

α -AMYLASES FROM THREE SPECIES OF STORED GRAIN COLEOPTERA AND THEIR INHIBITION BY WHEAT AND CORN PROTEINACEOUS INHIBITORS*

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Abstract—Two α -amylase isozymes were isolated from adults of both the rice weevil, *Sitophilus oryzae*, and the red flour beetle, *Tribolium castaneum*, and a single α -amylase from adults of the yellow mealworm, *Tenebrio molitor*. All of the purified enzymes had similar amino acid compositions as well as physical and chemical properties. The apparent molecular masses ranged from 53 to 58 kDa. Circular dichroism measurements revealed approx. 33% α -helical content. V_{max} and K_m values ranged from 1.33 to 5.98 mM $\text{min}^{-1} \text{mg}^{-1}$ and 0.76 to 5.57 mg ml^{-1} , respectively, using starch as the substrate. An α -amylase inhibitor from wheat (WRP-25) inhibited all five enzymes, whereas an inhibitor from corn inhibited only enzymes from the red flour beetle and yellow mealworm.

Key Word Index: insect; α -amylase; rice weevil; red flour beetle; yellow mealworm; *Sitophilus*; *Tribolium*; *Tenebrio*; inhibitor; wheat; corn

INTRODUCTION

Three common pests of stored grain are the rice weevil, *Sitophilus oryzae*, the red flour beetle, *Tribolium castaneum*, and the yellow mealworm, *Tenebrio molitor*. These insects are capable of causing extensive damage to cereal grains or their milled products (Anon., 1979). The starch of cereal grains can be degraded by amylases of the cereals themselves during germination or by the digestive amylases of stored product pests (Applebaum, 1964; Applebaum and Konijn, 1965; Baker, 1983; Kruger and Lineback, 1987). Also occurring in cereals are proteinaceous inhibitors of α -amylases, which may act as resistance factors against insect attack (Silano *et al.*, 1975; Buonocore *et al.*, 1977; Horber, 1983; Baker *et al.*, 1991; Feng *et al.*, 1991). We are interested in the properties of cereal proteinaceous inhibitors of insect α -amylases and their possible manipulation by genetic engineering methods for insect control purposes. We wished to determine the relative effectiveness of these inhibitors *in vitro* against α -amylases from several species of stored product insects. For this purpose, we isolated five α -amylases from *Tribolium castaneum*, *S. oryzae*, and *T. molitor* and studied their physical and enzymatic properties, and their differen-

tial inhibition by two purified inhibitors of α -amylase from wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.).

MATERIALS AND METHODS

Insects

S. oryzae was reared in whole wheat. *T. castaneum* and *T. molitor* were cultured in a mixture of 95% ground wheat and 5% brewer's yeast. Adults of mixed sexes and ages were collected, weighed, and stored at -70°C .

Chemicals

All chemicals were reagent grade and were obtained from Sigma Chemical Co., St Louis, Mo, U.S.A., unless otherwise noted.

Enzyme purification

Amylases were isolated essentially as described by Baker and Woo (1985), including a Mono-Q (Pharmacia) anion exchange high performance liquid chromatography (HPLC) step. Briefly, insects were homogenized twice in 1% NaCl (0.5 g wet wt/ml) using a Tekmar tissuemizer, and the total slurry was centrifuged at 5300 g for 20 min. The supernatant was adjusted to pH 5 by adding 0.1 vol of 10 \times assay buffer (1 \times : 20 mM sodium acetate pH 5.0, 20 mM sodium chloride, 0.1 mM calcium chloride) and filtered through glass wool. The filtrate was used for glycogen-amylase complexation directly, following the method of Loyter and Schramm (1962), except that glycogen was first suspended in assay buffer. After centrifugation, the pellet was dissolved in assay buffer and incubated at 37 $^{\circ}\text{C}$ for 3 h to digest the glycogen. The mixture was dialyzed extensively against 20 mM Tris-HCl (pH 7.5) and then applied to a Mono-Q HPLC column directly. Proteins were eluted with the salt gradient program shown in Fig. 1.

