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Extraction, Purification and Thermodynamic Characterization of Almond (*Amygdalus communis*) β-Galactosidase for the Preparation of Delactosed Milk

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Summary

Buffer type, pH and ionic strength, as well as the fraction of polyvinylpyrrolidone were optimized for efficient extraction of β-galactosidase from almond seeds. The enzyme was purified up to electrophoretic homogeneity employing $(NH_4)_2SO_4$ (15–60 %) fractionation, size exclusion and ion-exchange chromatography. Molecular mass of β-galactosidase as estimated by gel filtration and SDS-PAGE was approx. 62 kDa, confirming its monomeric nature. The optimum activity of the enzyme was at pH=5.5, and it was stable within the range of pH=5.0–6.0. Various kinetic parameters of β-galactosidase thermal inactivation were calculated: $\Delta H°$, $\Delta S°$ and $\Delta G°$ suggested that the enzyme undergoes significant processes of unfolding during denaturation. Using β-galactosidase from almond seed powder, lactose hydrolysis in milk up to approx. 50 % was observed. The findings indicate the potential use of almond seeds for the production of low/delactosed milk for lactose-intolerant population.

Key words: almond, β-galactosidase extraction, lactose intolerance, thermodynamic characterization

Introduction

Lactose, the principal carbohydrate in milk occurring at a fraction of approx. 5 %, has attracted the attention of researchers and dairy industry because of its associated nutritional (lactose intolerance), technological (crystallization) and environmental (whey disposal) problems (*1*). Lactose malabsorption/intolerance is common among approx. 70 % of the world's adult population and it is caused by the intestinal insufficiency of the enzyme β-galactosidase/lactase, EC 3.2.1.23 (*2,3*). Deficiency of this enzyme causes the accumulation of undigested lactose in small bowel, leading to increased influx of fluids inside the intestinal lumen. The unabsorbed lactose is passed into the large intestine, where in addition to increasing fluid volume of gastrointestinal content, is metabolized by the colonic bacteria, resulting in the production of short chain fatty acids and hydrogen gas, and associated symptoms of lactose intolerance (*4*).

Besides lactose, milk and dairy products provide calcium, protein, potassium and riboflavin in the human diet. Due to the gastrointestinal discomfort experienced after lactose consumption, lactose-intolerant individuals often avoid dairy products and thus eliminate a major source of calcium and energy from their diet, thereby inviting other complications like osteoporosis (*3*). However, the problem can be solved by removing lactose from the diet by the addition of exogenous β-galactosidase enzyme. The major products of hydrolysis, glucose

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and galactose, are sweeter, more soluble and more readily absorbed into the mammalian intestine (*5*).

β-Galactosidase is widely distributed in nature, it is found in numerous microorganisms, plant and animal tissues (*1,6,7*). However, before an enzyme preparation can be used in a food system, it must be granted GRAS (generally recognized as safe) status (*8*). Although various β-galactosidases have been granted GRAS status and purified from many microorganisms for the preparation of delactosed milk, many of them were inhibited by Ca^{2+}, an intrinsic milk component occurring at the concentration of 30 mM, indicating that lactose hydrolysis with these preparations is not effective (*1*). Therefore, to improve commercial applications, other sources are being explored in the search of a robust enzyme.

Almonds (*Amygdalus communis*) have been traditionally consumed with milk in India. While working on lactose intolerance, we found that almonds are very rich source of β-galactosidase. Although preparation of lactose-free milk has been tried using salt-fractionated almond proteins (*9*), to exploit the potential of an enzyme efficiently, it is necessary to absolutely purify and thoroughly characterize the enzyme in terms of its biochemical properties. Hence, in this study β-galactosidase from almond seeds has been extracted, purified and characterized in order to explore the possibility of preparation of delactosed milk for lactose-intolerant population using the purified enzyme.

Materials and Methods

Materials

Almond seeds were procured from the local market while milk was obtained from the Nandini Dairy, Mysore, Karnataka, India. Unless indicated otherwise, all chemicals used in the present investigation were of high quality analytical grade and were purchased from Sigma-Aldrich, St. Louis, MO, USA, and Sisco Research Laboratory Pvt. Ltd., HiMedia Laboratories Pvt. Ltd. and E. Merck Ltd., all from Mumbai, India.

Enzyme assay

The procedure described in our earlier report (*1*) was used for the estimation of enzyme activity. Hydrolytic activity of β-galactosidase was determined by measuring the release of *o*-nitrophenol (ONP) from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and the enzyme activity was expressed in international units (IU) where one IU is defined as the amount of enzyme that liberates 1.0 μmol of ONP per minute under the assay conditions. All the experiments were done in triplicate and the results are expressed as mean values±standard deviations (S.D.).

