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Adding value in barley malt rootlets as a source of 5'-phosphodiesterase: biochemical and thermodynamic evaluation of enzyme activity

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Abstract

A thermostable 5'-phosphodiesterase (5'-PDE, EC 3.1.4.1) was extracted from barley (*Hordeum distichum* var. Rex) malt rootlets. The purification procedure comprised acetone precipitation, S-Sepharose cation-exchange and DEAE-Sepharose anion-exchange chromatography. The enzyme was purified 101-fold with a recovery of 22% and a specific activity of 81.9 U mg⁻¹ protein. Optimum enzyme activity was obtained at 70 °C, and pH 8.9. The SDS-PAGE profiling of the purified protein exhibited molecular weight of 116 kDa and revealed three sub-unit fractions of 26, 43, and 56 kDa making up its active configuration. The kinetic constants K_m and V_{max} were determined as 0.25 mM and 0.816 mmol min⁻¹, respectively. Thermodynamic studies showed that the thermal inactivation of purified barley malt rootlets 5'-PDE followed the first-order kinetics, indicating inactivation energy (E_a) of 134 kJ mol⁻¹. The half-life ($t_{1/2}$) at 70 °C was estimated as 169 min. Thermodynamic parameters ΔH^* , ΔS^* and ΔG^* were determined as a function of temperature and were 131.15 kJ mol⁻¹, 37.01 kJ mol⁻¹ K⁻¹ and 118.4 kJ mol⁻¹, respectively. The purified enzyme has long half-life with 11 days at 0 °C, 37 hours at 4 °C and 11 hours at room temperature. These results provide useful information about the factors that affects the activity of barley malt rootlets 5'-PDE and suggests a good indication for application of this enzyme in pharmaceutical and food industry.

Keywords: barley malt rootlets, 5'-phosphodiesterase, pH stability, thermal stability, thermodynamic parameters

Introduction

The enzyme 5'-phosphodiesterase (5'-PDE, EC 3.1.4.1) is an exonuclease that cleaves 5'-nucleotides (5'-AMP, 5'-CMP, 5'-GMP, 5'-UMP) successively from 3'-hydroxy termini of 3'-hydroxy-terminated oligonucleotides originating from RNA (Steensma et al., 2004). 5'-Nucleotides are widely used in the pharmaceutical and food industries, especially as infant powder milk additives (Carver, 2003) and flavor enhancers (Sombutyanchit et al., 2001; Deoda and Singhal, 2003). Furthermore, the concern in 5'-PDE has grown over the last two decades due to the application of 5'-nucleotides in antiviral and anticancer treatment (Ying et al., 2006). As animal feed additives, they can increase the feed rate of pig, improving laying rate of the chicken, promoting the growth and reproduction of fishes and aquatic animals (Gardiner et al., 1995). Also, they can be used as plant growth regulator, used in agriculture to increase the yield and improve the quality of agricultural products (Roux et al., 2006).

All technologies create certain amounts of secondary products and waste materials. Malt sprouts are abundant and cheap by-products of the malting industry (Alexander et al., 1984), but there are the products obtained by the removal of sprouts from malted barley together with malt hulls and other parts of malt (official definition; Salama et al., 1997a). Malt sprouts, which constitute 3 to 5% of the malt are constituted of acrospires and rootlets. The rootlets which constituted about 40% of the malt sprouts, were found to be rich in protein (31.9%) of high biological value and moderate in fiber, vitamins and minerals content and used as useful food ingredients or generally incorporated into stock feeds (Kent and Evers, 1994; Salama et al., 1997b). Their market price is low, but still not fully assessed their value, and it is necessary to explore other possibilities of their exploitation (Pui et al., 2012).

5'-PDE has been reported to be found in different parts of plants such as carrot roots (Harvey et al., 1970), *Avena* leaf tissues (Udvardy et al., 1970) and sugar beet (Leach and Wolf, 1972). More recent studies have focused on germinating beans (Utami et al., 2011; Pui et al., 2012),

germinating barley seeds (Deoda and Singhal, 2003; Dhule et al., 2006), sorghum rootlets (Oriol and Kaid, 2009) and barley malt rootlets (Wang et al., 1993; Beluhan et al., 2003; Zou et al., 2008; Hua and Huang, 2011). Furthermore, *Penicillium citrinum*-based fermentation are also used as one the main 5'-PDE production methods (Okado et al., 2016), while Luo et al. (2017) suggest production of 5'-PDE by *Aspergillus fumigatus*.

Selection of the enzyme source for any industrial processes depends on factors such as the availability of primary raw materials or by-products, and the number of purification steps. After extraction step, 5'-PDE was found together with other enzymatic activities that become contaminants, particularly phosphomonoesterase (PME, EC 3.1.3.5), so purification is an essential step in order to obtain free 5'-PDE activity (Beluhan and Maric, 2011). Older methods for extraction and purification of 5'-PDE included one to several solvent precipitation steps followed by several chromatographic steps (Harvey et al., 1970; Shinshi et al., 1976; Benaiges et al., 1989; Wang et al., 1993). More recent methods usually involved one or two chromatographic steps or ultrafiltration (Lee et al., 2000; Deoda and Singhal, 2003; Dhule et al., 2006; Hua and Huang, 2011).

This study describes the purification and characterization of 5'-PDE from the barley malt rootlets in terms of temperature and pH optima and stability, kinetic parameters, effects of metal ions, chelating and reducing agents, estimation and evaluation of the thermodynamic parameters and determination of storage stability. To date, only sparse data are available on the thermodynamic properties of plant 5'-PDE so we found important to cover both biochemical and thermal characterization of purified barley malt rootlets 5'-PDE and reveal this enzyme as a potential candidate for industrial and biomedical applications.



Materials and Methods

Plant material, substrates and chemicals

Dried barley (*Hordeum distichum* var. *Rex*) malt rootlets were kindly obtained as a by-product of the malting industry, "Slavonija-Slad" (Nova Gradiska, Croatia). Separation of malt rootlets from remains of hulls was achieved by manual sieving using a commercial circular sieve with 1 mm diameter holes.

Thymidine 5'-monophosphate *p*-nitrophenyl ester (denoted as nitrophenyl-pT in this article) and *p*-nitrophenol were purchased from Sigma Chemical Co. (St. Louis, USA). Bovine serum albumin (BSA) and Tris base were purchased from Boehringer Mannheim (Germany). S-Sepharose and DEAE-Sepharose were purchased from Pharmacia AB (Sweden). Standard protein markers (low molecular weight) were purchased from Pharmacia Fine Chemicals (Sweden). All other reagents were of analytical grade.

