A β-galactosidase from chick pea (Cicer arietinum) seeds: Its purification, biochemical properties and industrial applications

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Abstract

A β-galactosidase from Cicer arietinum seeds has been purified to apparent electrophoretic homogeneity using a combination of various fractionation and chromatographic techniques, giving a final specific activity of 220 units mg⁻¹, with approximately 1840 fold purification. Analysis of the protein by SDS-PAGE revealed two subunits with molecular masses of 48 and 38 kDa, respectively. These bands were further confirmed with LC–MS/MS, indicating that Chick pea β-galactosidase (CpGAL) is a heterodimer. Molecular mass was determined to be 85 kDa by Superose-12 FPLC column, which is in agreement with the molecular mass suggested by mass spectroscopy to be 83 kDa. The optimum pH of the enzyme was 2.8 and it hydrolysed o-nitrophenyl β-D galactopyranoside (ONPG) with a K_m value of 1.73 mM at 37 °C. The energy of activation (E_a) calculated in the range of 35 to 60 °C, using Arrhenius equation, was determined to be 11.32 kcal mol⁻¹. The enzyme could also hydrolyse lactose, with an optimum pH of 4.0 at 40 °C, K_m and E_a for lactose hydrolysis was found to be 10 mM and 10.57 kcal mol⁻¹, respectively. The enzyme was found to be comparatively thermostable showing maximum activity at 60 °C for both ONPG and lactose. Galactose was found to be the competitive inhibitor. β-Galactosidase also exhibited glycoproteinaceous properties when applied on Con-A Sepharose column. The enzyme was localised in germinated seeds with X-gal activity staining and shown to be expressed prominently at grown radical tip.

1. Introduction

β-Galactosidase (EC 3.2.1.23, β-D-galactoside, galactohydrolase, lactase), a widespread family of glycosyl hydrolases from microorganisms to plantae and animalia kingdom, are characterised by their ability to hydrolyse terminal, non-reducing β-D-galactosyl residues from oligosaccharides and polysaccharides, as well as in glycoproteins and glycolipids (Nichtl, Buchner, Jaenicke, Rudolph, & Scheibel, 1998). Plant β-galactosidases exhibit significant differences from those isolated from microbes. Bacterial enzymes are generally tetrameric or monomeric (Marchesi, Steers, & Shifrin, 1969) and much larger than the plant enzymes, which are generally dimeric and much smaller (Simos, Giannakouros, & Georgatos, 1989). It also has been reported that the optimum pH of the plant enzyme lies in the acidic range (Mcgee & Murray, 1986), while those from bacteria lie near the neutral range (Marchesi et al., 1969). Plant β-galactosidase can be divided mainly into two classes, according to substrate specificity; one class that comprises exo-β-(1→4)-galactanases and specifically act on pectic β-(1→4)-D galactan, and a second class that prefers nitrophenyl-β-D galactoside (Kotake et al., 2005).

β-Galactosidase has been purified from various plant sources, like radish seeds (Sekimata, Ogura, Tsumuraya, Hashimoto, & Yamamoto, 1989), kiwifruit (Ross, Redgwell, & MacRae, 1993), apple (Ross, Wegrzyn, MacRae, & Redgwell, 1994), mangoes (Ali, Armugam, & Lazan, 1995), mung bean (Li, Han, Chen, & Chen, 2001), kidney beans (Biswa, Kayastha, & Seckler, 2003), carambola fruit (Balasubramaniam, Lee, Lazan, Othman, & Ali, 2005) and pea (Dwevedi & Kayastha, 2009) etc. Function of plants β-galactosidase includes fruit softening and ripening (Smith, Starrett, & Gross, 1998), seed germination (Li et al., 2001), and development of vegetative organs (Esteban et al., 2003). In higher plants, β-galactosidase is the only enzyme considered to hydrolyse galactosyl residues from cell wall polysaccharides and no enzyme capable of cleaving β-1,4-galactan in an endo fashion has been identified (Smith et al., 1998). Cell walls are mainly

Abbreviations: CpGAL, chick pea β-galactosidase; ONPG, o-nitrophenyl-β-D galactopyranoside; PNGP, p-nitrophenyl-β-D galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; DMF, N,N-dimethyl formamide; FPLC, fast performance liquid chromatography; PDB, Protein Data Bank; BLAST, Basic Local Alignment Search Tool; LC–MS/MS, liquid chromatography mass spectrometry.

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responsible for the integrity and texture of tissues and therefore determine fruit processing. β-Galactosidase activity increases drastically during seed germination in plants such as barley (Galnakourou, Karagjorgos, & Simos, 1991) and lupines (Buckeridge & Reid, 1994). The increase of activity during germination of barley seeds has been shown 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 (Galnakourou et al., 1991). Treatment with polyphenols and flavonoids, potent inhibitors of β-galactosidase, have shown delayed softening and improved shelf-life in apples (Dick & Bearne, 1988). β-Galactosidase in Cicer has been shown to be associated with cell wall pectin degradation function (Esteban et al., 2003).

