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THE a-GALACTOSIDASE ACTIVITY OF CALLOSOBRUCHUS CHINENSIS LARVAE*

by

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A B S T R A C T

a - galactosidase (E.C. 3.2.2.22) of Callosobruchus chinensis larvae was separated from sucrase by column chromatography. Its pH optimum is 5.2 - 5.6. Km for p-nitrophenyl-a-D-galac- topyranoside is 2.8 x 10^{-4}M, for raffinose 1.4 x 10^{-4}M and for stachyose 1.9 x 10^{-4}M. Hydrolysis of the nitrophenyl derivative is inhibited by stachyose. The activation energy is 11,600 cal/mole. The enzyme contains essential sulfhydryls but may require other free essential groups for activity.

Ag^{++} strongly inhibits, iodoacetamide not at all, and n-ethylmaleimide partly. The enzyme does not require cations for activity nor is it particularly activated by ethylene diamine tetraacetate Gluta- thione activates, but excess inhibits.

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The bruchid beetle Callosobruchus chinensis L. is specific for several legume seeds, which are noted for their unusually high content of oligosaccharides of the raffinose family (Courtois, 1959; Nigam & Giri, 1961; Podoler & Applebaum, 1971). Dietary raffinose has been demonstrated to stimulate development of C.chinensis (Podoler & Applebaum, 1971) and galactosidase activity in larvae of this beetle is evident from hydrolysis of all galactose residues present in a toxic heteropolysaccharide isolated from Phaseolus vulgaris beans (Applebaum & Guez,1971). Galactose itself is non-toxic to C. chinensis (Applebaum et al.,1970) and its release from reserve oligosaccharides should be nutritionally beneficial. Some properties of the enzymic activity of α-galactosidase, which is responsible for this hydrolysis, were accordingly investigated, and are herein reported on.

MATERIALS AND METHODS

Larvae of C. chinensis were reared at high population densities in chickpeas (Cicer arietinum) at 28° and 70% R.H. They were removed from the seeds after 15-17 days of development and served for the present experiments.

Enzyme preparations were initially prepared from the soluble intestinal content of larvae. Subsequently, after the presence of soluble α-galactosidase activity was demonstrated in the larval midgut, and its absence from haemolymph was established, a procedure was elaborated for obtaining larger amounts of activity. This involved the homogenation of whole larvae in cold distilled water, centrifugation for 0.5 hr at 105.000 g at 5° and retention of the supernatant. This enzyme solution was filtered through Whatman No. 1 filter paper, exhaustively dialysed in the cold against double-distilled water and recentrifuged as above at 105.000 g. The supernatant was further purified on an ECTEOLA-cellulose column as detailed in the results.

Specific α-galactosidase activity was assayed with p-nitrophenyl α-D-galactopyranoside (Sigma Chemical Co.) as substrate (Pollock, 1961) except that Na_2CO_3 substituted for K_2CO_3 in terminating the reaction mixture. Reactions were carried out at 30° and for periods up to 45 min, conditions under which activity was found to be linear. To the extent that non-reducing oligosaccharides served as substrates, activity was assayed as increase in reducing capacity with the 3,5-dinitrosalicylic acid reagent (Noelting & Bernfeld, 1948). Reactions were conducted in 5 mM ammonium acetate buffer, pH 5.6, at 37°.

Qualitative determinations of products of hydrolysis were carried out by thin-layer chromatography (TLC) on 20 x 20 cm^2 glass plates coated with Kieselgel G in 0.02 M sodium acetate and activated at 105° for 40 min prior to chromatography. The solvent system was composed of chloroform: methanol (6:4 v/v) (Pifferi, 1965). The chromatograms were developed by spraying with the following reagents: (A) Acidified anisaldehyde (reagent 9 in Stahl, 1965). (B) Acidified anthrone in ethanol (reagent 10 in Stahl, 1965). This reagent identifies ketoses and ketose-containing oligosaccharides. (C) Acidified diphenylamine-aniline in acetone (Bailey & Bourne, 1960) and developed at 85° for 10 min (Pifferi, 1965).

