The α -Amylase of the Beetle *Callosobruchus chinensis* PROPERTIES

By H. PODOLER AND S. W. APPLEBAUM
Faculty of Agriculture, The Hebrew University, Rehovot, Israel

(Received 14 July 1970)

C. chinensis larval amylase is activated by Ca^{2+} and inhibited by Cl^- and EDTA (K_i 6.7×10^{-3} M). GSH and 2-mercaptoethanol activate, presumably at different sites, as 2-mercaptoethanol interferes with Ca^{2+} activation, whereas GSH enhances it. The inhibition by iodoacetic acid and *N*-ethylmaleimide (K_i 1.55×10^{-2} M) suggest that free thiol groups are essential for activity. The pH optimum of 5.2–5.4 is moved to 5.6–5.8 by Ca^{2+} and 2-mercaptoethanol. The activation energy is 7270 cal/mol, and is not affected by Ca^{2+} and 2-mercaptoethanol. K_m for soluble starch is 2.3 mg/ml.

The purification procedure and action patterns of α -amylase (EC 3.2.1.1) from larvae of the Bruchid beetle *Callosobruchus chinensis* were described in the preceding paper (Podoler & Applebaum, 1971). The present paper deals with some properties of the purified enzyme, including its activation and inhibition.

METHODS

α -Amylase assay. This was usually performed (except when stated otherwise) in 0.01 M-sodium acetate buffer, pH 5.4. The reaction mixture usually contained 1% soluble starch (or other concentrations as stated) and was preincubated for 5 min without substrate. The reaction was initiated by adding the substrate and was continued for an additional 10 min at 37°C. Activators or inhibitors were assayed subsequent to a preincubation period of 20 min at 30°C. The reaction was terminated and hydrolysis determined with 3,5-dinitrosalicylic acid reagent as modified by Noelting & Bernfeld (1948). The alkaline 3,5-dinitrosalicylic acid reagent occasionally gives anomalous results, due to the effect of various ions on the reducing value (Robyt & Whelan, 1965). The experimental design of the experiments reported herein included appropriate controls in all cases. Maltose blanks were as a routine included in all experiments and contained activators and inhibitors to rule out the possibility of spurious results. In all cases, activity is related and calculated to maltose equivalent obtained in each experiment. Glutathione and 2-mercaptoethanol increased the colour obtained with the reagent, but this excess value was always subtracted to obtain net activity, and in experiments where more than one concentration was assayed, separate blanks were included for each concentration. The other activators and inhibitors did not affect the colour obtained with the dinitrosalicylic acid reagent at the concentrations used in this work.

RESULTS

Effect of pH on activity. Fig. 1 shows pH optima for the hydrolysis of soluble starch by the purified

amylase. The unactivated enzyme exhibits a pH optimum of 5.2–5.4, which is moved to 5.6–5.8 by the presence of calcium nitrate and 2-mercaptoethanol. After plotting of amylase activity as $\log v$ against pH values, the pK values of the unactivated and of the Ca^{2+} - and 2-mercaptoethanol-activated enzyme were calculated to be pK_{ES} , 4.7 and pK_{ES} , 6.5 for the unactivated amylase. Activation moved pK_{ES} to 5.2 but had no effect on pK_{ES} . A pK value of 4.7 is in the range that might be interpreted as indicating the presence of a carboxyl (glutamyl) group near the active centre, and pK 6.5 is in the range suggesting an imidazole (histidine) group, similarly situated (Cohn & Edsall, 1943).

Effect of temperature on activity. This was determined in the range of 10–60°C and in the presence or absence of Ca^{2+} and 2-mercaptoethanol. Maximal activity was obtained at 36–40°C. At temperatures above 45°C activity was drastically curtailed, and at 60°C the enzyme was almost completely inactivated.

The presence of activators does not alter the shape of the temperature-activity curve obtained or the stability of the amylase at extreme temperatures.

