



Purification and characterization of isoforms of β -galactosidases in mung bean seedlings

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Abstract

Five isoforms of β -galactosidase (EC 3.2.1.23), designated as β -galactosidases I–V, were isolated from five-day-old mung bean (*Vigna radiata*) seedlings. β -Galactosidases II and III were purified to electrophoretic homogeneity by a procedure involving acid precipitation, ammonium sulfate fractionation, chromatography on diethylaminoethyl-cellulose (DEAE-Cellulose) and con A-Sepharose, and chromatofocusing. β -Galactosidases I, II and III have the same molecular mass of 87 kDa, comprising two non-identical subunits with molecular masses of 38 and 48 kDa, while β -galactosidases IV and V have molecular masses of 45 and 73 kDa, respectively. All the enzymes were active against *p*-nitrophenyl- β -D-galactoside, and to a lesser extent, *p*-nitrophenyl- α -L-arabinoside and *p*-nitrophenyl- β -D-fucoside. The enzymes were inhibited by D-galactono-1,4-lactone, D-galactose, Hg²⁺, Ag⁺ and sodium dodecyl sulfate (SDS). β -Galactosidases I, II and III were shown to be competitively inhibited by either D-galactono-1, 4-lactone or D-galactose. Isoforms I, II and III have a common optimal pH of 3.6, while isoforms IV and V have pH optima at 3.8 and 4.0, respectively. Isoelectric points of isoforms I, II and III were 7.7, 7.5 and 7.3, respectively. Double immunodiffusion analysis indicated that β -galactosidases I, II, III and V are immunologically similar to each other, while β -galactosidase IV shares partially identical antigenic determinants with the other four isoforms. The purified β -galactosidases II and III were capable of releasing D-galactose residue from the hemicellulose fraction isolated from mung bean seeds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Vigna radiata*; Leguminosae; β -Galactosidase; Enzyme purification; Natural substrates

1. Introduction

β -Galactosidase (EC 3.2.1.23) is widely distributed in plant tissues. The enzyme has been implicated in a number of biological processes including plant growth and fruit ripening. One characteristic of fruit ripening and softening is the mobilization of cell wall components. The release of monomeric galactose from cell walls of ripening fruits has been described with apple, tomato, melon, avocado, kiwifruit and mango (Bartley, 1976; Pressey, 1983; Ranwala et al., 1992; Deveau et al., 1993; Ross et al., 1993; Ali et al., 1995). Molecular approaches were also used to unravel the role of β -galactosidases in fruit development and ripening. Ross et al. (1994) isolated a cDNA clone encoding an apple β -galactosidase. Northern blot analysis showed accumulation of a transcript

homologous to the cDNA clone during fruit ripening. A cDNA clone, a member of the β -galactosidase gene family, was isolated from a cDNA library of tomato fruit. This cDNA showed ripening-related gene expression in normal fruits, with lower levels apparent in the non-softening mutants (Carey et al., 1995). Another cDNA, corresponding to β -galactosidase II of tomato, was also cloned. Northern blot analysis revealed that the β -galactosidase II gene transcript was detectable at the breaker stage of ripeness, maximum at the turning stage, and present at decreasing levels during the later stages of normal tomato fruit ripening (Smith et al., 1998).

The correlation of β -galactosidases with plant growth and development has also been investigated. This included the isolation and characterization of β -galactosidases from cowpea, nasturtium, radish, mung bean, barley, carrot, rice shoots and lupins (Biswas, 1987; Edwards et al., 1985, 1988; Sekimata et al., 1989; Kundu et al., 1990; Giannakouros et al., 1991; Konno and Katoh, 1992; Konno and Tsumuki, 1993; Buckeridge and Reid, 1994).

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We have found that the activities of some glycosidases in germinating mung bean seeds increased to a high level between days 4–6. β -*N*-Acetylglucosaminidase, β -galactosidase and α -mannosidase showed the highest specific activity. The increase of β -galactosidase activity during seed germination was also reported in plants such as pinto beans (Agrawal and Bahl, 1968), castor beans (Harley and Beevers, 1985), nasturtium (Edwards et al., 1988), barley (Giannakouros et al., 1991) and lupin (Buckridge and Reid, 1994). The increase of β -galactosidase activity during the germination of barley seeds seems to be very moderate when compared with the many-fold increase in the activity of the same enzyme observed in the seeds of dicotyledon plants (Giannakouros et al., 1991). Thus, the developmental regulation of plant β -galactosidase during germination and growth has become evident. However, much less is known about the mechanism underlying this regulation. We investigated the structure and function of mung bean β -galactosidase as a first step towards studying its gene expression and regulation. This paper deals with the purification and characterization of five isoforms of β -galactosidase, and the activity of the two purified isoforms, II and III, of the enzyme towards cell wall polysaccharides of mung bean seeds. Separation by two-dimensional electrophoresis, followed by Western blot analysis of the two purified isoforms, II and III, as well as the immunological relationship of the five isoforms are also described.

2. Results

2.1. Purification

Acid precipitation removed a large portion of the contaminating proteins (about 65% of total proteins) in the cell-free extract, resulting in a 2.6-fold purification (Table 1). With the purification procedure shown in

Scheme 1, five β -galactosidases, I–V, were isolated as summarized in Table 1 and Fig. 1. β -Galactosidases I, II and III, which did not bind to either DEAE-cellulose or Con A-Sepharose, were purified by chromatofocusing. β -Galactosidases II and III were purified to electrophoretic homogeneity after repeating chromatofocusing twice, and whereas a faint contaminating band ran ahead of β -galactosidase I in a non-denaturing gel (Fig. 2), it did not indicate the actual molecular weights. The β -galactosidase IV, retained on the DEAE-cellulose (Fig. 1A) and Con A-Sepharose columns (Fig. 1C), was eluted in sequence from the columns. The eluted β -galactosidase IV was shown to be still contaminated with many proteins in a non-denaturing gel (data not shown). Thus, β -galactosidase V, which bound to Con A-Sepharose (Fig. 1B), was purified with this column. The purified isoform V contained approximately equal amounts of a contaminating protein that moved slightly faster than the enzyme in a non-denaturing gel (data not shown). The overall recovery of the five β -galactosidase isoforms was about 34%.

