An α -Amylase Inhibitor from Cranberry Bean (*Phaseolus vulgaris*): Its Specificity in Inhibition of Mammalian Pancreatic α -Amylases and Formation of a Complex with the Porcine Enzyme

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Summary A proteinaceous inhibitor that inhibits mammalian α -amylases was prepared from cranberry bean and examined for its reactivity with α -amylases from various origins. The cranberry bean α -amylase inhibitor (CBAI) exhibited inhibitory effects on pancreatic α -amylases from the following mammals: pig, dog, cat, horse, sheep, cow, rabbit, guinea pig, rat, and mouse. CBAI showed a maximal inhibition at pH 5.5 against porcine pancreatic α -amylase (PPA). It was confirmed by gel filtration that a complex was formed in the 1 : 1 ratio between CBAI and PPA when they were incubated at 37°C for 30 min at pH 5.5. A similar inhibition pattern was also observed at pH 6.9 that is optimal for the amylase reaction, but much higher concentrations of CBAI were required to give 50% inhibition at pH 6.9 than at pH 5.5. Especially, both bovine and rat α -amylases were virtually unreactive to CBAI at pH 6.9.

Key Words formation of complex, species-specificity, α -amylase inhibitor, pancreatic α -amylase, cranberry bean

Proteinaceous α -amylase inhibitors have been found in cereals(1-4), legumes(5-8), tubers(9,10), and bacteria(11) and characterized by the specificity towards animal α -amylases. Although the mechanism of inhibition is still obscure, the inhibitors are practically applicable to the control of post-prandial glucose level in the blood by disturbing digestion of starch in the intestine(12) and to the purification of salivary or pancreatic α -amylase by using as adsorbent for affinity chromatography(13). In a previous paper(14), we have reported the isolation of an amylase inhibitor from cranberry bean (*Phaseolus vulgaris*) (CBAI), and its

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physicochemical properties. The inhibitor was a glycoprotein, which acted effectively on porcine pancreatic α -amylase (PPA).

The present paper deals with the formation of a complex between CBAI and PPA and with the inhibition spectrum of CBAI against pancreatic α -amylase from various animals.

MATERIALS AND METHODS

Materials. CBAI was purified as previously described (14). Porcine pancreatic and human salivary α -amylases and amylases from *Aspergillus oryzae*, *Bacillus subtilis*, barley malt, and sweet potato were purchased from Sigma Chemical Co. Pancreas from pig, dog, cat, horse, sheep, cow, rabbit, guinea pig, rat, and mouse were also obtained as acetone powder from Sigma Chemical Co., from which α -amylases were purified in our laboratory by affinity chromatography with potato starch according to the method described by Tsujisaka (15). Other chemicals of analytical grade were commercially available and used without further purification.

Protein determination and molarity calculation. Protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as standard. The molar concentrations of PPA and CBAI were calculated on the basis of their molecular weights of 54,000 (17) and 45,000 (14), respectively.

Enzyme assay. The α -amylase activity was measured by a modification of the iodine method by Murao *et al.* (18). The reaction mixture containing an appropriate concentration of α -amylase, 0.5% soluble starch, 50 mM NaCl, 5 mM CaCl_2 and 0.01% ovalbumin in 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.9) was incubated at 37°C for 10 min. The reaction was stopped by adding 2.5 ml of an acidic solution containing 0.08 M HCl and 0.4 M acetic acid to 1.5 ml of the reaction mixture. To its aliquot (0.4 ml), 5 ml of an aqueous solution containing 0.005% iodine and 0.05% potassium iodine was added to measure the absorbance at 660 nm. One unit of enzyme was defined as the amount of enzyme capable of producing 0.8 decrease in the absorbance.

pH Dependence of inhibition by CBAI. A unit of PPA was incubated at 37°C for 30 min together with 1.2×10^{-8} M CBAI in various buffers composed of 10 mM sodium citrate (pH 4.0–5.5) or 10 mM sodium phosphate (pH 6.0–8.0) and 40 mM NaCl and 2 mM CaCl_2 . The residual α -amylase activity after the indicated incubation period was measured as mentioned above, and the extent of inhibition was expressed in percentage as the difference between the activities in the presence and absence of CBAI under the assay conditions.

Gel filtration. A mixture of PPA at 1.8×10^{-5} M and CBAI at 9.2×10^{-6} M in 50 mM acetate buffer (pH 5.5) containing 50 mM NaCl and 5 mM CaCl_2 that had been incubated at 37°C for 60 min was applied to a Toyo-Pearl HW-55 column (2.6 $\phi \times$ 95 cm) previously equilibrated with the same buffer, and the effluents were fractionated in each 4.0 ml. Aliquots (0.01 ml) of the fractions were assayed for their amylase and inhibitor activities.

