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Purification and Characterization of α-Galactosidase from White Chickpea (*Cicer arietinum*)

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ABSTRACT: Glycosylated α-galactosidase (melibiase) has been purified from white chickpea (*Cicer arietinum*) to 340-fold with a specific activity of 61 units/mg. *Cicer* α-galactosidase showed a M_r of 45 kDa on SDS-PAGE and by MALDI-TOF. The optimum pH and temperature with *p*NPGal were 4.5 and 50 °C, respectively. The K_m for hydrolysis of *p*NPGal was 0.70 mM. Besides hydrolyzing the *p*NPGal, *Cicer* α-galactosidase also hydrolyzed natural substrates such as melibiose, raffinose, and stachyose very effectively; hence, it can be exploited commercially for improving the nutritional value of soy milk. Galactose was found to be a competitive inhibitor. The property of this enzyme to cleave the terminal galactose residues can be utilized for converting the group B erythrocytes to group O erythrocytes.

KEYWORDS: *α-galactosidase, chickpea, MALDI-TOF, purification and characterization, erythrocytes*

INTRODUCTION

Glycosidases are enzymes that are involved in various important biological processes such as digestion, biosynthesis of glycoprotein, and catabolism of glycoconjugates.[1] α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) catalyzes the hydrolysis of terminal galactose residues and is widely distributed in microorganisms, plants, and animals. In plants α-galactosidase is maximally distributed in seeds, fruits, and leaves.[2−4] Several industrial applications of α-galactosidase are known, mainly in the sugar industry, where they improve the crystallization of sucrose by hydrolyzing the raffinose in beet sugar syrups.[5] In addition to this, the enzyme can also enhance the bleaching effect in the pulp and paper industry.[6] α-Galactosidase can also be used to improve the gelling properties of galactomannans used as food thickeners.[7] In humans, lysosomal α-galactosidase A is responsible for the metabolism of neutral glycosphingolipids that possesses α-galactosyl residues at the nonreducing terminus, predominantly globotriaosylceramide (Gb-3). Deficiency in enzyme activity results in Fabry disease, a lysosomal storage disorder caused by the accumulation of glycosphingolipids in body fluids and tissue lysosomes.[8] α-Galactosidase are known to remove the terminal galactose residues from glycans and thus are helpful in converting the B blood group to the more universally transferable type O group.[9]

Raffinose and stachyose (α-galactosyl derivatives of sucrose) are the main oligosaccharides responsible for flatulence production.[10] Because humans and other monogastric animals lack the enzyme α-galactosidase that cleaves the α-1,6-galctosyl linkages in their digestive tract, the intact oligosaccharide is not absorbed, and then these oligosaccharides accumulate in the large intestine, where anaerobic microorganisms ferment them and lead to flatus formation.[11] α-Galactosidase can be used for flatulence treatment.

Use of α-galactosidase for transglycosylation and reverse hydrolytic reactions for synthesis of α-galactosides is well-reported.[12] α-Galactosidase also plays a physiologically significant role in photoassimilate partitioning in cucurbit sink tissue.[3]

Chickpea belongs to the Fabaceae (or Leguminosae) family and is an excellent protein-rich source. Chickpeas are widely available and cheap, with high α-galactosidase activity. In this study, we report the purification and characterization of α-galactosidase from chickpea seeds and explore the possibility of using this enzyme for blood conversion and hydrolysis of Raffinose Family Oligosaccharides (RFOs).

MATERIALS AND METHODS

Plant Materials and Chemicals. Dry seeds of chickpea (*Cicer arietinum*) (Pusa 1053 variety) were purchased from a local market. They were surface sterilized with 1% (v/v) with sodium hypochlorite solutions for 10 min and then were washed thoroughly with Milli-Q water. All of the chemicals for buffers and other reagents were of analytical grade or electrophoresis grade. Unless otherwise specified, all of the chemicals and invertase (β-fructofuranosidase) were purchased from Sigma (St. Louis, MO, USA). Milli-Q water with a resistance of >18 MΩ cm was used throughout the experiments.

