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ARTICLE

A β-Galactosidase from Pea Seeds (*Ps*BGAL): Purification, Stabilization, Catalytic Energetics, Conformational Heterogeneity, and Its Significance

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A basic glycosylated β-galactosidase (*Ps*BGAL) has been purified from pea seeds by 910-fold with a specific activity of 77.33 μmoL min^{-1} mg^{-1} protein. The purified enzyme is an electrophoretically homogeneous protein consisting of a single protein band with an apparent M_r of 55 kDa, while the deglycosylated enzyme has a M_r of 54.2 kDa on SDS−PAGE under reducing conditions. According to MALDI-TOF measurements of the 55 kDa band, the enzyme showed a homology with BGAL from other sources present in the SWISS-PROT database, while it showed no resemblance to any lectin. The *N*-terminal sequence of *Ps*BGAL was determined as TIECK and showed a resemblance to BGAL from *Arabidopsis thaliana* (Q93Z24). The enzyme showed an unique property of multiple banding patterns on SDS−PAGE at 20 mA current, with tryptic digests of all bands having similar *m*/*z* values (using MALDI-TOF) while it showed only a single band at 10 mA current. *Ps*BGAL is effectively compartmentalized during seed maturation inside vacuoles (pH ∼ 5). The enzyme is capable of hydrolyzing pea seed xyloglucan, and it may be involved in modifying the cell wall architecture during seedling growth and development. The enzyme has a protonated carboxyl group at its active site as observed by ionization constant, thermodynamics, and chemical modification studies.

KEYWORDS: β-Galactosidase; MALDI-TOF; *Pisum sativum*; conformational heterogeneity; germination

INTRODUCTION

β-Galactosidase (BGAL) is widely distributed among microorganisms, plants, and animals. Primary structure and hydrophobic cluster analyses have shown that BGAL can be classified into four glycosyl hydrolase families 1, 2, 35, and 42 (*1*). Recently, gene expression analysis has revealed association of plant BGAL with senescence and abscission (*2*, *3*). A marked disparity exists between the relative expression levels of mRNAs and those of their corresponding proteins (*4*). It is insufficient to draw a link between transcript levels and a cell's protein complement alone (*5*). In order to draw a firm substantiation, thorough analyses regarding gene expression of BGAL and their corresponding protein complement have to be done.

Seed imbibition involves absorption of adequate quantity of water leading to softening of seed coat and allowing oxygen diffusion for seed respiration. Carbohydrate composition of cell wall polysaccharides (CWP) of seed coat and cotyledons which include celluloses, hemicelluloses, and pectin are the most crucial regulating factor responsible for the rate of water absorption. In the case of soybean seeds, variation in carbohydrate composition of CWP present in cotyledons was genetic, while it was environmental in the case of the seed coat (*6*). Osmotically stressed and dehydrated plant cells have a considerable increase in galactose and a decrease in glucose substituents of xyloglucans present in the cell wall (*7*, *8*). Mobilizations of pectins and xyloglucans during seed germination have been well documented by various authors (*9*−*12*). The presence of auxin is directly related with xyloglucan degradation during germination, but very little is known about the role of auxin during seed imbibition (*13*, *14*).

Lysosomal BGALs isolated from various animal tissues have been reported to form large functional multimeric aggregates through interaction with a glycoprotein known as protective protein under in vivo conditions. These interactions facilitate correct intracellular routing and lysosomal localization of BGAL (*15*). Dey and Pridham (*16*) have reported aggregation of α-galactosidase in mature seeds of *Vicia faba*, due to lectin-like activity of monomeric forms. This helps in effective compartmentalization of enzyme during seed maturation. According to cDNA analyses, plant BGALs have been characterized into two broad classes on the basis of the presence of C-terminal lectin domain. Biswas et al. (*17*) have isolated a complex containing BGAL and lectin from kidney beans. It is a matter of interest to study intensely about the association of lectin with enzyme.

Multiplicity of plant BGAL has been discussed in various reports (*18*−*25*). According to these reports, multiplicity was due to intrinsic proteolysis caused either accidentally during isolation, due to hydrolysis by SDS while doing SDS−PAGE, heterogeneity in glycosylation, or due to products of multigene families in several species. Products of multigene family have varied sites

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of localization inside the cell and further possess different biochemical properties including molecular mass, isoelectric point, etc. (*25*). It would be interesting to speculate on the rationale behind BGAL multiplicity during its isolation.

Here we present purification of BGAL to homogeneity from seeds of *Pisum sativum* (*Ps*BGAL). The active site studies further have revealed details about the mechanism employed by the enzyme during catalysis. The present study also provides some insight into the characteristics of the observed heterogeneity of the enzyme and its association with lectin-like activity.

MATERIALS AND METHODS

Plant Materials and Chemicals. Dry seeds of pea (*Pisum sativum* var. *arvense* AP-3) with moisture content of 8 and 12% were obtained from the Indian Institute of Vegetable Research (IIVR), Varanasi, India. For extraction of BGAL from these pea seeds, they were washed thoroughly with deionized water and subsequently surface sterilized using 0.1% $HgCl_2$. Seed germination was carried out under dark conditions at 25 °C over moist filter paper on a moist sand bed following seed imbibitions for 24 h in extraction buffer (25 mM sodium phosphate buffer, pH 6.8) at 4 °C. *Ps*BGAL was extracted from 100 g of seeds with 12% moisture content which were imbibed in extraction buffer. All the chemicals for buffers and other reagents were of analytical grade or electrophoresis grade. Unless stated, all the chemicals were purchased from Sigma (St. Louis, MO, USA). Milli Q quality water with a resistance of higher than 18 MΩ was used throughout the experiments.

Cell Wall Isolation and Fractionation. Pea seed cell walls were isolated and fractionated according to the slightly modified method of Nishitani and Masuda (*26*). Centrifugation during various steps of cell wall isolation was done at 8420*g* for 5 min, unless stated otherwise. Twenty-five grams of pea seeds were boiled in an open flask containing 500 mL of methanol for 15 min, and immediately cooled at room temperature and homogenized in 50 mL of ice cold Milli Q water. The homogenate was centrifuged at 30 °C and supernatant was discarded. The pellet was washed five times with ice cold water and dispersed in 1 M NaCl for 1 h at room temperature to remove wall bound proteins. After dispersion, it was centrifuged at 4 °C and pellet so obtained was washed successively with ice cold water, acetone, and chloroform−methanol mixture (1:1; v/v). This was done twice, later the pellet was air-dried at room temperature. The dried pellet was suspended in 3 mL of dimethyl sulfoxide (DMSO) and incubated for 24 h at room temperature. Subsequently, the slurry was centrifuged at 30 °C and the pellet was suspended in 1 mL of 20 mM ammonium oxalate-oxalic acid buffer (pH 4) and kept at 70 °C for 1 h to remove pectic polysaccharides and then centrifuged. This step solubilizes pectic polysaccharides which fractionated in the supernatant while xyloglucans remained in the pellet. The supernatant was further precipitated using ethanol (2 vol) and subsequently centrifuged at 4 °C. Finally the pellet containing only pectic polysaccharides was dissolved in 500 μL of 50 mM acetate buffer, pH 4.

