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CHARACTERIZATION OF β-GALACTOSIDASES FROM THE GERMINATING SEEDS OF *VIGNA SINENSIS*

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Key Word Index— *Vigna sinensis*; Leguminosae; seed germination; β-galactosidase; multiple forms.

Abstract—Four forms of β-galactosidase from the germinating seeds of *Vigna sinensis* were separated and partially purified by ammonium sulphate precipitation, ion exchange chromatography (DE-52) and gel filtration to more than 50% purity as judged by PAGE. The pH and temperature optima, stability, M_r, kinetic parameters and energy of activation of each enzyme have been determined. The four forms differed in their M_rs and ionic charges.

INTRODUCTION

Galactose-containing oligosaccharides present in seeds serve as a source of energy during the early phases of seed germination. The enzyme β-galactosidase (EC 3.2.1.23), responsible for hydrolysing β-galactosyl linkages, is found in a wide variety of sources [1, 2]. There is a wealth of data concerning *Escherichia coli* β-galactosidase [3]; however, only a few scattered reports deal with the characterization of plant β-galactosidases [4, 5]. Apart from its ability to split β-galactosidic linkages, its physiological functions are unknown. Recently, the molecular aspects of the plant protein has received increased attention [6, 7]. There is additional interest in β-galactosidases because some of them can modify the blood group specificity of intact human erythrocytes by removing galactose residues from their cell surface [8, 9]. The isolation and characterization of β-galactosidase may provide a tool to explore the nature of the antigenic determinants of a cell, and the enzyme might be used to investigate biochemical and clinical problems. In this paper, I report on the isolation, partial purification and characterization of multiple forms of β-galactosidase activity from the germinating seeds of *Vigna sinensis* (Linn) Savi. The results have been compared to the previously reported results for other β-galactosidases.

RESULTS AND DISCUSSION

Cotyledons of 96-hr-old germinating seeds of *V. sinensis* were used as a source of β-galactosidase. The enzyme protein was precipitated from the 10 000 *g* supernatant of a homogenate of the cotyledons with 40–60% ammonium sulphate and then further purified by chromatography on DEAE cellulose followed by Sephadex G-100 (Table 1). The first chromatographic step gave rise to four sets of active fractions: Gal-I (peak I), Gal-II (peak II), Gal-III (peak III) and Gal-IV (peak IV) (Fig. 1), indicative of the presence of four isoenzymes in the germinating seed. A summary of the quantitative aspects of the purification procedure is given in Table 1. During purification the total activity is divided into several fractions. So, the total recovery and fold of purification of each β-galactosidase is relatively low. Because of the low enzyme activity of Gal-III and Gal-IV further purification was not tried.

Purity

Electrophoresis on 7% polyacrylamide gel of the final purified β-galactosidases showed one major band and a few minor protein bands (Fig. 2). It should be noted that the pattern of protein bands in each class and their mobility through the gel are different.

Molecular masses

The M_rs of Gal-I, Gal-II, Gal-III and Gal-IV were determined by Sephadex G-150 column (1.4 × 90.0 cm) chromatography, using trypsin (23 800), ovalbumin (45 000), bovine serum albumin (67 000) and bovine intestinal alkaline phosphatase (140 000) as standards, to be about 43 600, 66 800, 54 300 and 79 400, respectively. These values are in the size range of the reported values of 68 000 for bovine testicular β-galactosidase [10], 65 000–75 000 for human liver β-galactosidase [11], 50 000 for 6-phospho-β-galactosidase from *Staphylococcus aureus* [12] and 75 000 for β-galactosidase from jack bean [4]. However, values of 540 000 for *E. coli* β-galactosidase [13], 125 000 and 250 000 for β-galactosidases from *H. pometia* [14] and 100 000 for β-galactosidase from *P. citrinum* [15] seem to form a larger size class of this enzyme.

Effect of temperature

At PH 5.0, Gal-II, Gal-III and Gal-IV were most active at 55°; Gal-I was most active at 60° (Fig. 3). Similar

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Abbreviations: PNPG, *p*-nitrophenyl β-D-galactoside,; ONPG, *o*-nitrophenyl β-D-galactoside; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethyl maleimide.