RESULTS AND DISCUSSION

Column chromatography of α-galactosidase activity: The 105.000 g supernatant of the larval homogenate exhibits hydrolytic activity on sucrose, and this activity is liable to complicate the assay of α- galactosidase activity on raffinose and stachyose. A midgut α-glucosidase, distinct from the sucrase activity, is also present in the homogenate, and can be demonstrated by TLC of digests of maltose and melezitose. It does not hydrolyse sucrose as far as could be ascertained, with the experimental procedure used. It hydrolyses the synthetic substrate p-nitrophenyl-α -D-glucopyranoside, but this activity does not interfere with the measurement of activity of α-galactosidase on either p-nitrophenyl-α -D-galactopyranoside or on sugars of the raffinose family, from which the only reducing sugar released is galactose. A procedure was therefore elaborated to separate the α-galactosidase activity from the sucrase activity: the 105.000 g supernatant was applied and adsorbed to an ECTEOLA-cellulose column (1 cm diameter x 48 cm height) equilibrated in 0.01 M ammonium acetate buffer, pH 5.6. The column was washed with 50 ml of this buffer and the α-galactosidase activity was subsequently eluted with 0.8 M NaCl in this buffer. The active fractions were collected, pooled and dialysed for 24 hrs against double-distilled water. Preparations were stored frozen at -20° C. The stepwise elution from the ECTEOLA-cellulose column essentially separates the α-galactosidase from sucrase which is present in soluble form in the 105.000 g supernatant. The sucrase does not elute from the column for all practical purpose in the above procedure.

Properties of the larval α-galactosidase

The pH optimum for the hydrolysis of p-nitrophenyl- α-D-galactopyranoside (0.008% in reaction mixture) is in the range of pH 5.2 - 5.6 (Fig. 1). Activity was assayed in citrate-phosphate buffer and is insensitive to the ionic strength of the buffers in the range employed. All subsequent assays were conducted at the optimal pH.

The midgut pH of C. chinensis larvae is 6.4 - 6.6, conditions under which the midgut α-galactosidase does not realize its full hydrolytic capacity.

The affinity of the larval α-galactosidase for several of its substrates is presented in Figs. 2-3. The apparent Michaelis constant (Km) for p-nitrophenyl- α-D-galactopyranoside, calculated from a Lineweaver-Burk plot, is 2.8×10^{-4} M (Fig. 2).

In comparison, the Km for raffinose is 1.4×10^{-4} M and that for stachyose is 1.9×10^{-4} M (Fig. 3).

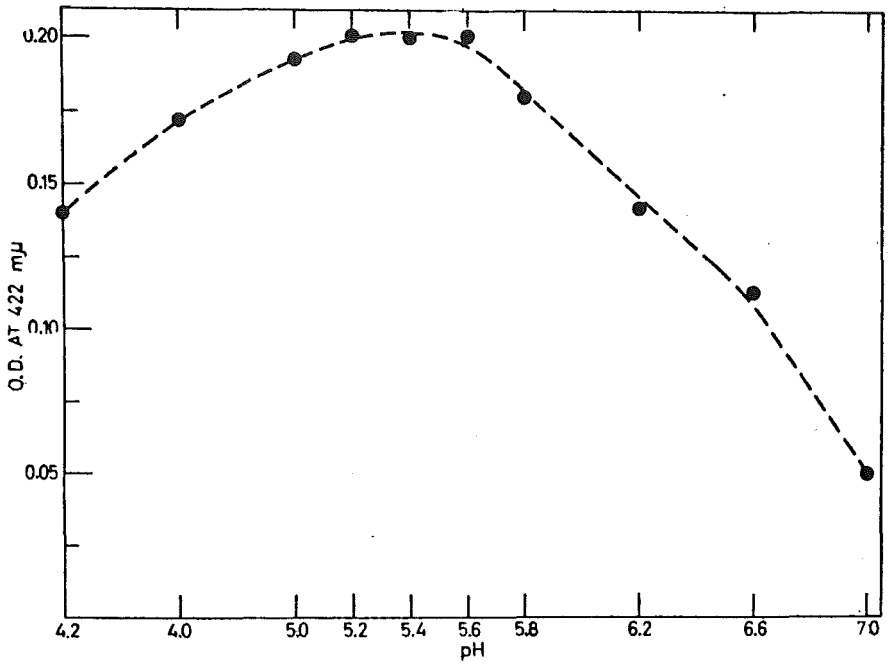

Fig. 1. Effect of pH on activity of C. chinensis larval α-galactosidase. Conditions of reaction: 0.008% p-nitrophenyl-α-D-galactopyranoside in citrate phosphate buffer for 30 min at 28.5°.