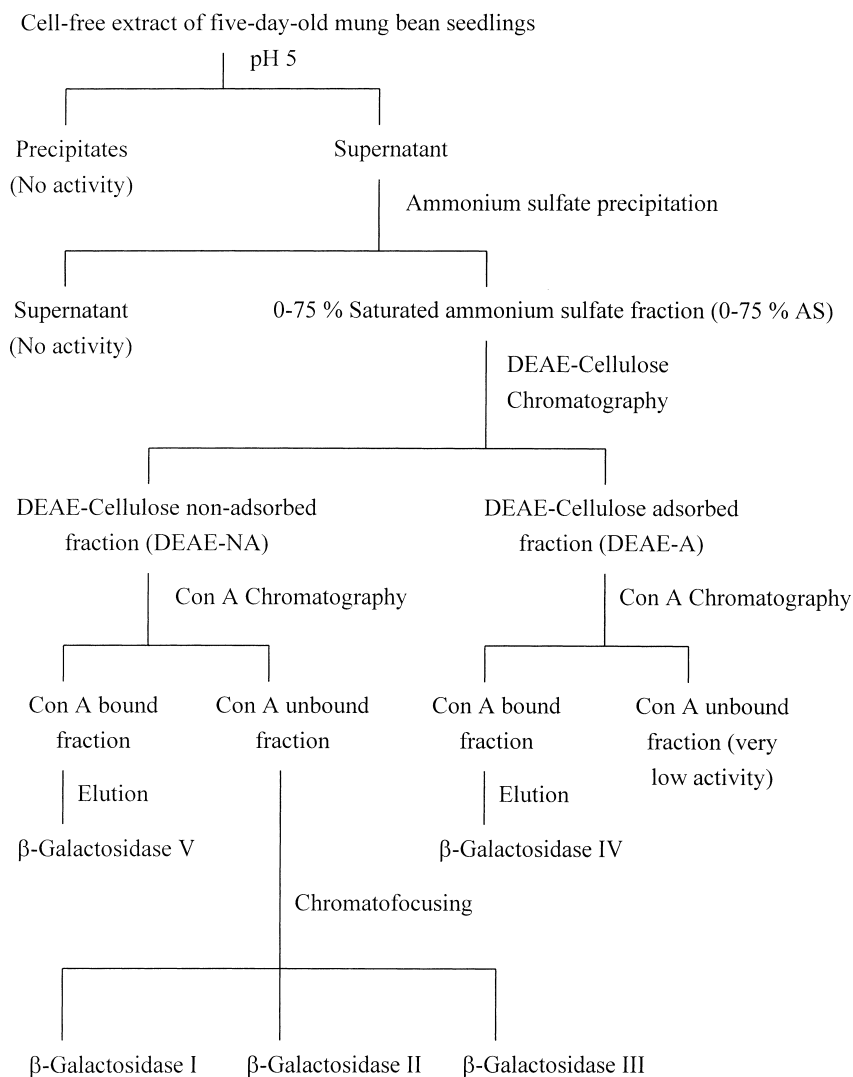
2.2. Characteristics of β -galactosidases

The molecular masses of β -galactosidases I, II, III, IV and V, estimated from their elution volumes from Sephacryl S-200, were 87, 87, 87, 73 and 45 kDa, respectively (data not shown). However, β -galactosidases I, II and III showed mobilities corresponding to 300–400 kDa in a non-denaturing gel (Fig. 2). A possible explanation for this discrepancy is that, firstly, the enzyme with isoelectric points in the range 7.3–7.7 might form aggregates during their concentration in the stacking gel (pH 6.8). Secondly, these isoforms are likely to be glycoproteins in nature, because they bound to a Con A column when NaCl was eliminated from the elution buffer. SDS-PAGE of β -galactosidases I, II and III showed that each form consisted of two non-identical subunits

Table 1
Purification of β -galactosidases from five-day-old mung bean seedlings^a

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Cell-free extract	11,206.0	645.5	0.0576	1.0	100
Acid precipitation	3946.3	580.2	0.15	2.6	89.9
Ammonium sulfate precipitation	2106.1	502.8	0.24	4.1	77.9
DE52 non-adsorbed	86.3	241.2	2.79	48.5	37.4
DE52 adsorbed	283.3	163.4	0.58	10.1	25.3
Con A non-adsorbed	5.4	139.1	25.76	447	21.5
Con A adsorbed (V)	12.9	45.9	3.65	63.4	7.1
Con A adsorbed (IV) (DE52 adsorbed)	33.2	51.8	1.56	27.1	8.1
Chromatofocusing					
I	0.48	19.1	39.79	691	2.96
II	0.51	48.7	95.49	1657	7.54
III	0.58	55.5	95.69	1661	8.59

^a A total of 4.1 kg of mung bean seedlings was used for the purification. Roman numerals I–V stand for β -galactosidases I–V, respectively.



Scheme 1.

with molecular masses of 38 and 48 kDa (Fig. 3). Since the SDS-PAGE provided only a rough estimation of molecular mass, these two figures (38 and 48 kDa) were more accurately determined by running these two sub-units through 0.1% SDS polyacrylamide gradient gel (10–20%) along with molecular weight standard proteins (data not shown). Two proteins bands, approximately 22 and 30 kDa, were also observed in β -galactosidase I and were probably due to contaminating proteins in the enzyme preparation (Fig. 3).

Effects of sugars and sugar derivatives on β -galactosidases I, II and III are shown in Table 2. D-Galacto-1,4-lactone was the most potent inhibitor followed by D-galactose and lactose. D-Galacto-1, 4-lactone at 12.5 mM almost completely inhibited the three isoforms (97.7–98.3% inhibition). The three isoforms were 71–72% inhibited by 50 mM D-Galactose, 26–28% inhibited by 50 mM lactose and slightly inhibited by 12.5 mM D-glucono- δ -lactone. D-Arabinose and D-fucose at 50 mM had no effect on the three enzymes. A kinetic analysis

indicated that D-galactono-1, 4-lactone and D-galactose acted as competitive inhibitors. K_i values for these two competitive inhibitors were determined by replots of data taken from double reciprocal plots. D-Galacto-1, 4-lactone has K_i values of 0.13, 0.085 and 0.17 mM for isoforms I, II and III, respectively, while D-galactose has K_i values of 11.4, 15 and 13 mM for I, II and III, respectively. The three isoforms have a common optimal pH of 3.6. Isoforms IV and V have pH optima at 4.0 and 3.8, respectively (Fig. 4). Isoforms I, II and III have temperature optima around 50–53°C (Fig. 5) and isoelectric points of 7.7, 7.5 and 7.3, respectively. All of the isoforms were stable from pH 4.0 to 8.0. Effects of metallic ions, EDTA, and SDS on β -galactosidase activities are shown in Table 3. Ag^+ , Hg^{2+} and SDS at 1 mM strongly inhibited the activities of isoforms I, II and III. The cell-free extract was active against 12 *p*-nitrophenyl glycosides tested. After purification, isoforms II and III retained the activities of β -galactosidase, β -D-fucosidase and α -L-arabinosidase, but were essentially devoid of activity