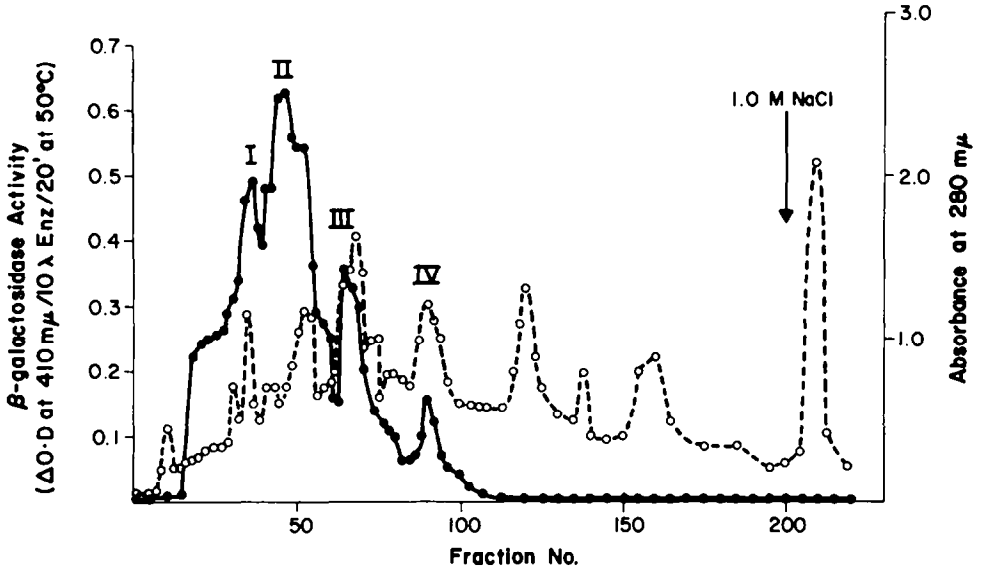

Fig. 1. Elution profile on DEAE-cellulose chromatography of β-galactosidase activity. A 15.0 ml solution of the 40–60% ammonium sulphate fraction containing 123 mg of protein was applied to the column (2.0 × 22.0 cm). The column was washed with 250 ml 10 mM Tris–HCl, pH 7.0, and then eluted with 500 ml buffer with a linear gradient of 0–250 mM NaCl (fraction 0–220). O——O, Absorption at 280 nm; ●——●, β-galactosidase activity. The different peak fractions were pooled separetely and designated as Gal-I (peak I), Gal-II (peak II), Gal-III (peak III) and Gal-IV (peak IV), respectively.

Table 1. Purification of β-galactosidases from 50 g of germinating seeds of *V. sinensis*

Step	Total protein (mg)	Specific activity*	Purification (-fold)	Recovery (%)
Crude homogenate	545.7	2.3		
40–60% $(NH_4)_2SO_4$ precipitation	123.0	7.4	3.2	71.5
De-52 chromatography				
Gal-I	0.8	72.9	31.4	4.6
Gal-II	3.7	33.9	14.6	9.9
Gal-III	2.5	5.8	2.5	1.1
Gal-IV	2.5	4.3	1.8	0.9
Sephadex G-100 gel filtration				
Gal-I	0.09	317.7	136.9	2.4
Gal-II	0.38	135.0	58.2	4.1
Gal-III	0.27	26.1	11.2	0.6
Gal-IV	0.15	27.3	11.8	0.3

*μmol *p*-nitrophenol liberated/hr/mg protein.

optimum temperatures for other β-galactosidases have been reported earlier [15, 16].

Effect of pH

The effect of pH on the activities was evaluated over pH ranges of 3.0–5.0 in 50 mM citrate buffer and 5.0–7.0 in 50 mM acetate buffer. The maximum activity was observed at pH 4.5 for Gal-I, Gal-II and Gal-III, and at pH 5.0 for Gal-IV. (The pH values for half-maximum activity were *ca* 3 and *ca* 5) These pH optima resemble some of the other plant β-galactosidases [4, 16–18] but differ from the pH optimum observed for bacterial β-galactosidase which tends to be at pH 7.0 [19]. *Neurospora crassa* has two isoenzymes of β-galactosidase with different pH optima [20].

