Interaction of a Purified Bean (*Phaseolus vulgaris*) Glycoprotein with an Insect Amylase[1]

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ABSTRACT

A glycoprotein α-amylase inhibitor from red kidney bean was purified by affinity chromatography, and its in vitro interaction with an amylase from *Tenebrio molitor* larvae was studied. Inhibition of the amylase by bean amylase inhibitor is dependent on pH, ionic strength, and temperature. The rate of combination of the amylase was 2.7×10^5 per mole per second at pH 5.4 and 30°C.

The presence of amylase inhibitors in various plant seeds has been well documented. The glycoprotein amylase inhibitors from *Phaseolus vulgaris* beans have been purified to homogeneity by either conventional techniques or affinity chromatography (Marshall and Lauda 1975; Pick and Wober 1978, 1979; Powers and Whitaker 1977a). The bean amylase inhibitors inhibit mammalian amylases but not microbial or plant amylases (Jaffe et al 1973, Powers and Whitaker 1977a). In one case, a crude insect amylase was shown to be susceptible to inhibition by inhibitor from light red kidney bean (Powers and Whitaker 1977a). Later research indicated that amylases from yellow meal worm (*Tenebrio molitor*) larvae, Mediterranean flour moth (*Anagasta kuhniella*) larvae, red flour beetle (*Tribolium castaneum*) adults, and both adults and larvae of confused flour beetle (*Tribolium confusum*) were inhibited by bean inhibitor (Powers and Culbertson 1982). The interaction of purified bean inhibitor and porcine pancreatic α-amylase has been studied (Marshall and Lauda 1975, Powers and Whitaker 1977b).

The interaction of pure bean amylase inhibitor with a purified insect amylase was examined. It has been suggested that the function of bean amylase inhibitors is as a protective agent against insects (Marshall and Lauda 1975). *Tenebrio molitor* was selected as a source of the enzyme because its amylase has been purified and characterized. Its interaction with wheat amylase inhibitors has also been studied (Buonocore et al 1976, 1980).

MATERIALS AND METHODS

Materials

Tenebrio molitor larvae were from a stock culture from the Department of Entomology, Washington State University. DEAE-cellulose (DE-32 microgranular lot 2432855) was from Whatman. Affi-Gel 10 (lot 20347) and electrophoresis reagents were from Bio-Rad. All other chemicals were of reagent grade. Distilled water was used throughout.

Purification of Inhibitor

Bean amylase inhibitor was purified from an ethanol precipitate of an aqueous extract of light red kidney bean (Powers and Whitaker 1977a) via affinity chromatography as described by Pick and Wober (1979). The affinity system entailed immobilization of porcine pancreatic α-amylase on Affi-Gel 10 at pH 7.5. Bean inhibitor was allowed to bind the immobilized amylase at pH 5.6 (60 m*M* sodium acetate) and was eluted at pH 2.8, using 50 m*M* glycine-HCl buffer.

Enzyme Purification

Amylase was extracted from whole *Tenebrio molitor* larvae using 10 m*M* sodium acetate-10 m*M* calcium chloride, pH 5.4, as described by Petrucci et al (1974). The enzyme was purified via 50–80% ammonium sulfate fractionation, dialysis, and DEAE-cellulose chromatography at pH 4.5 (50 m*M* sodium acetate-10 m*M* calcium chloride) with enzyme elution via a linear sodium chloride gradient. All steps were performed at 0–4°C.

Electrophoresis

Polyacrylamide disk gel electrophoresis of the purified inhibitor and enzyme was performed on 7.5% polyacrylamide slabs (1.5 mm thick) using the buffer system of Davis (1964). The gels were stained overnight at room temperature with 0.05% Coomassie blue R-250 in 50% ethanol-9.2% acetic acid. Gels were destained with 5% ethanol-7.5% acetic acid.

Assays

Amylase assays were done at pH 5.4 (40 m*M* sodium acetate-10 m*M* calcium chloride) and 30°C, using 0.5% reduced starch as substrate (Strumeyer 1967). Enzyme activity was followed by an increase in reducing groups, using dinitrosalicylic acid reagent (Bernfeld 1955). For enzyme-inhibitor interaction studies, enzyme and inhibitor were combined and aliquots removed to starch at specific intervals for amylase assay. To determine the effect of pH on the rate of enzyme inhibitor interaction, 40-m*M* acetate buffers from pH 3.9 to pH 5.7 were used. Potassium chloride was added to the enzyme-inhibitor mixture to a constant ionic strength of 0.04. After 5 min, aliquots were removed to starch at pH 5.4 for amylase assay.