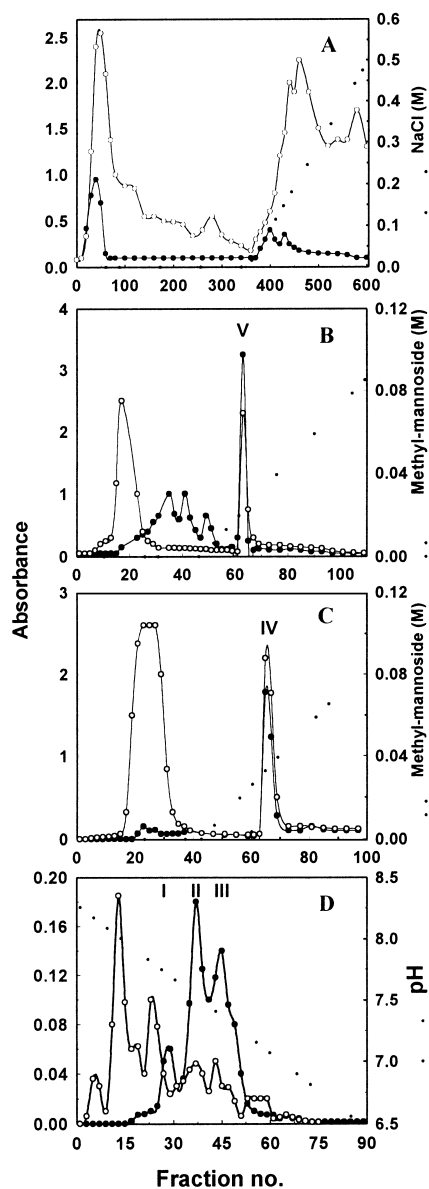


Fig. 1. Elution profiles of β -galactosidases on DEAE-cellulose columns (A), Con A-Sepharose columns (B, C) and chromatofocusing (D). The ammonium sulfate precipitated enzyme sample was applied to a DEAE-cellulose column (A). The DEAE-cellulose non-adsorbed fraction containing β -galactosidases I, II, III and V, and the adsorbed fraction (β -galactosidase IV), were then purified by Con A-Sepharose chromatography (B, C). The Con A-Sepharose adsorbed fraction is called β -galactosidase V. The non-adsorbed fraction containing β -galactosidases I, II and III was further purified by chromatofocusing. Experimental details are given in the Experimental section. Protein is expressed as absorbance at 280 nm (○) and β -galactosidase activity as absorbance at 420 nm (●).

towards the nine other *p*-nitrophenyl glycosides (less than 5%) (Table 4). Ratios of the activities of β -D-galactosidase, β -D-fucosidase and α -L-arabinosidase exhibited by isoforms II and III are 100, 35.7, 32.7% and 100, 31.2, 24.6%, respectively. Plant β -galactosidases with multiple glycosidase activities have been described (Conchie et al.,

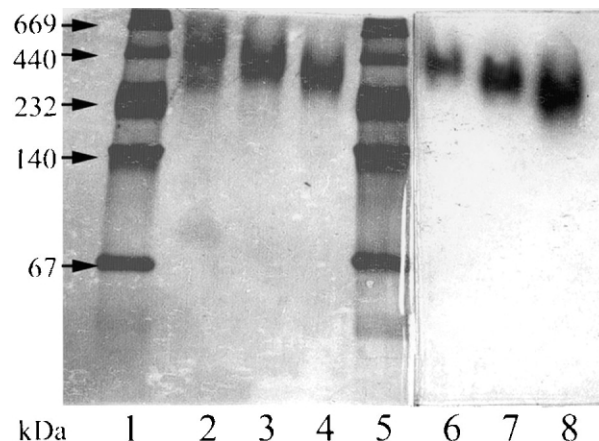


Fig. 2. The electrophoretic pattern of purified β -galactosidases I, II and III. After electrophoresis of the native enzymes (ca 5 μ g protein each), the left half of the gel (lanes 1–5) was stained with silver nitrate, and the right half (lanes 6–8), with activity staining. Lanes 1 and 5, molecular mass markers from top: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (67 kDa); lanes 2 and 6, β -galactosidase I; lanes 3 and 7, β -galactosidase II; lanes 4 and 8, β -galactosidase III.

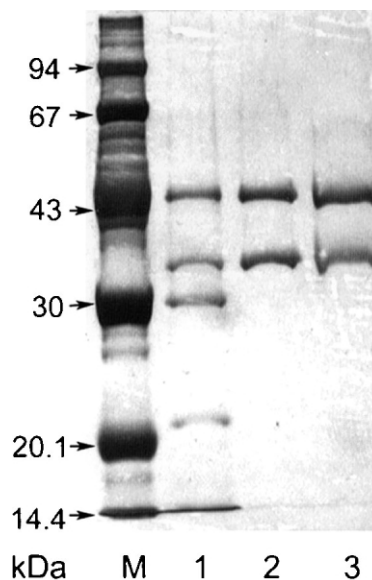


Fig. 3. SDS-PAGE of purified β -galactosidases I, II and III. Molecular mass markers from top: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). The numbers 1, 2 and 3 stand for β -galactosidases I, II and III, respectively. The gel was stained with silver nitrate.

1968; Kiss et al., 1981; Simos and Georgatsos, 1988). Since β -galactosidases II and III were purified to apparent homogeneity, cross-contamination from other glycosidases seems unlikely. Therefore, it is reasonable to suggest that the three glycosidase activities mentioned above reside in a single molecular species of β -galactosidases II and III.