Stability

Gal-I retained 44.5% of its initial activity after 60 min pre-incubation at 60° at pH 5.0, while Gal-II, Gal-III and Gal-IV retained 41.7, 29.2 and 35.3% of their initial activities, respectively (Fig. 4). But after pre-incubation at 60° for 60 min at pH 7.0, Gal-I, Gal-II, Gal-III and Gal-IV retained 33.3, 18.2, 16.7 and 12.1% of their initial activities (Fig 4). It appears that thermal stability is a function of pH.

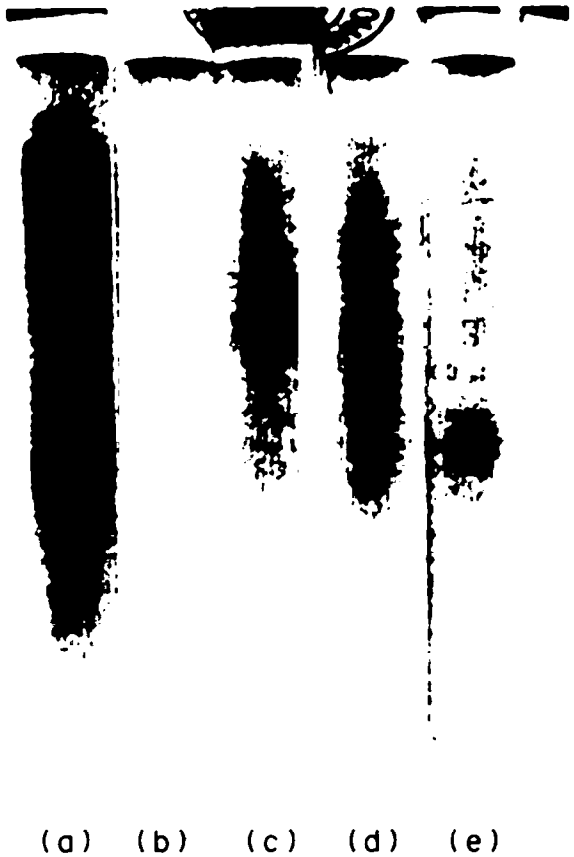

Fig. 2. PAGE of purified β-galactosidases. (A) Protein band of the 40–60% ammonium sulphate fraction; (B) protein band of purified β-galactosidase I; (C) protein band of purified β-galactosidase II; (D) protein band of purified β-galactosidase III; (E) protein band of purified β-galactosidase IV.

Effects of some metal ions and inhibitors

Except for Mg^{2+}, all the metal ions tested had inhibitory effects on the β-galactosidase activities (Table 2). There are some reports that a few galactosidases show an absolute requirement for Mg^{2+} and some do not [21, 22]. The extent of inhibition was greatest with Hg^{2+}, Co^{2+} and tungstate. The activity of Gal-IV was less inhibited by Zn^{2+} and arsenate in comparison with the others. The inhibition by the heavy metals suggests that a free sulphydryl group(s) is involved in enzyme activity. Similar observations were found in other systems [23, 24]. NaF, NaN_3, glucose and EDTA do not have any effect on the activities of β-galactosidases (Table 3). However, galactose, lactose and ascorbic acid have an inhibitory effect. *p*-Chloromercuribenzoate, iodoacetate and NEM also inhibited the activities. These results support the earlier suggestion that cysteine or other free sulphydryl group(s) are necessary for enzyme function. In contrast to these plant β-galactosidases, *p*-chloromercuribenzoate does not have any inhibitory effect on fungal β-galactosidase [15].

Kinetic studies.