Enzyme Activity Assays. For routine analysis and monitoring of activity during purification steps, α-galactosidase was assayed as described by Malhotra and Dey[13] using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal) as substrate with certain modification. Aliquots of 0.1 mL of *p*NPGal (2 mM) and 0.5 mL of 50 mM acetate buffer (pH 4.5) were preincubated at 37 °C for 2 min before the addition of 0.1 mL of suitably diluted enzyme to initiate the reaction. The reaction was terminated after 10 min by adding 3 mL of 0.1 M Na_2CO_3, and the released *p*-nitrophenol was determined spectrophotometrically at 410 nm. The activity toward melibiose was estimated in a reaction mixture containing 500 μL of 50 mM phosphate buffer (pH 6.0), 100 μL of suitably diluted enzyme, and 400 μL of 0.1 M substrate solution. After an incubation of 20 min at 40 °C, the reaction was stopped by heating the reaction mixture in a boiling water bath for 2 min. The released glucose was estimated using a commercially available kit (Span Diagnostics Ltd.) based on a glucose oxidase−peroxidase

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(GOD-POD) method as described by Dwevedi and Kayastha.[14] Control assays without enzyme or without substrate were included. The activities against raffinose and stachyose were assayed for 20 min at 40 °C using a reaction mixture containing 600 μL of 50 mM sodium acetate buffer (pH 5.0) and phosphate buffer (pH 6.0), respectively; 100 μL of suitably diluted α-galactosidase enzyme; 400 μL of a 0.1 M substrate solution; and 100 μL of suitably diluted invertase enzyme. The amount of reducing sugar produced was determined by adding 1 mL of 3,5-dinitrosalicylate reagent according to the method of Miller.[15] In raffinose/stachyose assay control was made with sucrose. The data presented for all α-galactosidase activity determinations are mean values of triplicate assays, and the standard deviations for all values were < ±5%.

One unit of α-galactosidase activity was defined as the amount of enzyme liberating 1.0 μmol of product per minute under the assay conditions. The specific activity is expressed in units of enzyme activity per milligram of protein.

Protein Estimation. Protein concentration was estimated according to the method of Lowry et al.[16] using bovine serum albumin (BSA) as standard. Fractions obtained during the chromatographic purification were screened for protein by absorbance measurement at 280 nm.

Enzyme Extraction and Purification. All purification steps were carried out at 4 °C, and centrifugation was done at 8420*g* for 20 min, unless stated otherwise. Buffers used in each step included the following additives: 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethanesulfonyl fluoride (PMSF). In a typical batch of purification, 75 g of seeds of *C. arietinum* was soaked in extraction buffer (50 mM sodium phosphate buffer, pH 7.0) for 24 h at 4 °C. The imbibed chickpea seeds were homogenized using a laboratory blender in 150 mL of chilled extraction buffer and then squeezed through two layers of muslin cloth; a clear supernatant was obtained by centrifugation, and the cell debris was discarded. The supernatant was stored at 4 °C and used for purification.

Acid Precipitation. The pH of the supernatant was lowered to 4.0, by dropwise addition of chilled 0.1 M citric acid with continuous stirring, and then the supernatant was incubated for 24 h at 4 °C, which led to precipitation of unstable proteins, and centrifuged; the pellet was discarded. The supernatant's pH was adjusted to 7.0 by dropwise addition of chilled 0.2 M NaOH with continuous stirring.

Ammonium Sulfate Fractionation. The supernatant was made 40% ammonium sulfate saturated and centrifuged, and the pellet was discarded. The supernatant was then made to 60% ammonium sulfate saturation, and the pellet from ammonium sulfate precipitation was recovered by centrifugation, dissolved in the minimum volume of 50 mM phosphate buffer, pH 7.0, and dialyzed against the same buffer.

Hydrophobic Interaction Chromatography (HIC). After dialysis, the protein sample (4 mL) was loaded to an octyl-Sepharose CL-4B column (8 cm × 2.5 cm), pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 2 M ammonium sulfate. The column was then washed with the same buffer containing 1 M ammonium sulfate, and elution was carried out with the same buffer containing 0.5 M ammonium sulfate at a flow rate of 20 mL/h; 5 mL fractions were collected. The active fractions were pooled, concentrated by Amicon (Millipore, USA), and dialyzed against 50 mM sodium acetate buffer, pH 4.0.

Ion-Exchange Chromatography. The dialyzed enzyme was loaded onto a CM-Cellulose cation exchange column (4 cm × 2 cm) pre-equilibrated with 25 mM acetate buffer, pH 4.0. Elution was performed with an increasing gradient 0–0.2 M NaCl prepared in equilibration buffer at the flow rate of 40 mL/h, and 2 mL fractions were collected. The active fractions were pooled, concentrated by Amicon (Millipore, USA), and dialyzed against 50 mM sodium phosphate buffer, pH 7.0.

Affinity Chromatography. Finally, the protein sample obtained from ion-exchange chromatography was loaded onto a Con A-Sepharose 4B column (2.5 cm × 1 cm) pre-equilibrated with sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$, respectively. After elution of unbound proteins, the enzyme was eluted with the same buffer containing 25 mM α-methyl-D-mannoside at the flow rate of 20 mL/h, and 1 mL fractions were collected. The active fractions were pooled, concentrated as above, and stored at −20 °C.