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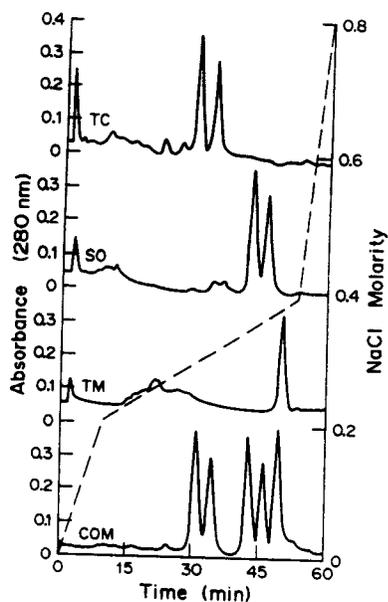


Fig. 1. HPLC separation of α -amylases from red flour beetle (TC), rice weevil (SO), yellow mealworm (TM), and mixture of all α -amylases (COM) by Mono-Q anion exchange column. Samples were prepared as described in Materials and Methods.

Inhibitor purification

The 12 kDa corn inhibitor (CI) (Swartz *et al.*, 1977; Mahoney *et al.*, 1984) was purified as follows. A 0.15 M NaCl extract of defatted corn meal was heated to about 60°C for 5 min in a boiling water bath (Chong and Reeck, 1987). The supernatant obtained by centrifugation at 10,000 *g* for 20 min was adjusted to 20% saturation by the addition of solid ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in water, and applied to a reversed-phase (RP) HPLC column (Lei and Reeck, 1986) for final purification of the 12 kDa corn inhibitor. Wheat amylase inhibitor WRP-25 was purified as described by Feng *et al.* (1991) and corresponded to HPLC peak number 25 of Fig. 3 in that study. WRP-25 is a member of the 0.28 subfamily of wheat amylase inhibitors (Silano, 1987).

Enzyme assay and kinetic analysis

Determination of α -amylase activity and kinetic parameters for hydrolysis of starch was made in 20 mM sodium acetate, pH 5.0, 20 mM NaCl, 0.1 mM CaCl₂ following the procedures of Baker and Woo (1985), except that different units of K_m and V_{max} were used and protein concentrations were determined by extinction coefficients (see below). Briefly, approx. 0.3 mM of each α -amylase was preincubated for 4 h in assay buffer at room temperature with 0.1, 0.2, 0.3, 0.5 and 0.7 mM inhibitor prior to enzyme assay. Three replicates were carried out. Michaelis constants and maximum velocities were determined by the method of Lineweaver and Burk (1934). Inhibition constants (K_i) were determined as described by Baker (1988).

Electrophoresis

Purified amylases were analyzed by electrophoresis in 10% non-denaturing polyacrylamide gels according to Davis (1964) and 7.5% SDS-PAGE gels as described by Laemmli (1970).

Sedimentation equilibrium centrifugation

Protein samples (about 3.5 mg ml⁻¹) were dialyzed against a mixture of 0.15 M sodium chloride, 20 mM sodium

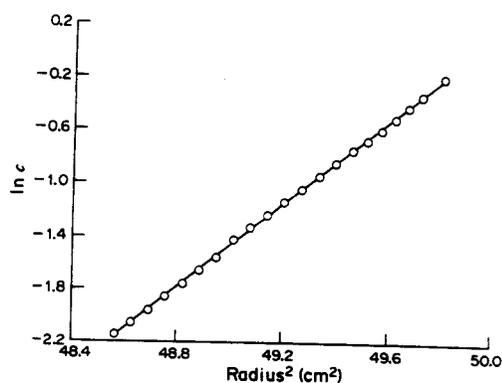


Fig. 3. Sedimentation equilibrium of rice weevil α -amylase SOA-1 in acetate buffer, pH = 5. The natural logarithm of the fringe displacement ($\ln c$) is plotted against the square of the radial distance (r^2). The plot for SOA-2 is essentially identical (not shown).

acetate (pH 5.0), 0.1 mM calcium chloride and centrifuged to equilibrium (32,000 rpm at 23°C for 48 h) using a Beckman model E analytical ultracentrifuge with an interference optical system. The data were collected and analyzed as described by Land (1985). Each isozyme was subjected to centrifugation three times. Partial specific volumes were calculated by the amino acid compositions (McKeekin and Marshall, 1952), and the density of the buffer was determined with a pycnometer.

