The α -Amylase of the Beetle *Callosobruchus chinensis*

PURIFICATION AND ACTION PATTERN

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(Received 14 July 1970)

Callosobruchus chinensis larval amylase was isolated and purified in five steps, which included co-precipitation with glycogen and column chromatography on ECTEOLA-cellulose. The enzyme was homogeneous by disc gel electrophoresis on polyacrylamide. The α -amylase nature was evidenced by the action on amylopectin β -amylase limit-dextrin, by the effect on the substrate-iodine complex and by the action pattern on several polysaccharide substrates. These action patterns are compared with those of other α -amylases.

α -Amylases (α -1,4-glycan 4-glucanohydrolase, EC 3.2.1.1) of insects have elicited only a limited amount of research. The α -amylase of the silkworm *Bombyx mori* was first investigated by Mori (1930) and *Tenebrio molitor* larval midgut α -amylase was first investigated by Applebaum, Jancović & Birk (1961). *Tenebrio* α -amylase exhibits an unusual action pattern, differing from plant α -amylases (Applebaum, 1964), and similar results were obtained for the α -amylase from the closely related beetle *Tribolium castaneum* (Applebaum & Konijn, 1965). Hori (1969) has reported on the properties of an α -amylase from the plant bug *Lygus disponst*.

The importance of this enzyme is paramount in insects developing in seeds rich in reserve starch, such as many of the legume seeds that serve as hosts for the beetle now investigated. From the scant information available, insect α -amylases seem to have some unusual properties. These are, however, difficult to evaluate, as all the published work refers to enzyme preparations that have been at the most only partially purified.

The present paper reports the isolation and purification of an α -amylase from larvae of *Callosobruchus chinensis* and describes the mode of action.

METHODS

α -Amylase assay. The hydrolysis of 1% soluble starch in 0.01M-sodium acetate buffer, pH 5.4, containing 0.05mM-Ca(NO₃)₂ and 5mM-2-mercaptoethanol, was assayed in a 2ml reaction mixture at 37°C, with the 3,5-dinitrosalicylic acid reagent as modified by Noelting & Bernfeld (1948). Ca(NO₃)₂ was included as an activator; 2-mercaptoethanol was included as a stabilizing agent although it does interfere with calcium activation to some extent. The reaction mixtures were preincubated for 5min before addition of the soluble starch for the 10min incubation period. The reaction mixture included

0.3 'amylase unit', 1 amylase unit being defined as the amount of enzyme that releases 1mg of maltose equivalent in 10min at 37°C.

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (Fraction V, B grade, Calbiochem) as standard.

Carbohydrate determination. This was done with the phenol-H₂SO₄ reagent of Dubois, Gilles, Hamilton, Rebers & Smith (1956).

Disc gel electrophoresis. This was performed on polyacrylamide gels at room temperature with a 7.5% (w/v) gel at several pH values. Electrophoresis was carried out at 4-5mA/tube (7.0cm $\times$ 0.5cm) for 40-60min, depending on the buffer. The gels were fixed in 15% (w/v) trichloroacetic acid for 30min and protein was detected by staining with 0.05% Coomassie Blue.

Paper chromatography. Paper chromatography of products of partial enzymic hydrolysis of polysaccharide substrates was performed as described by Robyt & French (1963) by multiple ascending chromatography, and quantified with the help of a Beckman Analytrol densitometer with an automatic integrator.

Thin-layer chromatography. This was performed according to Applebaum (1964).

Insect culture. *C. chinensis* was reared on chickpeas at 28°C and 70% relative humidity. Larvae were removed from the seeds when 17-18 days old and used for enzyme preparation.

RESULTS

Purification procedure. The various stages in the purification of α -amylase from *C. chinensis* larvae are detailed in the following steps.

Step 1 (homogenate). *C. chinensis* larvae (17-18 days old) were removed from chickpea seeds, washed with cold water, quickly dried with filter paper and immediately homogenized in 0.02M-sodium acetate buffer, pH 5.4, containing 0.05mM-calcium nitrate (standard buffer), made 40% (v/v)

with respect to ethanol. The proportion of larvae (fresh weight) to solvent was 1:10 (w/v). This homogenate was centrifuged at 3–5°C in an International ultracentrifuge at 60000g for 20 min. Activity was about 1 amylase unit per mg of larvae. The average weight of individual larvae, used for the enzyme preparation, was 6.5 mg. A typical extraction used 1000 larvae.