The activation energy of the enzymic activity is presented as an Arrhenius plot (Fig. 2). The value of E_a obtained from the slope of the line is 7270 cal/mol. The E_a can also be calculated from the equation:

$$E_a = \frac{4.560 T_1 T_2 (\log k_2 - \log k_1)}{(T_2 - T_1)}$$

where T_1 and T_2 are two temperatures in kelvin units and k_1 and k_2 are rate constants at these respective temperatures. This calculation leads to a value of 7600 cal/mol, consistent with the ΔH° (cal/mol) of an imidazole group suggested as

Bioch. 1971, 121

Fig. 1. Hydrolysis of 1% starch by *C. chinensis* larval α -amylase at different pH values, in the absence (●) or presence (○) of 0.05mM- $\text{Ca}(\text{NO}_3)_2$ +5mM-2-mercaptoethanol. The reaction mixtures were buffered with citrate-phosphate buffer as described by Gomori (1955). Buffers were prepared immediately before use. The α -amylase is presented as a percentage of the maximum activity in both cases (v_H/V_{max}).

positioned near the active centre by the pK_{ES_2} value. The E_a in the presence of Ca^{2+} and 2-mercaptoethanol is similar.

Stability of the enzyme at 37°C, which is in the optimum range for activity, was determined during 150min for the unactivated amylase and during 300min for the Ca^{2+} - and 2-mercaptoethanol-activated amylase. During both these periods activity increased linearly, indicating complete stability at this temperature for the periods of assay.

Effect of substrate concentration on activity. This was determined on soluble starch in the range 2.5–40mg/ml. The apparent Michaelis constant was determined by plotting s/v versus s in the

Fig. 2. Arrhenius plot of the effect of temperature on the reaction catalysed by *C. chinensis* larval amylase in the temperature range 10–60°C.

Fig. 3. Effect of substrate concentration on activity of *C. chinensis* larval amylase.

graphical presentation of Dixon & Webb (1964) of the mathematical formula of Hanes (1932) (Fig. 3). The intercept of the plotted line with the abscissa indicates K_m 2.3mg/ml. This K_m value was the same in the presence or absence of Ca^{2+} and 2-mercaptoethanol.

Activation and inhibition studies. An accepted property of α -amylases is their content of Ca^{2+} as an integral part of the enzyme molecule, and their consequent activation by Ca^{2+} and inhibition by chelating agents (Stein, Hsiu & Fischer, 1964). The effect of different concentrations of Ca^{2+} on amylolytic activity is presented in Table 1. NO_3^- was shown in preliminary experiments to be devoid of effect at the concentrations assayed. Mg^{2+} has been reported capable of substituting in some cases and to some extent for Ca^{2+} (Yamamoto & Fukumoto, 1960). Table 1 includes in addition results obtained with different concentrations of magnesium acetate. Maximal activation is obtained with 0.05mM-calcium nitrate and higher concentrations do not

inhibit. Mg^{2+} does not activate at comparable concentrations.

The chelating agent EDTA markedly affects amylolytic activity. The inhibitor constant, K_i , for EDTA was determined at several inhibitor and substrate concentrations and plotted according to the procedure of Dixon (1953) (Fig. 4). The calculated K_i for EDTA is $6.7 \times 10^{-3} M$ and the inhibition is competitive, as would be expected from its assumed mode of action.

Cl^- activates many but not all α -amylases. Its

effect on *C. chinensis* larval amylase is presented in Table 2. Cl^- inhibits the insect amylase. This inhibition clearly overcomes the activating effect of Ca^{2+} when amylolysis is assayed in the presence of calcium chloride. The inhibition by sodium chloride is reversible by dialysis.

Table 1. *Effect of different concentrations of calcium nitrate and magnesium acetate on activity of C. chinensis larval amylase*

Activity was assayed in 0.01 M-sodium acetate buffer, pH 5.4. The substrate was 0.5% soluble starch in the reaction mixture.

Concn. of $Ca(NO_3)_2$ (mm)	Amylase activity (% of control)	Concn. of magnesium acetate (mm)	Amylase activity (% of control)
0.001	154	0.05	100
0.005	198	0.1	100
0.025	235	0.2	115
0.05	250	0.4	100
0.2	250	0.8	105
0.8	260		

Table 2. *Effect of different concentrations of potassium chloride, sodium chloride and calcium chloride on activity of C. chinensis larval amylase*

Activity was assayed in 0.01 M-sodium acetate buffer, pH 5.4. Substrate concentration was 0.5% soluble starch in the reaction mixture.