Amino acid analysis

Amino acid analyses were conducted by reversed-phase HPLC after derivitization with phenylisothiocyanate (Bidlingmeyer *et al.*, 1984) as described by Chong and Reeck (1987). Norleucine was included as an internal standard. Appropriate corrections were made both for labile amino acids and for slowly released amino acids. Analysis were repeated three times. Cysteine and methionine were determined by the method of Hirs (1967), and tryptophan was determined spectrophotometrically from the ratio of tyrosine to tryptophan as described by Bencze and Schmid (1957) and Edelhoch (1967).

Compositions were compared quantitatively by calculating

$$C = \frac{\sum_{i=1}^{16} (X_{A,i} - 6.25)(X_{B,i} - 6.25)}{\sqrt{\left[\sum_{i=1}^{16} (X_{A,i} - 6.25)^2 \right] \left[\sum_{i=1}^{16} (X_{B,i} - 6.25)^2 \right]}}$$

where $X_{A,i}$ and $X_{B,i}$ are the contents in mole percent of amino acid i in proteins A and B, respectively. The summation is carried out over the 16 amino acids (not including Cys and Trp) whose values are obtained by amino acid analysis. The expression for C is the standard formula for a correlation coefficient indexed to treat the relevant circumstance of two 16-part observations, i.e. amino acid compositions of two proteins, as contrasted to the typical case in statistics of 16 observations from two populations. For two identical compositions, its value is 1.00.

Extinction coefficients

Extinction coefficients were calculated by determining the absorbances at 280 nm and the protein concentrations of solutions of the purified enzymes. Protein concentrations were determined by amino acid analysis, as described by Land (1985). Samples were dialyzed against 0.17 M ammonium acetate (pH 5.0), and absorbances were measured. 0.1 ml of the dialyzed sample was lyophilized together with 12.5 nmol norleucine. Then 0.1 ml water was added, and the sample was lyophilized again. The above procedure was