Native Molecular Mass. Native molecular mass of the purified α-galactosidase was determined with size exclusion chromatography (SEC) using a Sephacryl S-200 HR 16/60 column on AKTA FPLC (GE Healthcare Biosciences Ltd.), which was pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl. Five marker proteins with known molecular mass (12.5–250 kDa) were used for column calibration. The proteins were eluted with the same buffer at a flow rate of 30 mL/h. Along with absorbance measurement at 280 nm, the activity peak was checked by assaying the enzyme activity using *p*NPGal as substrate.

SDS-PAGE. Protein homogeneity of purified α-galactosidase (15 μg) from chickpea was assessed by 12% SDS-PAGE.[17] The proteins were stained with 0.25% Coomassie Brilliant Blue R-250, and molecular mass was determined by comparing the relative mobilities of appropriate protein markers. Medium molecular mass protein markers (Sigma Chemicals Co., USA) ranging from 29 to 205 kDa were used.

Zymogram Analysis. At 4 °C native-PAGE (8%) was carried out for α-galactosidase (0.5–0.6 U). For activity staining, the gel was incubated at 4 °C for 4 h in 50 mM sodium acetate buffer, pH 4.5. The gel then was covered with filter paper soaked in 4-methylumbeliferyl-α-D-galactopyranoside (MUGal; 10 mM) solution prepared in 50 mM sodium acetate buffer, pH 4.5, and incubated for 20 min at 37 °C, and activity bands were visualized as fluorescent bands under UV transilluminator.

Carbohydrate Determination. The carbohydrate content in purified α-galactosidase was estimated using the phenol–sulfuric acid method.[18]

Glycoprotein Staining. Schiff's reagent was used for glycoprotein staining using the earlier reported protocol.[19]

α-Galactosidase Deglycosylation. Deglycosylation was performed using trifluoromethanesulfonic acid (TFMS).[20]

Isoelectric Focusing (IEF). For determining isoelectric point (p*I*) IEF was performed with an Ettan IPG phor 3 instrument (GE Healthcare Biosciences Ltd.) using an 11 cm IPG strip (pH range 3–10) and accompanying reagents by following the protocol described elsewhere.[21] Protein was visualized using Coomassie Blue.

Kinetic Studies. For all kinetic studies, dialyzed enzyme obtained after affinity chromatography was used. Kinetic experiments were performed at 37 °C. The pH optimum was determined at pH values ranging from 3.0 to 8.0. Buffers used were 50 mM glycine–HCl buffer, pH 3.0–4.0; 50 mM acetate buffer, pH 4.0–5.6; 50 mM sodium phosphate buffer, pH 5.7–7.0; and 50 mM Tris-HCl, pH 7.1–8.5. The temperature optimum was determined by measuring the enzyme activity in the range of 20–80 ± 1 °C for 10 min. Activation energy (E_a) was calculated from the slope of the curve using an Arrhenius plot.[22] For thermal inactivation, the enzyme was maintained at 60 ± 1 °C in a water bath (Multitemp, Pharmacia, Sweden). Small aliquots were withdrawn at different time intervals, chilled immediately, and tested for enzyme activity using routine assay. Michaelis–Menten constants (K_m) for *p*NPGal, melibiose, raffinose, and stachyose hydrolysis were calculated using Lineweaver–Burk plots. The substrate concentrations ranged from 0.2 to 2 mM for *p*NPGal, from 2 to 20 mM for melibiose, and from 10 to 100 mM for raffinose and stachyose. The inhibition constant (K_i) for galactose was calculated using a Dixon plot. For K_i calculation the *p*NPGal concentrations used were in the range of 0.5–2 mM and the galactose concentration ranged from 2 to 20 mM.

Effect of Various Metal Ions and Compounds on α-Galactosidase Activity. The effect of different inhibitors, simple sugars, reducing agent, and metal ions on the activity of purified enzyme was examined. Enzyme was incubated with metal ions (2 mM) and different compounds (2 mM) for 24 h and assayed as described for *p*NPGal under Enzyme Activity Assays.

Substrate Specificity. The enzyme was tested for its ability to hydrolyze various synthetic and natural substrates. The activities against synthetic substrates (2 mM each), *o*-nitrophenyl-α-D-

Table 1. Purification of α-Galactosidase from *Cicer arietinum* Seeds

step	total activity (units)	total protein (mg)	specific activity (units/mg)	purification (fold)	yield (%)
crude	1044.00	5738.00	0.18		
acid fractionation	949.00	1082.00	0.87	4.80	90.90
ammonium sulfate (40−60%)	638.00	345.00	1.85	10.20	61.10
octyl-Sepharose CL-4B	310.00	10.00	31.00	171.30	29.70
CM-cellulose	145.00	3.50	41.43	228.90	13.90
Con A-Sepharose 4B	50.00	0.81	61.73	341.10	4.80

galactopyranoside (*o*NPGal), *m*-nitrophenyl-α-D-galactopyranoside (*m*NPGal), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPβGal), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPβGal), *m*-nitrophenyl-β-D-galactopyranoside (*m*NPβGal), and *p*-nitrophenyl-β-D-xylopyranoside (*p*NPβXyl) were taken under standard condition as described earlier for *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal) under Enzyme Activity Assays. The activities toward natural substrates raffinose (50 mM), stachyose (40 mM), and melibiose (2 mM) were determined as described earlier under Enzyme Activity Assays.