Table 2
Effects of sugars and sugar derivatives on β -galactosidase activities^a

	β -Galase activity (nkatal)		
	I	II	III
No inhibitor	3.76×10^{-2} (100%)	3.87×10^{-2} (100%)	4.41×10^{-2} (100%)
D-Galactono-1,4-lactone (12.5 mM)	8.35×10^{-4} (2.2%)	6.68×10^{-4} (1.7%)	1.0×10^{-3} (2.3%)
D-Glucono- δ -lactone (12.5 mM)	3.46×10^{-2} (92%)	3.44×10^{-2} (89%)	3.74×10^{-2} (85%)
D (+) Arabinose (50 mM)	3.72×10^{-2} (99%)	3.67×10^{-2} (95%)	4.28×10^{-2} (97%)
D (+) Fucose (50 mM)	3.64×10^{-2} (97%)	3.64×10^{-2} (94%)	4.19×10^{-2} (95%)
D (+) Galactose (50 mM)	1.05×10^{-2} (28%)	1.12×10^{-2} (29%)	1.24×10^{-2} (28%)
Lactose (50 mM)	2.71×10^{-2} (72%)	2.87×10^{-2} (74%)	3.22×10^{-2} (73%)

^a Sugars and sugar derivatives at a final concentration of 12.5 or 50 mM were pre-incubated with appropriate amount of enzyme in 50 mM sodium citrate buffer (pH 3.6) at 37°C for 10 min. PNP- β -D-galactopyranoside was then added to the mixture to assay the enzyme activity.

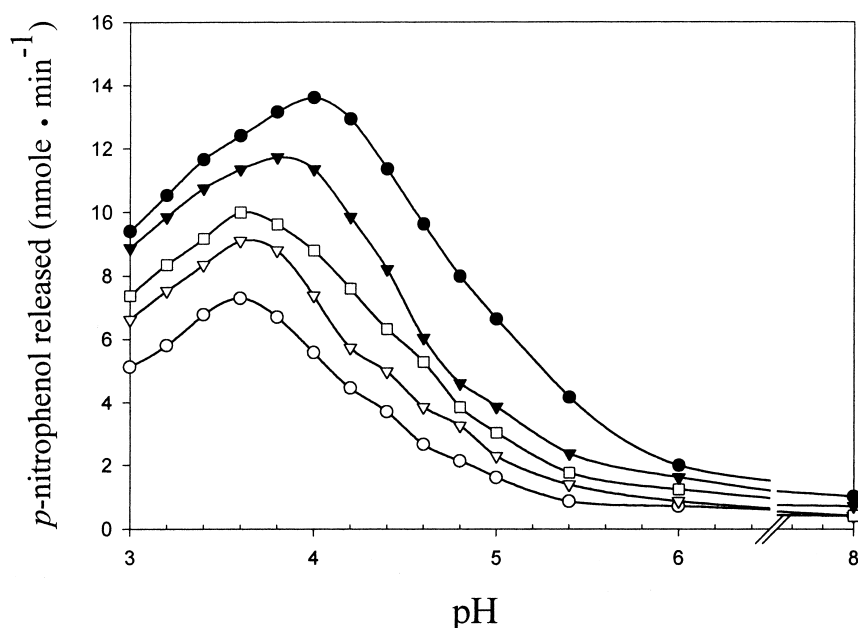


Fig. 4. Effect of pH on the purified β -galactosidases. McIlValine buffer (pH 3–6) and Na-phosphate buffer (pH 7–8) were used. The purified β -galactosidases I (○), II (▽), III (□), IV (●) and V (▼) were assayed at various pH by the method given in Experimental.

2.3. Immunochemical study

In order to investigate immunological relationships of the five isoforms of mung bean β -galactosidase, antisera were raised against the purified β -galactosidases II and III. Ouchterlony double immunodiffusion analysis indicated that all five β -galactosidase isoforms cross-reacted with anti β -galactosidase II antiserum (antiserum II) (Fig. 6A). The same result was obtained when anti β -galactosidase III antiserum (antiserum III), instead of antiserum II, was used (Fig. 6B). A spur was shown between the purified β -galactosidase IV and either β -galactosidase III or V. These results suggest that β -galactosidases I, II, III and V are immunologically similar to each other, while β -galactosidase IV shares partially identical antigenic determinants with the other four isoforms. In the purification of these β -galactosidases, isoform IV was the only β -galactosidase bound to the

DEAE-cellulose column equilibrated at a pH of 7.2 (Fig. 1A). The charge difference on the surface of isoform IV may in part account for its different immunological properties as compared with the other four isoforms.

Purified isoforms II and III were separated with two-dimensional electrophoresis using the method of O'Farrell (1975) and probed with antiserum II or III. The 2-D electrophoresis gel of isoform II is shown in Fig. 5. Similar results were obtained with isoform III. The results indicated that both isoforms II and III consisted of two groups of polypeptides with molecular masses of 38 and 48 kDa, the former containing at least six polypeptides and the latter four. The results are in accord with the finding that β -galactosidases II and III consist of two non-identical subunits (Fig. 3). The results also suggest that β -galactosidases in mung bean may be encoded by a multiple gene family. The β -galactosidases purified from cotyledons of mung bean

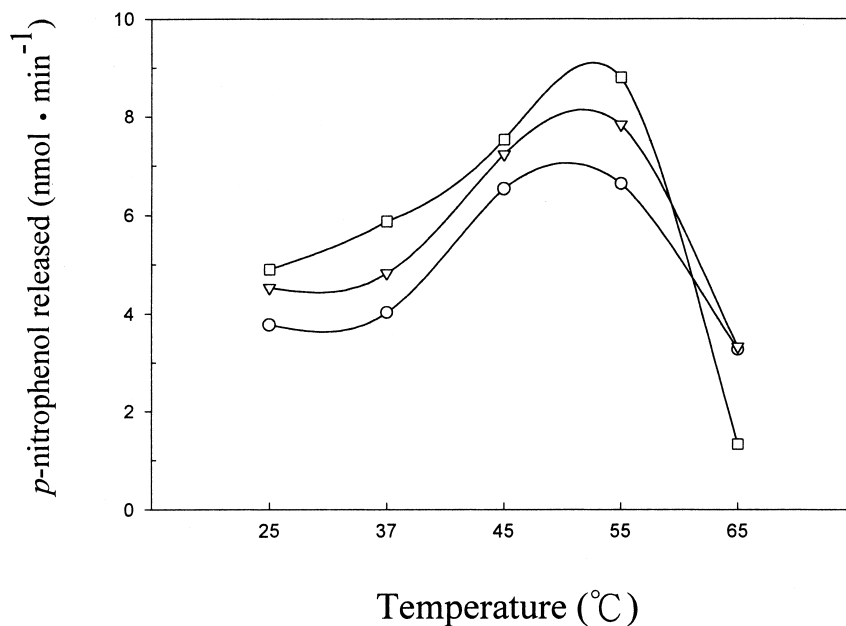


Fig. 5. Temperature optima of β -galactosidases I, II and III. Enzyme activities of the purified β -galactosidases I ($\circ$), II (∇) and III ($\square$) were assayed at various temperatures by the method given in the Experimental.