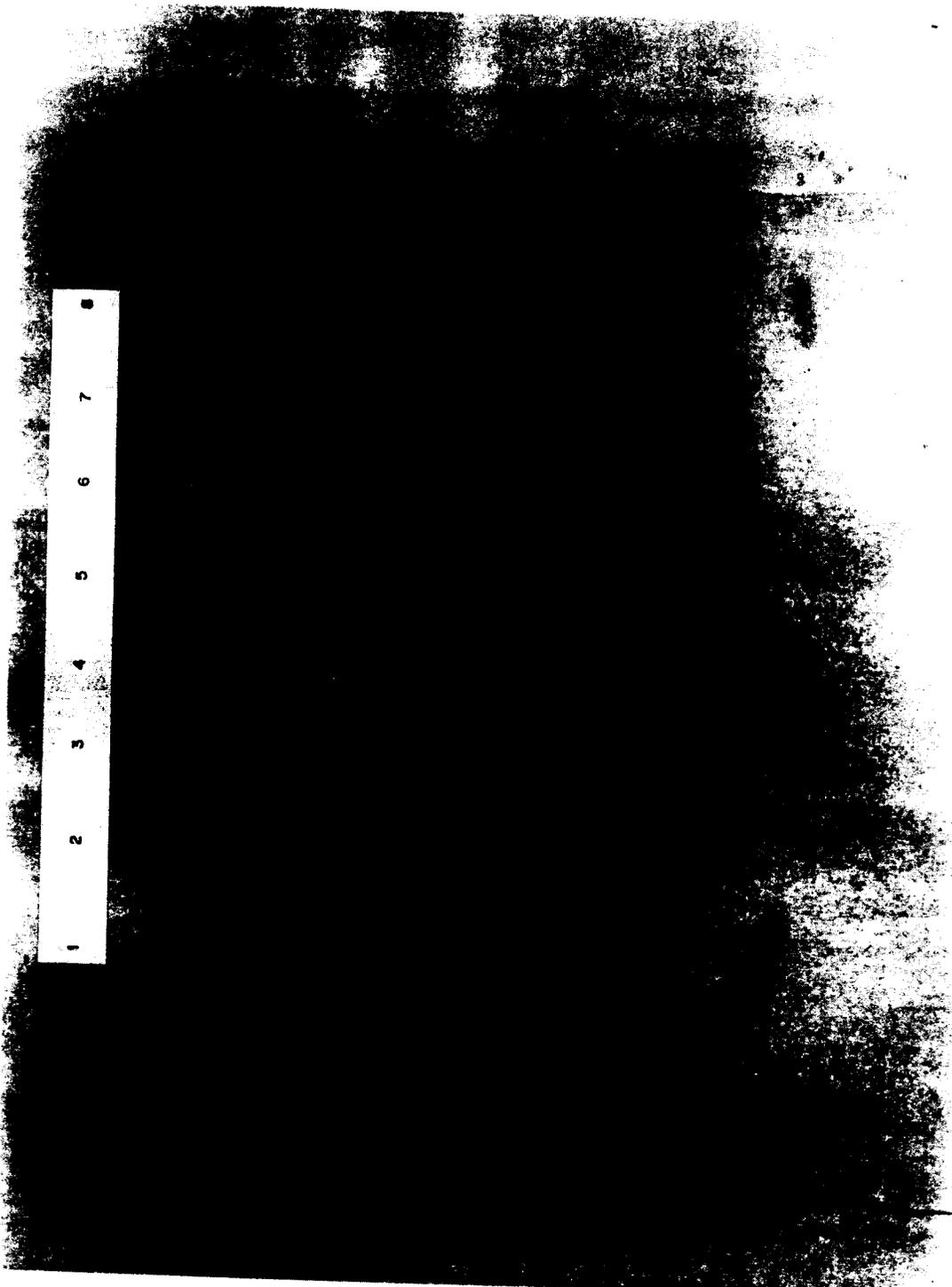


Fig. 2. Non-denaturing 10% polyacrylamide gel electrophoresis at pH 8.8 of α -amylases from yellow mealworm, red flour beetle, and rice weevil. Lane 1: TMA, lane 2: TCA-2, lane 3: TCA-1, lane 4: TCA-1 + TCA-2, lane 5: SOA-1, lane 6: SOA-2, lane 7: SOA-1 + SOA-2, lane 8: mixture of the five amylases above.

repeated three times in order to remove ammonium acetate from the sample. The samples were then hydrolyzed and analyzed as described above.

Average values of Asp, Glu, Gly, Ala, Leu, and Phe were selected for the calculation of extinction coefficients because these amino acids are stable during hydrolysis and are present at relatively high levels in the amylases.

Circular dichroism

Far ultraviolet dichroism spectra of the amylases were obtained in a 1 mm cell using a Cary CD model 6001 spectropolarimeter. Protein samples were treated similarly to those subjected to sedimentation equilibrium centrifugation. Helix content was calculated from the circular dichroism values between 206 and 260 nm, and β -sheet content was estimated according to the procedure of Siegal *et al.* (1980).

RESULTS

Enzyme purification

The method included glycogen complexation and precipitation, and anion-exchange HPLC. Two isozymes (designated TCA-1 and TCA-2) were purified from the red flour beetle. Two isozymes (SOA-1 and SOA-2) were similarly resolved from rice weevil extracts. In each case, the isozymes were effectively separated by anion-exchange chromatography (Fig. 1). The yellow mealworm contained only one α -amylase (Buoncore *et al.*, 1976), which we designated TMA, and it was also resolved by anion-exchange chromatography (Fig. 1). Polyacrylamide gel electrophoresis of the peak fractions from anion-exchange chromatography revealed a single band for each peak (Fig. 2), indicating that each HPLC peak contained an essentially homogeneous enzyme. Yields of the enzymes from 1 g of adults were quite similar and ranged from 500 to 900 μ g (0.05–0.09% of the wet wt).