5'-PDE extraction and purification

5'-PDE was extracted overnight from the barley malt rootlets with 10-fold (w/v) 0.1 M Tris-HCl buffer pH 8.9 at 37 °C. After extraction the sample was homogenized with gentle shaking and then squeezed through several layers of cloth and centrifuged for 20 min at 8000 rpm/min at 0 °C to remove barley malt rootlets. The clarified supernatant was used as the crude enzyme preparation.

The enzyme was purified from the crude supernatant by three steps procedure:

Step 1: The crude enzyme extract was brought to 70 °C and kept at this temperature for 20 min in a thermostatic water bath with continuous stirring, cooled and then centrifuged to remove insoluble materials. The enzyme solution was then stored at 4 °C until the enzymatic activity was determined. The control was crude enzyme preparation without thermal treatment.

Step 2: Acetone fractionation was used as reported earlier (Beluhan et al., 2003; Beluhan and Maric, 2011). The samples (fractions) were then dialyzed for 24 h at 4 °C against distilled water. After 24 hours the sample was analyzed by assaying the enzymatic activity.

Step 3a: Purification procedure of enzymatic preparation (1.45 mg) on activated S-Sepharose, previously equilibrated with 0.01 M Na-acetate buffer, pH 3.5, was conducted in the similar way. The proteins were eluted by increasing ionic strength of mobile phase by gradient of NaCl (0-1 M) in 0.1 M Na-acetate buffer pH 3.5 at the flow rate of 10 mL h⁻¹ and 1 mL fractions were collected.

Step 3b: The enzyme preparation (1.45 mg) obtained from the above step was applied to anion-exchange chromatography on DEAE-Sepharose column (1.5 cm × 8.5 cm) equilibrated previously with 1 M NaCl in 0.01 M Na-acetate buffer (pH 5.5). The column was washed with the binding buffer and eluted with a 0-1 M linear gradient of NaCl in 0.1 M Na-acetate buffer pH 5.5 at the flow rate of 10 mL h⁻¹ and 1 mL fractions were collected.

After ion-exchange column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm. These fractions showing high 5'-PDE activity were pooled and used for SDS-PAGE analysis.

5'-PDE activity determination and protein assays

The activity of 5'-PDE was determined according to the continuous-time method of Harvey et al. (1970) with some modifications. The reaction mixtures contained 50 mM Tris-HCl buffer pH 8.9, 2.0 mM nitrophenyl-pT, and enzyme preparation, in total volume of 3 mL. The increases of absorbance were measured at 405 nm, due to the enzymatic release of *p*-nitrophenol. Enzyme activity was determined at

55 °C for 15 min from the linear portion of absorbance versus time. All experiments were done in thermostable compartment of dual-beam Cary 3 spectrophotometer (Varian). 5'-PDE activity was calculated using a molar extinction coefficient of $1.85 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for *p*-nitrophenol. The enzymatically liberated *p*-nitrophenol was calculated from previously established standard curve, using *p*-nitrophenol as a standard. One unit enzyme activity was defined as the amount of enzyme producing 1 mmol of *p*-nitrophenol per min under standard assay conditions. Specific activities of the examined enzymes were expressed as U mg⁻¹ protein. Protein content was determined by using the method of Bradford (1976) with Bio-Rad dye reagent concentrate and bovine serum albumin as the standard.

Determination of purified 5'-PDE by SDS-PAGE

The molecular weight of the purified 5'-PDE was determined by SDS-PAGE. Purified enzyme preparations and standard molecular weight markers used were subjected to electrophoresis according to the method of Laemmli (1970) in a 12% (w/v) polyacrylamide slab gel and visualized by silver staining (Blum et al., 1987). The following low molecular weight standards were used as molecular mass standards: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α -lactalbumin (14.4 kDa). Protein molecular weight was determined from a standard curve of the relative protein mobility against log molecular weight.

Effect of temperature on 5'-PDE activity and stability

In order to determine the effect of temperature on the activity of 5'-PDE, the standard enzyme assay procedure at different temperatures, ranging from 37 to 80 °C (37, 65, 70, 75 and 80 °C). Prior to the addition of the purified 5'-PDE, the 2.0 mM nitrophenyl-pT was pre-incubated at 50 mM Tris-HCl buffer pH 8.9 and respective temperature for 10 min. The percentage of relative activities were expressed as the ratio of the 5'-PDE activity at a certain temperature to the maximum activity at the given temperature range.

The thermal stability of the purified 5'-PDE was investigated by measuring the residual enzyme activity after incubating the enzyme at the same temperature range, from 37 to 80 °C in a temperature-controlled compartment of spectrophotometer (Cary, Varian, Australia). The enzyme solution was placed in a pre-warmed tube at a specified temperature, and aliquots were withdrawn at 20 min time intervals, cooled in an ice water bath and residual activity (%) was assayed. The incubation was carried out in sealed vials to prevent evaporation of water.

Effect of pH on 5'-PDE activity and stability

The effect of pH on the 5'-PDE activity was determined by assaying the enzyme at different pH values ranging from 4.0 to 12.0. Following four buffer (50 mM) systems were used: sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 8.0-8.9), and sodium carbonate-bicarbonate (pH 9.2-12.0). The relative activities were based on the ratio of the activity obtained at a certain pH to the maximum activity obtained at that range and expressed as a percentage.

The pH stability of malt rootlets 5'-PDE was investigated in the same pH range and buffer systems. Therefore, 2 mL of the purified enzyme were mixed with 2 mL of the buffer solutions indicated above and incubated at optimum temperature for 2 h. After this period, aliquots of the mixtures were taken to measure the residual enzyme activity (%) with respect to control, under standard assay conditions.

Effect of metal ions, chelating and reducing agents

In order to determine the effects of metal ions on 5'-PDE activity, the enzyme was pre-incubated for 10 min at 55 °C with various metal ions such as Na⁺, Mg²⁺, Zn²⁺, Cu²⁺, Ca²⁺ and Mn²⁺, at concentrations of 1-10 mM. Also, the enzyme activity was estimated in the presence of 0.1, 1 and 10 mM EDTA, L-cysteine, glutathione and β-mercaptoethanol to investigate effects of chelating and reducing agents on 5'-PDE activity. The activity of the enzyme alone in Tris-HCl buffer pH 8.9 was taken to be 100%.