Optimization of conditions for β-galactosidase extraction

β-Galactosidase from almonds was extracted in buffers of different pH values such as sodium acetate (pH=4.0–6.0), sodium phosphate (pH=6.5–7.5) and Tris-HCl (pH=8.0) at 50 mM ionic strength. The enzyme activity was determined by extracting the enzyme with acetate buffer (pH=5.0) in a range of ionic strength of 50–200 mM. β-Galactosidase activity was determined by extracting the enzyme in different fractions of polyvinylpyrrolidone (PVP) in the range of 0.25–1.0 %.

Purification of enzyme

The crude enzyme was saturated with $(NH_4)_2SO_4$ (15–60 %), stored overnight at 4 °C, centrifuged at 10 000×*g* for 10 min and precipitates were dissolved in 0.1 M acetate buffer (pH=5.0). To make it free from sulphate ions, the $(NH_4)_2SO_4$ fraction was dialyzed using cellulose tubing (molecular mass cut-off of 10 kDa) against acetate buffer for 24 h with repeated changes of the buffer. The dialysate was concentrated by dialysis against solid sucrose and applied to a diethylaminoethyl (DEAE)-cellulose column (2.5×45 cm; Sigma-Aldrich) equilibrated with 0.1 M acetate buffer (pH=5.0). After the elution of unbound proteins, the bound proteins were eluted with a linear gradient of 0.5 M KCl in the same buffer at a flow rate of 30 mL/h and 3-mL fractions were collected. The fractions showing β-galactosidase activity were pooled and dialyzed against acetate buffer for 16–18 h. The dialyzed enzyme solution was concentrated and applied to a Sephadex G-100 column (85×1.5 cm; Sigma-Aldrich). The proteins were eluted with the acetate buffer (pH=5.0) at a flow rate of 20 mL/h and 2-mL fractions were collected. The active fractions were pooled, their molecular masses were determined and they were used in the kinetic study. The amount of protein in various samples was determined by Lowry method (*10*) using bovine serum albumin as the standard.

Molecular mass determination

The molecular mass of native β-galactosidase was determined by gel filtration on a Sephadex G-100 column. Using the proteins of known molecular masses, a calibration curve was first prepared and from the elution volume, the molecular mass of β-galactosidase was deduced. The molecular mass standards used were: alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

The molecular mass under denaturing conditions was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a gel system (Bio-Rad Laboratories, Hercules, CA, USA). The SDS-PAGE was performed with a 5 % acrylamide stacking gel (pH=6.8) and 10 % separating gel (pH=8.8) (*11*). Proteins were visualized by staining with Coomassie Brilliant Blue. The molecular mass of the β-galactosidase was calculated from the relative mobility of molecular mass markers run simultaneously.

Determination of kinetic parameters

To determine the maximum velocity (v_{max}) and Michaelis-Menten constant (K_m) of β-galactosidase, initial reaction rates were measured by using ONPG substrate at different concentrations (0–18 mM). K_m and v_{max} values were calculated by means of the Lineweaver-Burk plot (*12*).

Effect of pH and temperature on the activity and stability of β-galactosidase

The effect of pH on the activity of purified enzyme was determined in 50 mM HCl-KCl buffer (pH=2.0), glycine-HCl buffer (pH=2.5–3.0), citrate buffer (pH=3.5–5.5) and phosphate buffer (pH=6.0–8.0) using ONPG as the substrate. The optimum pH value obtained from these assays was used in all the other experiments. The effect of pH on the stability was performed by preincubation of the enzyme overnight at various pH values (pH=2.0–8.0).

Optimal temperature for β-galactosidase activity was determined by incubating the enzyme solution with the substrate in 100 mM acetate buffer (pH=5.5) for 30 min at various temperatures. The activation energy (E_a/(kJ/mol)) of the catalysis of β-galactosidase was determined from the slope of a plot between ln$v$ and 1/T (K) using the Arrhenius equation:

$$\text{Slope} = \frac{E_a}{R} \qquad /1/$$

where R is the universal gas constant.

The thermal stability of β-galactosidase was investigated at seven different temperatures between 40 and 60 °C for various periods of time in a temperature-controlled water bath. The enzyme solution was placed in a pre-warmed tube at the specified temperature and aliquots were withdrawn at 30-minute intervals, cooled and the residual activity was assayed. The stability of the enzyme was expressed as percentage of residual activity (RA/%). The incubation was carried out in sealed vials to prevent the change of volume of the sample, and hence the enzyme concentration due to evaporation. The data obtained from the thermal stability profile were used to analyze thermodynamic parameters related to the β-galactosidase activity. The experimental points were plotted according to the equation given below:

$$\ln\frac{A}{A_0} = k_d \cdot t \qquad /2/$$

where A_0 is the initial activity, A is the residual activity after heat treatment, k_d is thermal inactivation rate constant (min^{-1}) and t is the exposure time (min).