The effect of ionic strength on the rate of enzyme-inhibitor interaction was performed at pH 5.4. Potassium chloride was added to the enzyme-inhibitor mixture to adjust ionic strength. Aliquots of the mixtures were removed after 5 min and assayed for remaining amylase activity. In all cases, degree of interaction of enzyme and inhibitor is expressed in terms of percent inhibition based on assays identical except for deletion of inhibitor from the mixtures.

RESULTS AND DISCUSSION

Results of the polyacrylamide gel electrophoresis of the bean amylase inhibitor and *Tenebrio* amylase are shown in Fig. 1. The single band for the inhibitor is indicative of its purity, whereas the enzyme preparation shows a single major band with two minor bands. Figure 2 shows the time course for the interaction of the inhibitor and amylase at 30°C and pH 5.4, the pH optimum for this enzyme (Applebaum et al 1961). Replotted, these data indicate that the formation of enzyme-inhibitor complex is a second-order rate reaction. The second-order rate constant calculated was 2.7×10^5 $M^{-1}\text{sec}^{-1}$ at 30°C, pH 5.4. For this calculation, inhibitor was considered to be 100% active and enzyme 60% active, based on the endpoint percent inhibition at 60 min. The calculated rate constant is roughly 10 times greater than that reported for the interaction of the bean inhibitor with porcine pancreatic α-amylase at 30°C and pH 6.9, the pH optimum for that enzyme (Powers and Whitaker 1977b).

The effect of reaction mixture pH on the binding of the inhibitor

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and *Tenebrio* amylase is shown in Fig. 3. An increase in inhibition after 5 min of pre-incubation is noted as the pH is lowered from the pH optimum of the enzyme. At pHs below approximately pH 3.8, a very rapid and irreversible loss of enzyme activity was noted. The increase in inhibition as the pH is lowered from the enzymes pH optimum is similar to earlier observations on the interaction of bean amylase inhibitor and porcine pancreatic α-amylase (Marshall and Lauda 1975, Powers and Whitaker 1977b). The data presented here suggest that rate of expression of inhibition is dependent on a group with a pK_a near that of a carboxyl group. Whether this group is on the inhibitor or the enzyme is not known.

Data in Table I show the effect of ionic strength on the rate of interaction of enzyme and inhibitor at pH 5.4. The amount of inhibition at ionic strength 0.15 was four times greater than that at 0.04 ionic strength. The effect of ionic strength can be interpreted in several ways. Charged groups that interfere with the interaction of enzyme and inhibitor may be shielded by the increased number of ions in solution (ie, primary salt effect). The ionic strength effect and the pH effect could be due to a common factor, ie, the increase in extent of inhibition attained by lowering the pH from 5.4 could be considered equivalent to an increase in ionic strength at pH 5.4. Alternatively, ionic strength and pH effects may not involve specific charged groups involved in the enzyme-inhibitor complexation but may be due to alterations of equilibria between conformational forms of the free enzyme and/or inhibitor required to form the enzyme-inhibitor complex. That a conformational change may be important in formation of the complex is suggested by data that indicate that the rate of complex formation is three times greater at 29.3°C than at 20.0°C (E_a ~26 kcal/mole). Interaction of the inhibitor with porcine pancreatic α-amylase is temperature-dependent, with a reported E_a of 39.5 kcal/mole (Powers and Whitaker 1977b).

The in vivo significance of the data presented has yet to be assessed. If the bean amylase inhibitor can be shown by feeding studies to be related to resistance to insect pests, it could be valuable as an index for selecting and breeding of beans. Silano et al (1975) have shown that amylases from insects destructive of wheat grain are inhibited by wheat amylase inhibitor. Yetter et al (1979) have observed a correlation in some wheat varieties between in vivo resistance to *Sitophilus oryzae* (rice weevil) and the extent of in vitro inhibition of insect larval amylase by extracted inhibitors. The bean amylase inhibitor is especially attractive as an insect resistance factor; when fed in large amounts, it does not result in growth depression when fed to weanling rats on a diet containing starch (Savaino et al 1977).