Determination of kinetic parameters

The kinetic parameters of 5'-PDE were estimated using nitrophenyl-pT as standard substrate. The Michaelis-Menten constant (K_m) and maximum rate (V_{max}) values of the purified enzyme were estimated from the Lineweaver-Burke double-reciprocal plot (Lineweaver and Burk, 1934). The substrate concentrations range in the standard reaction mixture was from 0.036 to 1.667 mM.

Kinetics of thermal inactivation and estimation of the inactivation energy

The thermal inactivation kinetics of 5'-PDE was determined by incubating the purified enzyme at 37-80 °C temperature range in the absence of substrate. The residual activity is directly related to the thermal inactivation rate and was calculated by the first-order kinetic model and described in the following expression (Eq. 1):

$$\ln(A_t/A_0) = -k_d \cdot t \quad (1)$$

where A_t represents the value of enzyme activity at time t (min), A_0 is the initial enzyme activity at time zero, k_d is the inactivation rate constant at the process temperature (min^{-1}) and t is the exposure time (min).

The thermal inactivation rate constants k_d (min^{-1}) were obtained from slopes of a semi-logarithmic plot of residual activity against the treatment time. The half-life ($t_{1/2}$, min^{-1}) was determined from following relationship (Eq. 2):

$$t_{1/2} = \ln 2/k_d \quad (2)$$

For the first-order inactivation, decimal reduction time (D -value) and rate constants are directly related. The D -values were calculated using Eq. (3):

$$D\text{-value} = \ln_{10}/k_d \quad (3)$$

The temperature dependence of k_d was analyzed using the Arrhenius plot (Shuler and Kargi, 2002). Energy of inactivation (E_d) of 5'-PDE was estimated using the Arrhenius equation (4) as:

$$\ln(k_d) = \ln(k_0) - (E_d/R \times T) \quad (4)$$

Estimation of thermodynamic parameters

Using the values of the energy of inactivation (E_d) and Arrhenius rate constant (k_0) different thermodynamic parameters were determined. The enthalpy of inactivation (ΔH^* , kJ mol^{-1}) for each temperature was calculated according to Eq. (5):

$$\Delta H^* = \Delta E_d - RT \quad (5)$$

The values for the Gibb's free energy (ΔG^* , kJ mol^{-1}) of inactivation at different temperatures were calculated from the first-order constant of inactivation process by using following Eq. (6):

$$\Delta G^* = -RT \ln(k_d h/kT) \quad (6)$$

where h is a Plank's constant (6.626×10^{-34} J s), κ is a Boltzman's constant (1.38×10^{-23} J K⁻¹), R is universal gas constant (8.314 J mol⁻¹ K⁻¹) and T is the corresponding absolute temperature (K).

From Eqs. (5) and (6) the entropy of inactivation (ΔS^* , $\text{kJ mol}^{-1} \text{K}^{-1}$) of 5'-PDE was calculated from the Eq. (7):

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (7)$$

Determination of storage stability

For storage stability determination, purified enzyme was stored at 0 °C, 4 °C and room temperature in autoclaved polypropylene tubes. The activity of enzyme in 50 mM Tris-HCl buffer pH 8.9 was determined at regular time intervals (24 h) up to 12 days. The storage stability was evaluated by plotting activity profiles of enzyme preparations against storage time.

Results and discussion

Purification of 5'-PDE

The 5'-PDE from barley malt rootlets was purified by using the purification procedure described above and overall purification results obtained are presented in Table 1. After thermal inactivation at 70 °C and acetone precipitation (60% v/v), 5'-PDE activity recoveries were 96% and 88% of the total activity and purification were 7.2 and 32.7-fold, respectively. The obtained results showed that acetone fractionation resulted with a significant 5'-PDE purification degree (32.7-fold), as can be seen from the values of the initial solution. After the purification achieved in the first step by thermal treatment and acetone fractionation, cation and anion exchange chromatographic systems were assayed in order to increase 5'-PDE purity. Recovery of the purified 5'-PDE was 11% after both cation and anion exchange, but obtained specific activity and purification degree after anion exchange was 81.9 U mg⁻¹ and 101.1-fold, respectively, which present 11% better results than after cation exchange (72.9 U mg⁻¹ and 90-fold, respectively) (Table 1).



Table 1. The purification profile for barley malt rootlets 5'-PDE

Purification steps	Protein (mg)	Total activity (U*)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification fold
Crude extract	255.2	206.4	0.8	100	1.0
Heat-treatment	34.2	198.2	5.8	96	7.2
Acetone precipitation	6.9	182.5	26.5	88	32.7
Dialyzed sample	2.9	95.0	32.8	46	40.5
Cation exchange	0.6	45.2	72.9	22	90.0
Anion exchange	0.54	44.2	81.9	22	101.1

U*-activity units

When the final preparations of 5'-PDE after cation and anion exchange chromatography were subjected to SDS-PAGE, a triple of sharp, intense bands was detected at position which corresponded to 26, 43 and 56 kDa. The sum of the molecular weight of these three proteins was 116 kDa, and SDS-PAGE profiling revealed that 5'-PDE might be multimeric enzyme composed of three subunits. Somewhat, smaller specific activity after cation exchange is due to some impurities remained in the preparation as can be seen at Fig. 1.