Molecular approaches such as cDNA cloning and expression were also used recently to unravel the precise physiological role of β-galactosidase in plants. Chantarangsee, Tanthanuch, Fujimura, Fry, and Cairns (2007) cloned and sequenced the gene encoding two isoforms of β-galactosidase in germinating rice and demonstrated its localisation in embryo, aleurone layer, and in the radicle and shoot after germination. Esteban, Labrador, and Dopico (2005) described cloning and expression pattern of a family of three β-galactosidase cDNA in chickpea and established its role with high cell division rate in meristematic hook, young epicotyl, and apical internodes. Tomato β-galactosidase cDNA has shown ripening related gene expression in normal fruits, with lower apparent in the non-softening mutants (Carey et al., 1995).

β-Galactosidase is widely used in food technology, mainly in the dairy industry to improve sweetness, solubility, flavour, and digestibility of dairy products. The enzyme is utilised in the development of new products with hydrolysed lactose, which are suitable for lactose-intolerant people, for the improvement of technological properties of non-fermented milk products and for removing the lactose from whey. Industrial application of β-galactosidase is also in the production of galacto-oligosaccharides. These are applied in a wide variety of foods because of their positive effect on the intestinal bacterial microflora (Mlichova & Rosenberg, 2006). Initially, it was believed that lactose is the only substrate for the enzyme. Recently, it was observed that enzyme specificity is due to hydrolysable bond rather than substrate (Dwevedi & Kayastha, 2010).

In the present study, a β-galactosidase from Cicer arietinum seeds has been isolated and characterised kinetically and biochemically. Our further aim is to perform high resolution crystallographic studies with purified β-galactosidase form C. arietinum, as there is no structural data available in PDB for any plant β-galactosidase, till date.

### 2. Materials and methods

#### 2.1. Plant material and chemicals

Dry seeds of C. arietinum were purchased from local market. All the chemicals for buffers were of analytical or electrophoresis grade from Merck (Eurolab GmbH Darmstadt, Germany). Chromatographic materials (Octyl Sepharose-4B, DEAE-Sephalac and Con-A Sepharose), Trypsin profile IGD kit (for In-Gel digestion), substrate o-nitrophenyl-β-D galactopyranoside (ONPG), Bradford reagent and molecular markers for electrophoresis were purchased from Sigma (St. Louis, MO, USA). Chromatographic material SP-Sephadex C-50, Sephadex G-150 and molecular markers for FPLC were from Pharmacia, Sweden. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Biosynth AG, Switzerland. Milli Q (Millipore, Bedford, MA, USA) water with a resistance of higher than 18 MΩ cm was used all throughout the experiments.

#### 2.2. Enzyme and protein assays

β-Galactosidase activity was routinely measured by using ONPG and lactose substrate as described recently by Dwevedi and Kayastha (2009) with some modifications.

The reaction mixture for activity measurement against ONPG contained, in a final volume of 500 μl, 50 mM glycine–HCl (pH 2.8), 20 mM ONPG substrate and 10 μl of appropriately diluted enzyme. The reaction were carried out at 37 °C for 10 min. Liberated o-nitrophenolate was measured spectrophotometrically at 405 nm after stopping the reaction with the addition of 1.5 ml Sodium tetraborate (20 mM). Absorbance (A405) was proportional to the amount of enzyme in the reaction mixture under the assay conditions. The absorbance, A405, was also linear with time within the amount of enzyme used. One unit of β-galactosidase is defined as the amount required for releasing 1 μmol of o-nitrophenolate per min, under standard conditions (extinction coefficient of o-nitrophenolate equals 4.05 × 10³ M⁻¹ cm⁻¹).

Activity towards lactose was estimated in 50 μl of reaction mixture containing 50 mM lactose prepared in 50 mM acetate buffer, pH 4.0, and 10 μl of appropriately diluted enzyme. The reaction were carried out at 40 °C for 10 min. Reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. Glucose released was estimated using GOD-POD method, using a commercially available kit (Span Diagnostics Ltd., India). The reaction mixture (20 μl) was added to 500 μl of glucose reagent. Colour was developed for 10 min at 37 °C; absorbance was recorded at 505 nm. Glucose concentration was calculated by absorbance of test solution/absorbance of standard glucose solution (1 mg/ml) gives concentration in mg/ml, which is converted to concentration in μM. One unit of enzyme is defined as 1 μmol of glucose released per min at 40 °C.

Protein estimation was carried out by using the Bradford method Bradford (1976) using crystalline BSA as standard protein.

#### 2.3. Purification of β-galactosidase

All purification steps were carried out at 4 °C and centrifugation was performed at 8420 g for 20 min, unless stated otherwise. Chromatographic flow rates were controlled and monitored with Microperpex peristaltic pump (Pharmacia, Sweden). Buffers used in each step included additives (1 mM DTT and 0.5 mM PMSF) and the final preparation had a cocktail of commercial plant proteases inhibitors.

##### 2.3.1. Extraction

Dry seeds (70 g) were surface sterilised with 0.5% hydrogen peroxide, thoroughly washed with H2O and soaked in extraction buffer (25 mM sodium phosphate buffer, pH 6.8), for 24 h at 4 °C. Soaked seeds were homogenised in a laboratory blender in 140 ml chilled extraction buffer, and then squeezed through two layers of pre-washed muslin cloth. The resulting extract was centrifuged. The pellet containing cell debris was discarded and supernatant was collected.