Molecular weights

Using sedimentation equilibrium centrifugation, the same molecular weight, 53,000 \pm 900 kDa, was

obtained for both rice weevil α -amylase isozymes. Slopes of the $\ln c$ vs r^2 plots were linear (Fig. 3), indicating homogeneity of the preparations. Because SDS-PAGE indicated apparent molecular masses of 56 kDa for the rice weevil isozymes (data not shown), which is in essential agreement with the value from sedimentation equilibrium, it is clear that the enzymes exist in solution in monomeric form. SDS-PAGE yielded a similar apparent molecular mass for the α -amylases of the red flour beetle. The *Tenebrio* enzyme, on the other hand, migrated somewhat more slowly and had an apparent molecular mass of 58 kDa.

Amino acid compositions

Amino acid compositions of five purified beetle α -amylases are listed in Table 1, along with the compositions calculated from the amino acid sequences of *Drosophila* and mouse α -amylases. All of the enzymes were quite similar, as reflected by the C-values calculated from a comparison of amino acid compositions for all pairs of proteins (Table 2).

Circular dichroism

Far ultraviolet circular dichroism spectra of the rice weevil α -amylases are shown in Fig. 4. The other amylases exhibited similar spectra. A helix content of 33 \pm 2% was calculated for each of the enzymes. From this value, a β -sheet content of 31 \pm 5% was predicted by equation 7 of Siegal *et al.* (1980).

Extinction coefficients

We determined extinction coefficients as $A_{0.1\%}$ at 280 nm = 2.5 for the rice weevil α -amylases. Extinction coefficients for the other α -amylases were the same, based on determinations of protein concentration by the BCA method (Smith *et al.*, 1985) on solutions of known absorbances. These values were very similar to those of human saliva and porcine pancreatic α -amylases ($A_{0.1\%}$ = 2.6, Thoma *et al.*, 1971).

Table 1. Amino acid compositions of α -amylases from rice weevil (SOA), red flour beetle (TCA), yellow mealworm (TMA), fruit fly (DMA), and mouse*

Amino acid	Content (mole percent)						
	SOA-1	SOA-2	TCA-1	TCA-2	TMA	DMA	Mouse
Asx	14.5 \pm 0.2	14.9 \pm 0.4	12.6 \pm 0.8	12.9 \pm 0.5	12.4 \pm 0.7	14.1	16.3
Glx	7.5 \pm 0.1	8.6 \pm 0.2	9.3 \pm 0.2	9.0 \pm 0.5	8.2 \pm 0.4	6.6	6.3
Ser	9.3 \pm 0.3	8.5 \pm 0.2	7.7 \pm 0.2	6.9 \pm 0.3	6.7 \pm 0.2	10.4	5.7
Gly	9.7 \pm 0.1	9.4 \pm 0.1	10.1 \pm 0.4	9.2 \pm 0.1	9.0 \pm 0.5	12.1	9.3
His	2.7 \pm 0.1	2.6 \pm 0.1	2.6 \pm 0.1	3.0 \pm 0.2	2.2 \pm 0.1	2.3	2.6
Arg	3.2 \pm 0.3	3.6 \pm 0.2	5.8 \pm 0.3	5.6 \pm 0.1	4.9 \pm 0.1	4.3	6.1
Thr	6.3 \pm 0.1	7.2 \pm 0.1	4.4 \pm 0.1	4.6 \pm 0.2	4.6 \pm 0.2	5.0	4.1
Ala	6.8 \pm 0.5	6.6 \pm 0.3	5.9 \pm 0.2	6.2 \pm 0.1	6.2 \pm 0.4	7.5	6.5
Pro	4.9 \pm 0.1	4.0 \pm 0.2	3.7 \pm 0.2	4.1 \pm 0.0	4.2 \pm 0.2	3.3	3.9
Tyr	3.2 \pm 0.1	2.8 \pm 0.2	5.0 \pm 0.3	4.1 \pm 0.3	3.3 \pm 0.1	3.9	3.5
Val	6.4 \pm 0.2	6.0 \pm 0.1	3.9 \pm 0.2	4.9 \pm 0.2	7.0 \pm 0.3	7.7	7.7
Met	1.3 \pm 0.2	1.2 \pm 0.1	1.4 \pm 0.1	1.0 \pm 0.2	1.7 \pm 0.1	1.9	2.2
Ile	3.4 \pm 0.1	3.6 \pm 0.1	4.0 \pm 0.2	5.3 \pm 0.3	5.0 \pm 0.2	3.9	4.7
Leu	6.1 \pm 0.3	6.2 \pm 0.2	5.3 \pm 0.1	6.3 \pm 0.4	7.8 \pm 0.6	5.0	5.5
Phe	5.0 \pm 0.4	5.1 \pm 0.2	6.3 \pm 0.3	5.4 \pm 0.1	6.6 \pm 0.4	4.6	4.9
Lys	4.1 \pm 0.2	4.0 \pm 0.3	6.2 \pm 0.1	4.9 \pm 0.2	4.9 \pm 0.1	3.7	4.5
Cys	2.5 \pm 0.1	2.1 \pm 0.2	—	—	—	1.7	2.4
Trp	4.2 \pm 0.5	3.7 \pm 0.3	—	—	—	2.3	3.5