Mass Spectrometry. *In-Gel Digestion.* Identification and determination of molecular masses were performed by matrix-assisted laser desorption mass spectrometry (MALDI; Applied Biosystems, USA). The protein bands were excised from the destained SDS-PAGE gel. Gel slices were cut into smaller pieces and washed with 50 μL of 25 mM ammonium bicarbonate (ABC) and dehydrated with 50 μL of a 2:1 mixture of acetonitrile (ACN)/50 mM (ABC) repeatedly. Gel pieces were treated with 20−50 μL of 10−20 mM DTT and kept at 60 °C for 1 h. DTT was removed by pipetting out the added liquid, and gel pieces were rinsed with 50 μL of 25 mM ABC. An amount of 20−50 μL of 50−100 mM iodoacetamide (IAM) was added, and gel pieces were incubated at room temperature in the dark for 10−20 min. IAM was removed by pipetting out the added liquid, and gel pieces were rinsed with 50 μL of 25 mM ABC. Gel pieces were dried in a Speed Vac before addition of the digestion buffer in which Trypsin Gold, mass spectrometry grade from Promega, was used in a ratio of 1:50 trypsin to sample ratio, diluted in 25 mM ABC so that the gel pieces remained covered with the buffer. The sample was incubated at 37 °C overnight for digestion. The peptides were extracted from the gel by 50 μL of 50% ACN and 0.1% trifluoroacetic acid (TFA) solution three times. The sample was desalted with Zip Tip C_{18} (Millipore) prior to MALDI analysis.

Database Searching. There is no report for the amino acid sequence of α-galactosidase from *C. arietinum* in an available protein database. Therefore, the ratio of *m*/*z* values obtained from MALDI-TOF spectra corresponding to peptides of *Cicer* α-galactosidase was matched with α-galactosidase from other plant sources using Mascot (www.matrixscience.com). The NCBInr database was searched with Viridiplantae (Green Plants) taxonomy. Comparative analysis is based on parameters such as digestion with trypsin with 1 missed cleavage and the rest is by default. During our search, all of the peptide masses were assumed to be monoisotropic with a mass accuracy of ±1.2 Da.

Treatment of Red Blood Cells. Group B red blood cells (RBCs) were treated with *Cicer* α-galactosidase under conditions as described by Liu et al.[9] with minor modifications. Standard enzymatic conversion reactions were carried out in 1 mL of reaction mixture containing isotonic phosphate-buffered sodium chloride, pH 6.0, red blood cells and enzyme (50 units of *Cicer* α-galactosidase). Fresh blood was collected from group B donors under standard conditions in the Blood Bank of S. S. Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi. Packed RBCs were prepared by centrifugation under conditions that removed platelets and most leucocytes from the unit (1250*g* for 4.8 min) followed by three washings in saline and four washings in isotonic phosphate-buffered sodium chloride buffer, pH 6.0, in 1:3 ratio before the addition of enzyme.[23] The RBC−enzyme mixture was incubated for 1 h with gentle mixing at 30 °C. The enzyme was then removed by repeated washing with the same buffer, pH 7.3. The enzymatically converted O RBCs (ECO) were ABO typed, using licensed monoclonal antibody reagents (Tulip Diagnostics (P) Ltd.), Goa.

Enzymatic Hydrolysis of Oligosaccharides. A mixture of 50 μL of purified enzyme (containing 2.5 units) and 50 μL of raffinose (100 mM) in 50 mM acetate buffer, pH 5.0, and stachyose (100 mM) in 50 mM phosphate buffer, pH 6.0, was incubated for various time periods at 40 °C. The hydrolysates were analyzed qualitatively by thin-layer chromatography (TLC). About 5 μL of each of the sugar extracts was applied to silica gel G plates (20 cm × 20 cm) and developed by ascending chromatography using *n*-propanol/ethyl acetate/water (6:1:3; v/v) as the solvent system.[24] The sugar spots were located by incubating the plates at 90−100 °C after spraying 1% α-naphthol in absolute ethanol containing 10% of orthophosphoric acid.[25]

RESULTS AND DISCUSSION

Purification and Biochemical Properties. As summarized in Table 1, α-galactosidase was extracted from the seeds of *C. arietinum* and was purified 340-fold to a final specific activity of 61 U/mg. The purification protocol for α-galactosidase is highly reproducible with respect to yield as well as specific activity. About 170-fold purification was achieved by using HIC (octyl-Sepharose CL-4B). Purified α-galactosidase from germinating soybean (*Glycine max*) showed a specific activity of 0.89 U/mg with 12.7-fold purification.[26] Kang et al.[27] reported purified α-galactosidase from grape flesh with a specific activity of 51.8 U/mg.

