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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 (*Cp*GAL) 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 glycoproteineous 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 and seed coat. Sequence alignment of *Cp*GAL with other known plant β-galactosidase showed high amino acid sequence homology.

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, & Georgatsos, 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 (Biswas, 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: *Cp*GAL, chick pea β-galactosidase; ONPG, *o*-nitrophenyl-β-D galactopyranoside; PNPG, *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 (Giannakouros, Karagiorgos, & 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 (Giannakouros 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 degrading 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-Sephacel 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 (A_{405}) was proportional to the amount of enzyme in the reaction mixture under the assay conditions. The absorbance, A_{405}, 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 $4.05 \times 10^3\ M^{-1}cm^{-1}$).

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 H_2O and soaked in extraction buffer (25 mM sodium phosphate buffer, pH 6.8), for 24 h at 4 °C. Soaked seeds were homogenised using 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 bought 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