The half-life of β-galactosidase ($t_{1/2}$/min^{-1}) was determined from the following relationship:

$$t_{1/2} = \frac{\ln 2}{k_d} \qquad /3/$$

The D value (decimal reduction time or time required to preincubate the enzyme at a given temperature to maintain 10 % residual activity) was calculated from the following relationship:

$$D = \frac{\ln 10}{k_d} \qquad /4/$$

The z value (temperature rise necessary to reduce the D value by one logarithmic cycle) was calculated from the slope of the graph between log$D$ *vs.* temperature (°C) using the equation:

$$\text{Slope} = -\frac{1}{z} \qquad /5/$$

The activation energy for denaturation (E_d) of β-galactosidase was determined by an Arrhenius plot of log denaturation rate constants (lnk_d) *vs.* reciprocal of the absolute temperature T (K) using the equation:

$$\text{Slope} = -\frac{E_d}{R} \qquad /6/$$

The change in enthalpy [$\Delta H°$/(kJ/mol)], free energy [$\Delta G°$/(kJ/mol)] and entropy [$\Delta S°$/(J/(mol·K)] for thermal denaturation of β-galactosidase was determined using the following equations:

$$\Delta H° = E_d - RT \qquad /7/$$

$$\Delta G° = -RT \cdot \ln\frac{k_d\, h}{k_B\, T} \qquad /8/$$

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T} \qquad /9/$$

where E_d is the activation energy for denaturation (kJ/mol), T is the corresponding absolute temperature (K), R is the universal gas constant [(8.314 J/(mol·K)], h is the Planck constant ($11.04 \cdot 10^{-36}$ J/min), k_B is the Boltzman constant ($1.38 \cdot 10^{-23}$ J/K) and k_d is the deactivation rate constant (min^{-1}).

Effect of metal ions, chelator and reducing agents

The effect of various metal ions, chelator (EDTA) and reducing agents on β-galactosidase activity was determined by incorporating them in the standard assay mixture with the final concentrations ranging from 5 to 30 mM. The activity was expressed as relative activity compared to control.

Individual and combined effect of end products

To study the individual effect of end products, glucose and galactose were added into the reaction mixture in the range of 20–140 mM while their equimolar mixture (1:1) was added in the range of 40–280 mM to evaluate their combined effect on the enzyme activity.

Hydrolysis of milk lactose using purified β-galactosidase and almond seed powder

Skimmed milk was prepared by centrifuging cold milk at 10 000×g for 15 min. The fat layer was removed and treated with purified β-galactosidase (100 IU per 100 mL). In a separate set of experiments, different mass fractions of almond seed powder were tried for milk lactose hydrolysis at different temperatures. Aliquots of the reaction mixture were removed at different time intervals and hydrolysis was terminated by placing the samples in a boiling water bath for 5 min. The hydrolysis of lactose was calculated by estimating the amount of glucose released by the method described by Joshi *et al.* (*13*).

Results and Discussion

Effect of buffer pH, ionic strength, and PVP fraction on the extraction of β-galactosidase

Initially, the extraction was performed in phosphate buffer (0.05 M, pH=7.0). Once the enzyme activity was detected and the enzyme assay was developed, the extraction conditions were optimized to yield maximum β-galactosidase activity by manipulating buffer pH, ionic strength and PVP fraction.

Results pertaining to the extraction of the enzyme in buffers of different pH values are shown in Fig. 1a. As maximum activity was extracted in acetate buffer (0.05 M, pH=5.0), it was considered as the best extractant. Enzyme extraction in alkaline pH showed diminished activity, indicating the labile nature of the enzyme in alkaline conditions.