##### 2.3.2. Acid fractionation

The pH of supernatant was lowered to 4.0, by drop-wise addition of chilled 0.2 M HCl with continuous stirring and incubated for 4 h at 4 °C, leading to a thick, curdy precipitate of inactive proteins, which were removed by centrifugation. The clear supernatant was collected and its pH brought back to 6.8 using chilled 0.2 M NaOH.

##### 2.3.3. Ammonium sulphate fractionation

The supernatant was precipitated at 40–60% ammonium sulphate saturation. Protein precipitate as obtained from the above
2.3.4. Octyl Sepharose-4B chromatography

The enzyme so obtained was dialysed in the same buffer containing 2 M (NH4)2SO4 and passed through an assembly containing nitrocellulose membrane (0.45 µm) to get a clear solution and loaded onto Octyl Sepharose CL-4B column (10 × 3 cm), pre-equilibrated with 200 ml (5 bed volume) of 25 mM sodium phosphate buffer, pH 6.1. A given range was collected by centrifugation, dissolved in minimum volume of 25 mM sodium phosphate buffer, pH 6.1.

2.3.5. DEAE-Sepharose

The enzyme obtained was extensively dialysed to remove ammonium sulphate against 25 mM sodium phosphate buffer, pH 7.0 at 4 °C. Enzyme thus obtained was applied to a DEAE-Sepharose CL-4B column (8 × 3 cm), pre-equilibrated with 100 ml of 25 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted in flow through volume without getting adsorbed. Enzyme fractions (1.5 ml) were collected with a flow rate of 0.3 ml min⁻¹; fractions (2 ml) were collected and those having high specific activity were pooled and concentrated.

2.3.6. SP Sephadex C-50

The enzyme was dialysed against 25 mM sodium acetate buffer pH 4.0, for 4 h at 4 °C. The dialysed enzyme obtained from previous step was loaded onto SP-Sephadex C-50 column of dimensions (5 × 3 cm), pre-equilibrated with 4 column volumes of 25 mM sodium acetate buffer, pH 4.0. Column washing was done with 25 mM sodium acetate buffer, pH 4.0, containing 80 mM NaCl followed by elution with 160 mM NaCl, in the same buffer. Active fractions of 1.5 ml were collected with a flow rate of 0.3 ml min⁻¹, the enzyme obtained was concentrated and dialysed to remove NaCl.

2.3.7. Sephadex G-150

Concentrated enzyme from above step was loaded onto Sephadex G-150 column (25 × 1 cm), pre-equilibrated with 25 mM sodium acetate buffer pH 4.0 containing 50 mM NaCl. Active fractions of 1 ml were collected at 0.3 ml min⁻¹ flow rate, pooled, concentrated and stored with plant proteases inhibitor cocktail at 4 °C.

2.4. Determination of molecular mass

SDS-PAGE (2 cm, 5% stacking gel, pH 6.8, and 13 cm, 10% resolving gel, pH 8.8) was performed as described by Laemmli (1970), using a vertical gel electrophoresis apparatus (Monokin, India). An apparent subunit molecular mass of enzyme subunits were calculated by Alphalnotech (Alphalnauge, USA) from the data obtained by SDS-PAGE using protein marker with molecular mass ranging from 240 to 7 kDa. SDS gels were silver stained.

2.5. Native-PAGE and activity staining

Native-PAGE (2 cm, 5% stacking gel, pH 6.8, and 13 cm, 7.5% resolving gel, pH 8.8) was carried at 4 °C and at a constant current supply of 5 mA until samples were stacked and then the current was increased to 10 mA. Activity staining was done using substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) according to the procedure given by Biswas et al. (2003), with minor modifications. Half of the native-PAGE gel was stained with Coomassie Brilliant Blue R-250 and the other half was equilibrated with 50 mM Na-acetate buffer pH 4.0 for 1 h followed by incubation for 24 h in a solution of X-Gal (5 mg/ml). For preparation of X-Gal, 50 mg was first dissolved in 1 ml DMSO solution and then diluted to 10 ml by adding phosphate buffer at pH 7.0.

2.6. Native molecular mass determination by FPLC

This was done by FPLC (Pharmacia, Uppsala, Sweden) on a Superose-12 column pre-equilibrated with 25 mM sodium acetate buffer pH 4.0 containing 100 mM NaCl. Purified enzyme 200 µl was chromatographed at a flow rate of 0.4 ml min⁻¹. Fractions (0.5 ml) were collected and assayed for A280 and β-galactosidase activity. Native molecular mass was calculated from a plot of Ve/Vo against log of molecular mass, using the following protein standards: Ferritin (440 kDa), Catalase (232 kDa), Aldolase (158 kDa), Albumin bovine (66 kDa) and Chymotrypsinogen A (25 kDa). The void volume was determined by Blue Dextran.

2.7. Glycoprotein properties

Purified CpGAL was applied on Con-A Sepharose column (2 × 1 cm) to ascertain the glycoproteinaeous nature. Enzyme was dialysed extensively against 25 mM Citrate buffer, pH 5.0 containing 200 mM NaCl, 1 mM CaCl₂, and 1 mM MnCl₂ before being loaded onto the column, pre-equilibrated with the same buffer. Fractions were collected at a flow rate of 0.3 ml min⁻¹. Bound protein was eluted with the same buffer containing 100 mM glucose or 100 mM α-methyl mannoside, and β-galactosidase activity and absorbance at 280 nm were monitored.