It was found that the enzyme hydrolysed *p*-nitrophenyl β-D-galactoside and *o*-nitrophenyl β-D-galactoside but not *p*-nitrophenyl α-D-galactoside, *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside [23]. The K_m values for PNPG are 0.39, 0.57, 0.82 and 0.50 mM, for Gal-I, Gal-II, Gal-III and Gal-IV, respectively, whereas the K_m values for ONPG are 2.5 mM for Gal-I, Gal-III and Gal-IV, and 2.8 mM for Gal-II. These values are in agreement with some of the values obtained from other experimental studies. The reported K_m values for PNPG of β-galactosidases from various sources are 1.7 mM in *P. citrinum* [15], 0.51 mM in jack bean [4], 0.2 mM in human liver [25], 0.03 mM in *E. coli* [26], 0.52 mM in mouse [27] and 0.29 mM in bovine testes [10]. The K_m values of ONPG for β-galactosidase from different sources have been reported to 0.63 mM in jack bean [4], 9.26 mM in alkalophilic *Bacillus* [23], 1.7 mM in *P. citrinum* [18], 0.11 mM in *E. coli* [28] and 0.03 mM in bovine testes [13]. The V_{max} (in μmol/hr/mg protein) values obtained were 1000, 770, 176 and 104 for Gal-I, Gal-II, Gal-III and Gal-IV, respectively. The corresponding values when ONPG was used as substrate were 2000, 625, 125 and 125.

The type of inhibition and the inhibition constant of β-galactosidases by galactose, lactose, *p*-chloromercuri-

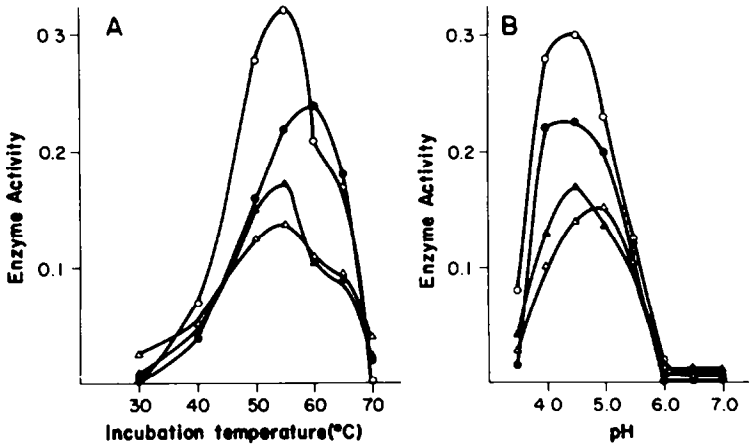

Fig. 3. Optimum temperature of β-galactosidases. ●——●, Gal-I; ○——○, Gal-II; ▲——▲, Gal-III; △——△, Gal-IV.

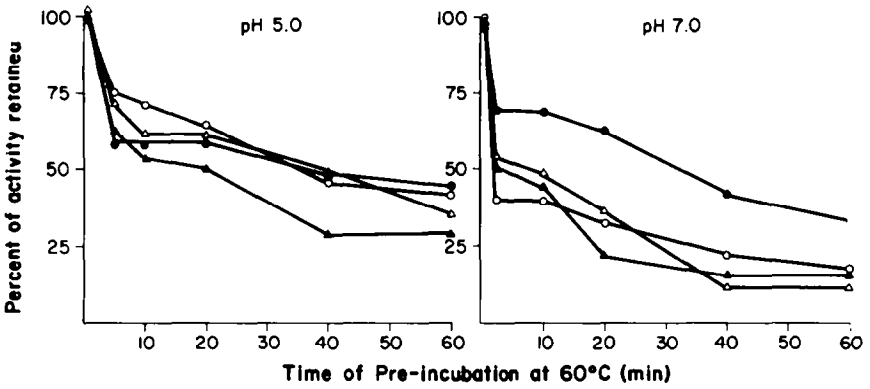

Fig. 4. Thermal stability of β-galactosidases. Enzyme samples in 50 mM acetate buffer, pH 5.0 or 7.0, were pre-incubated at 60° for different time periods and assayed for activity by adding PNPG as substrate. Activities at various times are expressed as a percentage of the appropriate zero time control. ●——●, Gal-I; ○——○, Gal-II; ▲——▲, Gal-III; △——△, Gal-IV.