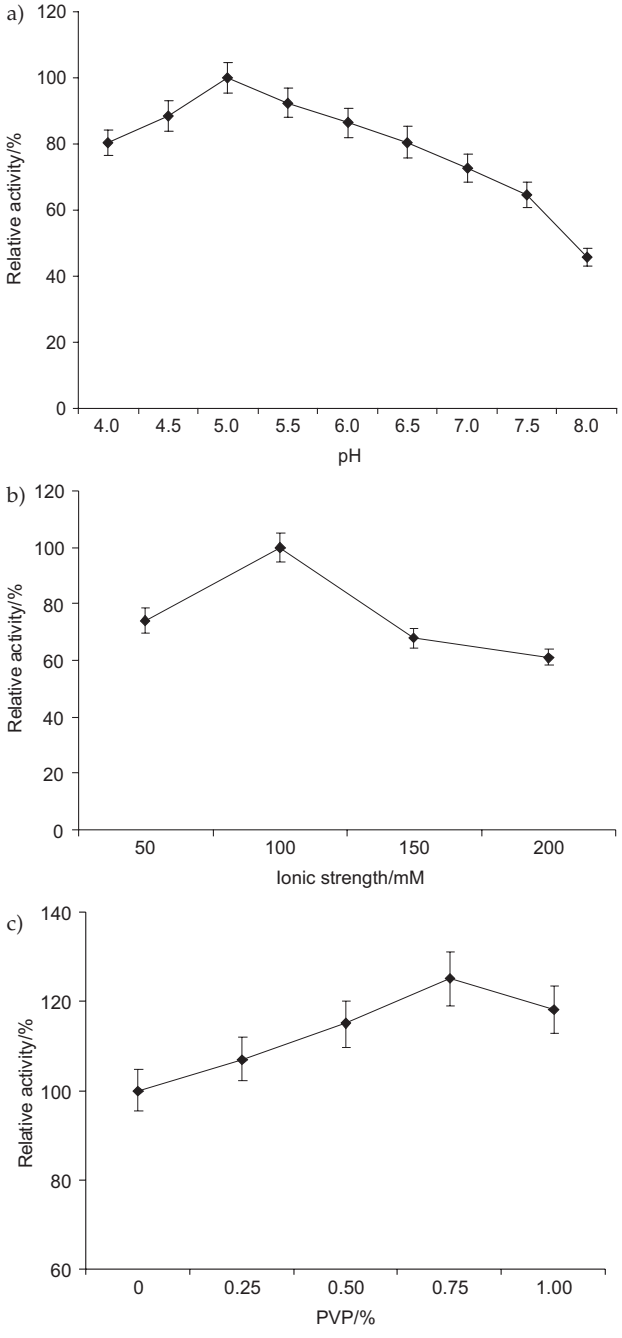

Fig. 1. Optimization of the extraction of β-galactosidase. The effect of: a) buffer pH, b) ionic strength, and c) PVP fraction on the extraction and activity of β-galactosidase

Acetate buffer (100 mM, pH=5.0) was found to be the best extractant for the almond β-galactosidase (Fig. 1b). However, the ionic strength required in the present study was high compared to that required for the extraction of other plant enzymes (*14,15*).

Most of the plant products are good sources of polyphenols, which are known to form insoluble complexes with enzymes. The complexes in turn reduce the extraction and activity of enzymes. PVP helps in the absorption of phenolic compounds after tissue disintegration and acts as enzyme stabilizer. Therefore, its effect on the extraction of β-galactosidase was studied and the results are shown in Fig. 1c. The maximum enzymatic activity was observed in the presence of 0.75 % PVP in the extraction mixture. The protective effect of PVP during enzyme extraction had also been observed in earlier studies (*16*).

Purification and characterization of β-galactosidase

Although numerous β-galactosidases have been purified from microorganisms (*1,3*), only few reports are available from plant sources. Moreover, the plant β-galactosidases have been studied for deciphering their role in plant growth and development and not for the production of delactosed milk. The β-galactosidase I and II were purified 7.4- and 53.5-fold from cowpea cotyledons (*17*), and more recently, another β-galactosidase has been purified from barley (*Hordeum vulgare*) by Hemavathi and Raghavarao (*18*).

In this work the β-galactosidase from almond seeds was purified up to electrophoretic homogeneity using a three-step chromatographic procedure. A summary of purification procedures is presented in Table 1. The crude enzyme extract having total activity of 146.8 IU and specific activity of 0.09 IU/mg was precipitated with $(NH_4)_2SO_4$ (15–60 %) at 4 °C. During this step, the enzyme was purified 4.2-fold with 81.54 % recovery. The precipitates recovered were then dissolved in 0.1 M acetate buffer (pH=5.0), dialyzed against the same buffer (diluted 1:10) and concentrated using solid sucrose. The concentrate was loaded onto the ion-exchange column and eluted with a linear gradient of 0.5 M KCl after the removal of unbound proteins. The enzyme got eluted at 0.18 M KCl and the level of recovery and purification in this step were 52.04 % and 26.3-fold, respectively. The active fractions were finally loaded on a pre-equilibrated Sephadex G-100 column for gel filtration chromatography. Elution profiles of the proteins and enzymes on DEAE-cellulose and Sephadex G-100 columns are presented in Fig. 2. This purification scheme resulted in the final enzyme preparation which was purified 50.9-fold with 33.72 % recovery of the total activity as compared to crude extract.

β-Galactosidases of different molecular masses have been reported from plant sources. Five isoenzymes of molecular masses of 87, 87, 87, 73 and 45 kDa were reported from mung bean seedlings (*19*), while a 45-kDa β-galactosidases were purified from radish seeds (*20*).