2.8. Mass spectrometry and database search

Gel band was excised, destained and dried before digestion with trypsin. Gel band of interest was digested overnight with MALDI grade trypsin (Sigma). Zip-Tip (Millipore, Bedford, MA) was used for concentrating the digested solution. The sample was mixed with equal volume of matrix solution (10 mg/ml R-cyano-4-hydroxycinnamic acid in same solvent), and spotted onto the MALDI target plate. MALDI-TOF-MS was performed using a Voyager-DE-STR instrument (Applied Biosystems, CA, USA). The mass spectra were subjected to sequence database search using MS-Fit (Protein Prospector) against NCBInr (release December, 2004), plant databases. Electrospray ion trap LC–MS/MS analysis was done using Q-Star Pulsar I (Applied Biosystems, USA). The spectra were analysed by Mascot sequence matching software (http://www.matrixscience.com). Protein identifications were evaluated on the basis of multiple variables such as score, number of peptides matched, percent coverage of the matched protein, quality of the peptide maps besides similarity of experimental and theoretical protein molecular masses. The mass peak was taken as input in MASCOT using following parameters: (a) fixed modifications carboxamidomethyl (C); (b) variable modifications: oxidation (M); (c) cleavage by trypsin: cuts C-term side of KR unless next residue is P.

2.9. Kinetic studies

Finally purified enzyme preparation was used for all kinetic studies. The pH optimum against ONPG and lactose was determined using varying buffers ranging from 2.0 to 7.0 (Glycine–HCl: pH 2.0–4.0, Acetate buffer: 4.0–5.5, Sodium phosphate: 5.5–7.0). Effect of pH upon stability of β-galactosidase was also determined by incubating the enzyme in different buffers, ranging from pH 3.0 to 9.0 (Glycine HCl: pH 3.0–4.0, Acetate buffer: 4.0–5.5, Sodium...
phosphate: 5.5–7.0, Tris–HCl: 7.0–9.0). The residual activity was regularly checked at an interval of 24 h up to a week under standard assay condition. The optimum temperature against ONPG and lactose were determined by assaying the enzyme at different temperatures ranging from 25 to 70 °C (±1 °C) for 10 min in a water bath (Multitemp; Pharmacia, Sweden). Thermal inactivation studies were also studied by incubating enzyme at various temperatures for different time intervals, followed by residual activity assay under standard condition.

For determination of Michaelis–Menten constant (K_{m}), enzyme was assayed in the presence of varying concentrations of ONPG (0.2–40 mM) and lactose (2–40 mM) under standard assay conditions. Data was plotted and analysed using Lineweaver–Burk plot. Activation energy (E_a) was calculated from the slope of the curve using Arrhenius law. Inhibition constant (K_i) for galactose was also determined using Dixon plot. Different concentration of additives (DTT, PMSF, EDTA and β-mercaptoethanol), salts (CaCl_2, MgCl_2, NaCl, KCl, HgCl_2, and FeCl_3), detergents (SDS and Triton-X) and sugars (galactose and glucose) were added and residual activity measurement was monitored on day to day basis, till one week.

All reported parameters are the mean of three experiments performed independently or in parallel.

### 2.10. Localisation of β-galactosidase in germinating seed

Sterilised seeds of *C. arietinum* were imbibed in 25 mM phosphate buffer (pH 6.8) for 24 h. Germination of imbibed seeds was carried out on a moistened filter paper in dark at room temperature up to 48 h. Germinated seeds were fixed with 3% glutaraldehyde solution, prepared in 25 mM phosphate buffer (pH 6.0) for 2 h at 4 °C. Fixed germinated seeds were then incubated directly in staining solution or preincubated in 50 µM 2,4-dinitrophenol (D-D-deoxy-2-fluoroglucoside) for 2 h at room temperature to inhibit other β-glucosidase activity. It was then incubated in the staining solution (5 mg ml^{-1} X-Gal solution in 25 mM phosphate buffer, pH 6.0) for 2 h and distilled in 50% methanol for 10 min. Imibed seeds were also tested for staining with X-Gal after soaking in 1 mM FeCl_3. Semi-thin sections from radical were cut and mounted on a 0.1% (w/v) polylysine-coated glass slide for microscopic visualisation using Nikon light microscope (DS – Fi1).

### 2.11. Sequence alignment

MASCOT CpGAL sequence was taken as input and its homologous sequences were searched using BLAST (Basic Local Alignment Search Tool). Retrieved sequences including CpGAL were aligned by BioEdit Sequence Alignment Editor.

### 3. Results and discussion

#### 3.1. Purification

β-Galactosidase from *C. arietinum* (CpGAL) was purified up to 1841 fold to a final specific activity of about 220 units mg^{-1} and an overall recovery of 12% using various fractionation and chromatographic techniques, as shown in Table 1A. The purified enzyme showed a typical absorption spectrum with λ_{max} at 280 nm and A_{280}/A_{260} ratio equal to 1.56 (data not shown), similar to the kidney bean β-galactosidase (*Biswas et al., 2003*) and apparently free from nucleic acid and other nucleotides.