Step 2 (supernatant). The supernatant was kept overnight in the cold. An additional precipitate formed. The precipitate was found to be devoid of amylolytic activity and was discarded after centrifugation as in step 1.

Step 3 (glycogen complex). The supernatant was co-precipitated as a glycogen–amylase complex by the method of Loyter & Schramm (1962) with glycogen previously purified as described by Stevenson & Wyatt (1964). The amount of glycogen added was in the proportion of 1 mg of glycogen/50 amylase units. The mixture was kept at 5°C for 10 min with occasional stirring and centrifuged at 7000g for 10 min to collect the complex. The precipitate was dispersed and washed three times in the standard buffer made 40% (v/v) with respect to ethanol. Each wash was with 10 ml of the 40% ethanol–buffer.

Step 4 (glycogen digestion). The precipitate was dissolved in 2–3 ml of standard buffer and incubated for 1 h at 37°C, to allow for complete dissociation and amylolysis of the glycogen. The solution was centrifuged at 7000g for 10 min and the clear supernatant was exhaustively dialysed against standard buffer.

The degree of purification of the larval α -amylase in steps 1–4 is presented in Table 1.

Step 5 (ECTEOA-cellulose column chromatography). The dialysed supernatant in standard buffer was applied to a column (1.7 cm $\times$ 30 cm) of the anion-exchanger ECTEOA-cellulose (Carl Schleicher & Schuell Co., Keene, New Hampshire, U.S.A.), equilibrated with standard buffer, and cooled by circulating ice-cold water through a glass jacket. The amount of protein applied in each chromatographic run was 0.7–1.2 mg. The column was first eluted with 80–100 ml of standard buffer and then with a gradient of sodium chloride in standard buffer (see Fig. 1).

Fig. 1 in addition presents the distribution of protein (E_{280} automatically recorded), the distribution of amylolytic activity and the distribution of carbohydrate, the last associating with a major peak of protein in the chromatographic separation that is, however, devoid of amylolytic activity. The amylase was eluted at sodium chloride concentrations of 0.05M–0.14M. Tubes 43–50 were collected from such chromatographic runs and the contents concentrated on Pellicon membranes (Millipore; 7 atm N_2 , flow rate 0.5 ml/min, recovery

Table 1. Purification of larval α -amylase from *C. chinensis*

Assays were done in standard buffer at pH 5.4.

Step	Recovery (%)	Specific activity (amylase units/mg of protein)
1 (Homogenate)	100	33
2 (Supernatant)	97	62
3 (Glycogen complex)	54	430
4 (Glycogen digestion)	54	590

Fig. 1. Gradient elution of *C. chinensis* larval α -amylase from a column (1.7 cm $\times$ 30 cm) of ECTEOA-cellulose in 0.01M-sodium acetate buffer, pH 5.4, containing 0.05M- $Ca(NO_3)_2$. Protein was continuously monitored at 280 nm (—), and 3.5 ml fractions were collected and assayed for carbohydrate content (E_{480}) (---) and amylolytic activity (E_{550}) (○). The applied enzyme was from step 4 of the purification procedure.

of activity 50%). The concentrated solutions were submitted to disc gel electrophoresis on 7.5% (w/w) polyacrylamide gels in three buffer solutions: (1) 0.01M-sodium acetate buffer, pH 5.4; (2) 0.35M- β -alanine–acetic acid buffer, pH 4.5 (Reinsfeld, Levis & Williams, 1962); (3) 0.37M-glycine–tris buffer, pH 8.9 (Orenstein & Davis, 1961). The protein did not enter the gel at pH 4.5. At pH 5.4 and 8.9 all the applied protein migrated as a single sharp band toward the anode, which suggests the homogeneity of the purified enzyme.

Mode of action. The action pattern of the purified α -amylase was determined by controlled partial enzymic hydrolysis of the following polysaccharide substrates: soluble starch, amylopectin β -amylase limit-dextrin (prepared as described by Applebaum, 1964), glycogen and amylopectin.