The molecular mass of the α-galactosidase was estimated to be 40 kDa by size exclusion chromatography and 45 kDa on SDS-PAGE under reducing conditions (Figure 1, lane b). A single band was also observed in the case of native-PAGE (Figure 1, lanes c,d). The purified protein also showed a molecular mass of 45.7 kDa by MALDI-TOF analysis. Thus, *Cicer* α-galactosidase is a monomeric protein. This was in good agreement with the other α-galactosidases purified from different sources.[28,29] *Cicer* α-galactosidase contains about 8−10% carbohydrate content. Kotwal et al.[30] reported α-galactosidase from *Humicola* sp. to have 8.3% carbohydrate content. Native-PAGE stained with Schiff reagent confirmed that the enzyme is a glycoprotein (data not shown). Probably because of this property, the protein band in SDS-PAGE was slightly diffused, and in the case of native-PAGE, the protein migrates as a broad band. This was also reported in the purification of α-galactosidase from *Aspergillus oryzae*.[31] Deglycosylation of *Cicer* α-galactosidase with TFMS marks the loss of activity, and no band was observed in zymogram analysis. This led to the conclusion that glycosylation is essential for *Cicer* α-galactosidase activity. The p*I* of *Cicer* α-galactosidase was 5.60 (data not shown). α-Galactosidase isolated from *Bacteroides ovatus*[32] showed the same p*I*.

Kinetic Studies. The maximum activity was observed at pH 4.5 with *p*NPGal for *Cicer* α-galactosidase in sodium acetate buffer (Figure 2). This was in good agreement with the other α-galactosidase purified from other sources.[29,33] *Cicer* α-galactosidase showed maximum activities with raffinose and

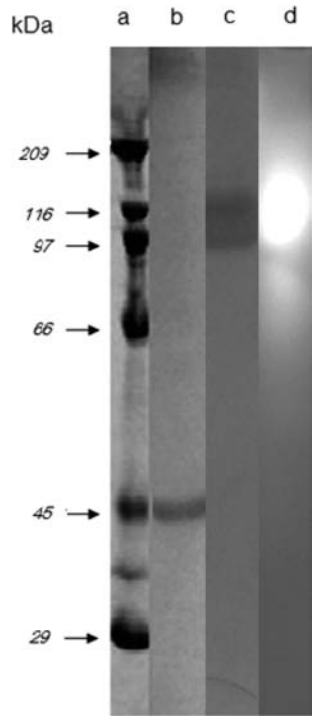

Figure 1. SDS-PAGE and zymogram of *Cicer* α-galactosidase. Lanes: (a) molecular size marker; (b) Coomassie staining of SDS-PAGE; (c) native-PAGE; (d) activity staining.

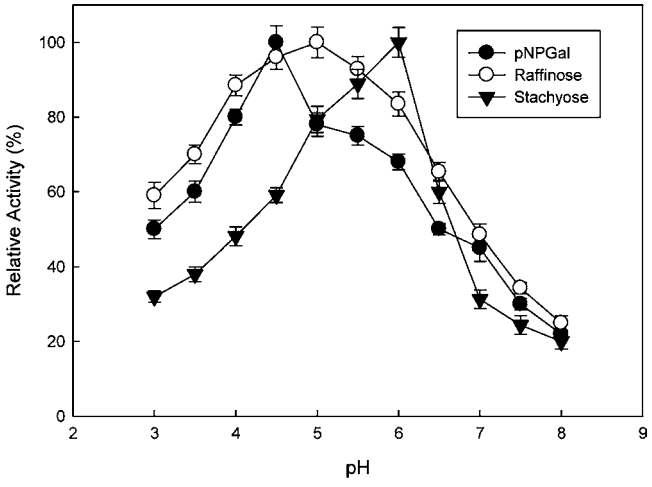

Figure 2. Effect of pH on the activity of *Cicer* α-galactosidase with *p*NPGal, raffinose, and stachyose.

