification or denaturation of CBAI during the purification process.

Limited occurrence of α-amylase inhibitor in leguminous seeds

The aqueous extracts from 13 kinds of bean cultivars were examined for their precipitin reaction with the rabbit anti-CBAI serum. Immunoreactive among various bean cultivars so far tested were seven kinds of *Phaseolus vulgaris*. A quite similar pattern was observed for the inhibitor activity. Figure 2 depicts the results of immunoassay, in which each single precipitin line was completely fused into that toward CBAI. Needless to say, the preimmune serum gave no precipitin reaction with any aqueous extracts from beans. Incidentally, Pick and Wöber (1978*b*) have reported that broad beans (*Vicia faba*), not having any inhibitor effect on mammalian α-amylase, are inactive in immunoreactivity with rabbit antiserum against white kidney bean α-amylase inhibitor.

In-vitro digestibility of CBAI; changes in inhibitor activity and antigenicity

The stability of CBAI against treatment with proteolytic enzymes such as pepsin, trypsin and chymotrypsin was assessed by measuring its time-dependent losses in the inhibitor activity as well as the antigenicity. CBAI was virtually resistant to pepsin digestion (37°C, 3 h) despite the enzyme concentration of 10-fold molar ratio to CBAI (Fig. 3(a)). The tendency was exactly as in the presence of trypsin at a 100-fold molar concentration (Fig. 3(b)). In either case, a structural change might have been rather reflected in the loss of antigenicity than in that of inhibitor activity. In contrast to its resistance to digestion by pepsin or trypsin, CBAI was relatively sensitive to chymotrypsin digestion and its inhibitor activity was almost absent within 2 h (Fig. 3(c)). Nevertheless, the antigenicity of CBAI was maintained by a half even after complete disappearance of inhibitor activity. SDS-electrophoresis showed that chymotrypsin digestion produced little or no fragments other than three subunits (data not shown). Even so, a partial cleavage of the peptide bond may have occurred in the reactive site of CBAI or its vicinity. This is the reason why the antigenicity does not correspond to the inhibitor activity regarding their decrease rates.

In any case, it seems likely that CBAI is emptied from the stomach without any accompanying loss of the inhibitor activity and easily combines with pancreatic α-amylase in the intestinal lumen, when both the resistance of CBAI to pepsin digestion and the presence of a large amount of dietary protein in chyme are taken into consideration. This problem will be referred to in detail in another paper.

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