Extraction of Xyloglucan. The pellet containing xyloglucans obtained from the above step was used further to fractionate it into structural and reserve xyloglucans by dissolving overnight at room temperature in 1 mL of 4% and 24% KOH, respectively, and then centrifuged at 30 °C. Each alkali extract was further acidified with 1 mL of 5% acetic acid and 1 mL of 33% acetic acid, respectively, and subsequently centrifuged at 30 °C. Supernatant was collected and precipitated using ethanol (2 vol), centrifuged at 30 °C, and the pellet was dissolved in 500 μL of acetate buffer (50 mM), pH 4.

Enzyme Assays. The activities against synthetic substrates and lactose were assayed similarly as given by Dwevedi and Kayastha (*27*). In the case of synthetic substrates, one unit of BGAL activity was defined as the amount of enzyme required for releasing 1 μmoL of *o*-, *m*-, or *p*-nitrophenol produced min^{-1} mL^{-1} at 37 °C (ε: *o*-nitrophenol: 4.05×10^3 M^{-1} cm^{-1}, *p*-nitrophenol: 1.53×10^3 M^{-1} cm^{-1}, *m*-nitrophenol: 1.56×10^3 M^{-1} cm^{-1}). One unit of enzyme activity in the case of lactose was 1 μmoL of glucose released min^{-1} mL^{-1} at 37 °C (also defined as 1 μmoL of galactose released min^{-1} mL^{-1} at 37 °C, as glucose is equivalent to galactose according to stoichiometry). Activities toward natural substrates were determined by incubating 50 μL of substrates (2 mg mL^{-1}) (hemicellulosic polysaccharides, low and high molecular weight xyloglucans) prepared in sodium acetate buffer (50 mM, pH 4) and enzyme with a final concentration of 25 μg mL^{-1} for 24 h at 37 °C. The liberated sugar (galactose, as BGAL acts on galactosyl residues attached to oligo- and polysaccharides with β-linkages) was determined reductometrically using dinitrosalicylic acid. The control in each case was prepared by excluding enzyme from the reaction mixture. One unit of enzyme is defined as 1 μmoL of galactose released min^{-1} mL^{-1} at 37 °C. In the case of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and lactose, the amount of *o*-nitrophenol (ONP) and glucose released were equivalent to galactose released according to stoichiometry (ONPG → ONP + galactose; lactose → glucose + galactose). Therefore, % hydrolysis of various substrates including natural and synthetic substrates was calculated using following equation:

$$\frac{\mu\text{mol of galactose released min}^{-1}\text{ mL}^{-1}\text{ mg}^{-1}\text{ of }Ps\text{BGAL at 37 °C towards various substrates}}{\mu\text{mol of galactose released min}^{-1}\text{ mL}^{-1}\text{ mg}^{-1}\text{ of }Ps\text{BGAL at 37 °C towards ONPG}} \times 100$$

Enzyme Extraction and Purification. All purification steps were carried out at 4 °C and centrifugation was performed at 8420*g* for 20 min unless stated otherwise. Buffers used in each step included additives: 1 mM DTT, 0.02 mM EDTA, and 1 mM PMSF. In a typical batch of purification, imbibed pea seeds were homogenized using laboratory blender in 200 mL chilled extraction buffer (25 mM sodium phosphate buffer, pH 6.8), then squeezed through two layers of muslin cloth and the extract was centrifuged. The pellet containing cell debris was discarded and supernatant was collected. The pH of supernatant was lowered to 3.5, by dropwise addition of chilled 0.2 M HCl with continuous stirring and incubated for 24 h at 4 °C, leading to a thick, curdy precipitate of inactive proteins, removed by centrifugation. Proteins precipitating in the range 40−55% saturation of ammonium sulfate were collected by centrifugation. Pellet obtained was dissolved and dialyzed in 25 mM sodium phosphate buffer, pH 6.1. The enzyme so obtained was passed through an assembly containing nitrocellulose membrane (0.45 μm) and loaded onto Octyl Sepharose CL-4B column (50×5 cm), which was equilibrated with 200 mL (5× column volume) of 25 mM sodium phosphate buffer, pH 6.1 containing 2 M $(NH_4)_2SO_4$. The bound proteins were eluted with 25 mM sodium phosphate buffer, pH 6.1 containing 1 M $(NH_4)_2SO_4$ with a flow rate of 0.3 mL min^{-1}; fractions (5 mL) were collected and those having high specific activity were pooled, concentrated, and dialyzed against Tris-HCl (50 mM, pH 8) for 2−3 h at 4 °C. Enzyme thus obtained was applied to a DEAE-Sephacel column (12×2 cm), which was equilibrated with 100 mL of Tris-HCl buffer (50 mM, pH 8.0). The column was washed with 1× column volume with equilibration buffer and then the enzyme containing fractions of 1 mL were collected with a flow rate of 0.3 mL min^{-1} using same buffer. The active fractions with high specific activity were pooled and concentrated. Enzyme so obtained was dialyzed against 50 mM acetate buffer (pH 5) and concentrated.

Native Gel Electrophoresis and Activity Staining. Purified *Ps*BGAL (20 μg) was electrophoresed through 0.75 mm thick gel (2 cm, 5% stacking gel, pH 6.8, and 13 cm, 8% resolving gel, pH 8.8) using the alkaline gel system of Davis (*28*) and Ornstein (*29*). It was performed at a constant current supply of 5 mA until samples were stacked and then the current was increased to 10 mA as described by Goldenberg (*30*). After electrophoresis, the gel was equilibrated with 50 mM Na-acetate buffer (pH 4). Staining using substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was performed according to Biswas et al. (*17*).

Isoelectric Focusing. Isoelectric focusing was performed using the method described earlier (*31*) with a slight modification in the pH range 5−9. The gels were subjected to a prerun before sample loading at a constant current of 1 mA per tube gel for 2 h to develop the pH gradient. Protein sample (100 μg) containing 10% (v/v) ampholine and 25% glycerol was prepared (stock), and 25 μg of protein was loaded on each tube gel and electrophoresed at a constant current of 2 mA per gel tube for 4 h. After the run, gels were fixed and stained using Coomassie brilliant blue R-250.

Determination of M_r. *Native Molecular Mass*. It was determined using Superdex S-200 HR 10/30 column on AKTA fast protein liquid chromatography (Amersham Biosciences). Column was calibrated with markers of molecular weight: 440 kDa, 206 kDa, 66 kDa, and

Table 1. Purification of *Ps*BGAL from Seeds of *Pisum sativum*[a]

steps	total activity[b] (U)	total protein (mg)	specific activity (U mg^{-1})	purification[c] (fold)	recovery[c] (%)
crude extract	380.95	4481.76	0.085	1	100
acid step	289.58	603.29	0.480	6	76.02
ammonium sulfate (40−55%)	188.05	143.55	1.31	15	49.36
octyl Sepharose-4B	118.07	3.05	38.71	455	30.99
DEAE-Sephacel	74.78	0.967	77.33	910	19.63

[a] 100 g seeds of *Pisum sativum* with 12% moisture content were imbibed in extraction buffer (25 mM sodium phosphate buffer, pH 6.8) for 24 h under dark conditions at 4 °C. [b] BGAL activity was determined using ONPG, and protein estimation was done using the Bradford method. [c] Recovery at a particular purification step was expressed as a percentage of initial activity, and purification factors were calculated on the basis of specific activities.