stachyose at pH 5.0 and 6.0, respectively (Figure 2). α-Galactosidase from melon fruit showed pH optima of 5.0−5.5 with raffinose,[3] and grape flesh α-galactosidase has a pH optimum of 6.0 with stachyose.[27] The observed pH optimum shift in the case raffinose/stachyose was due to change in pK_a. The released *p*-nitrophenol has lower pK_a (7.1) than the pK_a of released glucose/fructose (12−12.5), which led to a shift of pH toward the acidic side with *p*NPGal. The variation of the pH with different substrates has been observed with some other glycosidases, such as β-galactosidase.[34]

The hydrolyzing activity of *Cicer* α-galactosidase was monitored at different temperatures from 20 to 80 °C. The rate of the enzyme-catalyzed reaction increases to an optimum value with increasing temperature until inactivation of the enzyme occurs. *Cicer* α-galactosidase showed maximal activity at 50 °C (Figure 3), and this optimum value is in good

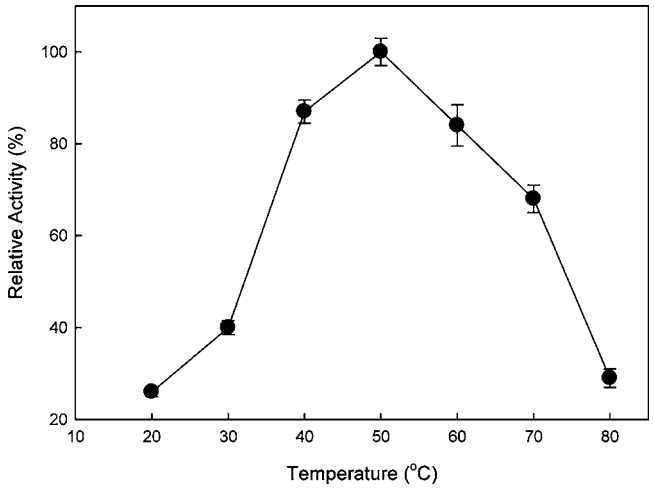

Figure 3. Effect of temperature on the activity of *Cicer* α-galactosidase.

agreement with reported α-galactosidases from other sources.[28,29] The energy of activation (E_a) was found to be 35.69 kJ/mol for *Cicer* α-galactosidase, similar to values reported from other sources.[35] *Cicer* α-galactosidase was quite stable at 50 °C, but at 70 °C it showed around 90% loss in activity (Figure 4).

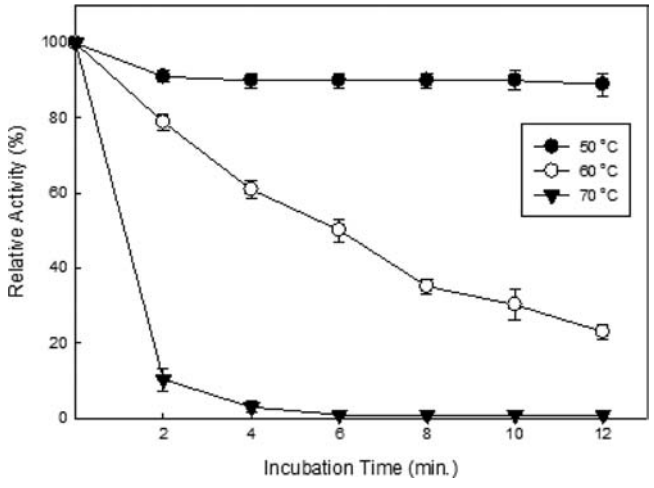

Figure 4. Effect of temperature on the stability of *Cicer* α-galactosidase.

The thermal inactivation at 60 °C followed first-order kinetics with $t_{1/2}$ equal to 6 min and first-order rate constant (k) equal to 0.115 min^{-1}.

Effects of Metal Ions and Various Compounds on *Cicer* α-Galactosidase Activity. The sensitivity of purified *Cicer* α-galactosidase to various group-specific reagents was tested (Table 2). Almost 88% activity was retained by the enzyme against *N*-ethylmaleimide and 76% activity retained on using iodoacetamide. This suggests that the enzyme does not have a sulfydryl group at its active site. This is in agreement with the α-galactosidase isolated from sweet almonds.[36] Thus, not all α-galactosidases require a −SH group for their activity. The inhibition due to *p*-chloromercuribenzoate (*p*-CMB) was probably because of the presence of mercuric ions present in it. The enzyme was completely inhibited by $Hg^{2+}$and Ag^{+}. The