Table 1. Purification and activity of β-galactosidase

Fraction	Total activity	*m*(total protein)	Specific activity	Purification	Yield
	IU	mg	IU/mg	fold	%
Crude extract	146.8	1642.2	0.09	1	100
Ammonium sulphate precipitation (15–60 %)	119.7	314.5	0.38	4.2	81.54
Ion-exchange chromatography	76.4	32.2	2.37	26.3	52.04
Gel permeation chromatography	49.5	10.8	4.58	50.9	33.72

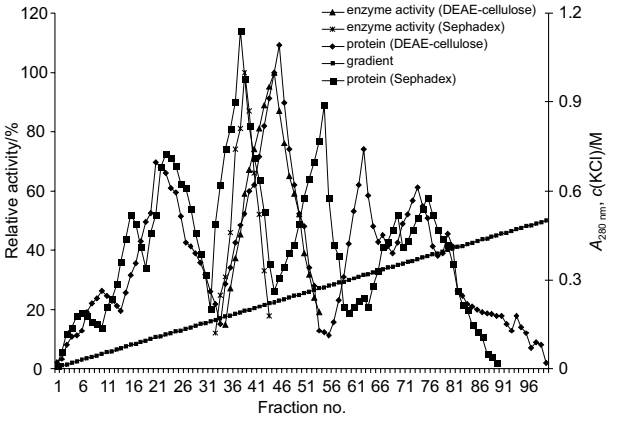

Fig. 2. Elution profile of β-galactosidase on DEAE-cellulose and Sephadex G-100 columns

The molecular mass of purified β-galactosidase from almond seeds as determined by gel filtration was approx. 62 kDa. The purified preparation yielded a single protein band on SDS-PAGE (Fig. 3) and migrated with an apparent molecular mass of approx. 62 kDa, as determined by relative mobility. The molecular mass determined by gel filtration and SDS-PAGE confirms the monomeric nature of β-galactosidase from almonds.

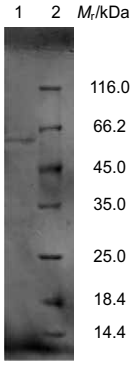

Fig. 3. SDS-PAGE analysis of purified β-galactosidase. Lane 1: purified β-galactosidase, lane 2: molecular mass marker

Kinetic parameters, thermal and pH activity and stability of β-galactosidase

With increasing concentration of substrate in an otherwise standard assay mixture, the enzyme showed a typical hyperbolic velocity saturation curve revealing that it followed Michaelis-Menten kinetics. From the double reciprocal Lineweaver-Burk plot, K_m of the enzyme for ONPG was calculated as 10.53 mM with a v_{max} of 2.67 IU/mL.

The enzyme was highly active in a wide range of pH, showing more than 50 % of the maximum activity in the pH range from 3.5 to 6.5. The optimum pH of the purified β-galactosidase was 5.5 (Fig. 4a). The results indicate that the enzyme was quite stable over the broad pH range and is suitable for hydrolysis of lactose present in whey or milk where pH varies from 4.5 to 6.8. It has been reported earlier that the pH optima of plant β-galactosidases are in the acidic pH range while those derived from bacteria are in the neutral pH range (*21*).

A pH stability study is an essential part of any enzyme characterization before it can be exploited commercially. The results show that pre-incubation of β-galactosidase in the pH range from 5.0 to 6.0 did not have any effect on the enzyme activity measured at pH=5.5. However, pre-incubation at 5.0>pH>6.0 resulted in decreased activity of β-galactosidase (Fig. 4a). Thus, the decline in the activity between pH=5.0 and 6.0 must result from the formation of an improper ionic form of β-galactosidase and/or substrate. When the enzyme is pre-incubated at pH>6.0 or pH<5.0, full activity is not regained at pH=5.5. Thus, part of the decline in the activity above pH=6.0 and below pH=5.0 results from irreversible enzyme inactivation. Reports are available about the pH stability of β-galactosidase but without interpretation.

The initial reaction rates were determined at temperatures between 30 and 80 °C. The enzyme activity increased with the increase in temperature with maximum activity at 60 °C (Fig. 4b). On further increase in temperature, the enzyme activity declined gradually and at 80 °C, the enzyme exhibited 21 % of the maximum activity. The loss of activity of the enzyme at higher temperatures could be attributed to its unfolding and subsequent loss of active site (*9*). A similar optimum temperature for catalysis has been reported for β-galactosidases of *Vigna unguiculata* (*22*), while the isozymes of β-galactosidase from mung bean seedlings have been shown to exhibit temperature optima in the range of 50–53 °C (*19*). Activation energy (E_a) calculated using the Arrhenius plot was 10.23 kcal/mol (42.86 kJ/mol), which is in the range that is characteristic of a typical enzymatic reaction. It was also obvious from the Arrhenius plot that the enzyme had a single conformation up to the transition state.