Specific activity of CpGAL increases to a maximum within 48 h following inhibition at 4 °C. Initial studies to determine the appropriate pH and amount of seeds to extraction buffer ratio was carried out before attempting any further purification (data not shown). It was found that a 2:1 ratio of imbibed seeds to extraction buffer (25 mM phosphate buffer, pH 6.8) was ideal and retained most of the activity in the supernatant. The crude extract was thick slurry, which was cleared with acid fractionation that removed a large portion of other plant proteins. At acidic pH, most of the insoluble and acid unstable proteins get precipitated, which could be removed easily by centrifugation, leaving clear supernatant with about three fold purification. Enzyme was further selectively fractioned with ammonium sulphate in the range of 40–60% saturation.

Lactosyl Sepharose-4B has been widely used as an affinity material for chromatographic purification procedure from various sources (*Ross et al., 1993; Ross et al., 1994; Simos et al., 1989*). However, in the present case, enzyme did not bind to Lactosyl Sepharose-4B over a broad range of pH (4.0 to 8.0). Therefore, the enzyme obtained from ammonium sulphate fractionation was applied to the Octyl Sepharose CL-4B column. Enzyme was eluted in a single peak from Octyl Sepharose-4B column after washing and gave about 15-fold purification from the previous step. Recently, *Dwevedi and Kayastha* (2009) efficiently employed hydrophobic interactions for purification of β-galactosidase from peas (*Pisum sativum*) and achieved 30-fold increase in purification.

As the next step of purification, anion-exchange chromatography (DEAE-Sephalcel) was used for the concentrated and dialysed enzyme fraction obtained from Octyl Sepharose CL-4B column. Almost complete enzyme came in flow through volume, without getting adsorbed onto it. This step was found to be important in terms of significant recovery and with two fold purification from previous step. Enzyme was next added to SP-Sephadex C-50 (cationic exchanger), as it completely ionised over a wide pH range. Most of the enzyme (>90%) binds to the SP-Sephadex C-50 column through cationic interaction, but a small fraction of enzyme did not bind. This fraction could be possibly the isomers of β-galactosidase as suggested by *Esteban et al. (2003)*, which was left unattended due to very low activity as well as specific activity and presence of other unbound proteins. Enzyme after SP-Sephadex C-50 column was nearly homogeneous on SDS-PAGE with a minor faded band in the lower portion on the gel which was further clarified by Sephadex G-150 column. Enzyme eluted as a single peak when finally added to Sephadex G-150 column. This step however did not yield any further increase in specific activity, thereby confirming the homogeneity of the enzyme.

The final specific activity of CpGAL was found to be 220 units mg^{-1} against ONPG with a purification of 1841 fold. These values are comparatively higher than those reported earlier from various plant sources. Purified β-galactosidase from peas (*P. sativum*) showed a specific activity of 77.33 units mg^{-1} and a purification of 910 fold (*Dwevedi & Kayastha, 2009*). Five isofoms of β-galactosidase (I-V) were isolated from mung bean seedlings, among which β-galactosidase III had maximum specific activity of 95.69 units mg^{-1} and overall yield of 12%. Apple β-galactosidase was shown to have 23.2 units mg^{-1} specific activity with 180 fold recovery (*Ross et al., 1994*). *Sekimata et al. (1989)* reported β-galactosidase from radish seeds with specific activity of 5.62 units mg^{-1}.

Purified β-galactosidase applied on Superose-12 FPLC column eluted as a single peak corresponding to a native molecular mass of 85 kDa (Fig. 1A). SDS–PAGE of CpGAL showed two non-identical polypeptides with molecular masses of 48 and 38 kDa, suggesting that the native protein is a heterodimer of two non-identical subunits (Fig. 1B and C). These bands were submitted for mass spectrometry analysis (LC MS/MS) and showed significant matches against β-galactosidase from *C. arietinum* and other plant β-galactosidase (supporting information). Sorted matched peptide from...
C. arietinum with ion score is given in Table 1B. The identified trypsin digested peptides are identical in both subunits, possibly due to insufficient separation of the peptide before trypsinolysis. These values are similar to those of dimeric \( \beta \)-galactosidase from various plant sources (Balasubramaniam et al., 2005; Biswas et al., 2003; Li et al., 2001; Ross et al., 1994; Simos et al., 1989) but different from tea leaf, radish and tomato enzymes, which have been shown to be monomeric proteins of 60, 45 and 75 kDa, respectively (Carey et al., 1995; Halder & Bhaduri, 1997; Sekimata et al., 1989). Recently, \( \beta \)-galactosidase from peas (\( P. \) sativum) revealed a single protein band of lower molecular mass of 55 kDa on SDS–PAGE (Dwevedi & Kayastha, 2009).

Molecular mass determined by FPLC size exclusion chromatography (FPLC-SEC) is in agreement with the data obtained by mass spectrometry (83 kDa). The band of native-PAGE corresponded to the X-gal activity stained band. Most of the times, the protein bands in PAGE were not sharp under reducing and non-reducing conditions. Such diffusion of bands could be due to glycosylation of the protein, as was also confirmed by Con A Sepharose chromatography.