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Table 2. Effect of Metal Ions and Various Compounds on the Activity of *Cicer* α-Galactosidase

effector[a]	relative activity[b] (%) ± SD
control	100 ± 0.92
$HgCl_2$	nd[c]
NaCl	98.0 ± 2.09
SDS	33.0 ± 0.50
PMSF	96.0 ± 1.73
$CaCl_2$	89.0 ± 1.29
N-ethylmaleimide	88.0 ± 2.02
KCl	99.8 ± 2.16
iodoacetamide	76.0 ± 1.90
Na_2SO_4	94.0 ± 1.65
KSCN	95.0 ± 1.81
$AgNO_3$	nd
p-CMB	5.0 ± 0.04
sucrose	91.0 ± 1.80
D-glucose	96.0 ± 2.04
D-galactose	85.0 ± 2.12
$MgCl_2$	97.0 ± 2.42
$MnCl_2$	97.0 ± 2.42
lactose	94.0 ± 1.88
$CoCl_2$	96.0 ± 1.82
EDTA	100.0 ± 1.50

[a]The final concentrations of all effectors were 2 mM. [b]Relative activities were calculated in relation to *p*NPGal activity, determined in the reaction without effectors, which was considered as 100%. [c]Not detected.

Ag^+ was probably due to its reaction with carboxyl and/or histidine residues. The Hg^{2+} strongly reacts with thiol group but in addition to this it is also known to react with amino and imidazolium groups of histidine and with peptide linkages.[37] A reduction in α-galactosidase activity by $Hg^{2+}$and Ag^+ has been reported for α-galactosidase isolated from *Tachigali multijuga* seeds.[28] From Table 2, it is evident that incubation with NaCl/KCl maintains the enzyme activity; this may have been due to a salt stabilization effect.[38] Other cations did not significantly increase or decrease the activity. The anionic detergent sodium dodecyl sulfate (SDS) was a powerful enzyme inhibitor. SDS is an extremely effective denaturing agent for proteins; in its presence, most proteins lose their activity due to the disruption of tertiary and quaternary structures.[39] SDS leads to loss of 70% of activity rather than complete loss (due to loss of tertiary structure). This may be due to the glycoproteinaceous nature of *Cicer* α-galactosidase, leading to poor binding activity of SDS. Sugars such as galactose, one of the products of the catalytic function of *Cicer* α-galactosidase on α-D-galactosides, were found to be potent competitive inhibitors with K_i of 1 mM as obtained from Dixon plot, whereas other sugars have no significant role in increasing or decreasing the activity. α-Galactosidases from *T. multijuga* seeds were also competitively inhibited by galactose.[28] EDTA did not inhibit *Cicer* α-galactosidase enzyme activity, indicating that *Cicer* α-galactosidase is not a metalloenzyme. This agrees with the result reported for α-galactosidase isolated from soybean seeds.[26] It is also evident that PMSF did not inhibit *Cicer* α-galactosidase, indicating the absence of a serine group at or near active site of enzyme.

Substrate Specificity. Purified *Cicer* α-galactosidase was used to hydrolyze various natural and synthetic substrates (Table 3). *p*NPGal was hydrolyzed most efficiently by the enzyme. The rate of hydrolysis was in the order *p*- > *o*- > *m*-nitrophenyl derivatives of α-D-galactopyranosides. This suggests that probably the para configuration of the substrate might make it better accessible to the active site of enzyme. The rapid hydrolysis of α-D-Gal and low hydrolysis of β-D-Gal suggest that the *Cicer* α-galactosidase is α-anomer specific. Natural substrates, such as melibiose, raffinose, and stachyose, were also good substrates for this enzyme (Table 4).

Table 3. Hydrolysis of Substrates by *Cicer* α-Galactosidase

substrate	concentration (mM)	relative activity[a] (%) ± SD
*p*NPGal	2.0	100.0 ± 0.25
*o*NPGal	2.0	40.0 ± 0.18
*m*NPGal	2.0	32.0 ± 0.20
*p*NPβGal	2.0	10.0 ± 0.10
*o*NPβGal	2.0	6.0 ± 0.08
*m*NPβGal	2.0	2.0 ± 0.05
raffinose[b]	50.0	42.5 ± 0.40
melibiose	2.0	62.1 ± 0.60
stachyose[b]	40.0	52.2 ± 0.65
*p*NPβXyl	2.0	nd[c]

[a]Relative activities were calculated in relation to *p*NPGal activity, which was considered as 100%. [b]Raffinose and stachyose at 2 mM concentration showed no activity. [c]Not detected.

Table 4. Kinetic Values for *Cicer* α-Galactosidase

substrate	K_m (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*p*NPGal	0.70 ± 0.02	52.50 ± 0.50	75.00
melibiose	10.0 ± 0.12	1.50 ± 0.05	0.15
raffinose	50.0 ± 0.35	0.50 ± 0.01	0.01
stachyose	40.0 ± 0.20	1.20 ± 0.02	0.03

[a]Turnover number (k_{cat}) is defined here as the number of moles of product s^{-1} mol^{-1} of α-galactosidase, given by the relationship $V_{max}/[E]_t$.