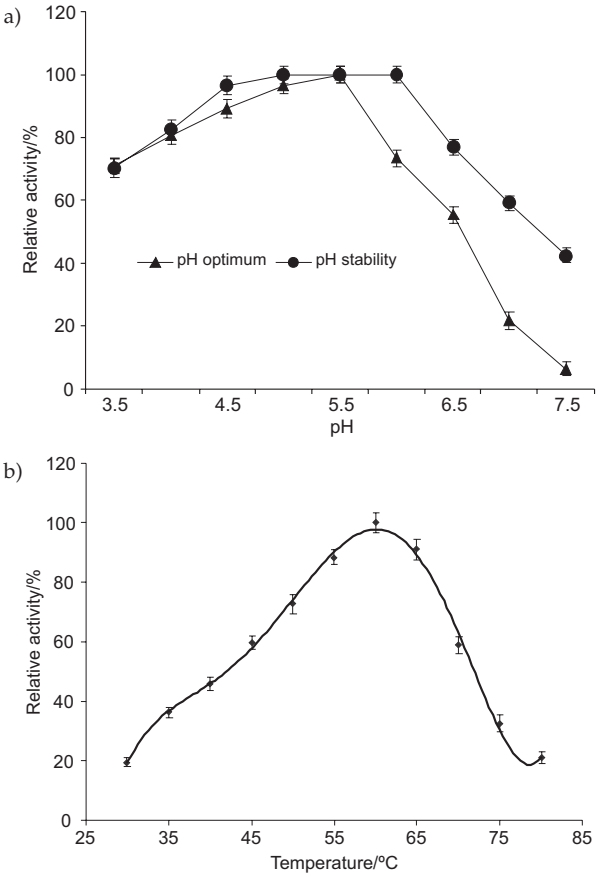

Fig. 4. Activity and stability of β-galactosidase activity influenced by: a) pH (9 mM ONPG, 0.1 M buffer) and b) temperature

β-Galactosidase showed no loss in the activity after incubation at 35 °C during the test period of 6 h. At 40 °C and above, the activity decreased with increasing temperature. The plots of residual activity *vs.* incubation time for the enzyme were linear, with R^2>92 %, indicating that the inactivation could be expressed as first order kinetics in the temperature range of 40–60 °C (Table 2). The half-life ($t_{1/2}$) determinations are more accurate and reliable especially when computing the stability properties of an enzyme at different temperatures. With increasing temperature, the $t_{1/2}$ and D value decreased and the first order thermal deactivation rate constants (k_d) increased (Table 2). It is clear from the results that the enzyme is less thermostable at higher temperatures. A higher rate constant means the enzyme is less thermostable (*23*). The z value of β-galactosidase, calculated from the slope of the graph between log$D$ *vs.* temperature, was 15.8 °C. In general, high z value indicates more sensitivity to the duration of heat treatment, while lower z value indicates more sensitivity to the increase in temperature (*24*).

The activation energy (E_d) of the thermal inactivation mechanism is equal to 29.72 kcal/mol (124.53 kJ/mol). The higher value found for the E_d in comparison with E_a means that a higher amount of energy is needed to initiate denaturation as compared to catalysis (*24*). The enzyme had an enthalpy of denaturation ($\Delta H°$) in a range of 121.93 to 121.76 kJ/mol at 40–60 °C, showing a decreasing trend with the increase in temperature (Table 2). The high values of $\Delta H°$ obtained for the thermal inactivation of β-galactosidase indicate that the enzyme undergoes a considerable change in conformation during denaturation (*25*). The fact that $\Delta H°$ value decreases with the increase in temperature reveals that less energy is required to denature the enzyme at high temperature (*26*). The value of free energy of thermal denaturation ($\Delta G°$) for β-galactosidase was 104.96 kJ/mol at 40 °C, which initially increased and then decreased with the increase in temperature. When entropy of inactivation ($\Delta S°$) was calculated at each temperature, it showed positive values, which indicates that there are no significant processes of aggregation, since had this happened, the values would have been negative (*25*). In contrast to the present study, we have earlier reported that xylanase purified from *Aspergillus niger* DFR-5 undergoes significant processes of compaction/aggregation with increase in the temperature (*27–29*). A decreasing $\Delta H°$ and negative $\Delta S°$ with an increase in the temperature has also been observed by Rajoka and Riaz (*30*) in xylosidase of parent and mutant *Kluyveromyces marxianus* PPY 125. It is also worth mentioning that ΔG° values, which are measures of the spontaneity of inactivation processes, are lower than the $\Delta H°$ values. This is due to the positive entropic contribution during the inactivation process (*31*).