43 kDa (Amersham Biosciences) before use. 500 μL of purified *Ps*BGAL (200 μg mL^{-1}) was loaded on the column equilibrated with 50 mM Na-acetate buffer (pH 5).

Denatured Molecular Mass. An apparent subunit molecular mass *Ps*BGAL was calculated by linear regression analysis from the data obtained by SDS−PAGE using 12% gel (*32*) at a constant current supply of 10 mA using markers: 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 14.2 kDa (Sigma). Purified *Ps*BGAL (10 μg) was prepared in 6× loading buffer with the final concentration on loading of 1× and heated for 3 min at 85 °C and centrifuged at 8420*g* for 2 min, 4 °C. 6× loading buffer contains 0.3 M Tris-HCl, pH 6.8, 0.01% bromophenol, 10% glycerol, 6% SDS, 5% DTT, and 0.6% (v/v) protease inhibitor cocktail (Calbiochem, Canada).

Analytical Methods. Protein was estimated using Bradford reagent calibrated with crystalline bovine serum albumin (*33*). Lectin activity of *Ps*BGAL was determined using trypsinized sheep erythrocytes according to the method of Lis et al. (*34*). Reducing sugar (galactose) was estimated using dinitrosalicylic acid (*35*). Carbohydrate content (%) was estimated using phenol/H_2SO_4 method (*36*), whereas tryptophan and tyrosine content (%) were calculated using the protocol of Edelhoch (*37*) with a protein concentration of 10 μg mL^{-1}, in each case. *Ps*BGAL deglycosylation was performed using trifluoromethanesulphonicacid (TFMS) (*38*). Purified single band of *Ps*BGAL on SDS−PAGE (12%) was identified using *N*-terminal sequencing and mass spectrometry. The band was blotted onto a PVDF membrane and submitted for *N*-terminal sequencing. For mass spectrometry, the band was in-gel digested using trypsin as given by Schleuder (*39*) and submitted for MALDI-TOF for its identification.

Database Searching. There is no report for the amino acid sequence of BGAL from *Pisum sativum* in an available protein database. Therefore, the ratio of *m*/*z* values obtained from MALDI-TOF spectra corresponding to peptides of *Ps*BGAL were matched with BGAL from other plant sources (whose amino acid sequence is available in a protein database) using PeptideMap (a tool of PROWL). PeptideMap is based on a Bayesian algorithm available at http://prowl.rockefeller.edu/. The software is based on direct comparison of a protein sequence with the monoisotropic masses of peptides obtained by MALDI-TOF. It is useful especially in the cases where the sequence of the protein to be identified is not available in the protein database. Comparative analysis based on parameters as- (Option: Chemistry): Digestion with trypsin with 1 missed cleavage, (Option: Global modifications): Complete was unmodified, partial was acrylamide (C) and rest by default, (Option: Local modifications): all are by default, (Option: Cross-link): none and rest by default. During our search, all the peptide masses were assumed to be monoisotropic with a mass accuracy of ± 1.0 Da with minimum peptide match of 10 considered during our search. The PROWL software showed the % resemblance of *Ps*BGAL with other BGALs from various plant sources.

Determination of Active Site Groups. Using Lineweaver−Burk plots, K_m was determined at different pH values using glycine-HCl buffer (50 mM) ranging from pH 2.7 to 3.3 with ONPG as a substrate. The two tangents were drawn (broken lines) with slopes of 0 and 1 on the plot (pK_m versus pH) crossing on a point, which gave the value of ionization constant pK_a as the abscissa of the point of intersection. Values of pK_a obtained at different temperatures were plotted with pK_a versus temperature. From the slope of plot (ΔH^o/2.303) based on the van't Hoff equation: $d(\ln K)/dT = \Delta H^o/RT^2$, standard dissociation enthalpy change (ΔH^o) was calculated. The value corresponding to ΔH^o gives the groups present at the active site of *Ps*BGAL.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used for further confirmation of groups present at the active site of *Ps*BGAL and estimation of their number. Stock solution of purified *Ps*BGAL (250 μg mL^{-1}) was mixed with the aliquot of EDC (40−200 mM) prepared in 0.1 M Na-acetate buffer, pH 4.5 with the final concentration of protein as 125 μg mL^{-1} at 4 °C for the indicated time intervals. Five microliters of samples were withdrawn at regular intervals, and residual enzyme activity was determined after diluting the aliquot with assay buffer using ONPG as substrate. Rates of reaction (*k*) for inactivation by EDC at different concentrations were determined from the slope of the plot of log % residual activity versus different time intervals. Values obtained for "*k*" were plotted as log *k* versus log [EDC]. From the slope of the plot, the number of protonated carboxylates present at active site of *Ps*BGAL was estimated.

RESULTS AND DISCUSSION

Purification and Homogeneity of Purified *Ps*BGAL. As summarized in **Table 1**, BGAL was extracted from seeds of *P. sativum* (*Ps*BGAL) following their imbibition. The enzyme was purified to homogeneity using five steps with a 910-fold purification and a yield of approximately 15%, and specific activity of 77.33 μmoL min^{-1} mg^{-1}. Hydrophobic interaction chromatography (HIC) using Octyl Sepharose CL-4B (**Figure 1A**) was the most effective step used during purification leading to an increase in fold purification by ∼500 (**Table 1**). From various reported purification schemes using either ion exchange or affinity chromatography, the yields ranged between 0.71 to 16.5%, and specific activity ranged between 3.9 to 95.69 μmoL min^{-1} mg^{-1} (*24*, *40*, *41*).

Purified *Ps*BGAL produced a single peak on a Superdex S-200 10/30 HR column corresponding to a molecular mass of 228 kDa at pH 5.0 (**Figure 2A**). A single band was observed in case of Native-PAGE and isoelectric focusing. The band of Native-PAGE corresponded to the activity stained using X-gal (**Figure 2B, i**). The isoelectric point of purified *Ps*BGAL was found to be 8.8 (**Figure 2B, ii**) similar to previous reports (*9*, *19*, *24*, *42*). The pI of protein provides information regarding the possible niche of the protein inside the cell (*43*). The basic pI of *Ps*BGAL indicates that the enzyme is provided with an acidic environment inside the cell, possibly inside vacuoles (∼pH 5.0). It is further supported by maximum stability of *Ps*BGAL at the respective pH (data not shown).