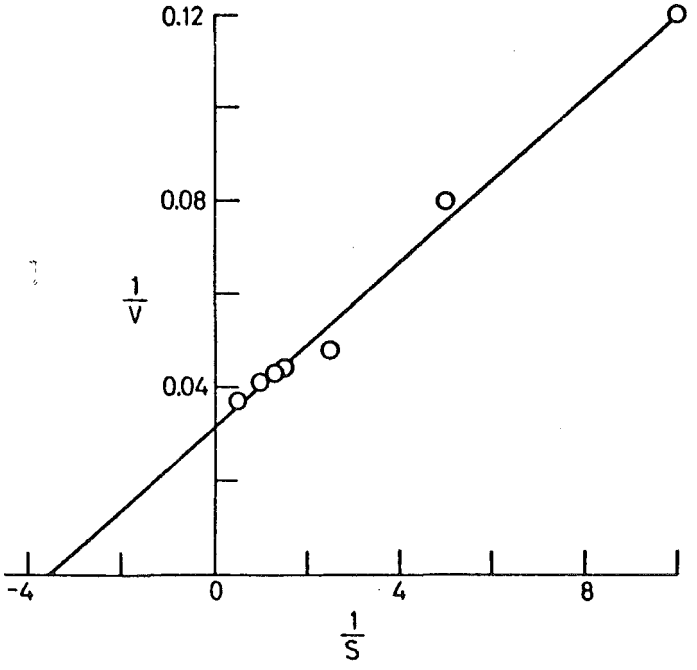

Fig. 2. Lineweaver-Burk plot of the effect of p-nitrophenyl-α-D-galactopyranoside concentration on activity of C. chinensis larval α-galactosidase. Conditions of reaction: substrate in citrate-phosphate buffer pH 5.5, for 30 min. at 31°.

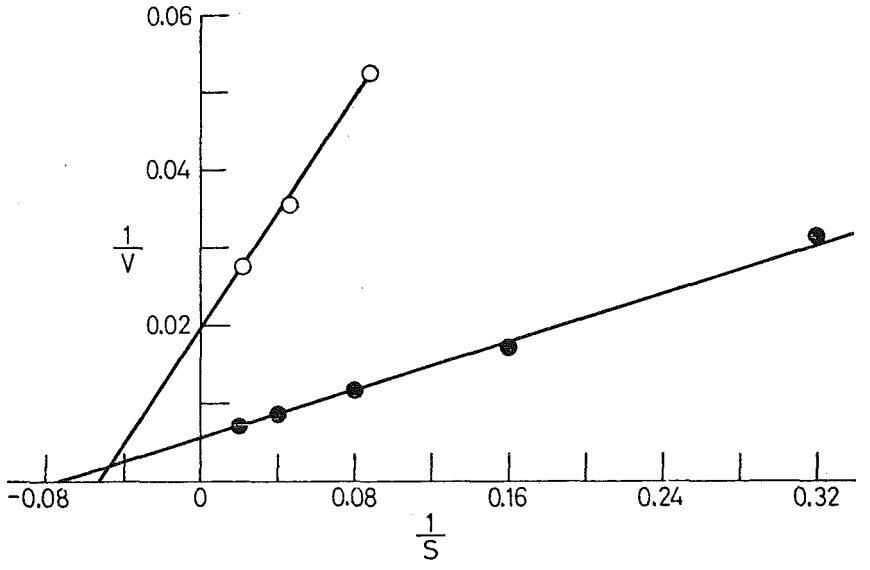

Fig. 3 Lineweaver-Burk plot of the effect of raffinose and stachyose concentration on activity of C. chinensis larval α-galactosidase. Conditions of reaction: substrates in citrate-phosphate buffer pH 5.6, for 20 min at 37°. (●——● = raffinose; o —— o = stachyose).

Although the affinity of the enzyme for its two naturally-occurring substrates--raffinose and stachyose--is similar, the rate of reaction is several-fold higher on the former substrate than on the latter ($\frac{\text{Vmax raffinose}}{\text{Vmax stachiose}} = 4.7$). This observation is reflected in the fact that stachyose in fact inhibits hydrolysis of the synthetic substrate p-nitrophenyl-α-D-galactopyranoside (Fig. 4).

The hydrolysis of this synthetic substrate by sweet-almond α-galactosidase is inhibited by the disaccharide melibiose (Malhotra & Dey, 1967).

The effect of temperature on activity was determined in the range of 20° to 60°. Maximal activity was obtained at 55°, with activity only slightly reduced at 60°. The activation energy (Ea) of the enzymic activity is presented as an Arrhenius plot (Fig. 5). The value of apparent Ea calculated from the slope of the line is 11,660 cal/mole. The limited degree of purification of the α-galactosidase precludes an unambiquous evaluation of functional groups at the active site, as other proteins in the enzyme solution might affect the apparent activity at the various temperatures.