In initial purification trials, samples stored over a few weeks showed some other bands in the lower portion of SDS–PAGE, prominently at 25 kDa and 15 kDa positions, representing the proteolytic fragments, possibly due to the presence of traces of endogenous proteases activity. To prevent this proteolysis, 1% (v/v) protease inhibitor cocktail (Calbiochem, Canada) and 30% glycerol was added in the purified preparation and stored at \(-20^\circ C\). CpGAL stored with protease inhibitor cocktail did not produce any

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**Table 1A**

Purification of \( \beta \)-galactosidase from \( C. \) arietinum seeds (70 g).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity* (units mg(^{-1}))</th>
<th>Purification fold(^b)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>384.12</td>
<td>3224.33</td>
<td>0.119</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Acid fractionation</td>
<td>319.23</td>
<td>886.34</td>
<td>0.36</td>
<td>3.02</td>
<td>83.10</td>
</tr>
<tr>
<td>Ammonium sulphate fractionation</td>
<td>297.51</td>
<td>378.60</td>
<td>0.78</td>
<td>6.59</td>
<td>77.45</td>
</tr>
<tr>
<td>Octyl sepharose-4B</td>
<td>220.65</td>
<td>18.25</td>
<td>12.09</td>
<td>101.5</td>
<td>57.44</td>
</tr>
<tr>
<td>DEAEE-sephacel</td>
<td>212.57</td>
<td>8.89</td>
<td>23.89</td>
<td>200.57</td>
<td>55.34</td>
</tr>
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<td>SP sephadex C-50</td>
<td>86.51</td>
<td>0.396</td>
<td>218.46</td>
<td>1833.77</td>
<td>22.52</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>48.26</td>
<td>0.22</td>
<td>219.38</td>
<td>1841.51</td>
<td>12.57</td>
</tr>
</tbody>
</table>

*Enzyme activity was determined using ONPG (\( \alpha \)-nitrophenyl-\( \beta \)-D galactopyranoside), and protein determination was done using Bradford method.

\(^b\) Fold purification was calculated with respect to the specific activity of the crude extract.

**Fig. 1.** (A) The semi logarithm plot of elution volume versus molecular mass. (B) Electrophoresis pattern of purified CpGAL. Slab a and b shows silver stained molecular weight markers and purified enzyme, respectively under reduced condition (SDS–PAGE). Slab c shows silver stained Native-PAGE under non denaturing condition. Slab d shows activity staining of the enzyme using X-gal as a substrate. (C) Mass spectrum (LC–MS/MS) fragment from matched peptide of CpGAL.

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**Table 1B**

Sorted peptide according to their residue number and masses along with the peptide match for band.

<table>
<thead>
<tr>
<th>Band (kDa)</th>
<th>Start – end</th>
<th>Observed</th>
<th>( M_s ) (expt)</th>
<th>( M_s ) (calc)</th>
<th>Delta ( \Delta )</th>
<th>Ion score</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>303–326</td>
<td>874.43</td>
<td>2620.24</td>
<td>2620.23</td>
<td>0.01</td>
<td>36</td>
<td>RSAGGPHITSYDYAPIDEYGLR.E</td>
</tr>
<tr>
<td>38</td>
<td>303–326</td>
<td>874.43</td>
<td>2620.26</td>
<td>2620.23</td>
<td>0.03</td>
<td>62</td>
<td>RSAGGPHITSYDYAPIDEYGLR.E</td>
</tr>
</tbody>
</table>
unwanted proteolytic fragment up to two months as checked by SDS–PAGE.

3.2. Effect of pH and acid stability

The enzyme was fairly stable for a week in the crude preparation at 4 °C and retained more than 80% residual activity when assayed with ONPG substrate. Purified preparation was found to be kinetically very stable, and retained more than 85% residual activity over a month, when stored in 25 mM acetate buffer, pH 4.0 at 4 °C. Stability of purified enzyme was also checked at various dilutions (10 µg ml⁻¹–2 mg ml⁻¹), and was found to be almost independent of protein concentration up to a week at 4 °C, pH 4.0.

Mass spectrometry analysis showed a pI value of 8.63 for 
CpGAL. pI value in the basic pH range had been observed in β-galactosidase from other fruits, such as kiwifruit, tomatoes, carambola fruit and pea seeds (Balasubramaniam et al., 2005; Carey et al., 1995; Dwevedi & Kayastha, 2009; Ross et al., 1993). Basic pI of β-galactosidase may facilitate its binding to the acidic cell wall as suggested by Balasubramaniam et al. (2005).

3.3. Effect of temperature and thermostability

CpGAL is fairly stable at 50 °C with no significant loss up to 10 min but lost about 20% activity when kept at 55 °C for 10 min (data not shown). Temperature optimum for ONPG and lactose hydrolysis was found to be 60 °C, for both (Fig. 2B). This value is consistent with previously reported values for plant β-galactosidases (Biswas et al., 2003; Dwevedi & Kayastha, 2009). A higher temperature optimum suggests the formation of stable enzyme-substrate complex that protects the enzyme from denaturation by heat (Dwevedi & Kayastha, 2009).