The purified *Ps*BGAL showed a single band corresponding to 55 kDa on SDS−PAGE under reducing conditions at 5 mA (**Figure 5A, II**). *m*/*z* values corresponding to tryptic digests of the 55 kDa band on SDS−-PAGE showed resemblance to other plant BGALs present in the SWISSPROT database, by using PROWL tool (**Table 2**). The *N*-terminal sequence of *Ps*BGAL was found to be (TIECK), and the sequence showed the highest number of hits against BGAL from *Arabidopsis thaliana* (Q93Z24) using an NCBI BLAST search. cDNA analyses of BGAL (Q93Z24) revealed that the sequence does not have any lectin-like domain at its C-terminus (*25*). Thus, it can be concluded that *Ps*BGAL belongs to the second category of BGAL classification, having no lectin domain. It is further supported by

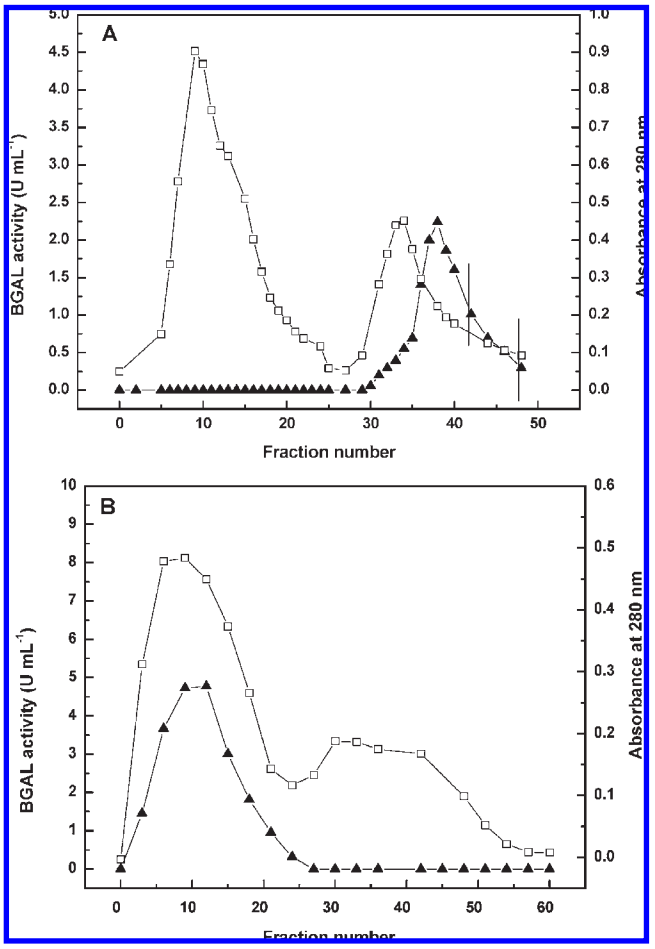


Figure 1. (A) Chromatography of dialyzed precipitate of 40−55% ammonium sulfate on Octyl Sepharose CL-4B column (50 × 5 cm). The fractions of 5 mL volume were collected at a flow rate of 0.3 mL min^{-1} and assayed for protein content (□) and BGAL activity using ONPG as substrate (▲). Fractions corresponding to bars were taken and pooled. **(B)** Chromatography of high specific activity fractions of Octyl Sepharose-4B on DEAE-Sephacel (12 × 2 cm). The fractions of 1 mL were collected at flow rate of 0.3 mL min^{-1} and assayed for protein content (□) and BGAL activity using ONPG as substrate (▲).

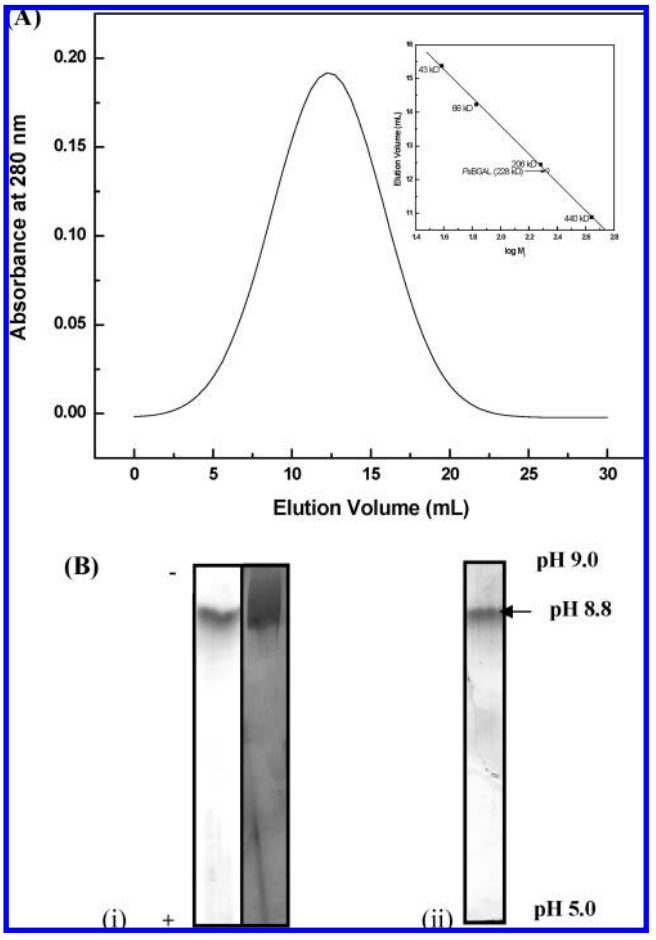


Figure 2. (A) Gel filtration of the purified *Ps*BGAL (200 μg mL^{-1}) using a Superdex S-200 HR 10/30 column. Inset: The semilogarithm plot of elution volume versus molecular weight. The filled and open circles represent standard molecular markers and *Ps*BGAL, respectively. **(B)** Electrophoretic patterns of purified *Ps*BGAL **(i)** Native PAGE (8%). **(ii)** Activity staining using X-gal as substrate. Isoelectric focusing using pH 5−9 ampholines.

a negative hemeagglutination test using sheep erythrocytes (data not shown).

***Ps*BGAL Stability under in Vitro Conditions.** The enzyme was fairly stable for a week in the crude preparation at 4 °C with a residual activity of more than 60%. Dilute purified *Ps*BGAL preparation with a protein concentration < 0.1 mg mL^{-1} was unstable with a $t_{1/2}$ of ∼13 h, 4 °C at pH 5.0. At a protein concentration of 0.1 mg mL^{-1}, the enzyme has a $t_{1/2}$ of ∼10 days, 4 °C at pH 5.0. The enzyme retained 86.26% of its activity when kept for 10 min at 40 °C; however it lost 90.85% of its activity at 50 °C and 94.89% at 60 °C, respectively. The BGAL reported from other sources (*40*−*42*) were also found to be thermolabile. As high temperatures do not prevail in vivo in these systems, the enzyme is better adapted with a temperature stability at lower temperatures.

Biochemical Properties of *Ps*BGAL. *(i). Effect of pH.* *Ps*BGAL has a pH optimum at 3.2 with ONPG as the substrate and at pH 4 with lactose (data not shown). The obtained pH optimum is ideal for xyloglucan hydrolysis (*44*) taking place during cell division. The observed shift in optimum pH in the case of lactose was due to a change in pK_a. According to Price and Stevens (*45*), the pK_a of the functional group in the enzyme can be shifted either to the left or right of the determined value due to solvent polarity of the solution. The solution of ONPG has greater solvent polarity than lactose solution; moreover, released *o*-nitrophenol has a lower pK_a than the neutral product glucose that leads to a shift of pK_a toward the acidic side with ONPG.