Stoichiometry in formation of a complex. For the purpose of stoichiometric investigation on the formation of a complex between PPA and CBAI, a mixture of PPA at a fixed level (1.6×10^{-8} M) and CBAI at two fixed levels (6.5×10^{-9} M and 1.3×10^{-8} M) was incubated at pH 5.5 and 37°C and its aliquots (1.0 ml) were withdrawn at stated intervals during incubation to measure the residual α -amylase activity. Subsequently, PPA (1.6×10^{-8} M) was incubated at pH 5.5 and 37°C for 60 min together with CBAI at varied concentrations of 1.4×10^{-9} M to 1.4×10^{-8} M, and then, the residual α -amylase activity was likewise measured.

Reactivity of CBAI with various amylases. CBAI (1.2×10^{-7} M) was allowed to act on amylases from various sources such as animal, plant, fungus, and bacteria in 50 mM acetate buffer (pH 5.5) or in 20 mM PIPES buffer (pH 6.9) at 37°C for 30 min. The residual amylase activity was measured at pH 6.9 with α -amylases from animals and at pH 5.5 with amylases from other sources. The specificity of CBAI towards pancreatic α -amylases was investigated using the acetate buffer (pH 5.5) and the PIPES buffer (pH 6.9), in which both incubation and activity measurement were carried out.

RESULTS AND DISCUSSION

A unit of PPA underwent 25% inhibition of the initial activity after incubation of 30 min at 25°C and pH 6.9 in the presence of 6.2×10^{-8} M CBAI. The degree of inhibition by CBAI increased with increasing temperature, and the preincubation at 40°C caused a threefold increase in inhibition relative to that at 25°C . In this connection, Marshall and Lauda (5) and Powers and Whitaker (19) have referred to

Fig. 1. Effects of pH on inhibition of PPA by CBAI. PPA was incubated at various pHs with CBAI for 30 min at 37°C . Controls were run without inhibitor at each pH simultaneously (○). The extent of inhibition (●) at different pHs was calculated by setting α -amylase activity in each control incubation at 100%.

Fig. 2. Gel filtration of the mixture of PPA and CBAI. PPA and CBAI were incubated in 50 mM acetate buffer (pH 5.5) containing 50 mM NaCl and 5 mM CaCl_2 for 60 min at 37°C. The mixture was applied to a Toyo-Pearl HW-55 column (2.6 ϕ × 95 cm) and then eluted with the same buffer. Left and right arrows indicate where free PPA and CBAI were eluted, respectively. Amylase activity (○), absorbance at 280 nm (●).

the more rapid formation of a complex between PPA and kidney bean α -amylase inhibitor at 25°C than at 37°C, while Buonocore *et al.* (20) have found that there is a decrease in inhibition of chicken pancreatic α -amylase by wheat kernel inhibitors at above 30°C. Nevertheless incubation was carried out at 37°C in our experiment.

Figure 1 shows the effects of pH on inhibition of PPA by CBAI; CBAI exhibited the highest inhibitory effect on PPA at pH 5.5. The drop in inhibition below or above pH 5.5 was considered not due to denaturation of CBAI, because the inhibitor was stable over the pH ranges of 3 to 7 (21). The optimum pH of inhibition against PPA was in the range of 4.5 to 5.5 with α -amylase inhibitors from many beans (5, 22), while wheat inhibitors were most inhibitory at pH 6.4–8.4 and pH 5.8–7.0 against human salivary and pancreatic α -amylases, respectively (1).

Figure 2 shows a typical gel filtration pattern of the CBAI and PPA mixture that had been previously incubated under the conditions optimal for inhibition. In the presence of α -amylase excess to inhibitor, two peaks appeared on the chromatogram. Neither α -amylase nor inhibitor activities were detected in the first peak. Taking into account the elution volume, there is little doubt that the first peak component is responsible for a complex formed between PPA and CBAI. Tanizaki and Lajolo (23) have reported that the complex between PPA and kidney bean α -amylase inhibitor is partially dissociative at acidic pH or above pH 6.5. On either side of the optimum pH, a similar dissociation may have occurred in the complex between PPA and CBAI. The second peak component was attributable mainly to PPA by activity measurement.

Fig. 3. Time course of inhibition of PPA by CBAI. PPA (1.6×10^{-8} M) was incubated with two different levels of CBAI at pH 5.5 and 37°C , respectively. After varying times of incubation, residual α -amylase activity was determined. 6.5×10^{-9} M of CBAI (○), 1.3×10^{-8} M of CBAI (●).

Fig. 4. Stoichiometry of inhibition of PPA by CBAI. PPA (1.6×10^{-8} M) was incubated at pH 5.5 and 37°C for 60 min with CBAI at varied concentrations of 1.4×10^{-9} M to 1.4×10^{-8} M, and then, the residual α -amylase activity was measured.