Salt	Concn. (mm)	Amylase activity (% of control)
KCl	0.05	77
	0.1	69
	0.2	53
	0.4	51
NaCl	0.4	59
	2.0	52
$CaCl_2$	0.001	110
	0.005	111
	0.025	89
	0.1	55
	0.8	33

Fig. 4. K_i for EDTA at inhibitor concentrations of 2.5–10 mm and three substrate concentrations (Δ , 0.25%; $\bullet$, 0.50%; $\circ$, 0.75%) on *C. chinensis* larval amylase.

The effect of the reducing agents GSH and 2-mercaptoethanol are presented in Table 3, including the co-operative effect of these agents with Ca^{2+} .

The effect of 2-mercaptoethanol differs from that of GSH. The former shows a fairly constant extent of activation in the range 1–10mM, and this activation is not enhanced by addition of Ca^{2+} ; indeed the extent of co-operative activation is somewhat lower. On the other hand, GSH, though activating to a similar degree in the range 0.5–2mM, shows diminished activation at 10mM. The co-operative activation of GSH and Ca^{2+} is more than the sum of the individual activations by each alone. Even here 10mM-GSH shows considerable diminution of activation, lowering the extent of activation below that evidenced with Ca^{2+} alone.

Effect of thiol reagents on activity. The following compounds reactive with thiol groups were assayed: (1) silver nitrate, which inhibited completely at a

concentration of 0.01mM in the reaction mixture; (2) iodoacetic acid, which inhibited to the extent of 36% at a concentration of 1mM and completely at 5mM; (3) *N*-ethylmaleimide, which was examined more thoroughly (see Fig. 5): K_i was determined at several inhibitor and substrate concentrations, and the calculated K_i was $1.55 \times 10^{-2}\text{M}$; this inhibitor is of the competitive type.

DISCUSSION

C. chinensis larval amylase exhibits several unusual characteristics compared with what is known about α -amylases of other sources. The change in pH optimum in the presence of the activators calcium nitrate and 2-mercaptoethanol might be comparable with the reported activation of the salivary α -amylase of the plant bug *Lygus disponsi* by Cl^- and NO_3^- (Hori, 1969), but differs in that Ca^{2+} is the activator of the *C. chinensis* amylase (NO_3^- is devoid of activity at the concentrations used), and Cl^- is inhibitory. However, in both cases the change in pH optimum approaches the pH of the gut. The activation energy of *C. chinensis* larval amylase is similar to those reported for garden pea α -amylase (7600 cal/mol) (Swain & Dekker, 1966), and barley malt α -amylase (7050 cal/mol) (Fischer & Haselbach, 1951), but is much lower than that reported for all other α -amylases of animals and micro-organisms (Bernfeld, 1962).

The K_m of *C. chinensis* amylase for soluble starch is higher (2.3 mg/ml) by one order of magnitude than that obtained with garden-pea α -amylase (0.2 mg/ml) (Swain & Dekker, 1966). Most α -amylases exhibit the higher affinity for starch. The only other known α -amylase with a K_m close to that of this insect has been reported from *Bacillus stearothermophilus* (K_m 1 mg/ml) (Manning & Campbell, 1961). The activation of *C. chinensis* larval amylase by Ca^{2+} is comparable with the activating and stabiliz-

Table 3. *Effect of different concentrations of GSH and 2-mercaptoethanol on activity of C. chinensis larval amylase in the absence and presence of 0.05mM-calcium nitrate*

Compound assayed	Concn. (mM)	Amylase activity (% of control)	
		No $\text{Ca}(\text{NO}_3)_2$	+ $\text{Ca}(\text{NO}_3)_2$
2-Mercaptoethanol	1.0	182	163
	2.5	210	155
	5.0	215	166
	10.0	235	176
GSH	0.1	134	480
	0.5	220	510
	1.0	205	510
	2.0	206	460
	10.0	120	174
Control		100	248

Fig. 5. K_i for *N*-ethylmaleimide at inhibitor concentrations of 2.5–10mM and three substrate concentrations (Δ , 0.25%; $\bullet$, 0.50%; $\circ$, 0.75%) on *C. chinensis* larval amylase.

ing effect of this ion on *Tenebrio molitor* larval amylase (Applebaum, 1964).