(ii). Effect of Temperature. Temperature optimum for *Ps*BGAL was found to be 60 °C (data not shown), similar to previous reports (*40*, *42*, *46*). Energy of activation (E_a) found using Arrhenius plot (data not shown) for the reaction catalyzed by *Ps*BGAL was 11.68 ± 0.23 kcal mol^{-1} of enzyme. The temperature optima so determined is considerably higher than what would prevail inside the cell. Lower temperature stability and higher temperature optima suggest that the formation of an enzyme−substrate complex protects the enzyme from denaturation/inactivation by heat, as suggested for glyceraldehyde 3-phosphate from mung beans (*47*).

(iii). Effect of Salts, Detergents, and Sugar. These were studied by incubating the purified *Ps*BGAL for 24 h at 4 °C, pH 5. From the **Table 3**, it is evident that incubating the enzyme with monovalent salts has increased the enzyme stability by 10−15%, but there was a drastic decrease in enzyme activity in the presence of divalent salts. In the presence of EDTA, there was an increase in stability of the enzyme even at low concentrations, such as 0.10 mM. These results suggest that *Ps*BGAL is not a metallo-

Table 2. Resemblance of Isolated *Ps*BGAL to Other BGALs from Plant Sources[a]

organism	accession no.	no. of matches per total of tryptic digest matched	% sequence coverage
Arabidopsis thaliana	Q9FVW7	24/43	50
	Q9CAR2	18/43	48
	Q8GX69	27/43	52
	O49609	20/43	48
Cicer arietinum	O82670	17/43	44
Oryza sativa	Q7G642	16/43	33
Solanum lycopersicon	P48980	21/43	42
Vigna radiata	Q9M5J4	19/43	45
Brassica oleracea	P49676	16/43	22

[a] It was performed using PROWL (Bioinformatics tool) available at http://prowl.rockefeller.edu/. The software performs matching of theoretical *m/z* values of the tryptic digest of the amino acid sequence of BGAL from plant sources available at SWISS-PROT database to experimentally determined *m/z* values of the tryptic digest of *Ps*BGAL. Peptide matching greater than 10 at nonstringent conditions (mass error allowed ± 1 Da) validates the identity of the isolated *Ps*BGAL to other plant BGALs (see Material and Methods for further details).

Table 3. Effect of Various Salts, Sugars, EDTA, and Detergents on *Ps*BGAL

additives	concentration	% residual activity[a]
salts		
NaCl	2 mM	108.67
	10 mM	110.36
	50 mM	123.47
NaF	1 mM	116.90
	5 mM	124.87
	10 mM	132.65
$CaCl_2$	1 mM	88.29
$MgCl_2$	1 mM	85.18
$\begin{pmatrix} CaC1_2+ \\ MgC1_2+ \\ NaCl \end{pmatrix}$	$\begin{pmatrix} 1\ mM \\ 1\ mM \\ 0.15\ mM \end{pmatrix}$	73.20
sugars		
methylmannoside	50 mM	no effect
galactose	10 mM	50.0
chelating agent (EDTA)	0.02 mM	127.3
	0.05 mM	118.5
	0.10 mM	107.8
detergents		
Triton-X	0.10%	96.0
	1.0%	83.0
	10.0%	61.0
CTAB	0.25%	148.0
	0.50%	145.0
	1.0%	129.0
SDS	0.1%	12.69

[a] % Residual activities were determined using equation: *Ps*BGAL activity (using ONPG as substrate) in the presence of various additives divided by *Ps*BGAL activity of control (with no additives) × 100 (see Materials and Methods for further details).

zyme, while an increase in stability with monovalent salt may have been due to a salt stabilization effect (*48*).

Effect of anionic surfactant (SDS, 0.1%; w/v) at pH 5.0 leads to an unusual loss of nearly 87% of activity rather than a complete loss (due to the loss of 3D structure of the enzyme protein). This may be due to the glycoproteinaceous nature of *Ps*BGAL, leading to poor binding of SDS (*46*, *49*, *50*). On the other hand, a cationic surfactant [cetyltrimethylammonium bromide (CTAB), 1%; (w/v)] caused an increase in *Ps*BGAL activity. *Ps*BGAL has surface positive charge at pH 5.0 (pI 8.8), such that CTAB was unable to bind completely to the enzyme. Further, enhanced ionic strength of the solution in the presence of CTAB led to an increase in enzyme activity. Akins and Tuan (*51*) also demonstrated retention of enzymatic activity in the presence of CTAB in many proteins. Nonionic surfactant (Triton-X, 10%; v/v) caused a decrease in *Ps*BGAL activity by 39%.

Table 4. Kinetic Values of *Ps*BGAL at 37 °C, pH 3.2

substrate[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
ONPG	0.97 ± 0.05	293.85 ± 11.35	302.94
lactose	3.03 ± 0.12	55.10 ± 3.47	18.18

[a] The reactions were carried out at pH 3.2 (50 mM glycine-HCl) and pH 4.0 (50 mM sodium acetate-acetic acid) with ONPG and lactose as substrates, respectively, at 37 °C. The reaction mixture containing enzyme and substrate (0−20 mM ONPG, 0−50 mM lactose) was incubated for 10 min, and rate of reactions were determined as described in Material and Methods. Using the Hanes-Woolf plot, K_m and V_{max} were determined. Turnover number (k_{cat}) is defined here as the number of moles of product produced s^{-1} mol^{-1} of *Ps*BGAL, given by the relation $V_{max}/[E]_t$.

Sugars, like galactose, was found to be a potent competitive inhibitor with K_i of 4.5 mM (obtained from Dixon plot), whereas methylmannoside has no effect even at 50 mM. These results suggest that these sugars have no physiological significance; as such high concentrations are not present inside the cell.

(iv). Tryptophan, Tyrosine, and Carbohydrate Content. Tryptophan (W) and tyrosine (Y) contents of *Ps*BGAL were found to be 3.7% and 4.5%, respectively. The *Ps*BGAL has 11.3% carbohydrate content with respect to neutral sugars. Obtained values were similar to other plant BGALs available at SWISS-PROT and NCBI nonredundant public protein databases.

Substrate Specificity. Purified *Ps*BGAL was used to hydrolyze various natural and synthetic substrates, under experimental conditions described in Material and Methods. It has been observed that the enzyme showed maximum turnover with ONPG and a K_m of 0.97 ± 0.05 mM, while that for lactose was found to be 3.03 ± 0.12 mM (**Table 4**). The enzyme hydrolyzed low molecular weight β-D-galactopyranosides but not α-D-galactopyranosides (**Table 5**). The rate of hydrolysis was on the order of o → *p* → *m*-nitrophenyl derivatives of β-D-galactopyranosides. This suggests that probably the *ortho* configuration of the substrate might make it more accessible to the active site of enzyme. This is an unique capability of *Ps*BGAL especially in plants, as ONPG was found to be a better substrate in the case of bacterial and fungal BGALs (*52*). On comparing the hydrolysis of natural substrates (namely, hemicellulosic fractions), structural xyloglucans were least hydrolyzed compared to reserve xyloglucans and pectic polysaccharides (**Table 5**). The ability of *Ps*BGAL to hydrolyze cell wall pectins further helps us understand the function of pectin in the cell wall and in cell wall porosity. Even limited BGAL activity on cell wall pectins has a significant effect on pectin solubility, by decreasing the ability of pectin molecules to aggregate (*53*). Pectins act as physical barriers between xyloglucan and hydrolytic enzymes (*54*), and these enzymes are