Table 2. The effects of metal ions on the activities of β-galactosidases

Metal	Gal-I		Gal-II		Gal-III		Gal-IV	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
$MgCl_2$	90	95	88	90	68	67	68	100
$CuSO_4$	13	51	28	67	21	67	11	94
Mo_2O_7	15	15	16	21	21	39	21	61
$ZnSO_4$	56	84	61	87	53	83	100	89
$PbCl_2$	21	57	30	80	37	78	42	83
$HgCl_2$	5	3	0	0	0	0	0	0
Tungstate	0	0	0	0	0	0	0	0
$CoCl_2$	13	6	12	0	21	0	21	5
Arsenate	10	30	54	64	21	31	53	67

Metal ion and enzyme were mixed in 10 mM acetate buffer, pH 5.0, and allowed to stand for 10 min at room temperature. PNGP was added to the reaction mixture and the enzyme activity was determined. Activity is expressed as the percentage of the activity in the absence of metal ions. (a) Enzyme was pre-incubated with 1 mM metal ions; (b) enzyme was pre-incubated with 1 mM metal ion and then with 10 mM EDTA.

Table 3. The effects of some chemical agents on the activities of β-galactosidases

Inhibitor	Final concn (mM)	% of activity retained			
		Gal-I	Gal-II	Gal-III	Gal-IV
NaF	50	100	112	100	100
NaN_3	50	100	100	79	100
Galactose	50	20	22	35	43
Glucose	50	100	93	86	100
Lactose	50	72	71	59	43
Ascorbic acid	10	72	56	83	71
PCMB	0.1	3	3	7	0
Iodoacetate	10	3	3	0	7
NEM	10	49	32	31	36

Chemical agent and enzyme were mixed in 10 mM acetate buffer, pH 5.0, and allowed to stand for 10 min at room temperature. PNGP was added to the reaction mixture and the enzyme activity was determined. Activity is expressed as a percentage of the activity in the absence of chemical.

benzoate and iodoacetate were determined from initial velocity measurements at various substrate concentrations (0.12–0.75 mM) and three different inhibitor concentrations. The results were calculated from Lineweaver–Burk plots. The common intercept on the $1/V$ axis indicated that galactose and lactose inhibit the enzyme competitively. The K_i values obtained from the secondary plots are about 3.0, 4.0, 5.0 and 9.0 mM (galactose) and 85.0, 50.0, 45.0 and 35.0 mM (lactose) for Gal-I, Gal-II, Gal-III and Gal-IV, respectively. Similarly the different intercept on the $1/V$ axis indicated that the inhibition of the enzyme activities by *p*-chloromercuribenzoate and iodoacetate is non-competitive. The K_i values obtained from the secondary plots are about 0.07, 0.05, 1.6 and 0.6 μM (PCMB) and 30, 36, 120 and 300 μM (iodoacetate) for Gal-I, Gal-II, Gal-III and Gal-IV, respectively.

Energy of activation

At the optimum pH, the rate of PNPG hydrolysis by the four β-galactosidases was determined as a function of the temperature. The activation energies for the hydrolysis of PNPG are 5.9, 8.6, 8.4 and 7.0 kcal/mol for Gal-I, Gal-II, Gal-III and Gal-IV, respectively. The activation energy of PNPG for β-galactosidase was reported as 18 kcal/mol in sugar cane [29] and 12.4 kcal/mol in *P. vulgaris* [20].

In conclusion, I have shown that there are at least four forms of galactosidase in the germinating seeds of *V. sinensis*. They show identity or a close relationship in all the properties tested except for a small variation of their M_r. However, they are easily separated by ion-exchange column chromatography; this could be explained by the existence of multiple enzyme forms differing in charge. The physiological role of these β-galactosidases in seed germination remains to be established.

EXPERIMENTAL

Materials. Seeds of *V. sinensis* were collected from a local market. *p*-Nitrophenyl β-D-galactoside (PNPG), *o*-nitrophenyl β-D-galactoside (ONPG), NaF, *N*-ethylmaleimide (NEM) and DEAE-Cellulose (DE-52) were purchased from Sigma, U.S.A. Allaxan, Triton X-100 and polyethylene glycol 6000 were purchased from BDH, U.K.