*Mean values \pm SEM ($n = 3$). Cys and Trp were not determined for TCA-1, TCA-2, or TMA. Cys and Trp for TCA and TMA were assumed to be 2 and 4 mol percent, respectively. The compositions of DMA (*D. melanogaster* α -amylase) and mouse α -amylase were calculated from the amino acid sequences determined by Boer and Hickey (1986).

Table 2. *C*-values for pair-wise comparisons of the amino acid compositions of beetle, fly, and mouse α -amylases

	SOA-2	TCA-1	TCA-2	TMA	DMA	MA
SOA-1*	0.99	0.87	0.91	0.91	0.95	0.89
SOA-2		0.98	0.93	0.92	0.92	0.89
TCA-1			0.96	0.89	0.85	0.83
TCA-2				0.95	0.87	0.90
TMA					0.87	0.91
DMA						0.88

*SOA = *S. oryzae* α -amylases, TCA = *T. castaneum* α -amylases, TMA = *T. molitor* α -amylase, DMA = *D. melanogaster* α -amylase, MA = mouse α -amylase.

Kinetic parameters

Analysis of Michaelis–Menten plots of the starch hydrolyzing activity of the five beetle α -amylases yielded the kinetic parameters listed in Table 3. V_{\max}/K_m ratios varied by less than a factor of 4. This result indicated that the catalytic efficiency was relatively similar for all of the enzymes studied.

Effects of wheat and corn inhibitors

Differential sensitivity to the WRP-25 inhibitor from wheat and the 12K inhibitor from corn was exhibited by the enzymes, with the former being more effective than the latter in all cases examined (Table 4). Except for rice weevil isozyme SOA-1, the wheat amylase inhibitor inhibited the α -amylases quite effectively (with K_i values ranging from 0.02 to 0.09 μ M). SOA-1 was approx. 10-fold less sensitive to WRP-25 than were the other enzymes. The corn 12 kDa inhibitor was most effective against the red flour beetle isozymes. It did not inhibit either of the rice weevil isozymes and only weakly inhibited the yellow mealworm α -amylase.

DISCUSSION

We have isolated five α -amylases from whole body homogenates of adults of the rice weevil, red flour beetle, and yellow mealworm. We presume that these enzymes are derived primarily from the alimentary canal, although they may also be derived from the salivary gland. In the case of the *Sitophilus* enzymes, there is rather clear evidence to support the former tissue as the major source (Baker, 1987). Baker has studied α -amylases from both rice weevil adult whole body homogenates and larval midguts and was able to equate the α -amylases isolated from the two sources. Our rice weevil isozymes (SOA-1 and SOA-2), in turn, clearly correspond to the enzymes Baker isolated from the adult whole body homogenates. There is also good reason to believe that the yellow mealworm α -amylase we have studied is from the

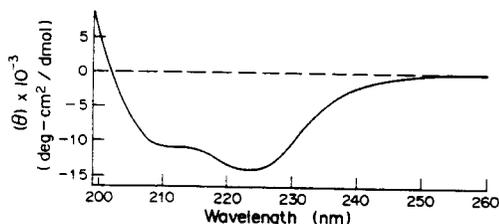


Fig. 4. Far ultraviolet circular dichroism spectrum of rice weevil α -amylase SOA-1. Spectra for SOA-2, TCA-1, TCA-2, and TMA are essentially identical (not shown).