Table 5. Substrate Specificity of *Ps*BGAL towards Natural and Synthetic Substrates

substrate[a]	% relative activity[b]
natural substrate	
structural xyloglucans	0.57
reserve xyloglucans	1.80
pectic polysaccharides	1.27
lactose	11.70
synthetic substrate	
o-nitrophenyl-β-D-glucopyranoside	3.27
p-nitrophenyl-β-D-glucopyranoside	0.47
p-nitrophenyl-α-D-glucopyranoside	0.90
p-nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside	1.18
o-nitrophenyl-β-D-galactopyranoside	100
o-nitrophenyl-α-D-galactopyranoside	1.62
p-nitrophenyl-β-D-galactopyranoside	34.49
p-nitrophenyl-α-D-galactopyranoside	0.51
m- nitrophenyl-β-D-galactopyranoside	0.13
p-nitrophenyl-α-D-mannopyranoside	0.31
p-nitrophenyl-β-D-mannopyranoside	0.90
p-nitrophenyl-α-L-arabinoside	0.74
p-nitrophenyl-β-L-arabinoside	0.21
p-nitrophenyl-β-D-glucouronide	0.69
p-nitrophenyl-β-D-xylopyranoside	0.31

[a] Natural substrates (2 mg mL^{-1}), lactose (50 mM), and synthetic substrates (20 mM) were incubated with purified *Ps*BGAL at a concentration of 25 μg mL^{-1} (natural substrates and lactose) and at 5 μg mL^{-1} (ONPG) at 37 °C. [b] % Relative activity is expressed as Activity of *Ps*BGAL toward various substrates (estimated using galactose released in μmol mL^{-1} min^{-1} mg^{-1}) divided by 77.33 μmoL min^{-1} mL^{-1} mg^{-1} (Activity of *Ps*BGAL toward ONPG) × 100 (see equation given in Materials and Methods).

larger than wall pores, whose size is determined by the pectin matrix (*55*). Thus, a localized enlargement of these pores through changes in pectin architecture could allow hydrolases to access their substrate. Further, slow hydrolysis of natural substrates indicates that in addition to BGAL various other enzymes are also involved in hydrolyzing these natural substrates during seed growth and development. Similar observations were made by Nunan et al. (*56*) in the case of grape berry development. It has been shown that expression of various cell modifying enzymes, such as α-galactosidase, β-galactosidase, pectin-methylesterase, cellulase, xyloglucanase, galactanase, were increased during grape berry development. Because of the cumulative effect of all cell modifying enzymes, the hydrolysis is probably taking place with very high turnover under in vivo conditions.

BGAL in Seeds. As shown in **Figure 3**, there was a significant increase in total BGAL activity and its specific activity isolated from mature seeds with 8% moisture content during their imbibitions as compared to seeds with 12% moisture content. It was found by Iraki et al. (*57*) that seeds with a lower moisture content have a higher percentage of galactose present in pectins and xyloglucans. It can be predicted from the higher BGAL activity in seeds that lower moisture content was due to the presence of higher galactose content in them. According to some authors (*7*, *8*) xyloglucans possess hydrodynamic properties (important in imbibition and xeroprotection) besides acting as a germination reserve. Alteration in galactose content present in xyloglucans by *Ps*BGAL under in vivo conditions makes it a key determining factor in seed imbibitions. Treatment of pea seeds with actinomycin D (transcription inhibitor) and cycloheximide (translation inhibitor) at a concentration of 100 and 150 μg mL^{-1}, respectively, have no effects on total *Ps*BGAL activity and specific activity extracted from imbibed and germinated seeds. It indicates that enzyme was present even before seed imbibition and germination.

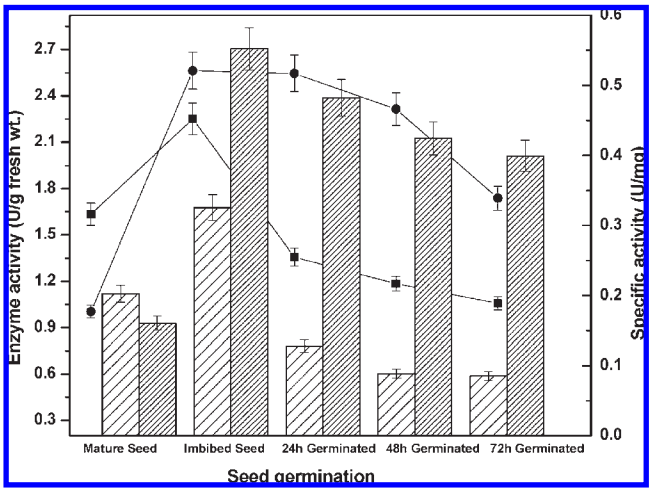


Figure 3. Changes in *Ps*BGAL activity and its specific activity during different stages of seed germination. Bars represent total BGAL activity/g (using ONPG as substrate) of fresh weight of seeds. White hatched bars and darker hatched bars indicate changes in total BGAL activity of the seed with 8% and 12% moisture content, respectively. Line plot represents changes in the specific activity of BGAL present in the seed with 12% (■) and 8% (●) moisture content, respectively. Vertical bars represent the standard error (SE) during the experiment.

Active Site Studies. Figure 4A, shows a van't Hoff plot of pK_a versus temperature where pK_a values were determined using a plot of pK_m versus pH at different temperatures (insets in **Figure 4A**). From the slope of the van't Hoff plot, the standard dissociation enthalpy change (ΔH^o) for *Ps*BGAL catalyzed reaction was found to be 4.60 ± 0.08 kcal mol^{-1}. The standard enthalpy change for protonation of a carboxylate is 1.9−2.2 kcal mol^{-1} and for hydrogen bonding is 1.5−2.0 kcal mol^{-1}. *Ps*BGAL was irreversibly inhibited in the presence of EDC. Therefore, it can be postulated that *Ps*BGAL has either aspartic acid or glutamic acid at its active site. The single carboxylate group of either amino acid present at the active site of the enzyme gets protonated, with the formation of hydrogen bonding with the substrate during the transition step of catalysis (*58*).

The time-course for inactivation of *Ps*BGAL at different concentrations of EDC (500−1000 fold molar excess over the enzyme) is shown in **Figure 4B**. Plot of log % residual activity against time was linear, indicating that modification of enzyme followed pseudo-first-order kinetics. Analysis of the order of reaction by the method of Levy et al. (*59*) yielded a slope of 1.2 (inset in **Figure 4B**). Therefore, it can be postulated that a single carboxyl group of aspartic acid or glutamic acid present at the active site of *Ps*BGAL plays an important role during catalysis as also supported by standard dissociation enthalpy studies.