Enzyme assay. β-Galactosidase activity was estimated by measuring the release of *p*-nitrophenol from the substrate PNPG according to the method described in ref. [2]. The reaction mixture (total vol. 1.0 ml) contained 25 mM acetate buffer, pH 5.0; 0.25 mM PNPG; and the enzyme. It was incubated at 50° for 20–40 min. The reaction was stopped by adding 4.0 ml 0.1 M NaOH, and the absorbance of the *p*-nitrophenol released was measured at 410 nm. One unit of β-galactosidase activity is defined as the amount of enzyme which hydrolyses 1 μmol PNPG/hr under the conditions described above. The sp. act. of the enzyme was expressed as units/mg protein.

Estimation of protein. Protein concn was estimated by the method of Lowry *et al.* [30] with bovine serum albumin as standard or by UV absorption measurement at 280 nm.

Enzyme purification. (a) *Germination of seeds.* The seeds (50 g) were washed with distilled H_2O and immersed in 0.02% $HgCl_2$ for 10 min followed by several changes with distilled H_2O. They were then decoated and allowed to germinate in the dark at 28°.

(b) *Preparation of cell-free extract.* Cotyledons of 96-hr-old germinating seeds were separated from the embryo and kept under ice-cold conditions. After washing with distilled H_2O, the cotyledons were homogenized in a blender with ice-cold buffer A (10 mM Tris–HCl, pH 7.42; 9 vols.) containing 0.1% Triton X-100. The homogenate was kept overnight on ice and then strained through two layers of cheesecloth to remove the coarser cell debris. The homogenate was centrifuged at 2000 *g* for 10 min at 4° to remove the remaining cell debris. The supernatant thus obtained was further centrifuged at 10000 *g* for 20 min. The pellet was discarded and the supernatant was used as the starting material for purification of β-galactosidase activity. Unless otherwise indicated, all operations during the enzyme purification were carried out at 4°.

$(NH_4)_2SO_4$ *fractionation.* Solid $(NH_4)_2SO_4$ was added slowly to the extract with constant stirring to obtain 40% saturation. The mixture was stirred for an additional 60 min and left to stand for 2 hr. The ppt. was removed by centrifugation and discarded. The supernatant was further fractionated by adding more solid $(NH_4)_2SO_4$ to 60% saturation. After standing overnight, the precipitated protein containing β-galactosidase activity was collected by centrifugation and dissolved in a minimum vol. of buffer A. This fraction was dialysed against the same buffer for 18 hr followed by centrifugation to remove the undissolved materials and denatured proteins. This is referred to as the 40–60% $(NH_4)_2SO_4$ fraction.

Chromatography on DEAE-cellulose. A DE-52 column (2.0 × 22.0 cm) was equilibrated with buffer A, and the dialysed 40–60% $(NH_4)_2SO_4$ fraction containing 123 mg protein loaded onto it. The column was then washed with 250 ml buffer A. Elution was carried out with 500 ml buffer A with a linear gradient of 0–250 mM NaCl. The flow rate of the column was adjusted to 30 ml/hr. Fractions containing 2.5 ml were collected. The enzyme activity of each fraction was assayed. The active fractions were pooled into four classes according to the peaks of activity and designated as Gal-I (peak I), Gal-II (peak II), Gal-III (peak III) and Gal-IV (peak IV) (Fig. 1). Each group of pooled fractions was dialysed successively against buffer A for 18 hr with polyethylene glycol 6000 and buffer A.

Gel filtration on Sephadex G-100. The concd pooled fractions from DE-52 chromatography were loaded separately onto a Sephadex G-100 column (1.4 × 96 cm) which had been equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 6 ml/hr. The fractions containing β-galactosidase activity were pooled and stored at −20° until use.

PAGE. Gel electrophoresis was carried out at pH 8.3 by the method of Davis [31] on 7.0% polyacrylamide gels with bromophenol blue as tracking dye. A current of 3 mA/gel was applied. Protein was stained with Coomassie Blue.

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