Table 3
Effect of metal ions, EDTA and SDS on β -galactosidase activities

	β -Galase activity (nkatal)		
	I	II	III
Control	4.43×10^{-2} (100%)	3.17×10^{-2} (100%)	4.81×10^{-2} (100%)
Fe^{2+}	3.76×10^{-2} (84.9%)	2.79×10^{-2} (87.9%)	4.19×10^{-2} (87.2%)
Ag^{2+}	3.67×10^{-3} (8.3%)	4.01×10^{-3} (12.6%)	2.34×10^{-3} (4.9%)
Hg^{2+}	2.17×10^{-3} (4.9%)	1.67×10^{-3} (5.3%)	4.34×10^{-3} (9%)
Ca^{2+}	3.76×10^{-2} (84.9%)	2.79×10^{-2} (87.9%)	4.19×10^{-2} (87.2%)
Cu^{2+}	3.39×10^{-2} (76.6%)	3.06×10^{-2} (96.3%)	4.04×10^{-2} (84%)
Mn^{2+}	3.49×10^{-2} (78.9%)	3.14×10^{-2} (98.9%)	4.79×10^{-2} (99.7%)
Mg^{2+}	3.74×10^{-2} (84.5%)	3.26×10^{-2} (102.6%)	4.73×10^{-2} (98.3%)
Zn^{2+}	4.46×10^{-2} (100.8%)	2.94×10^{-2} (92.6%)	4.86×10^{-2} (101%)
SDS	6.68×10^{-4} (1.5%)	8.35×10^{-4} (2.6%)	3.17×10^{-3} (6.6%)
EDTA	4.02×10^{-2} (90.9%)	3.06×10^{-2} (96.3%)	4.86×10^{-2} (101%)

Table 4
Enzyme activities of the purified β -galactosidases towards various *p*-nitrophenyl glycoside substrates

Substrate	β -Galase activity (nkatal)				
	I	II	III	IV	V
PNP- β -D-galactopyranoside	9.87×10^{-2} (100%)	1.22×10^{-1} (100%)	1.31×10^{-3} (100%)	1.65×10^{-1} (100%)	1.54×10^{-1} (100%)
PNP- α -L-arabinopyranoside	1.99×10^{-2} (20.1%)	4.01×10^{-2} (32.7%)	3.21×10^{-2} (24.6%)	2.07×10^{-2} (12.5%)	2.86×10^{-2} (18.5%)
PNP- β -D-fucopyranoside	2.72×10^{-2} (27.6%)	4.38×10^{-2} (35.7%)	4.07×10^{-2} (31.2%)	2.45×10^{-2} (14.8%)	4.04×10^{-2} (26.2%)
PNP- α -D-mannopyranoside	6.68×10^{-4} (0.70%)	1.67×10^{-3} (1.4%)	5.01×10^{-4} (0.4%)	8.18×10^{-2} (49.4%)	8.35×10^{-4} (0.5%)
PNP- β -D-mannopyranoside	6.68×10^{-4} (0.7%)	1.0×10^{-3} (0.8%)	8.35×10^{-4} (0.6%)	7.01×10^{-3} (4.2%)	8.35×10^{-4} (0.5%)
PNP- α -D-glucopyranoside	1.12×10^{-2} (11.3%)	6.01×10^{-3} (4.9%)	2.67×10^{-3} (2.0%)	1.34×10^{-2} (8.1%)	1.05×10^{-2} (6.8%)
PNP- β -D-glucopyranoside	1.34×10^{-3} (1.4%)	1.17×10^{-3} (1.0%)	4.68×10^{-3} (3.6%)	7.35×10^{-3} (4.4%)	2.51×10^{-3} (1.6%)
PNP- <i>N</i> -acetyl- β -D-glucosaminide	2.0×10^{-3} (2.0%)	5.85×10^{-3} (4.8%)	1.84×10^{-3} (1.4%)	1.72×10^{-2} (10.4%)	2.17×10^{-3} (1.4%)
PNP- β -D-xylopyranoside	1.5×10^{-3} (1.5%)	2.67×10^{-3} (2.2%)	2.17×10^{-3} (1.7%)	7.85×10^{-3} (4.7%)	3.51×10^{-3} (2.3%)
PNP- α -D-galactopyranoside	2.0×10^{-3} (2.0%)	3.67×10^{-3} (3.0%)	1.0×10^{-3} (0.8%)	6.51×10^{-3} (3.9%)	2.17×10^{-3} (1.4%)
PNP- α -L-fucopyranoside	1.34×10^{-3} (1.4%)	1.34×10^{-3} (1.1%)	1.5×10^{-3} (1.2%)	7.68×10^{-3} (4.6%)	2.51×10^{-3} (1.6%)
PNP- β -D-glucuronide	4.68×10^{-3} (4.7%)	4.68×10^{-3} (3.8%)	1.0×10^{-3} (0.8%)	2.59×10^{-3} (15.6%)	9.85×10^{-3} (64.0%)

Table 5

Activity of the purified β -galactosidases II and III toward cell wall polysaccharides of mung bean seeds^a

Substrate	Enzyme activity (mkatals/kg)		
	Cell-free extract	Galase II	Galase III
Hot water soluble pectin	23.5 $\pm$ 0.67	7114.2 $\pm$ 2120.9	4024.7 $\pm$ 317.3
Oxalate soluble pectin	18.2 $\pm$ 1.0	7732.1 $\pm$ 3406.8	1553.1 $\pm$ 33.4
4% KOH soluble hemicellulose	71.0 $\pm$ 9.0	16,082.1 $\pm$ 3089.5	14,228.4 $\pm$ 935.2
24% KOH soluble hemicellulose	74.5 $\pm$ 9.0	24,114.8 $\pm$ 2872.4	22,261.1 $\pm$ 2471.6
Xyloglucan	18.2 $\pm$ 0.3	12,057.4 $\pm$ 1553.1	12,675.3 $\pm$ 935.2
Lactose	103.7 $\pm$ 9.0	92,785.2 $\pm$ 8032.7	159,585.2 $\pm$ 3089.5