The disappearance of substrate–iodine complex colour was monitored at set intervals in reaction mixtures and the fall in iodine value for several substrates during partial amylolysis was plotted against the increase in reducing value. The achroic

point is attained after the apparent hydrolysis of 31% of the soluble starch (180 min), 35% of β -amylase limit-dextrin (240 min) and 60% of amylopectin (300 min) (Fig. 2).

The degree of partial enzymic hydrolysis was concurrently determined at similar time-intervals

with portions of the same reaction mixtures as above. The action patterns of amylase activity were examined by t.l.c. and quantitatively determined by paper chromatography (Table 2).

DISCUSSION

An amylase present in larvae of the beetle *C. chinensis* has been obtained by a five-step procedure in a high state of purity, as evidenced by a single protein band obtained at two pH values in disc gel electrophoresis.

The α -amylase nature of this insect enzyme is evident from the data. β -Amylase limit-dextrin prepared by exhaustive digestion of amylopectin with β -amylase is effectively hydrolysed by the insect amylase. In addition, the iodine value falls to zero after apparent hydrolysis of 31–60% (Fig. 2), whereas in digestion by β -amylase the iodine value falls to a constant value that is attributed to the resistance of residual β -amylase limit-dextrin to further hydrolysis. Finally, the action pattern of amylolysis obtained with *C. chinensis* larval amylase is consistent with its identification as an α -amylase. The action pattern obtained with several substrates (Table 2), in which fractions of at most four glucose units accumulate, with three-glucose units initially predominant, is similar to that obtained with *Tenebrio* larval α -amylase (Applebaum, 1964) but differs from that of sorghum α -amylase, in which higher oligosaccharides (six- to eight-glucose units) accumulate (Dube & Nordin, 1962), and from the α -amylase of *Bacillus subtilis*, which released mainly three- and six-glucose units (Robyt & French, 1963). At later stages of hydrolysis, but before the achroic point is reached, maltose

Fig. 2. Decrease in iodine value of reaction mixtures containing: soluble starch (Δ), amylopectin β -amylase limit-dextrin ($\bullet$) and amylopectin ($\circ$) as substrates, and *C. chinensis* larval α -amylase as enzyme, plotted against increase in reducing value. The initial substrate concentration was 1% and enzyme concentration was 0.15 amylase unit/ml. The reaction mixtures were buffered in 0.01 M-sodium acetate buffer, pH 5.4, containing 0.05 mM- $\text{Ca}(\text{NO}_3)_2$ and 5 mM-2-mercaptoethanol.

Table 2. Amount of oligosaccharides released during the hydrolysis of several substrates by *C. chinensis* α -amylase

The amount of oligosaccharides was calculated from the colour intensity of the spots obtained by paper chromatography and determined with the aid of a Beckman Analytrol densitometer.

Substrate	Degree of polymerization	Amount of saccharide released (μmol)					
		40 min	60 min	90 min	120 min	240 min	360 min
Amylopectin	G ₁	0.01	0.02	0.03	0.04	0.10	0.12
	G ₂	0.02	0.04	0.06	0.09	0.21	0.24
	G ₃	0.08	0.06	0.07	0.09	0.16	0.20
	G ₄	0.05	0.04	0.02	0.03	0.09	0.11
Amylopectin β -amylase limit-dextrin	G ₁	0.02	0.02	0.03	0.05	0.08	0.08
	G ₂	0.03	0.03	0.05	0.06	0.09	0.12
	G ₃	0.04	0.06	0.06	0.06	0.07	0.08
	G ₄			0.04	0.02	0.02	0.04
Glycogen	G ₁		0.01	0.03	0.03	0.04	0.05
	G ₂		0.03	0.03	0.03	0.05	
	G ₃		0.03	0.06	0.03	0.03	

accumulates as the predominant sugar, which suggests that three- and four-glucose units are being further hydrolysed. This is similar to the second stage of α -amylolysis reported by Walker & Whelan (1960) for salivary α -amylase. Maltose itself was not further hydrolysed by *C. chinensis* larval amylase.

The authors are grateful to Professor Z. Avidov for his interest and suggestions during this work, and to Mrs Nitza Levin for technical assistance.

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