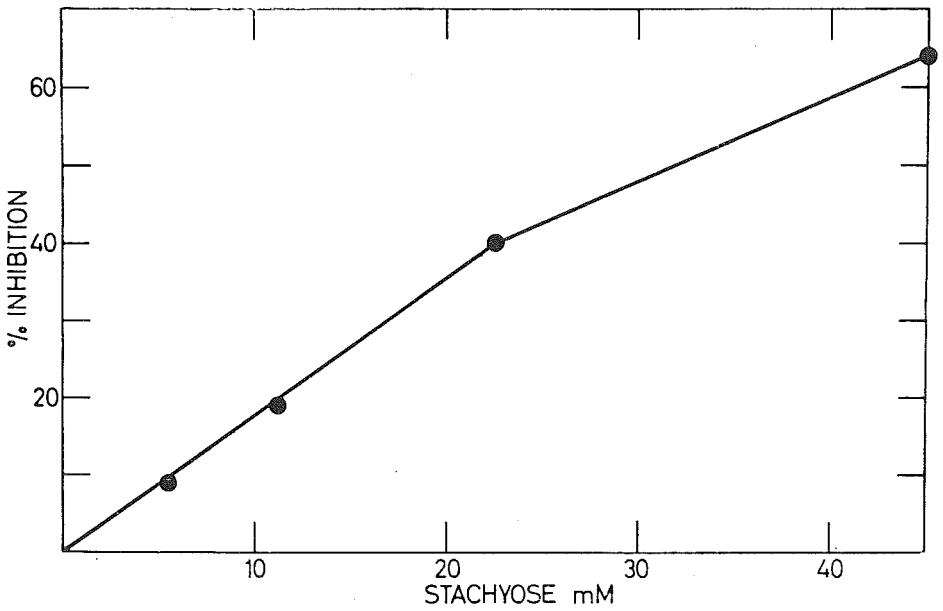

Fig. 4.

Inhibitory effect of stachyose on activity of C. chinensis larval α-galactosidase. Conditions of reaction: 5 mM p-nitrophenyl-α-D-galactopyranoside as substrate, in 5 mM ammonium acetate buffer pH 5.6, for 40 min at 37°.

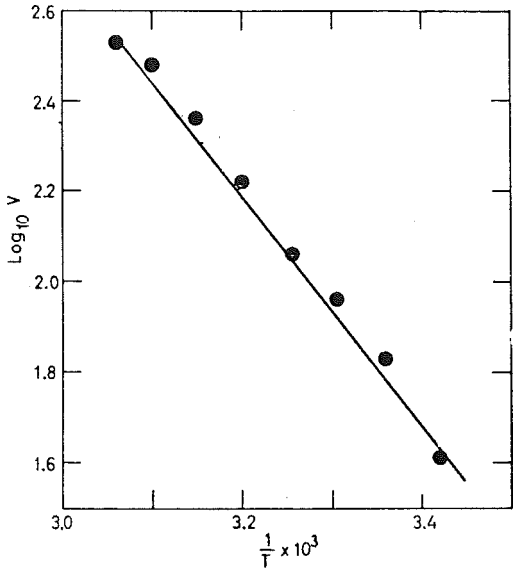

Fig. 5

Arrhenius plot of the effect of temperature on the reaction catalyzed by C.chinensis larval α-galactosidase. Conditions of reaction : 0.8 mM p-nitrophenyl-α-D galactopyranoside as substrate, in citrate phosphate buffer pH 5.5, for 15 min in the range of 20° - 55°.

Activation and inhibition studies were conducted in order to assess the importance of free sulfhydryl groups for the hydrolytic activity of the larval α-galactosidase, and compare its response to α-galactosidases from other sources (Wallenfels & Malhotra, 1961; Sheinin & Crocker, 1961).

Several thiol reagents were first assayed for their effect on the α-galactosidase activity (Table I). Ag^{+} inhibited completely at high concentrations (1 mM) but had only partial effect at 0.01 mM. It should be noted that this cation may inhibit carboxyl groups in addition to its reaction with free sulfhydryls (Dixon & Webb, 1964). Iodoacetamide did not inhibit at all at 1 mM concentration. Fungal α-galactosidase is similarly insensitive to iodoacetamide (Li & Shetlar, 1964). N-ethylmaleimide--the most specific of these three reagents--inhibits 50% of the activity at 1 mM.

TABLE 1

Effect of several thiol reagents as inhibitors of activity of C.chinensis larval α-galactosidase

Compound assayed	Concentration in reaction mixture	% inhibition
$AgNO_3$ *	1 mM	100
"	0.1 mM	87
"	0.01 mM	19
Iodoacetamide **	1 mM	1
n-ethylmaleimide **	1 mM	50

* Conditions of reaction: 0.5 mM p-nitrophenyl-α-D-galactopyranoside in 5 mM ammonium acetate buffer pH 5.6, for 30 min at 37°.