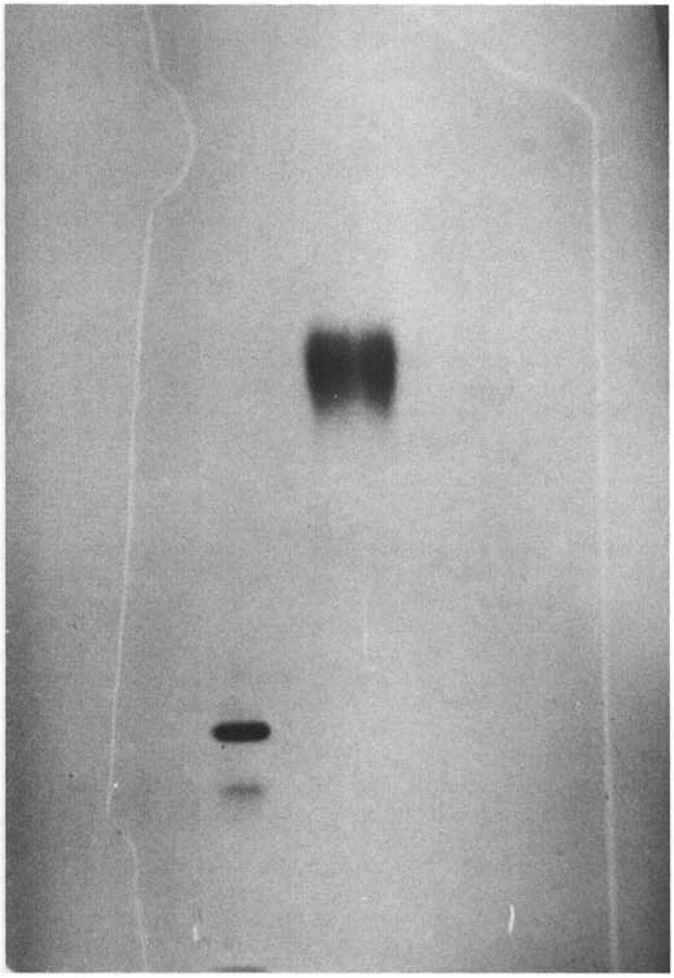

Fig. 1. Polyacrylamide slab gel of purified inhibitor (right) and enzyme. Bottom is anode.

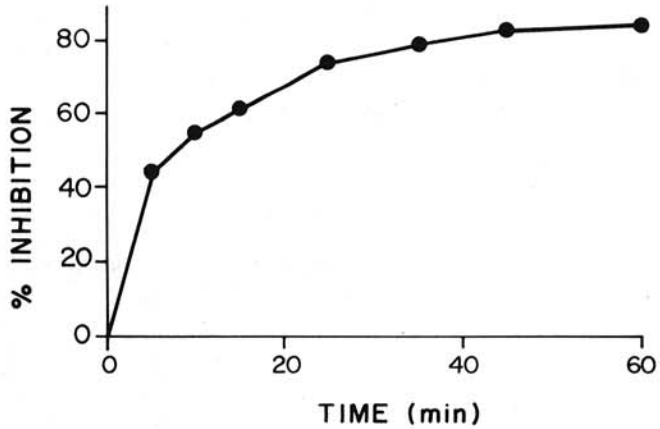

Fig. 2. Time course of reaction of 0.385 μg/ml inhibitor and 0.891 μg/ml enzyme (final concentrations) at 30°C and pH 5.4.

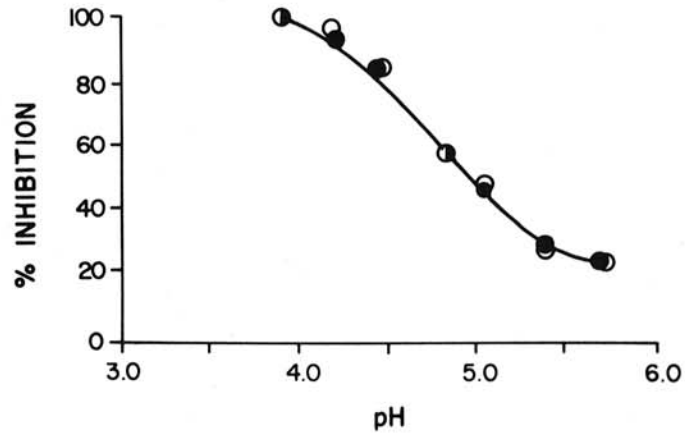

Fig. 3. Effect of pH of medium on expression of inhibition after 5 min of pre-incubation.

TABLE I
Effect of Ionic Strength on Inhibition

Ionic Strength[a]	Percent Inhibition[b]
0.04	9
0.06	16
0.08	22
0.10	25
0.12	29
0.15	35

[a] Adjusted by addition of KCl.
[b] Percent inhibition of amylase after 5 min of pre-incubation with inhibitor.

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