^a Experimental conditions are given in Experimental. Enzyme activity is expressed as μ mol of D-galactose liberated per min per mg protein. The data are expressed as mean $\pm$ S.D. from three determinations.

were previously shown to be homodimers (Kundu et al., 1990). A question arises whether the 38 kDa subunit is the degradation product of the 48 kDa subunit. To clarify this point, cell-free extracts of the 5-day-old germinated mung bean seeds, the supernatant fraction of acid (pH 5.0) precipitated fraction and 0–70% saturated ammonium sulfate of the cell-free extract were separated by SDS-PAGE and subjected to Western blot analysis. The results showed that all three samples exhibited two polypeptides with molecular masses corresponding to 38 and 48 kDa, which cross-reacted with antiserum II (data not shown).

2.4. Degradation of cell wall polysaccharides by β -galactosidases II and III

β -Galactosidase activity was determined during the course of mung bean germination. The enzyme activity increased from day 1 after imbibition in the dark, approached a high level around day 5 and remained about the same until day 9 (data not shown). To understand whether β -galactosidases II and III potentially participate in the mobilization of cell wall polysaccharides, the two purified enzymes and cell-free extract were examined for their hydrolytic activity towards cell wall polysaccharides isolated from mung bean seeds.

The results indicated that both β -galactosidases II and III were capable of releasing monomeric galactose from the cell wall polysaccharides in vitro (Table 5). Rates of galactose liberation by β -galactosidase II were high with 4 and 24% KOH soluble hemicellulose as substrates, and moderate with xyloglucan, hot water soluble and oxalate soluble pectins. While exhibiting high activity towards 24 and 4% KOH soluble hemicellulose and xyloglucan similar to β -galactosidase II, β -galactosidase III showed relatively lower activity on hot water soluble and oxalate soluble pectin. The cell-free extract exhibited higher activity on 24 and 4% KOH soluble hemicellulose than the other cell wall polysaccharides. The release of monomeric D-galactose from 4 or 24% KOH soluble hemicellulose by purified β -galactosidase II amounted to 2.5–2.6 μ g after prolonged incubation for

more than 6 h. This is approximately equivalent to 3.3–3.5% of total polysaccharides (75 μ g) in the reaction mixture. Purified β -galactosidase III gave similar results (data not shown). Both enzymes also have high activity against lactose (Table 5). Thus, purified β -galactosidases II and III were capable of efficiently releasing D-galactose residue from the hemicellulose fraction in vitro.

3. Discussion

In this report, we have isolated and characterized five isoforms of β -galactosidase from etiolated mung bean seedlings. β -Galactosidases II and III were purified to electrophoretic homogeneity, while I, IV and V were partially purified. Optimal pH (3.6–4.0) and temperature (50–53°C), isoelectric points (7.3–7.7) and molecular mass (87, 87, 87, 73, 45 kDa) of these enzymes were comparable with those of other plant β -galactosidases previously described (Li et al., 1975; Biswas, 1987; Edwards et al., 1988; Kundu et al., 1990), with the exception that the β -galactosidase isolated from mature seeds of mung bean had molecular masses of 162 kDa (Dey, 1984).

The results of SDS-PAGE (Fig. 3) and two-dimensional electrophoresis (Fig. 5) indicate that β -galactosidases II and III are composed of two non-identical subunits (38 and 48 kDa). Fig. 7 also shows that the 38 and 48 kDa

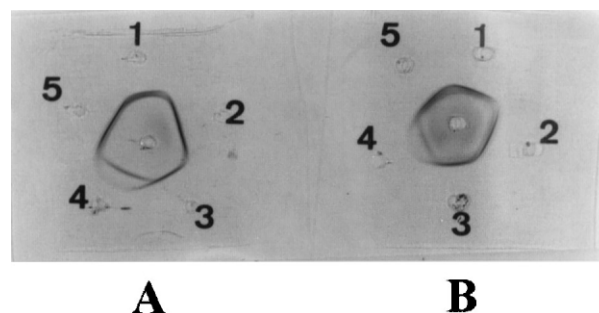


Fig. 6. Double immunodiffusion analysis of β -galactosidases I–V. Panel A: centre well, anti β -galactosidase II antiserum; wells 1–5, the purified β -galactosidases I–V, respectively. Panel B: centre well, anti β -galactosidase III antiserum; wells 1–5, the same as panel A.

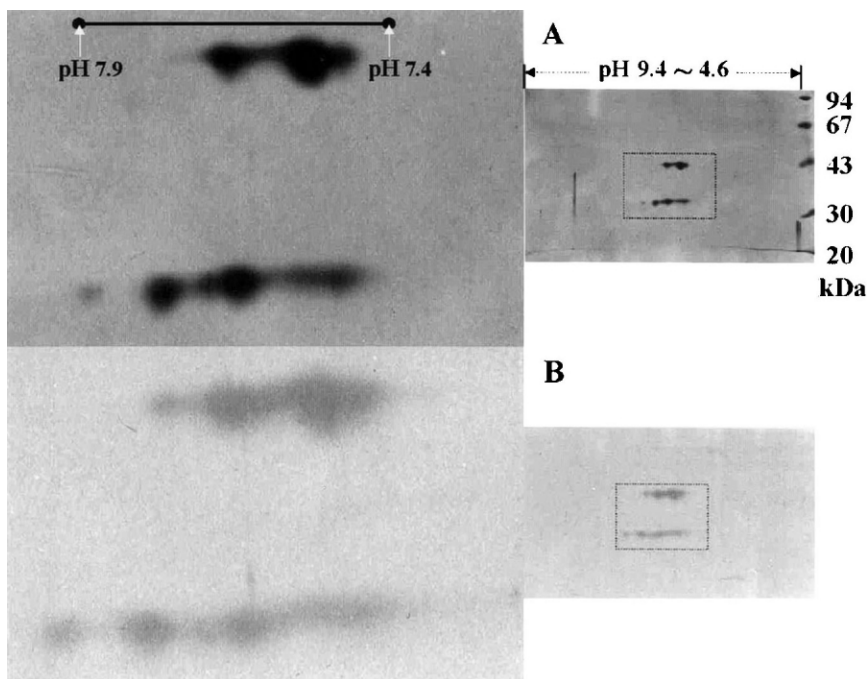


Fig. 7. Two-dimensional gel electrophoresis of β -galactosidase II. A, silver staining; B, Western blotting. The areas marked with dotted lines are enlarged at left.

subunits were further separated into six and four polypeptides, respectively, which differed only in isoelectric point values. These results, together with the previous reports (Simos et al., 1989; Giannakouros et al., 1991), suggest that the multiplicity of plant β -galactosidases was at least in part due to different isoelectric point values.