** Conditions of reaction: 1% raffinose in 5 mM ammonium acetate buffer pH 5.6, for 40 min at 37°.

The divalent cations Ca^{++}, Mg^{++}, Cu^{++} and Zn^{++} do not appreciably alter the apparent rate of activity. Pb^{++} only partially inhibits (Table II). The lack of activation by ethylene diamine tetraacetate agrees with these observations. Bacterial α-galactosidase is reported to be inhibited by Cu^{++} (Li et al., 1963).

TABLE II

Effect of several inorganic compounds on activity of C. chinesis larval α-galactosidase

Compound assayed	Concentration in reaction mixture	% inhibition or activation
$Ca(NO_3)_2$ *	1 mM	+ 8
$Mg(CH_3COO)_2$ **	1 mM	- 12
$ZnSO_4$ **	1 mM	- 6
$CuSO_4$ **	0.2 mM	- 10
$Pb(CH_3COO)_2$ **	1 mM	- 35
Ethylene diamine tetraacetate **	2 mM	+ 12

* Conditions of reaction as in Table I

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The reducing reagent glutathione exhibits ambivalent effect on the apparent α-galactosidase activity. Whereas it activates at lower concentrations, in excess activity is inhibited (Fig. 6).

These results can be explained by assuming that some free -SH groups are essential for activity, but that an excess of glutathione, by cleaving additional disulfide bonds, adversely alters the configuration of the enzyme. An activation of 25% is observed in the presence of 1 mM 2-mercaptoethanol, and the inhibitory effect of 1 mM n-ethylmaleimide is largely negated by addition of 1 mM glutathione.

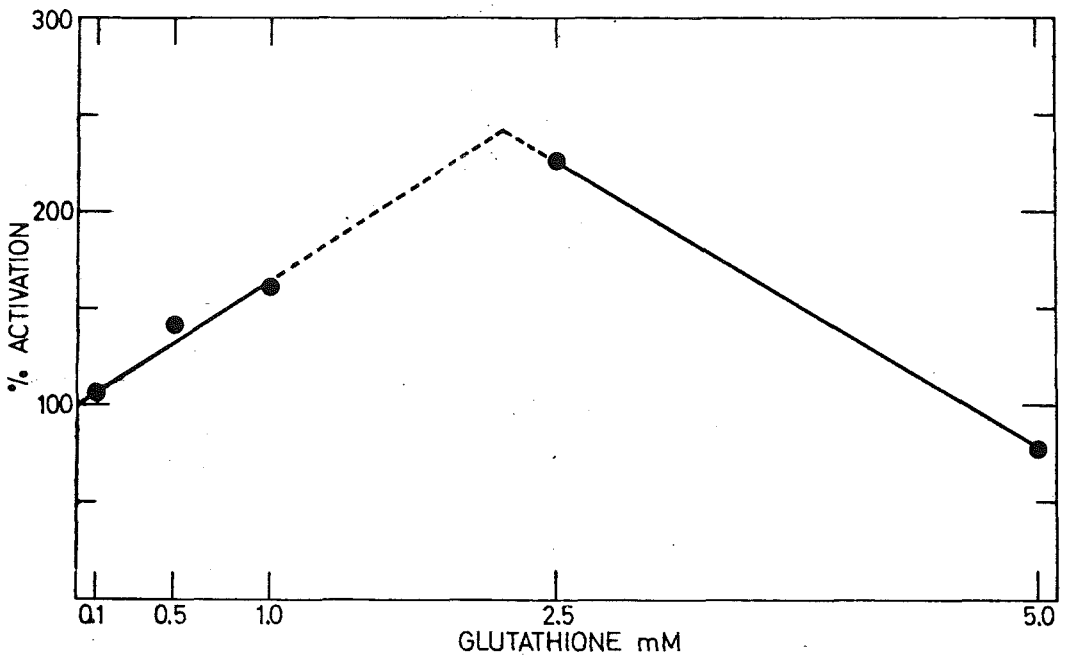

Fig. 6. Effect of glutathione on activity of C. chinensis larval α-galactosidase: 40 min preincubation of glutathione and enzyme prior to reaction on 1% raffinose in 5 mM ammonium acetate buffer pH 5.6, for 40 min at 37°.

The relatively strong inhibitory effect of Ag^{++} may be due to its reaction with essential carboxyl groups, more than to its effect on sulfhydryl groups. Broad-bean α-galactosidase I reportedly contains essential carboxyl groups (Dey & Pridham, 1969) but does not seem to require thiol groups for enzymic activity.

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