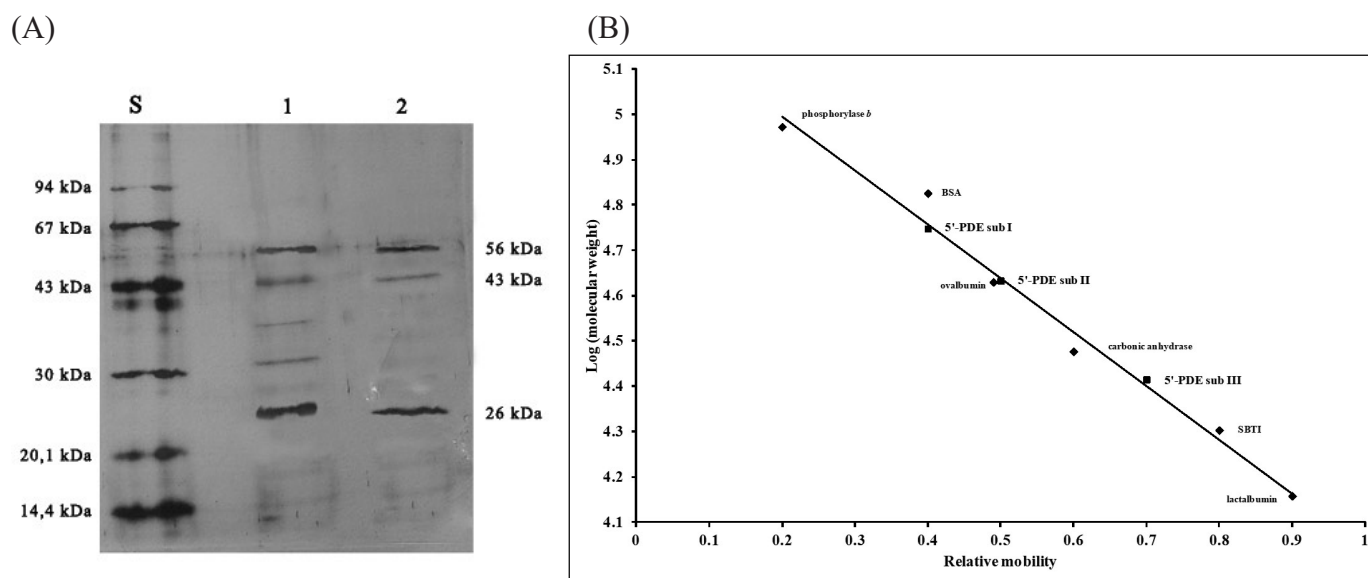


Figure 1. A) SDS-PAGE of purified 5'-PDE. Line S; molecular weight markers, line 1; purified 5'-PDE after cation exchange chromatography (S-Sepharose), line 2; purified 5'-PDE after anion exchange chromatography (DEAE-Sepharose). Protein bands were visualized by silver staining. B) Molecular weight estimation of purified 5'-PDE by comparing its relative mobility with standard markers used.

The molecular weight of the enzyme was nearly similar to results of Wang et al. (1993), who find the molecular weight of barley rootlets 5'-PDE was approximate 100 kDa, composed of two subunits essentially with the molecular weight of 34 and 52 kDa subunits, and a minor band with a molecular weight around 16 kDa. Since the enzyme originates from the different varieties of barley, differences in the values of molecular weight subunits are probably a consequence of small differences in post-synthetic modification of proteins. The molecular weight of the 5'-PDE from other plant origins, sugar beet leaves or carrots is the same, or not quite different, approximately 110 kDa (Lerch and Wolf, 1972; Harvey et al., 1970). The two enzymes, and also the 5'-PDE from *Avena* leaves, are apparently closely related and are, in all their enzymatic properties, very similar to snake venom phosphodiesterase, whereas a 5'-PDE from germinated barley appears to have somewhat different properties (Dhule et al., 2006).

Enzyme characterization

The purified 5'-PDE activity studied as a function of temperature in a temperature range between 37 and 80 °C showed that the enzyme was highly active (more than 70 %) over a broad temperature range of 55 to 80 °C, with the optimum at 70 °C (Fig. 2A). There are various literature reports on optimum temperature for 5'-PDE activity. Exactly the same optimal temperature determined Hua and Huang (2011) for barley malt rootlets 5'-PDE. As a closest comparison, Dhule et al. (2006) reported that the optimum temperature of the 5'-PDE from germinated barley was at 60 °C and the same optimum temperature determined Luo et al. (2017) for *Aspergillus fumigatus* 5'-PDE.

Thermal stability of purified 5'-PDE was determined by studying the time-dependent thermal inactivation of enzyme, as shown in Fig. 2B. For thermal stability estimation, enzyme preparations were incubated for 120 min at various temperatures ranging from 37 to 80 °C. The study reveals that the enzyme was totally inactivated within 30 min at 37 °C, but showed great thermal stability at temperature range between 65 and 75 °C. It retained more than 80% of its activity after two hours of incubation at above mentioned temperatures. This confirms that the barley malt rootlets 5'-PDE prefers higher temperatures and this results makes a good prospect for industrial applications of this enzyme.