Table 3. Kinetic parameters for α -amylases from rice weevil (SOA), red flour beetle (TCA), and yellow mealworm (TMA)*

Enzyme	V_{\max} (mM min ⁻¹ mg ⁻¹)	K_m (mg ml ⁻¹)	V_{\max}/K_m
SOA-1	5.57 ± 0.11	3.71 ± 0.01	1.5
SOA-2	1.33 ± 0.04	0.76 ± 0.03	1.8
TCA-1	4.45 ± 0.05	5.57 ± 0.03	0.8
TCA-2	5.44 ± 0.07	1.90 ± 0.03	2.8
TMA	5.98 ± 0.04	1.98 ± 0.02	3.0

*Mean values ± SEM ($n = 3$).

alimentary canal, because Terra *et al.* (1985) showed that essentially all of the α -amylase activity in *T. molitor* is associated with the gut (80% with the midgut and 20% with the foregut).

It is interesting to compare the beetle α -amylases that we isolated to the *Drosophila* enzymes, the genetics and biochemistry of which have been well documented (Gemmill *et al.*, 1986; Singh *et al.*, 1982; Boer and Hickey, 1986). Most strains of *Drosophila* have two closely linked genes for α -amylase and correspondingly produce two α -amylase isozymes. Some strains, however, produce a single α -amylase because one of the gene copies is inactivated (Boer and Hickey, 1986). Evidence indicates that the two α -amylases present in *S. oryzae* are the products of two separate genes (Baker and Halliday, 1989; Baker *et al.*, 1990). The very high *C*-value for these two proteins (Table 2) suggests that the enzymes are very similar in amino acid sequence. Whether the *Tribolium* enzymes are allelic or derived from separate genes is unknown. *Tenebrio* appears to have only one active gene for α -amylase, because only one form of the enzyme is found in that species (Buonocore *et al.*, 1976 and this study). If allotypes exist for this gene, they are inseparable by our methods.

The amino acid compositions of the beetle α -amylases are rather similar to each other as well as to the *Drosophila* and mouse enzymes. The high *C*-values (Table 2) suggest that the enzymes will also exhibit substantial similarity in amino acid sequences. The *Drosophila* and mouse α -amylases are identical at 55% of the positions, when their sequences are aligned (Boer and Hickey, 1986). Any pair of α -amylases with a *C*-value equal to or higher than that for these two enzymes (0.88) can be expected to be at least as similar in sequence as the *Drosophila* and mouse enzymes are. In fact, all pairs of beetle enzymes have *C*-values of ≥ 0.87 and, thus, would be expected to exhibit $> 50\%$ sequence identity.

Therefore, beetle enzymes appear to be part of a family of α -amylases that includes enzymes from microorganisms, insects, and mammals. We suggest that the beetle α -amylases are likely to have three-dimensional structures very similar to those that have

Table 4. Inhibitor constants for wheat (WRP-25) and corn (12 kDa) inhibitors of α -amylases from the rice weevil (SOA), red flour beetle (TCA), and yellow mealworm (TMA)

Enzyme	K_i (μ M)	
	Wheat inhibitor (WRP-25)	Corn inhibitor (12 kDa)
SOA-1	0.98 ± 0.08	No inhibition
SOA-2	0.05 ± 0.01	No inhibition
TCA-1	0.05 ± 0.01	0.08 ± 0.01
TCA-2	0.02 ± 0.01	0.08 ± 0.02
TMA	0.09 ± 0.02	0.38 ± 0.05

been determined by X-ray crystallography for porcine pancreatic α -amylase and Take- α -amylase (Matsuura *et al.*, 1984; Buisson *et al.*, 1987). The molecular weights of the beetle enzymes are similar to those of the mammalian and *Drosophila* enzymes, the amino acid compositions of the beetle enzymes strongly resemble those of the *Drosophila* and mouse enzymes (Table 1), and our circular dichroism spectra indicate that contents of helix and sheet rather closely correspond to those observed in the mammalian enzyme.