Influence of metal ions, chelator and reducing agents on the activity of β-galactosidase

The divalent metal ions such as Ca^{2+} and Mn^{2+} stimulated the enzyme activity by 62 and 49 %, respectively, while monovalent cations like Na^+ and K^+ had low

Table 2. Kinetic and thermodynamic parameters of β-galactosidase inactivation

Temperature	k_d	R^2	$t_{1/2}$	D	$\Delta H°$	$\Delta G°$	$\Delta S°$
°C	min^{-1}	%	min	min	kJ/mol	kJ/mol	J/(mol·K)
40	0.0012	99.7	577	1919	121.93	104.96	54.21
45	0.0015	99.1	462	1535	121.89	106.09	49.68
50	0.0040	92.8	173	576	121.85	105.16	51.67
55	0.0071	95.7	98	324	121.80	105.26	50.43
60	0.0208	93.3	33	111	121.76	103.94	53.51

k_d=thermal inactivation rate constant, R^2=coefficient of correlation, $t_{1/2}$=half-life, D=decimal reduction time, $\Delta H°$=variations in enthalpy, $\Delta G°$=variations in free energy, $\Delta S°$=variations in entropy

Table 3. Effect of metal ions, chelator and reducing agents on β-galactosidase activity

Additives	Enzyme activity/%					
	c/mM					
	5	10	15	20	25	30
Ca^{2+}	122.8±7.8	124.6±6.2	126.5±4.7	162.2±12.2	ppt	ppt
Mn^{2+}	121.3±5.4	129.3±5.8	138.8±6.2	149.1±8.9	148.5±6.2	147.0±8.5
Mg^{2+}	100.5±5.2	101.3±4.3	104.7±4.9	110.1±4.6	119.9±5.4	123.4±3.9
K^{+}	102.5±4.9	106.8±5.8	109.8±3.8	112.5±4.8	115.8±4.9	119.4±5.2
Na^{+}	100.9±5.2	104.3±4.2	106.2±4.1	108.1±6.2	111.5±6.7	112.8±4.2
Li^{+}	92.6±4.6	86.3±6.2	83.9±3.7	78.8±4.7	72.9±4.3	64.2±3.7
EDTA	79.5±3.4	67.2±4.8	60.4±4.2	54.8±2.9	49.8±3.4	46.7±2.8
ascorbic acid	102.5±6.7	106.8±7.1	110.4±4.8	109.6±7.5	108.7±7.2	109.2±4.9
vanillin	104.8±5.9	109.1±3.8	112.6±4.2	115.5±4.9	116.8±5.8	114.8±8.2
cysteine	102.6±4.8	104.8±4.8	103.8±7.5	104.2±3.8	103.8±4.6	104.3±6.4
glutathione	101.5±6.1	103.8±7.5	104.8±6.4	105.2±4.8	105.8±6.1	105.2±5.7
β-ME	100.9±5.6	101.5±6.4	100.8±4.8	101.5±3.7	101.3±4.8	100.7±6.7
DTT	106.9±3.8	105.8±4.8	104.6±6.4	105.6±5.8	104.9±2.9	105.8±6.7

Values are mean±S.D. of 3 experiments
ppt=assay mixture was precipitated, β-ME=β-mercaptoethanol, DDT=dithiothreitol

stimulating effect on the activity at various concentrations. Li^{+} was found to inhibit the enzyme activity substantially (Table 3). The addition of EDTA inhibited the enzyme activity, suggesting that metal ions are needed for the enzymatic reaction.

Ca^{2+} is one of the important intrinsic components of milk and stimulation of enzyme activity by this divalent cation is good from the practical point of view since it will facilitate the hydrolysis of lactose. In contrast to almond β-galactosidase, microbial β-galactosidases have been reported to be inhibited by Ca^{2+}, thereby restricting their application (*1*).

The effect of a number of reducing agents, most widely used for the reduction of disulphide bonds, was tested on β-galactosidase activity (Table 3). At a concentration of 15 and 25 mM in the reaction mixture, ascorbic acid and vanillin activated the enzyme activity maximally to an extent of 10 and 16 %, respectively. The other reducing agents such as dithiothreitol (DTT), glutathione, cysteine, and β-mercaptoethanol (β-ME) also displayed identical behaviour but the effect was not substantial. The stimulation of β-galactosidase activity by reducing agents indicates that there is a relationship between the reduced form of the cysteine residues and the activity of the β-galactosidase.