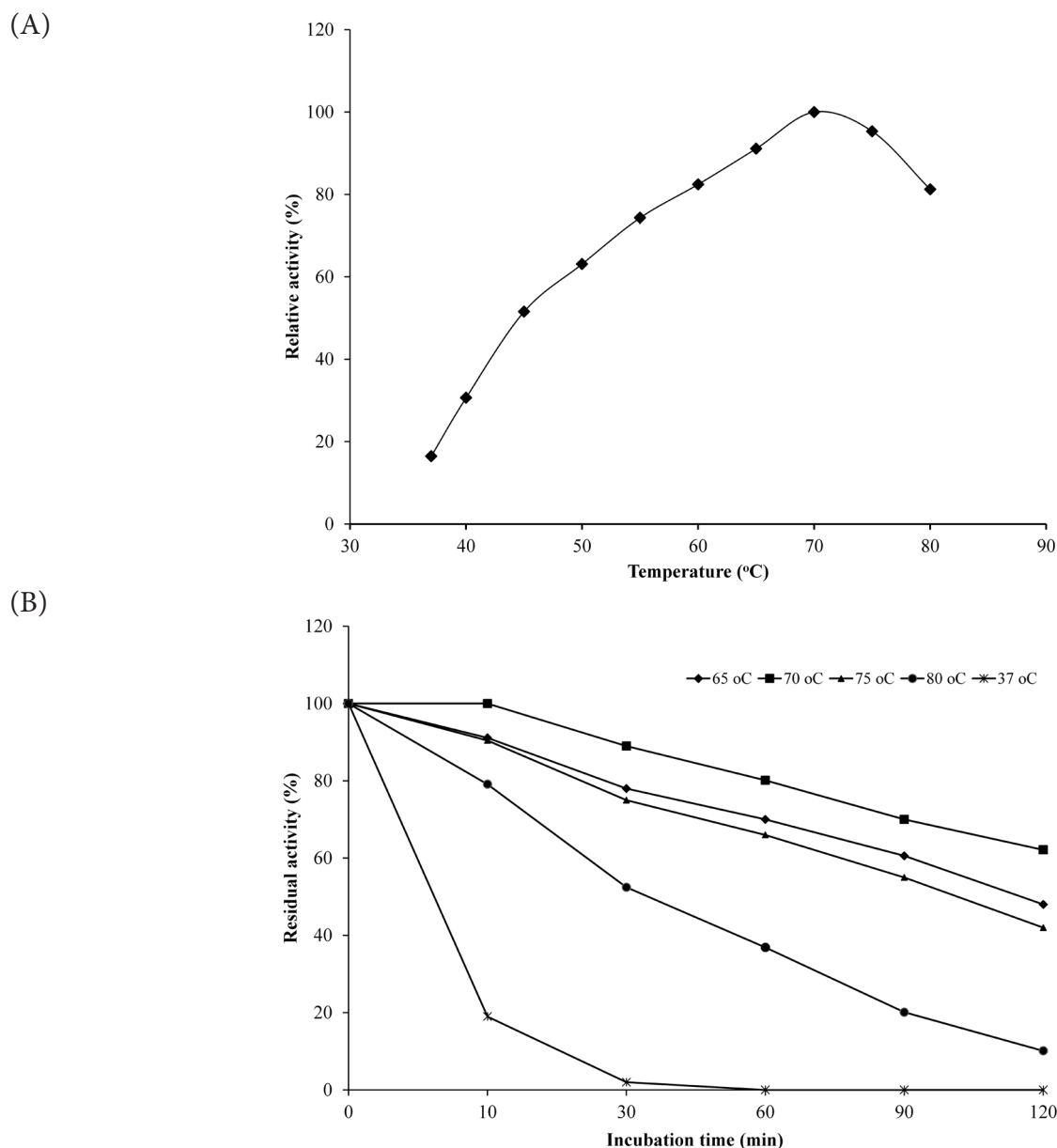


Figure 2. A) Effect of temperature on purified 5'-PDE activity: effect of temperature was evaluated by incubating the assay mixture at different temperature ranging from 37 to 80 °C. B) Thermal stability of purified 5'-PDE: for stability determination, enzyme preparations were incubated at temperature in the range of 37 to 70 °C for 120 min at optimum pH (8.9) and residual activity (%) was determined at regular time intervals of 20 min. Each data point represents the mean±standard deviation from three analyses.

The pH optima were determined in four buffer systems. As is seen from Fig. 3A, the enzyme showed good activity over a broad of pH range (7.0-



11.0) with more activity toward the alkaline range than toward acidic range. Optimum 5'-PDE activity was observed at pH 8.9. At pH 8.0 and 10.0, the enzyme showed about 85% of the activity obtained at pH 8.9 (Fig. 3A). The pH stability of enzyme was determined by incubating at 70 °C for 2 h, at pH values in the range of 4.0-12.0. The enzyme activity remained stable for 2 h over a wide pH range (5.0-12.0) and showed about 98% (at pH 8.9) activity after treatment at 70 °C for 2 h (Fig. 3B).

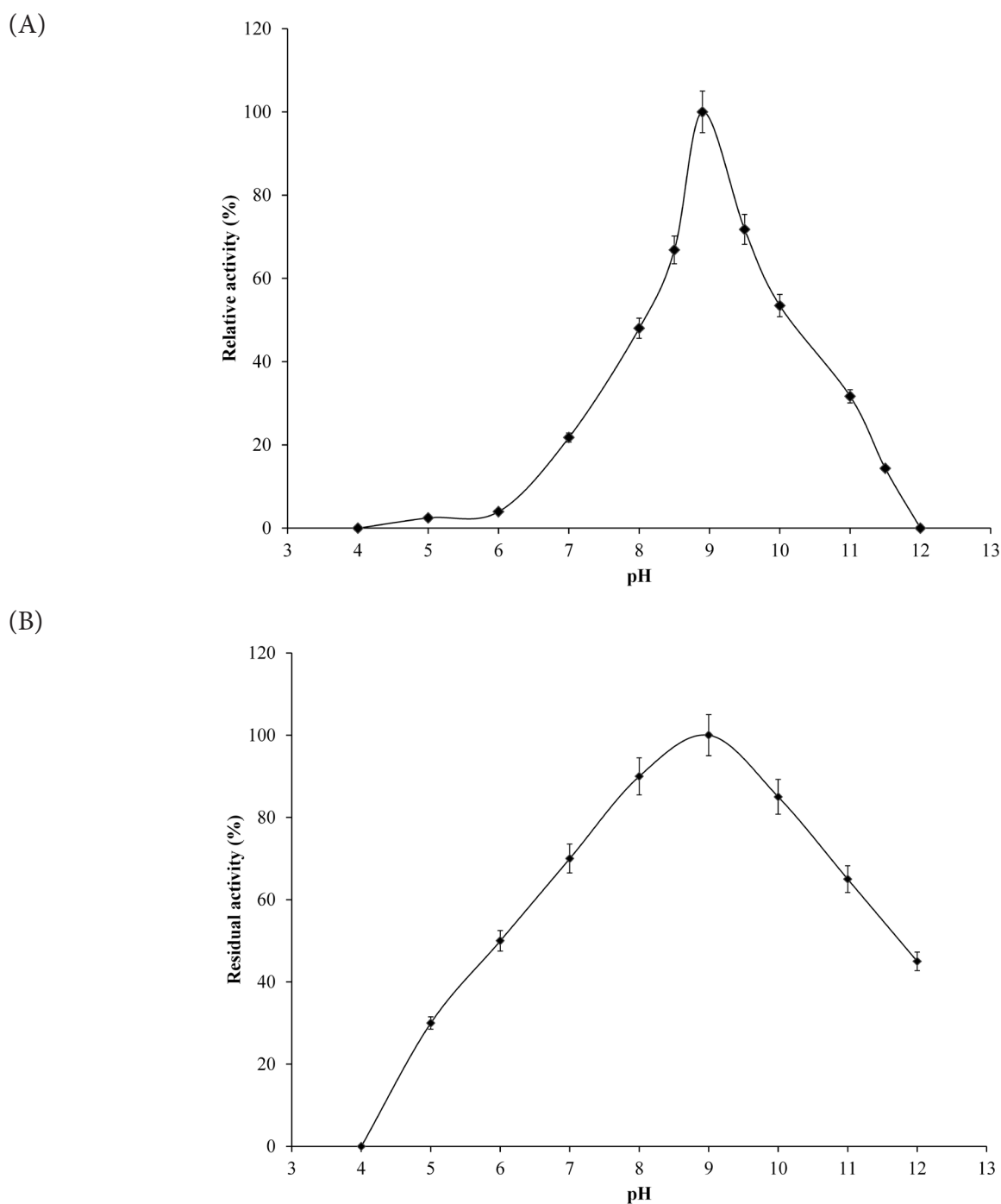


Figure 3. A) Effect of pH on purified 5+-PDE activity: rate of digestion of nitrophenyl-pT with purified 5'-PDE was evaluated at various pH values (4.0-12.0). B) Effect of pH on purified 5'-PDE stability: enzyme preparations were incubated at pH values (4.0-12.0) for 2 h, at optimum temperature and residual activity was determined at regular time intervals of 20 min. Each data point represents the mean±standard deviation from three analyses.