EDTA inhibits amylases that contain Ca^{2+} as an essential component of the enzyme molecule, presumably because of its chelating properties (Fischer, Sumerwell, Junge & Stein, 1958), and *C. chinensis* larval amylase is similarly affected, the inhibition by EDTA being competitive.

The inhibitory activity of Cl^- is unique. All other known α -amylases are either activated by this anion or are indifferent to its presence (Bernfeld, 1962).

Low-molecular-weight reducing reagents such as GSH and 2-mercaptoethanol are regarded as acting by altering protein configuration as a consequence of disulphide-bond cleavage, protecting essential thiol groups in their reduced state or binding heavy-metal ions. The effect of these two compounds on *C. chinensis* larval amylase indicates that more than one of these three possibilities is of importance for enzyme activity (Table 3). Thus 2-mercaptoethanol seems to interfere with Ca^{2+} activation, suggesting either the proximity of their site of action or critical configurational changes occurring as a consequence of reduction of disulphide bonds. GSH, in contrast, does not interfere with Ca^{2+} activation, and even enhances it. That thiol groups are in fact essential for enzymic activity is evident from the inhibitory effect of thiol-blocking reagents on the unactivated enzyme. Ag^+ , although strongly inhibitory, is not regarded as specific for thiol groups only, and may inhibit carboxyl groups as well (Dixon & Webb, 1964). Iodoacetic acid, which serves for the alkylation of thiol groups, is regarded as more specific, although it too may affect other groups under certain conditions (Stark, Stein & Moore, 1961). *N*-Ethylmaleimide, on the other hand, is regarded as highly specific, and reacts especially well with freely available thiol groups.

The presence of essential thiol groups is unusual in α -amylases (e.g. Manning & Campbell, 1961; Bernfeld, 1962). In comparison *Tenebrio molitor* and *Tribolium castaneum* larval amylases are

clearly not thiol enzymes, for they are inhibited by reagents such as 2-mercaptoethanol or GSH (Applebaum, 1964; Applebaum & Konijn, 1965).

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

REFERENCES

- Applebaum, S. W. (1964). *J. Insect Physiol.* **10**, 897.
 Applebaum, S. W. & Konijn, A. M. (1965). *J. Nutr.* **85**, 275.
 Bernfeld, P. (1962). In *Comparative Biochemistry*, vol. 3, p. 379. Ed. by Florkin, M. & Mason, H. S. New York and London: Academic Press.
 Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*, p. 445. New York: Reinhold Publishing Corp.
 Dixon, M. (1953). *Biochem. J.* **55**, 170.
 Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., pp. 68, 345. New York: Academic Press Inc.
 Fischer, E. H. & Haselbach, C. H. (1951). *Helv. chim. Acta*, **34**, 325.
 Fischer, E. H., Sumerwell, W. N., Junge, J. & Stein, E. A. (1958). *Proc. 4th int. Cong. Biochem., Vienna*, vol. 8, p. 124.
 Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 141. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Hanes, C. S. (1932). *Biochem. J.* **26**, 1406.
 Hori, K. (1969). *J. Insect Physiol.* **15**, 2305.
 Manning, G. B. & Campbell, L. L. (1961). *J. biol. Chem.* **236**, 2952.
 Noelting, G. & Bernfeld, P. (1948). *Helv. chim. Acta*, **31**, 286.
 Podoler, H. & Applebaum, S. W. (1971). *Biochem. J.* **121**, 317.
 Robyt, J. F. & Whelan, W. J. (1965). *Biochem. J.* **95**, 10p.
 Stark, G. R., Stein, W. H. & Moore, S. (1961). *J. biol. Chem.* **236**, 436.
 Stein, E. A., Hsiu, J. & Fischer, E. H. (1964). *Biochemistry, Easton*, **3**, 56.
 Swain, R. R. & Dekker, E. E. (1966). *Biochim. biophys. Acta*, **122**, 75.
 Yamamoto, T. & Fukumoto, J. (1960). *Bull. agric. chem. Soc. Japan*, **24**, 16.