The rate of enzymatic hydrolysis of ONPG is temperature-dependent and obeys Arrhenius relationship in the range of 35 to 60 °C, with energy of activation (Eₐ) equal to 11.32 kcal mol⁻¹ of enzyme. Energy of activation for lactose hydrolysis was calculated to be 10.56 kcal mol⁻¹. Energy of activation for ONPG hydrolysis with kidney bean β-galactosidase catalysed reaction were equal to 14.8 kcal mol⁻¹ (Biswas et al., 2003), whereas for peas, the value was 11.68 kcal mol⁻¹ (Dwevedi & Kayastha, 2009).

3.4. Steady state kinetics

The kinetics of ONPG and lactose hydrolysis was also studied under standard experimental conditions according to Michaelis–Menten equation using Lineweaver–Burk plot. It has been observed
Table 2

<table>
<thead>
<tr>
<th>Synthetic Substrate</th>
<th>% Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Nitrophenyl-p-o-galactopyranoside</td>
<td>100.00</td>
</tr>
<tr>
<td>o-Nitrophenyl-p-L-arabinopyranoside</td>
<td>0.90</td>
</tr>
<tr>
<td>o-Nitrophenyl-p-o-glucopyranoside</td>
<td>0.76</td>
</tr>
<tr>
<td>p-Nitrophenyl-o-o-mannopyranoside</td>
<td>0.86</td>
</tr>
<tr>
<td>p-Nitrophenyl-o-o-arabinopyranoside</td>
<td>0.78</td>
</tr>
<tr>
<td>p-Nitrophenyl-o-o-galactopyranoside</td>
<td>42.00</td>
</tr>
<tr>
<td>p-Nitrophenyl-p-o-xylopyranoside</td>
<td>0.76</td>
</tr>
<tr>
<td>p-Nitrophenyl-p-o-mannopyranoside</td>
<td>0.67</td>
</tr>
<tr>
<td>p-Nitrophenyl-p-o-glucopyranoside</td>
<td>0.48</td>
</tr>
<tr>
<td>p-Nitrophenyl-p-o-fucopyranoside</td>
<td>0.00</td>
</tr>
<tr>
<td>p-Nitrophenyl-p-o-galactopyranoside</td>
<td>0.20</td>
</tr>
<tr>
<td>m-Nitrophenyl-o-o-galactopyranoside</td>
<td>0.12</td>
</tr>
<tr>
<td>m-Nitrophenyl-p-o-galactopyranoside</td>
<td>0.02</td>
</tr>
<tr>
<td>p-Nitrophenyl-2-acetamido-2-deoxy-p-o-glucopyranoside</td>
<td>0.47</td>
</tr>
<tr>
<td>4-Methylumbelliferyl-o-o-galactopyranoside</td>
<td>0.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>14.27</td>
</tr>
</tbody>
</table>

Purified enzyme was incubated with synthetic substrates (20 mM) in a final volume of 500 μL, 50 mM glycine–HCl (pH 2.8) at 37 °C for 10 min. Enzyme activity against o-nitrophenyl-p-o galactopyranoside was taken as control to calculate percentage residual activity with rest of the synthetic substrates, as listed above.

that the enzyme showed a maximum turnover for ONPG with Vmax and Km values to be 29.56 units ml⁻¹ and 1.73 mM, respectively (Fig 2C), while that for the lactose, these values were found to be 1.67 units ml⁻¹ and 10 mM, respectively. Recently, β-galactosidase from peas showed a Km value of 0.97 mM with ONPG (Dwevedi & Kayastha, 2009). Km value of kidney bean β-galactosidase was found to be 0.63 mM for p-nitrophenyl β-p galactopyranoside (PNPG), 0.74 mM for ONPG and 50 mM for lactose (Biswas et al., 2003). The apparent Michaelis constant for jack bean β-galactosidase for each substrate was determined to be: PNPG 0.51 mM, ONPG 0.63 mM, lactose 12.3 mM. For radish seed enzyme PNPG 0.46 mM, ONPG 1.19 mM, lactose 8.55 mM, while the tea-leaf enzyme also showed orders of magnitude similar to other plant enzymes Halder & Bhaduri, 1997; Sekimata et al., 1989. The Km for different isoforms of Cajanus indicus and mango fruit also showed similar values for PNPG (Ali et al., 1995; Dey & Dixon, 1974). Lupin seed enzyme hydrolysed some synthetic β-galactosides rapidly, but lactose very slowly (Buckeridge & Reid, 1994).

Inhibition studies were also carried out with glucose and galactose. Galactose was found to be the competitive inhibitor, whereas glucose does not inhibit the enzyme significantly even at 50 mM concentration. Inhibition constant Ki for galactose was calculated to be 2.44 mM using Dixon plot. Generally, galactose is a competitive inhibitor of β-galactosidases. Recently, (Dwevedi and Kayastha (2009)) showed the competitive inhibition of peas β-galactosidase by galactose with a Ki value of 4.5 mM. Three isoforms of carambola β-galactosidase was also shown to be inhibited by galactose with Ki values of 11.4, 15 and 13 mM, respectively (Balasubramaniam et al., 2005). β-Galactosidase from Coffea arabica was shown to be inhibited by galactose with a Ki value of 0.26 mM (Golden, John, & Kean, 1993).