The pH is one of the most important parameter capable of altering the enzymatic activities in aqueous solution. There are a number of distinct effects that a change in pH can have on enzyme catalyzed reactions. An enzyme with high activity over a wide range of pH is advantageous, because it will eliminate the necessity for pH adjustment during enzyme action (Tanaka et al., 1985). The pH optimum of purified barley malt rootlets 5'-PDE observed at pH 8.9 is different with that reported by Dhule et al. (2006) who found to be 5.0 for partially purified 5'-PDE from germinated barley, with slight elevation in activity at pH 8, and close with pH 9.5 for purified barley malt rootlets 5'-PDE reported by Wang et al. (1993) and pH 9.3 for 5'-PDE from *Avena* leaf tissues (Udvardy et al., 1970). The pH optimum of purified *Aspergillus fumigatus* 5'-PDE reported by Luo et al. (2017) was found to be 5.0. The 5'-PDE activity of broad pH range and pH stability confirms its potential use in biotechnological applications.

Various metal ions at 1-10 mM concentrations were tested on purified barley malt rootlets 5'-PDE. Among them, only Na⁺ reduced the enzyme activity for about 40%, a slight inhibition was observed in the presence of Zn²⁺, Cu²⁺, Mn²⁺, and Ca²⁺, while, in the presence of Mg²⁺, the enzyme was activated up to 169% of the original activity (Fig. 4). The overall metal ions study reveals that the 5'-PDE activity had inhibitory effects to certain degrees but not completely except Mg²⁺ ions which increase enzyme activity. However, stability of an enzyme in presence of metal ions plays a vital role in their application in industries.

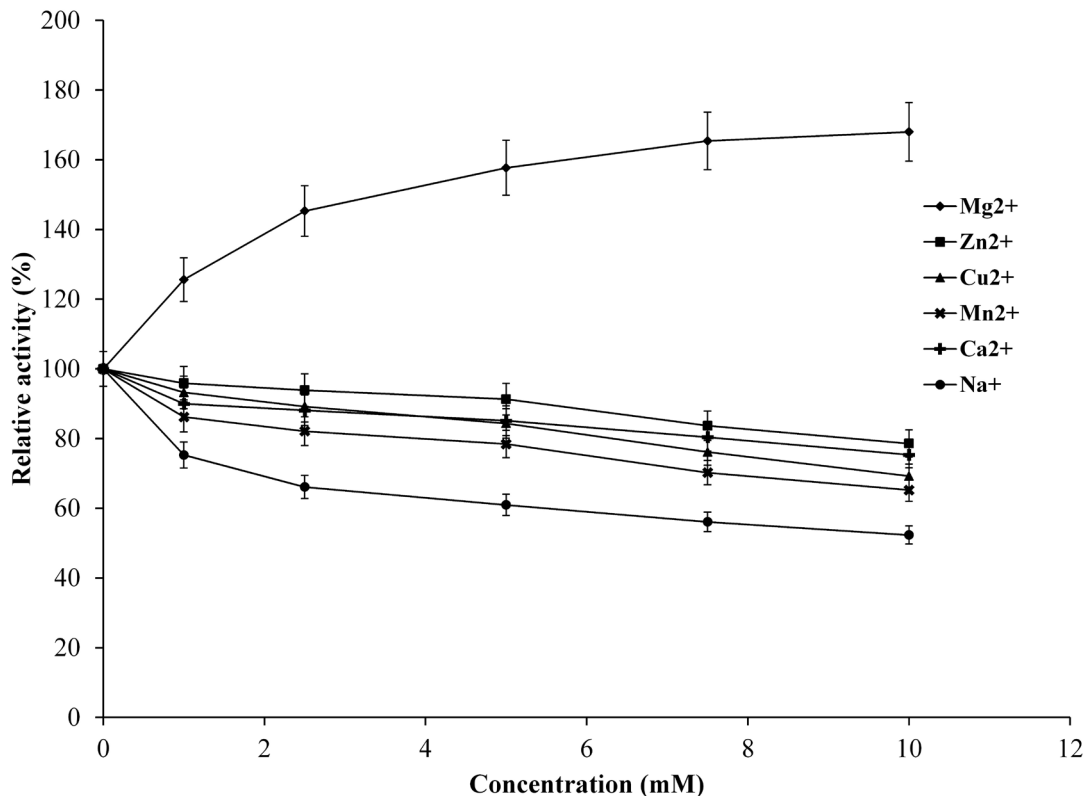


Figure 4. Effect of various metal ions (Mg²⁺, Zn²⁺, Cu²⁺, Mn²⁺, Ca²⁺ and Na⁺) on purified 5'-PDE activity. Each data point represents the mean±standard deviation from three analyses.

Chelating agent (10 mM EDTA) caused 91% inhibition of the enzyme activity (Fig. 5). Inhibition due to addition of EDTA indicated that barley malt rootlets 5'-PDE is metalloenzyme. Nevertheless, it may be interesting to note that, although EDTA inhibited 5'-PDE activity significantly, barley malt rootlets 5'-PDE in this work appears to be the first purified enzyme which is active at alkaline pH even in the presence of 10 mM EDTA. The effect of reducing agents on the purified barley malt rootlets 5'-PDE is also shown in Fig. 5. On the reducing agents tested, all of them acted inhibitory on 5'-PDE activity; nevertheless β-mercaptoethanol and L-cysteine inhibited the enzyme activity most strongly, indicating that 5'-PDE is a disulfide stabled protein (Valerio et al., 2002). Similar effects of reducing agents on 5'-PDE activity from carrot (Harvey et al., 1970), sugar beet leaves (Leach and Wolf, 1972), tobacco cells (Shinshi et al., 1976), and *Avena* leaf tissues (Udvardy et al., 1970) had been reported.