Conformational Heterogeneity of *Ps*BGAL. According to SDS−PAGE, a single band (55 kDa) was observed at 10 mA, while three bands (55 kDa, 38 kDa, and 30 kDa) were observed at 20 mA of constant current supply (**Figure 5A**). The tryptic digests of all the bands produced at 20 mA have similar *m*/*z* values obtained from MALDI-TOF spectrometry (**Figure 5B−D**). It indicates that all bands belong to a single protein species. A sample of purified *Ps*BGAL was prepared in loading buffer containing protease inhibitor cocktail (see Materials and Methods) before loading onto SDS−PAGE. Therefore, the possibility of multiple banding due to intrinsic proteolytic activity could be completely ruled out. On carrying out deglycosylation of *Ps*BGAL

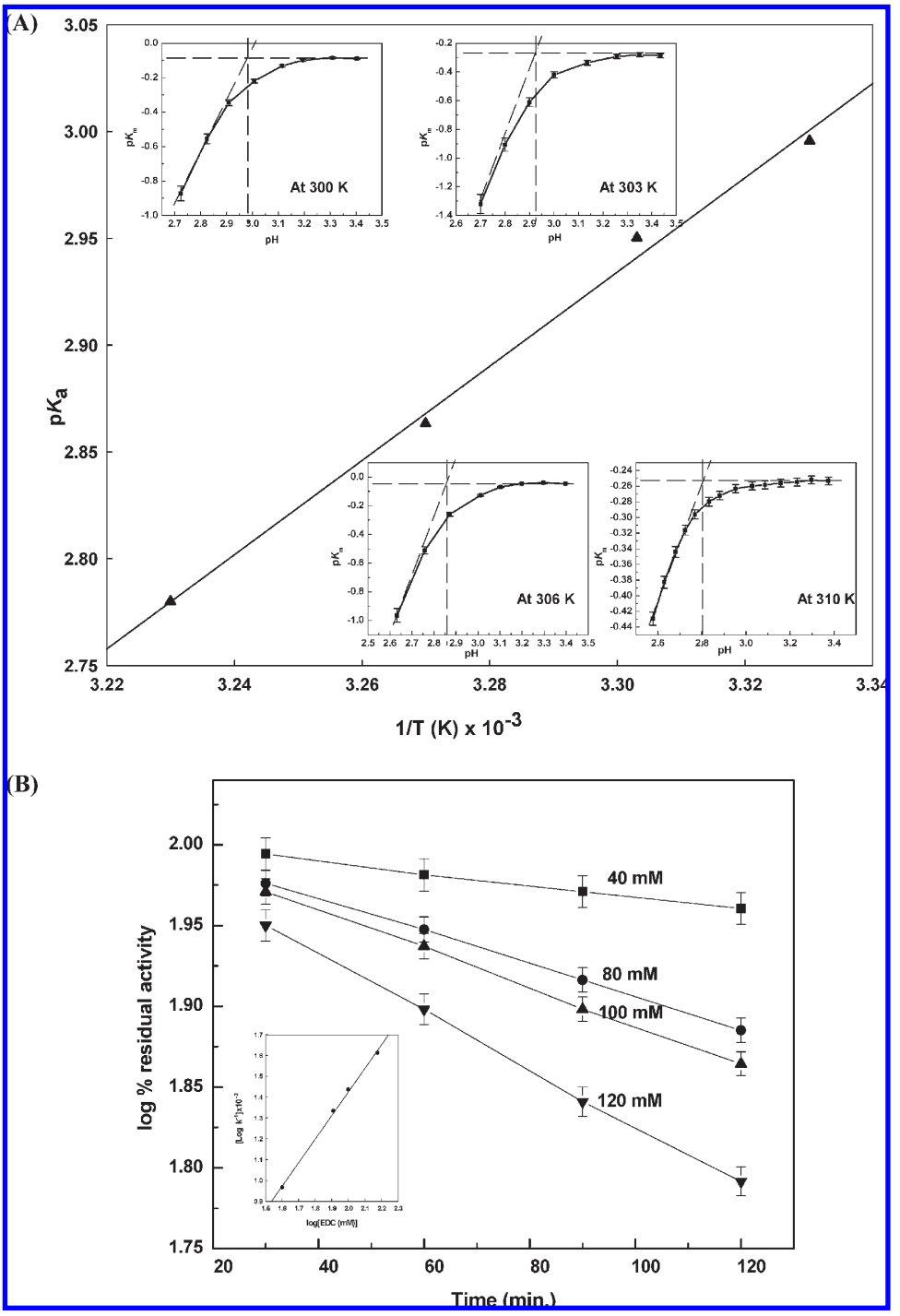
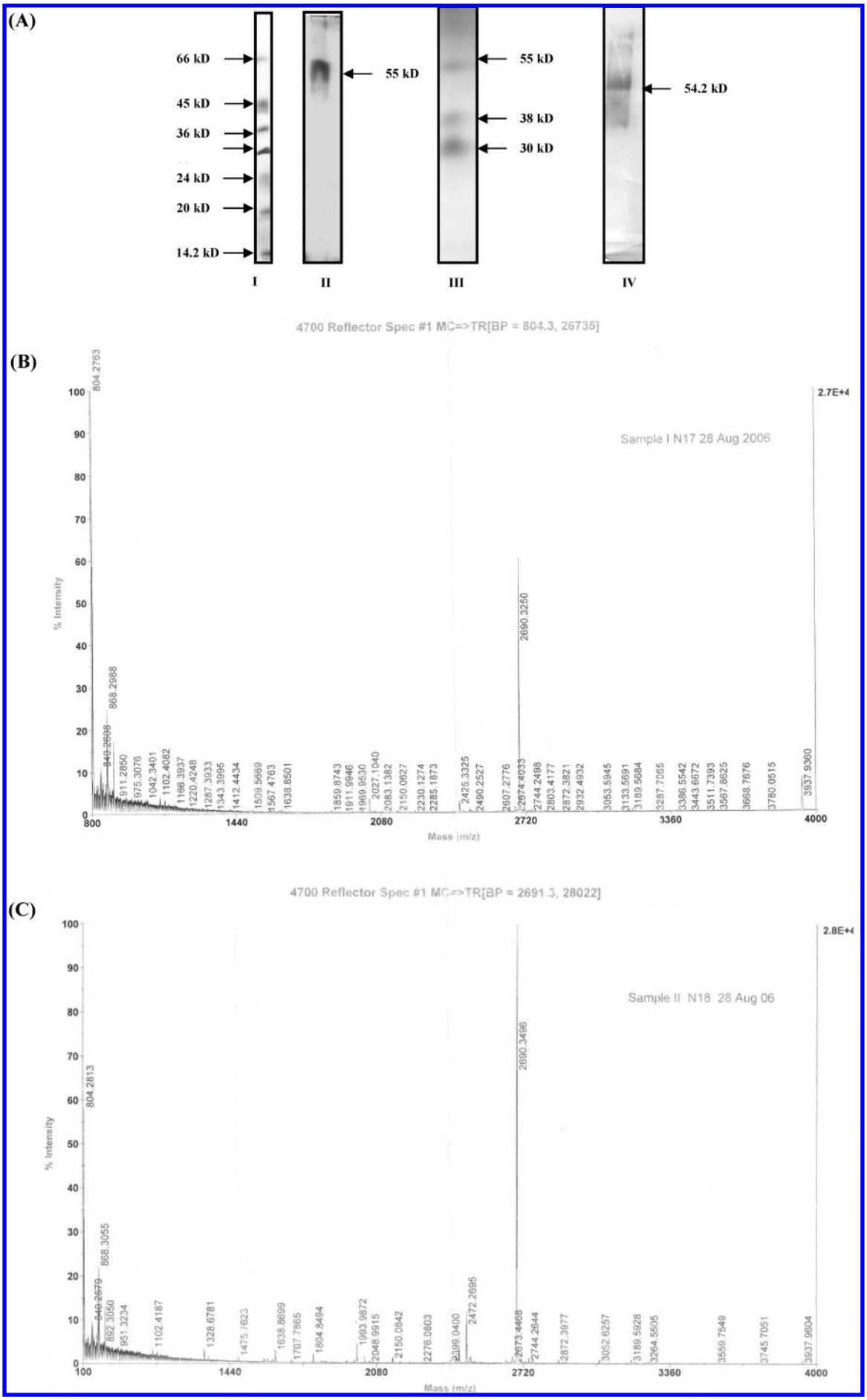