The enzymatic properties of the beetle α -amylases are also similar to those for mammalian and *Drosophila* enzymes. K_m values for human pancreatic and salivary α -amylases with various substrates (oligosaccharides) ranged from 1.9 to 6.8 mg ml⁻¹ and from 2.3 to 12.1 mg ml⁻¹, respectively (Saito *et al.*, 1979). K_m values for *Drosophila* amylases with soluble starch as substrate ranged from 0.9 to 2.9 mg ml⁻¹ (Hoorn and Scharloo, 1978). The K_m values we determined for beetle α -amylases with soluble starch as substrate ranged from as low as 0.8 mg ml⁻¹ for SOA-2 to as high as 5.6 mg ml⁻¹ for TCA-1. Baker (1987) determined K_m values of 2.4 mg ml⁻¹ and 0.7 mg ml⁻¹ for *S. oryzae* Amy-1 and -2, respectively, whereas we measured values of 3.7 mg ml⁻¹ and 0.8 mg ml⁻¹ for our preparations of the two allozymes for *S. oryzae*. Buonocore *et al.* (1976) measured a $K_m = 1.8$ mg ml⁻¹ for *T. molitor* α -amylase, and we determined a value of 2.0 mg ml⁻¹ for our preparation of the same enzyme.

Our characterization and those of others make the coleopteran α -amylases rather well-defined physical and chemical species. Their physical properties, ease of isolation, and stability during storage allow them to be used as target enzymes for studying inhibitors. Our group and others have reported differential responses of insect, mammalian, microbial, and avian α -amylases to several of the amylase inhibitors extracted from plants (Silano *et al.*, 1975; Buonocore *et al.*, 1977, 1980; Gatehouse *et al.*, 1986; Campos *et al.*, 1989; Lemos *et al.*, 1990; Feng *et al.*, 1991 and unpublished data). In this study, we evaluated the inhibition of the beetle enzymes by two protein inhibitors: WRP-25 from wheat (a member of the 0.28 inhibitor subfamily) and the 12 kDa corn inhibitor. The two inhibitors are quite different in their selectivities, i.e. the range of α -amylases that they inhibit. The 12 kDa corn inhibitor inhibits α -amylases from *Tribolium* and *Tenebrio* but not the *Sitophilus* enzymes. Furthermore, the corn inhibitor does not inhibit human salivary or porcine pancreatic α -amylases (Reeck *et al.*, unpublished results). However, inhibition of the *Tribolium* and *Tenebrio* enzymes does establish the corn inhibitor as bifunctional, because the inhibition of bovine trypsin and human activated Hageman factor is already well documented (Swartz *et al.*, 1977; Mahoney *et al.*, 1984; Chong and Reeck, 1987). WRP-25 strongly inhibited both *Tribolium* isozymes, but, interestingly, it strongly inhibited only one *Sitophilus* α -amylase, SOA-2, and weakly inhibited SOA-1. Perhaps this property reflects an evolutionary adaptation of *Sitophilus* to have roughly half of its overall α -amylase activity relatively unaffected by a major inhibitor present in wheat.

We are aware of only one other report of a single amylase inhibitor being selective for one of two isozymes from the same species. O'Donnell and McGeeney (1983) found that a 26 kDa wheat protein inhibitor caused 90% inhibition of human salivary amylase, whereas only 20% inhibition of human pancreatic amylase was observed over a 10-fold activity range under the same conditions. Whether that inhibitor is related to WRP-25 is unknown. However, the two wheat inhibitors appear to be different because WRP-25 inhibited human salivary amylase only 6% and porcine pancreatic amylase only 12% under conditions where it inhibited insect amylases >88% (Feng *et al.*, 1991).

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