It has been suggested that β -galactosidases play a vital role in plant growth and development, the β -galactosidases described here, which are capable of efficiently hydrolysing the hemicellulose fractions of mung bean seeds (Table 5), are apparently similar to a number of broad specificity β -galactosidases found in cotyledons and germinated seeds. The mung bean β -galactosidases are developmentally regulated as suggested by the finding that the activity of the β -galactosidases rapidly increased to a high level between day 4 and day 6 of germination. Our results, together with the previous findings (Edwards et al., 1985; Konno and Tsumuki, 1993), suggest that a number of β -galactosidases differing in substrate specificities are synthesized or activated to mobilize, or modify cell wall components during seed germination and plant growth, and that the temporal expression of the β -galactosidase genes are subjected to developmental regulation.

4. Experimental

4.1. Plant material and enzyme source

Mung bean seeds (*Vigna radiata* VC 3890 Sel No. 5) were submerged in distilled water at 25°C for 2 h and

surface-sterilized with 0.5% hydrogen peroxide, thoroughly washed with H₂O and germinated in the dark at 28°C. Five-day-old germinated seeds were thoroughly washed with ice-cold H₂O and homogenized with Polytron (Kinematica) in 50 mM Tris-HCl (pH 7.2) containing 10 mM 2-mercaptoethanol and 1 mM EDTA. Unless otherwise indicated, all steps and the purification procedure were conducted at 5°C. The resultant slurry was squeezed through three layers of cheesecloth, phenyl methylsulphonyl fluoride (PMSF) was added at a final concentration of 1 mM, and centrifuged at 12,800×g for 30 min. The supernatant was dialysed against the same buffer solution containing 1 mM PMSF. The dialysed enzyme solution was referred to as cell-free extract.

4.2. Enzyme purification

The overall procedure for the purification of mung bean β -galactosidases is shown in Scheme 1. The cell-free extract was adjusted to a pH of 5.0 to precipitate large amounts of contaminating proteins, while retaining most of the β -galactosidase activity in the supernatant. Solid ammonium sulfate was added to the supernatant to obtain a 0–75% saturated ammonium sulfate fraction, which contained most of the β -galactosidase activity. The 0–75% saturated ammonium sulfate fraction was applied to a DE-52 column. Both DEAE-cellulose adsorbed (DEAE-A) and non-adsorbed (DEAE-NA) fractions were shown to contain β -galactosidase activity. These two fractions, therefore, were further subjected to Con A affinity column chromatography. After

Con A chromatography, almost all of the β -galactosidase activity in the DEAE-A was recovered in the Con A bound fraction. The β -galactosidase eluted from this column is named β -galactosidase IV. On the other hand, β -galactosidase activity in the DEAE-NA was separated into Con A bound and Con A unbound fractions. The former was eluted as a single protein peak that is called β -galactosidase. The latter was further separated into three activity peaks by chromatofocusing. These three isoforms were called β -galactosidases I, II and III, based on the order of their emergence from the column. β -Galactosidases II and III were purified to apparent homogeneity by repeated chromatofocusing on the same column. The experimental conditions for each purification step are described as follows.

The cell-free extract was adjusted to pH 5.0 with 3.5 M acetic acid, allowed to stand at 5°C for 2 h, and the precipitates were removed by centrifugation. Solid ammonium sulfate was added to the supernatant until 75% saturation was reached. The sample obtained from 75% saturated ammonium sulfate precipitation was redissolved in a small volume of 10 mM Tris-HCl (pH 7.2) containing 10 mM 2-mercaptoethanol (Buffer A), dialysed overnight against the same buffer solution and applied to a DEAE-cellulose (DE-52, Whatman) column (4.4×15 cm) previously equilibrated with Buffer A. The column was washed with 10 volume of Buffer A and eluted with a linear gradient (2 l total volume) of 0–0.5 M NaCl in 10 mM Tris-HCl (pH 7.2) containing 10 mM 2-mercaptoethanol. Fractions of 8 ml each were collected at a flow rate of 2 ml/min. Active fractions were pooled and concentrated using an Amicon ultrafiltration device (Ya 1000 membrane). This sample was called the DEAE-cellulose adsorbed fraction (DEAE-A). The column washing solution that still had β -galactosidase activity was concentrated and referred to as the DEAE-cellulose non-adsorbed fraction (DEAE-NA).

For purification of the DEAE-NA, Con A-Sepharose column (2.6×10 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 7.2) containing 0.3 M NaCl, 1 mM CaCl_2 and 1 mM MnCl_2 (Con A affinity buffer). The DEAE-NA were pooled, concentrated with the Amicon ultrafiltration device, and dialysed against the Con A affinity buffer. The non-dialysable solution was placed on the Con A-Sepharose column. After washing with the Con A affinity buffer, the column was eluted with a linear gradient (250 ml total volume) of 0–0.2 M α -methylmannoside in 20 mM Tris-HCl (pH 7.2) containing 0.3 M NaCl. Fractions of 6 ml were collected. It was found that the effluent and washing showed β -galactosidase activity that had not bound to Con A-Sepharose under the chromatographic conditions used. The fractions exhibiting β -galactosidase activity in the effluent and washing were pooled and designated as a Con A-Sepharose non-adsorbed fraction that was shown to contain β -galactosidases I, II and III. β -

Galactosidase V bound on Con A-Sepharose was eluted from the column.

The DEAE-A was also purified with the Con A-Sepharose column. The procedure was essentially as described above except that the column was equilibrated with 20 mM Tris-HCl (pH 7.2) containing 1 M NaCl. There was no detectable β -galactosidase activity in the washing solution and effluent. The protein peak that coincidentally came off the column with β -galactosidase activity after elution was referred to as β -galactosidase IV.