3.5. Glycosylation

Carbohydrate moieties in glycoproteins play an important role in protecting the protein from degradation, enhance thermal stability, as well as solubility and facilitate effective transport inside the cell (Yadav, Pande, & Jagannadham, 2006). CpGAL was found to have properties of glycoprotein as it was bound to an affinity Con-A Sepharose column and could be eluted with 100 mM glucose or 100 mM α-methyl mannoside. This step does not lead to any further gain in specific activity of the enzyme; hence substantive purification to apparent homogeneity. Glycoproteinaceous nature of other plant β-galactosidase is well-documented in mango, carambola fruits, mung bean seedling, and peas (Ali et al., 1995; Balasubramaniam et al., 2005; Dwevedi & Kayastha, 2009; Li et al., 2001).

3.6. Effect of salts and chemicals

Effect of various salts, detergent and chemicals were studied by incubating the purified enzyme for 24 h and a week at 4 °C (25 mM acetate buffer, pH 4.0). EDTA has a stabilising effect on CpGAL and increases the relative activity over time period at 0.1 mM concentration. Stabilisation effect of EDTA on β-galactosidase is well-documented (Dwevedi & Kayastha, 2009; Li et al., 2001). Enzyme activity does not affect much at low salt concentration but increases in the presence of higher salt concentration (Fig. 2D). Moreover, divalent cations have comparatively better activation effect on enzyme than monovalent cations. These results suggested that CpGAL is not a metallo-enzyme, while an increase in the activity of the enzyme with salts could be due to salt activation effects. HgCl2 and FeCl3 were found to be the potent inhibitor of enzyme and results in complete loss of enzymatic activity at 0.1 mM concentration. SDS completely inhibited the enzyme activity at 0.01 mM concentration, while Triton-X (up to 10%) did not inhibit the enzyme.

3.7. Substrate specificity

Substrate specificity studies towards some of the synthetic substrates shows that the purified enzyme had no α-galactosidase, α-mannosidase and β-glucosidase activity, but retained β-galactosidase and α-L-arabinosidase activity (Table 2). Plant β-galactosidase with multiple glycosidase activities have already been reported from various sources. Ratio of activities of β-D-galactosidase, β-D-fucosidase and α-L-arabinosidase exhibited by mung bean β-galactosidase are 100, 35.7 and 32.7% (Li et al., 2001). To a limited extent, carambola β-galactosidase also degraded arabino-galactoside (Balasubramaniam et al., 2005).

Fig. 3. Localisation of β-galactosidase enzyme in germinating seed (A) and radical (B). The β-galactosidase enzyme was spotted through X-gal staining.
3.8. Localisation of \( \beta \)-galactosidase in germinating seed

The localisation of \( \beta \)-galactosidase enzyme was spotted through activity staining with X-Gal. As X-gal itself is a colourless chemical, the presence of blue coloured product substantiates the presence of active \( \beta \)-galactosidase. The activity of \( \beta \)-galactosidase was mainly present in the seed coat and growing radical. Transverse section of seed also revealed...
low level of X-gal staining in the peripheral tissue (Fig. 3A). Epidermal and endodermal cells of radical were shown to be heavily stained by X-Gal staining (Fig. 3B). A potent $\beta$-galactosidase activity was also detected in the vascular bundle; whereas intercellular spaces of cortical cells also revealed moderate level of $\beta$-galactosidase activity. Pre-incubation with 2,4 dinitrophenol $\beta$-D-deoxy-2-fluoroglucoside, which completely inhibit $\beta$-glucosidase activity, did not bring any change in staining pattern. In addition, FeCl$_2$ treated seeds exhibited no X-Gal staining, signifying that the staining was exclusively due to $\beta$-galactosidase enzyme. Therefore, it is confirmed that the tissue specific X-Gal staining is a representation of targeted $\beta$-galactosidase localisation and it is unlikely to be due to the non-specific reaction of $\beta$-glucosidase. Cellular localisation also shed light on its physiological role in plants. The presence of $\beta$-galactosidase in cell wall signifies its association with cell wall degradation during germination and growth.

Sekimata et al. (1989) were first to demonstrate the localisation of $\beta$-galactosidase in the cell wall of radish radical, using X-gal as a substrate. Chantarangsee et al. (2007) also used X-gal staining to demonstrate the localisation of $\beta$-galactosidase in the embryo, aleuronic layer and surrounding tissues, and in the radical and shoot after germination.

3.9. Multiple sequence alignment

A full-length peptide sequence of CpGAL (Accession CAA10128) was used for BLAST (Basic Local Alignment Search Tool) using National Centre for Biotechnology Information website (http://
4. Conclusions

The specific activity of CpGal was found to be comparatively high as compared to other known β-galactosidases from plant sources. The purified preparation was also stable for a month at its optimal pH on storage. The kinetics of CpGal with such a high specific activity and other enzymatic properties indicate that it could serve as a new GRAS (Generally recognised as safe, a FDA of USA) material for lactose hydrolysis and galacto-oligosaccharide (GOS) production at an industrial scale. Thermal stability with wide range of pH and temperature operability also makes it more suitable to perform in adverse industrial environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.03.032.

References