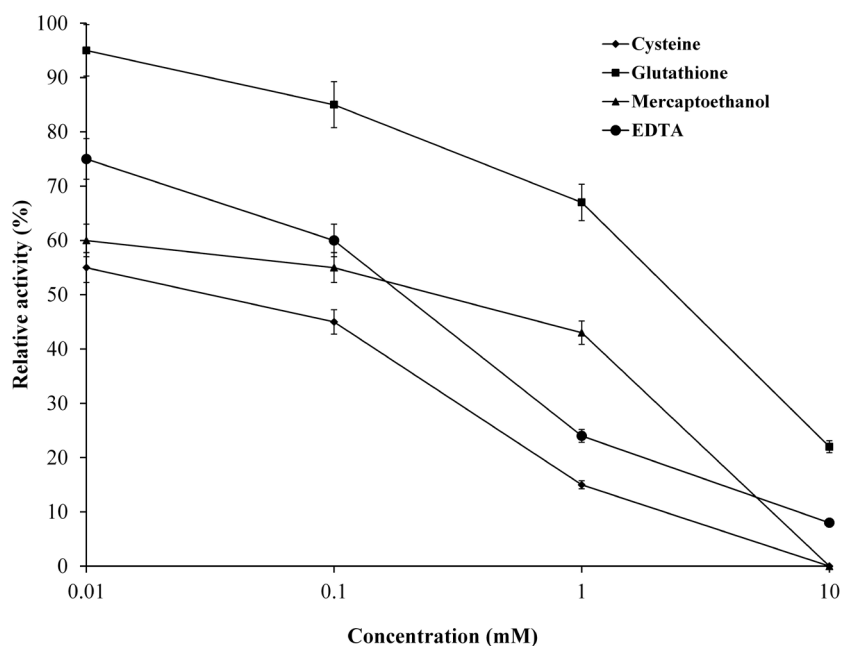


Figure 5. Effect of chelating agent (EDTA) and reducing agents (L-cysteine, glutathione and β -mercaptoethanol) on purified 5'-PDE activity. Each data point represents the mean \pm standard deviation from three analyses.

Enzyme kinetics, K_m and V_{max} are significant coefficients in guiding scientific research and engineering design. The kinetic parameters of barley malt rootlets 5'-PDE, describing its affinity towards nitrophenyl-pT at pH 8.9 and 55 °C were obtained by a typical double reciprocal Lineweaver-Burk plot. The K_m and V_{max} values of the 5'-PDE were 0.25 mM and 0.816 mmol min^{-1} of protein, respectively (Fig. 6). The estimated K_m value was close to K_m (0.28 mM) of purified 5'-PDE from barley rootlets (Wang et al., 1993). Dhule et al. (2006) were found that K_m value of purified 5'-PDE from germinated barley was 0.33 mM. Compared with the K_m values of the 5'-PDE from other plant sources, this K_m is relatively high. For nitrophenyl-pU, the alkaline 5'-PDE of sugar beet leaves (Lerch and Wolf, 1972) had K_m value of 0.19 mM. The acid 5'-PDE of cultured tobacco cells had K_m value of 0.17 mM (Shinshi et al., 1976) and K_m values of 5'-PDE from carrot (Harvey et al., 1970) for nitrophenyl-pT was 0.02 mM.

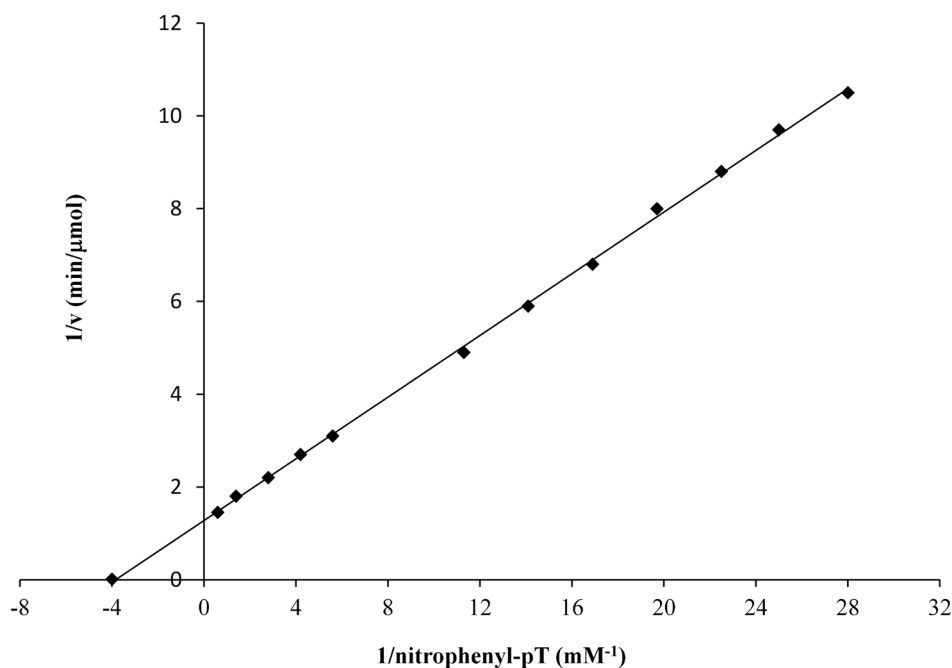


Figure 6. Lineweaver-Burk plot of purified 5'-PDE activity on nitrophenyl-pT as a substrate.

Kinetics of thermal inactivation of 5'-PDE

The process whereby the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds is defined as inactivation. The thermal inactivation parameters of malt rootlets 5'-PDE are presented in Table 2. The first order inactivation rate constants (k_d) at 37, 65, 70, 75 and 80 °C, decreased with increasing temperature, indicating that the enzyme was much thermostable at higher temperatures. The half-life ($t_{1/2}$) is another important parameter used in the characterization of enzyme stability. The variation in half-life with temperature is a measure of the temperature stability of the enzyme. The half-life ($t_{1/2}$) of inactivation of purified 5'-PDE at 70 °C (optimum temperature) was about 170 min and was 18, 1.4, 1.7 and 4.5 times higher than the half-life values at 37, 65, 75 and 80 °C, respectively (Table 2). This issue reveals the thermal stability of this enzyme not only at 70 °C, but also at 5 °C lower and up to 15 °C higher temperatures, and the 5'-PDE easy inactivation at low temperatures (37 °C). The decimal reduction time (D -value) is the time, at given temperature, needed for 90% reduction of the initial enzyme activity. D -values obtained in this work ranged between 561.6 and 31.3 min at the temperature studied (Table 2).