MALDI-TOF MS Analysis. High-quality MALDI-TOF peptide mass spectrum was obtained after tryptic digestion of *Cicer* α-galactosidase. The mass of the peptide was searched against relevant databases in NCBI. The most significant match was found with α-galactosidase from *Cucumis sativus* (gil 84310212). The matched peptide sequence was **TTNDINDSWES MISR** (230−244) with a score of 76.

Seroconversion of RBC Cells. The blood conversion was carried out at pH 6.0, because at acidic pH, cells were less stable and lysis of the cells might occur. Thus, seroconversion by *Cicer* α-galactosidase at pH 6.0 is a compromise between cell viability and enzyme activity. Enzyme cleaved the terminal α-linked galactose from blood group B, converting it to blood group O, which was checked by a blood testing kit. B ECO cell population showed almost no reactivity with anti-B (Figure 5). By using *Cicer* α-galactosidase, enzymatic conversion of type B to type O erythrocytes can be done conveniently.

Enzymatic Hydrolysis of Oligosaccharides. Raffinose and stachyose were digested for 20, 40, 60, and 120 min, respectively, showing that the amount of complex oligosaccharides decreased, whereas the spots of sucrose on TLC became more apparent (Figure 6). Thus, it can be used for improving the nutritional value of soy milk, which is commonly used by persons who experience lactose intolerance. Many α-galactosidases from microorganisms have been reported to degrade these complex oligosaccharides from soy milk,[40] but

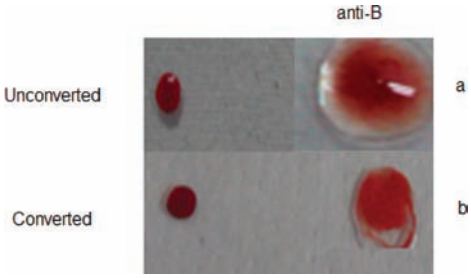

Figure 5. Blood group test before and after enzymatic conversion. Unconverted RBCs of group B blood agglutinated strongly with anti-B (a) but did not agglutinate after enzymatic conversion (b).

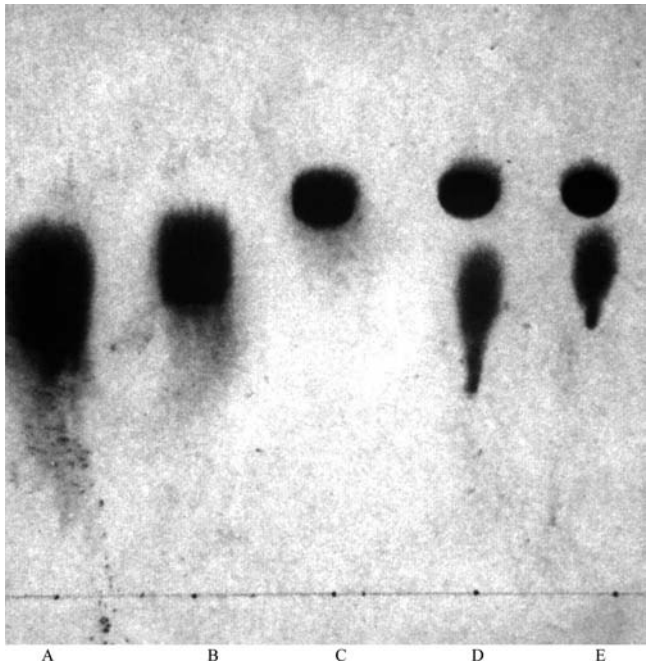

Figure 6. Thin-layer chromatography of the hydrolysis of raffinose and stachyose: (A) standard stachyose; (B) standard of raffinose; (C) standard sucrose; (D) enzymatically treated stachyose; (E) enzymatically treated raffinose.

these enzymes do not have GRAS (Generally Regarded As Safe) status. Therefore, *Cicer* α-galactosidase may prove to be a better alternative.

In conclusion, *Cicer* α-galactosidase was purified to homogeneity from *C. arietinum*. The enzyme was acid stable, and the production of viable group O cells by using enzyme offers great opportunity in the management of blood inventories. This will not only help in maintaining a continuous supply of the O (universal) blood group but also make greater use of the type B blood group, which is sometimes unused and discarded. Here, a simple method for the conversion of B blood group to O blood group has been presented. *Cicer* α-galactosidase can also be used for increasing the nutritional value of soy foods.

SAFETY

Acrylamide is neurotoxic and carcinogenic; therefore, it was handled with safety gloves and discarded only in polymerized form. All other experiments were carried out with maximum precaution using only Milli-Q water.

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