Figure 4. (A) Determination of groups present at the active site of *Ps*BGAL using dissociation energy. Insets: plots of pK_m (negative log of Michaelis−Menten constant using LB plot) versus pH at different temperatures. Vertical bars represent standard error during experiment. (B) Determination of groups and their number at active site of *Ps*BGAL (250 μg mL^{-1}) using EDC modification. Inactivation by EDC followed first-order kinetics in each case; from the slope of each plot the rate constant (k) was determined. Inset: The plot of log [rate constant (k)] versus log [EDC]. Vertical bars represent the standard error during the experiment.

using triflouromethanesulphonicacid (TFMS), a single band was observed on SDS−PAGE even at 20 mA of constant current supply. This indicates that in the present case multiplicity is due to the existence of varied glycosylation of the enzyme protein.

According to Native-PAGE results (**Figure 2B, i**), a single band was produced with a current supply similar to the results described by Goldenberg (*30*) (see Materials and Methods). According to Goldenberg (*30*), a low current supply during protein loading is beneficial as under native conditions protein exists as a mixture of interconverting conformations. If the electrophoretic separation is rapid compared to the rate of conformational interconversion it may be possible to resolve the different intermediate species. If the $t_{1/2}$ for interconversion between conformations is less than about 1/10th the duration of the electrophoresis, there will be no separation. Thus, a single band will be observed with the mobility that is the weighted average of the mobilities of the two forms. At the other extreme, if the $t_{1/2}$ of the interconversion is more than about 10 times the


(A)
66 kD
45 kD
36 kD
24 kD
20 kD
14.2 kD
55 kD
55 kD
38 kD
30 kD
54.2 kD
I
II
III
IV
(B)
4700 Reflector Spec #1 MC=>TR[BP = 804.3, 26735]
Sample I N17 28 Aug 2006
% Intensity
Mass (m/z)
(C)
4700 Reflector Spec #1 MC=>TR[BP = 2691.3, 28022]
Sample II N18 28 Aug 06
% Intensity
Mass (m/z)

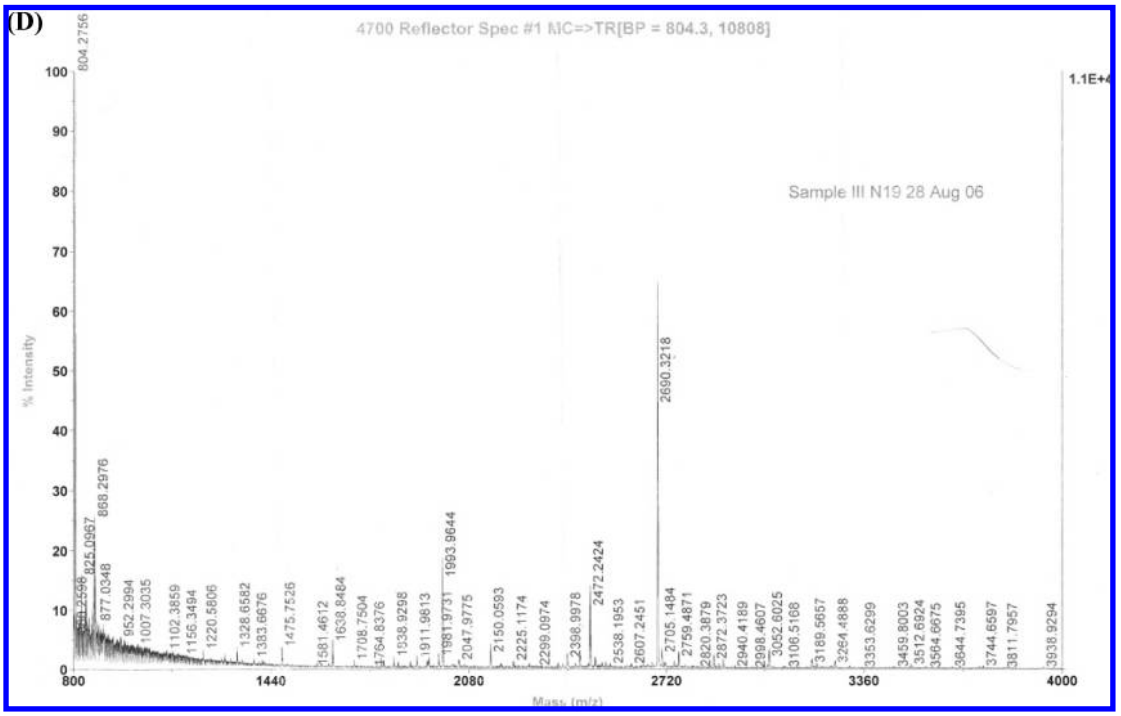


Figure 5. **(A)** Conformational heterogeneity shown by purified *Ps*BGAL on SDS−PAGE (12%) as a function of current supply. Marker protein **(I)**, purified glycosylated *Ps*BGAL electrophoresed at 10 mA **(II)**, glycosylated **(III)** and deglycosylated *Ps*BGAL electrophoresed at 20 mA **(IV)**, respectively. **(B)** MALDI-TOF profile of 55 kD *Ps*BGAL band on SDS-PAGE after cleavage with trypsin. **(C)** MALDI-TOF profile of 38 kD *Ps*BGAL band on SDS-PAGE after cleavage with trypsin. **(D)** MALDI-TOF profile of 30 kD *Ps*BGAL band on SDS-PAGE after cleavage with trypsin.

electrophoresis time, different conformations exist and are clearly separated. At intermediate rates of interconversion, only a fraction of molecules will interconvert during the separation, leading to smear of protein between the positions of two forms. Therefore, a low current supply during protein loading is helpful in determination of the number of proteins present in the given sample. This might be the reason that isoelectric focusing which is run under native conditions is carried out at a low current supply (2 mA per tube gel; Materials and Methods).

In the case of SDS−PAGE, all the interconverting forms of nonglycosylated protein bind to SDS and gain a similar charge density. Therefore, it produces a single band on SDS−PAGE even at a high current supply. The glycosylation of glycoprotein interferes with SDS binding to the protein (*49*). Thus, interconverting glycoforms are seen even in the case of SDS−PAGE, especially when a high current supply is used.

CONCLUSION

*Ps*BGAL was purified to homogeneity from *Pisum sativum*. The enzyme was acid stable via multimerization at lower pH and lacks lectin-like activity. Enzyme plays a significant role during seed imbibitions by allowing an appropriate amount of water absorption and in hydrolysis of food reserves required for seedling growth.

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