Table 2. Kinetic parameters for thermal inactivation of purified barley malt rootlets 5'-PDE

T (K)	k_d (1/min) ^a	$t_{1/2}$ (min)	R ²	D -value (min)
37 (310)	0.0738	9.40	0.981	31.20
65 (338)	0.0057	121.60	0.985	403.96
70 (343)	0.0041	169.06	0.994	561.61
75 (348)	0.0068	101.93	0.987	338.62
80 (353)	0.0183	37.88	0.992	125.82

^a k_d , thermal inactivation rate constant; $t_{1/2}$, half-life; R², coefficient of correlation;

D -value, decimal reduction time

The stability of an enzyme in extreme conditions such as high temperature and low or high pH value is the decisive factor for its industrial application. Thermal stability of an enzyme is defined as the ability to resist thermal unfolding in the absence of substrates. However, the high thermal stability of the enzyme is advantageous for industrial application, because the high temperature of processing leads to denaturation of possible thermolabile contaminating enzyme activities (Bowles, 1991). Purified barley malt rootlets 5'-PDE showed remarkable stability under conditions where most enzymes lose their activity.

The thermodynamic parameters during inactivation of 5'-PDE

The information about thermodynamic parameters is a good tool to analyze the stability of enzyme and to reveal any secondary stabilization or destabilization effects that might be missed if only the half-life times are taken into consideration (Anthon and Barrett, 2002).

Inactivation energy (E_d) of the 5'-PDE was calculate to be 134 kJ mol⁻¹ from the slopes of the linear curve plotted by $1/T$ against $\ln k_d$ using Eq. (2). This value was in close approximation to the values (120-160 kJ mol⁻¹) estimated for many plant enzymes (Van Loey et al., 2003).

A positive ΔH^* and ΔS^* were determined in the temperature ranges studied. With increase of temperature, a slight decrease in ΔH^* and a marked increase in ΔS^* were observed. This suggested thermal denaturation of the enzyme. The increase in ΔS^* also indicated an increase in number of protein molecules in transition activated stage, resulting in lower values of ΔG^* at 37 and 65 °C (Table 3).

The enthalpy and entropy of activation and the Gibb's free energy data can provide some clues about proteins and thermostability. Low enthalpy values indicate a highly effective transition state, while low entropy indicates the transition complex of the substrate and that the enzyme is highly ordered (Kikani and Singh, 2012). The changes in enthalpy (ΔH^*) and entropy (ΔS^*) were calculated according to Eqs. (5), and (7) for the thermal inactivation of 5'-PDE (Table 3).

A positive ΔH^* and ΔS^* were determined in the temperature ranges studied. With increase of temperature, a slight and marked decrease in ΔH^* and ΔS^* were observed, respectively. The decrease in ΔS^* indicated a decrease in number of protein molecules in transition activated stage, resulting in higher values of ΔG^* . Also, positive entropy values suggested that enzyme unfolding might be the rate determining step for the irreversible thermal inactivation of 5'-PDE. Moreover, solvent and structural effects are reported to be the two major factors influencing the numerical values of ΔH^* and ΔS^* . Particularly, ΔS^* values are known to provide information regarding the degree of solvation and the degree of compactness of protein molecule (Dogan and Tari, 2008). An exact comparison of our values with those published in the literature, however, is not possible because no thermodynamic parameters for catalysis of other plant 5'-PDE have been reported. In fact, this is the positive outcome since we demonstrated that the purified barley malt rootlets 5'-PDE in the current study is very close in biochemical, as well as in thermal characteristics to commercial enzymes currently used.

**Table 3.** Thermodynamic parameters of thermal inactivation of purified barley malt rootlets 5'-PDE

Temperature (°C/K)	ΔH^* (kJ mol ⁻¹)	ΔG^* (kJ mol ⁻¹)	ΔS^* (kJ mol ⁻¹ K ⁻¹)
37/310	131.42	93.43	122.57
65/338	131.19	109.31	64.73
70/343	131.15	118.45	37.01
75/348	131.11	118.78	35.43
80/353	131.07	110.84	57.29

Storage stability

Achieving high storage stability is one of the most important parameters which should be considered in the purification procedure. Purified proteins often need to be stored for an extended period of time while retaining their original structural integrity and activity. The extend of enzyme storage half-life can vary from a few days to more than a year and is depend on the nature of the protein and the storage conditions used.

In our study purified enzyme solution was stored at 0 °C, 4 °C and room temperature for 12 days. The degradation kinetics was studied by measuring the activity every 24 h. Semi-log plots of residual activity against time at all temperatures were linear, indicating a first order degradation kinetics. The half-life ($t_{1/2}$), calculated from slope Eq. (1) clearly indicated that maximum activity and stability of enzyme were at 0 °C (Table 4). As clearly shown in Table 4, the highest storage stability of purified barley malt rootlets 5'-PDE was obtained after 270 h storage at 0 °C. The stabilities of 5'-PDE activity at 4 °C and room temperature were much less as compared to activity at 0 °C. In addition, high storage stability at pH 8.9 indicates that the active site of the enzyme is stable at alkaline pH and could interact properly with substrates after storage time.

Table 4. Slope (k_d) and half-life ($t_{1/2}$, h) of the purified barley malt rootlets 5'-PDE stored 12 days at various temperatures

Temperature (°C)	Regression equation ^{a,b}	Slope (k_d)	$t_{1/2}$ (h)
0	$y = -0.0616x + 98.552$ ($R^2 = 0.9246$)	0.0616	270.0
4	$y = -0.4455x + 95.359$ ($R^2 = 0.9882$)	0.4455	37.2
24	$y = -1.5625x + 97.5$ ($R^2 = 0.9868$)	1.5625	10.56

^aRegression equation correlating $\log U$ mL⁻¹ and the storage time in days at different temperatures

^bValues within parenthesis indicates the correlation coefficient.

Conclusions

The biochemical characterization, thermodynamic properties and some factors that affect the activity and stability of purified barley malt rootlets 5'-PDE were investigated. The purified enzyme showed high thermal stability, wide pH resistance, long shelf life and good storage stability, which are important properties for industrial and biomedical applications. Moreover, the value obtained for activation energy and change in enthalpy of inactivation suggest that the enzyme is stable toward thermal denaturation. The good storage stability of barley malt rootlets 5'-PDE showed the suitability of using purified enzyme to obtain commercial preparation. Thus, it seems that the barley malt rootlets obtained as a by-product after malting processing could be used as source to obtain 5'-PDE for industrial, pharmaceutical or research purposes.

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