Figure 3 shows the time course of inhibition of PPA at a fixed level by CBAI at two fixed levels. The complete inhibition took place in 30 min at pH 5.5. However, the inhibition was not completed at pH 6.9 even after incubation of 6 h (data not shown). In this regard, it took 1 h at optimum pH for kidney bean inhibitors to completely inhibit PPA (5) but over 6 h at pH 6.9 for black kidney bean inhibitors to completely inhibit PPA (22). On the other hand, a *Streptomyces* inhibitor and PPA associated to form the complex within a few minutes (24). The ratio between the degrees of PPA inhibition by CBAI at two different levels suggests that a stoichiometric relationship is valid for their complex. This relationship was also

Table 1. Inhibition of various amylases by CBAI.

Source of amylase	Inhibition (%)
α -Amylases	
Porcine pancreas	100
Human salivary	100
Barley malt	0
<i>Aspergillus oryzae</i>	0
<i>Bacillus subtilis</i>	0
β -Amylase	
Sweet potato	0
Amylase	
Cranberry bean ^a	0

^aThe extract from cranberry bean germinated for 7 days was used. Other enzymes were obtained from Sigma.

Table 2. Reactivity of CBAI with mammalian pancreatic α -amylases.

Source of pancreatic α -amylase ^a	Amount of CBAI (nM) required for 50% inhibition	
	(pH 6.9)	(pH 5.5)
Pig	40	7.3
Dog	28	7.7
Cat	22	8.9
Horse	30	8.0
Sheep	250	6.7
Cow	> 3,000	100
Rabbit	160	9.5
Guinea pig	180	12
Rat	> 3,000	27
Mouse	150	8.4

^aPreparations from pancreatic acetone powder were used as amylases.

ascertained by using various concentrations of CBAI. As shown in Fig. 4, the residual activity of PPA decreased in inverse proportion to CBAI concentration and the molar ratio of CBAI to PPA to give a complete inhibition was obtained as 1 : 1 by extrapolation of the linear plot to the abscissa.

Table 1 summarizes the inhibitory effects of CBAI on amylases from various sources. The CBAI was as effective in inhibition against PPA as against human salivary α -amylase, while it had no inhibitory effect on amylases from plant, fungus and bacteria.

Table 2 compares the concentrations of CBAI to yield 50% inhibition against

pancreatic α -amylases from mammals. Although mammalian pancreatic α -amylases corresponding to one unit were used at either pH, the concentration of CBAI to give 50% inhibition was much lower at pH 5.5 than at pH 6.9 for most of the enzymes. Under the physiological pH conditions, α -amylases from pig, dog, cat, and horse were most effectively inhibited by CBAI, to which the enzymes from sheep, rabbit, guinea pig, and mouse followed. Noteworthy, the enzymes from cow and rat were little inhibited by CBAI even at a level of more than 3.0×10^{-6} M under the same conditions. There was a clear difference in optimum pH between the catalytic activity of pancreatic α -amylase and the inhibitory activity of its inhibitor. The concentrations of CBAI to give 50% inhibition against pancreatic α -amylases from cow and rat were 1.0×10^{-7} M and 2.7×10^{-8} M, respectively, at pH optimal for the inhibitory activity, pH 5.5. The reactivity of inhibitor with amylase has been hitherto evaluated at pH optimal for the amylase activity (24-26). Probably for this reason, multi-forms of wheat α -amylase inhibitors did not act on guinea pig pancreatic α -amylase (25), nor did microbial inhibitors on rabbit pancreatic α -amylase (24), nor did inhibitors from red gram, sorghum, pearl millet, sanwa millet and proso millet on horse pancreatic α -amylase (26). Such inhibitors may also become more inhibitory against α -amylases when assayed at weakly acidic pH.

CBAI even at sufficiently high concentrations showed little inhibition against pancreatic α -amylases from cow and rat at neutral pH, suggesting that CBAI would not always serve as an inhibitor in the intestinal environments of these animals. In relation to this possibility, Savaiano *et al.* (27) reported that administration of red kidney bean α -amylase inhibitor to rats did not affect their growth. On the contrary, Marshall and Lauda (5) described that human pancreatic α -amylase was as much inhibited by white kidney bean α -amylase inhibitor as PPA at pH 5.5. Layer *et al.* (28) have recently observed that perfusion of human duodenum with an aqueous solution containing kidney bean α -amylase inhibitor leads to a considerable decrease in the amylase activity remaining in the lumen. This observation supports the possibility of use of α -amylase inhibitor as an antidiabetic drug. Amylase-inhibitor interaction and physiological justification of the naturally occurring α -amylase inhibitor remain further investigated.

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