The Con A-Sepharose non-adsorbed fraction containing β -galactosidases I, II and III was concentrated to 3–4 ml and purified by chromatofocusing according to the instruction manual of Pharmacia. Polybuffer Exchanger PBE 94 (Pharmacia) column (1.0×15 cm) was equilibrated with 25 mM Tris-HCl (pH 8.3). Polybuffer 96 (Pharmacia) was diluted 13-fold with deionized water and adjusted to pH 6.8 with 6 M HCl (eluent). Eighteen milliliters of the eluent was introduced to the column to create a pH gradient from 6.8 (top) to 8.3 (bottom). The concentrated enzyme solution was dialysed against the eluent and placed on the column. β -Galactosidases I, II and III were eluted in this order from the column with 90 ml of the eluent. In order to obtain homogeneous enzyme, chromatofocusing was conducted twice more for each β -galactosidase peak.

4.3. Enzyme assays

β -Galactosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl (PNP)- β -D-galactopyranoside according to the method of Agrawal and Bahl (1968). The reaction mixture contained, in a final volume of 0.5 ml, 50 mM sodium citrate buffer (pH 3.6), 5 mM substrate, and varying amounts of enzyme. The reactions were carried out at 37°C for 10 min. Liberated *p*-nitrophenol was measured spectrophotometrically at 420 nm after stopping the reaction with the addition of 0.7 ml of 0.2 M sodium carbonate. Absorbance (A_{420}) was proportional to the amount of enzyme in the reaction mixture under the assay conditions. The absorbance, A_{420} , was also linear with time within the amounts of enzyme used. One unit of β -galactosidase is defined as the amount that hydrolyses 1 μmol of PNP- β -D-galactopyranoside per min at pH 3.6 and 37°C. One katal is defined as the amount of β -galactosidase that hydrolyses 1 mol of PNP- β -D-galactopyranoside per second under the same condition. Other glycosidase activities were determined by the use of the respective PNP-glycoside. The reaction conditions were essentially the same as those described above.

Release of D-galactose from cell wall polysaccharides was determined using β -D-galactose dehydrogenase (Sigma) (Kurz and Wallenfels, 1974). Protein concentration was determined by the methods of Bradford (1976) using bovine serum albumin as a standard.

4.4. Preparation of cell walls

Cell walls of mung bean seeds were prepared according to the method described by Jarvis et al. (1981). Mung bean seeds were homogenized in ice-cold H₂O (5 l/kg) containing 5 g/l sodium deoxycholate and 10 g/l *n*-octanol in a Waring blender. The homogenate was filtered through three layers of cheesecloth and washed on a 120-mesh sieve with ice-cold H₂O (4 l/kg). The crude cell walls were suspended in distilled water (650 ml/kg), homogenized for 2 min with Polytron (Kinematica) and washed with distilled water (8 l/kg). The homogenization and sieving was repeated until no more starch grains were released. Lipids were removed by stirring with 800 ml/kg chloroform-methanol (1:1 v/v), and the cell walls removed by filtration and freeze-dried.

4.5. Cell wall fractionation

The dry cell walls were sequentially extracted with hot water, 0.25% ammonium oxalate-oxalic acid, 4% KOH and 24% KOH by the method of Kato and Matsuda (1976). Xyloglucan was prepared from mung bean seeds according to the procedure previously described (Edwards et al., 1985).

4.6. Degradation of cell wall polysaccharides

Each of the two pectic fractions (hot water and oxalate soluble fractions), the two hemicellulosic fractions (4% KOH and 24% KOH soluble fractions), and xyloglucan obtained above was dissolved in 0.2 M sodium citrate buffer (pH 3.6) at a final concentration of 1.5 mg/ml and used as substrate for purified β -galactosidases II and III, and the cell-free extract. The reaction mixture contained, in a final volume of 0.83 ml, 75 μ g substrate and an appropriate amount of enzyme or cell-free extract. The reaction mixture was incubated at 37°C and aliquots were taken at various intervals for determination of D-galactose with galactose dehydrogenase (Kurz and Wallenfels, 1974).

4.7. Preparation of antisera

Purified β -galactosidases II and III, which are homogeneous on disc polyacrylamide gel electrophoresis, were used as the antigens. Two rabbits were injected subcutaneously four times with 150–180 μ g each of β -galactosidases II and III emulsified with Freund's complete adjuvant. Intervals between injections were 2–3 weeks. The rabbits were killed one month after the final injection. The blood clots were centrifuged and the clear serum lyophilized and stored at –70°C.

4.8. Immunological methods

Ouchterlony double immunodiffusion was performed at room temperature in a 2% agarose gel as previously

described (Ouchterlony and Nilsson 1973). Western blot analysis was performed according to the method of Burnette (1981) with some modification.

4.9. Electrophoresis

Purified β -galactosidases were analysed by electrophoresis in non-denaturing conditions using 4% acrylamide stacking gel and 5–20% acrylamide gradient separating gel (Hames, 1981), SDS-PAGE (Laemmli, 1970) and two-dimensional electrophoresis (O'Farrell, 1975). Proteins were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 or silver nitrate (Blum et al., 1987). Activity staining of β -galactosidase was performed according to the method described by Spielman and Mowshowitz (1982) using 6-bromo-2-naphthyl- β -D-galactoside (Sigma) as a substrate.

4.10. Enzyme characterization

The molecular mass of the native β -galactosidases were determined by gel filtration on a Sephacryl S-200 column (1.6×90 cm). The column was calibrated with Blue Dextran and standard proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, albumin, aldolase, catalase, ferritin, and thyroglobulin) (Sigma). The molecular mass of the denatured enzymes was estimated by SDS-PAGE. The pH optima of the purified enzymes against PNP- β -D-galactopyranoside was determined in McIlValine buffer covering a pH range of 3–6 and Na-phosphate buffer covering a pH range of 7–8. The isoelectric point of the purified enzyme was estimated from the elution profile of chromatofocusing. In the inhibition study of β -galactosidase activity by sugars, sugar derivatives, metallic ions and SDS, β -galactosidase was pre-incubated for 10 min at 37°C with each compound or metallic ion at a concentration of 1 mM (for metallic ion and SDS) or 12.5 mM (for sugar and sugar derivatives), then assayed as normal. In order to understand the mechanism of inhibition of β -galactosidase, either by D-galactono-(1→4)-lactone or by D-galactose, β -galactosidase activity was assayed in the presence of varying concentrations of the inhibitor. The data were analysed with a double reciprocal plot.

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