Individual and combined effect of end products of lactose hydrolysis

Glucose and galactose are the end products of β-galactosidase-catalyzed hydrolysis of lactose and therefore their effect on β-galactosidase activity is worth studying. Activity of β-galactosidase from almonds was considerably decreased in the presence of high concentrations of glucose and galactose, indicating that lactose hydrolysis is slowed down as the reaction products build up (Fig. 5). It has been observed earlier that the end product of hydrolysis, particularly galactose, competitively inhibits

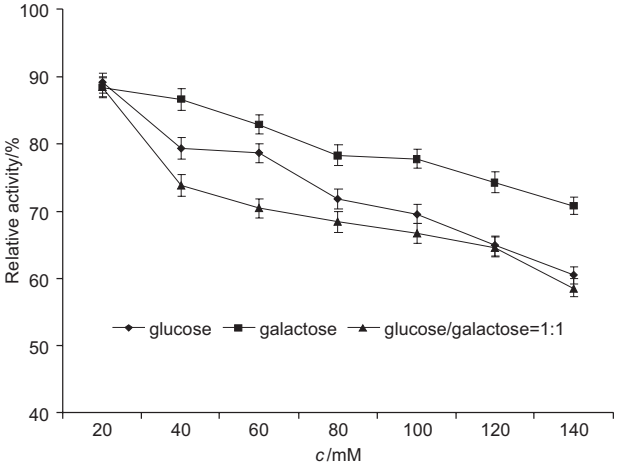

Fig. 5. Individual and combined effects of glucose and galactose on β-galactosidase activity

the enzyme activity (*1,7*). When glucose and galactose were incorporated at a ratio of 1:1 in the reaction mixture, neither additive nor synergistic effect on the enzyme activity was observed (Fig. 5). This may be due to the well established second activity, *i.e.* transgalactosylation activity of this bifunctional enzyme by which it synthesizes galactooligosaccharides (GOS) using glucose as a primer. So, in the presence of both glucose and galactosidase, GOS are synthesized, thereby decreasing the effective concentration of these metabolites and subsequent enzyme inhibition.

Hydrolysis of milk lactose using purified β-galactosidase and almond seeds

Lastly, lactose hydrolysis in skimmed milk was studied using purified β-galactosidase, and hydrolysis up to the extent of 95 % was observed after 4 h of incubation

at room temperature (data not shown). The 100 % hydrolysis could not be estimated probably because once the end products build up, the transgalactosylation activity dominates, which blocks glucose molecules in GOS, thereby making it unavailable for estimation.

To prepare low-lactose/delactosed milk, powdered almond seeds were added into the milk instead of purified β-galactosidase and the obtained results are shown in Fig. 6. Our study shows that approx. 50 % lactose hydrolysis can be achieved by incubating 100 mL of milk with 10 g of almond seed powder for 5 h at 42 °C. Apart from low lactose content, the antioxidant activity of β-galactosidase from almonds will additionally make the milk more nutritious. Moreover, the proteolytic enzymes from almonds might partially digest the milk protein to further aid its easy digestion.

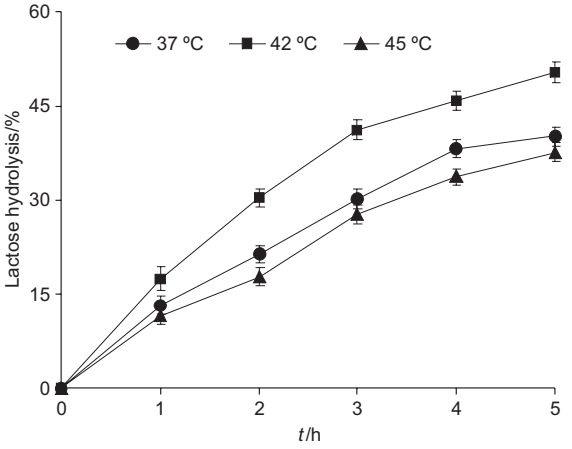

Fig. 6. Lactose hydrolysis catalyzed by almond seed powder at different temperatures

Conclusions

A 50.9-fold purification and 33.72 % recovery of β-galactosidase was achieved from almond seeds using neutral salt fractionation (15–60 %), gel permeation (Sephadex G-100 column) and ion-exchange chromatography (DEAE-cellulose column). The enzyme was monomeric in nature with molecular mass of approx. 62 kDa as estimated by gel filtration and SDS-PAGE. $\Delta H°$, $\Delta S°$ and $\Delta G°$ of thermal denaturation revealed that the enzyme undergoes significant processes of unfolding during denaturation. Milk lactose up to the extent of 95 % could be hydrolyzed using purified β-galactosidase, while approx. 50 % hydrolysis was observed using β-galactosidase from almond seed powder. The findings indicate that the almond seeds can be successfully employed for the production of low-lactose/delactosed milk for lactose-intolerant people.

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