Partial Purification and Biochemical Characterization of α-Glucosidase from Corn by Three-Phase Partitioning

α-Glukozidazın Mısırdan Üçlü-Faz Ayırma ile Kısmî Saflaştırılması ve Biyokimyasal Karakterizasyonu

Research Article

Başak Bilen, Hasan Bayraktar, Seçil Önal*
Biochemistry Department, Faculty of Science, Ege University, Bornova-İzmir, Turkey.

ABSTRACT

α-Glucosidase was first purified from corn by three-phase partitioning(TPP) and then characterized. Ammonium sulfate and t-butanol were used in order to obtain three phases. Effect of different process parameters such as; ammonium sulfate concentration, enzyme to t-butanol ratio and pH required for efficient purification of the corn α-glucosidase was studied to get highest purification fold and activity recovery. Optimum purification parameters of the TPP system were determined as 50% (w/v) ammonium sulfate saturation with 1.0:0.75 (v/v) ratio of crude extract: t-butanol at pH 4.5. Under optimized conditions α-glucosidase was purified with 3.8 purification fold and 60% activity recovery. The molecular weight of α-glucosidase was determined approximately as 29 kDa by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis(SDS–PAGE). Characterization studies showed that, optimum pH and temperature of α-glucosidase were pH 4.5 and 55°C, respectively. The purified enzyme was found to be very stable at a temperature range of 25-60°C and a pH range of 3.5-5.5. Kinetic constants (K_M and V_{max}) were calculated from Lineweaver-Burk plot as 0.64 mM and 0.45 U, respectively. With 10 mM of CaCl2 concentration α-glucosidase activity was significantly increased to 130%. TPP is a useful strategy to concentrate and purify α-glucosidase for its applications.

Key Words
Three-phase partitioning (TPP), α-glucosidase, corn, purification.

ÖZ

α-Glukozidaz önce mısırdan üçlü-faz ayırma (TPP) tekniği ile saflaştırıldı ve ardından karakterize edildi. Üç faz elde etmek için amonyum sülfat ve t-butanol kullanıldı. Mısır α-glukozidazının yüksek saflaştırma katı ve aktivite geri kazanımı ile etkin bir şekilde saflaştırılması için çeşitli proses parametrelerinin örneğin; amonyum sülfat konsantrasyonu, enzim: t-butanol oranı ve pH etkisi çalışıldı. TPP sisteminin optimum saflaştırma koşulları %50(w/v) amonyum sülfat saturasyonu, 1.0:0.75 (v/v) ham ekstrakt:t-butanol oranı ve pH 4.5 olarak belirlendi. Optimize koşullar altında α-glukozidaz %60 aktivite verimi ile 3.8 kat saflaştırıldı. α-Glukozidazın molekül kütlesi sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE) ile yaklaşık 29 kDa olarak belirlendi. Karakterizasyon çalışmaları, enzimin optimum pH ve sıcaklığının pH 4.5 ve 55°C olduğunu gösterdi. Saflaştırılan enzimin 25-60°C sıcaklık aralığında ve pH 3.5-5.5 aralığında oldukça kararlı olduğu bulundu. Kinetik sabitler (K_M ve V_{max}) Lineweaver-Burk diyagramından 0.64 mM ve 0.45 U olarak hesaplandı. α-Glukozidaz aktivitesi 10 mM CaCl2 konsantrasyonunda oldukça arttı (%130). TPP α-glukozidazın uygulamaları için konsantre edilmesi ve saflaştırılması için kullanışlı bir stratejidir.

Anahtar Kelimeler
Üçlü-faz ayırma (TPP), α-glukozidaz, mısır, saflaştırma.

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Correspondence to: S. Onal, Biochemistry Department, Faculty of Science, Ege University, Bornova-İzmir, Turkey.
Tel: + 90 232 311 54 23 Fax: + 90 232 311 54 85 E-Mail: secil.onal@ege.edu.tr

INTRODUCTION

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is an exo-α-1,4-glucosidase that catalyzes the hydrolysis of terminal, non reducing 1,4-linked α-D-glucose residues present in maltooligosaccharides, α-glucosides and α-glucans [1]. Some of α-glucosidases are also involved in transglucosylation reactions that are very useful in the food industry. Both the hydrolase and transferase activity of α-glucosidases are very important for their industrial applications [2,3]. α-Glucosidases are widely used in various biotechnological processes such as; in the production of glucose syrup and maltooligosaccharides as foodstuff, in brewing and starch processing. They are widespread in mammals, plants, insects, fungi and bacteria. α-Glucosidases have been isolated and purified from different enzyme sources employing various traditional separation and purification methods like; ammonium sulfate precipitation, ion-exchange and gel filtration [4-7].

In spite of various methods have been developed for separation and purification of α-glucosidases, most of them involved a number of steps, furthermore the scale up of these methods is difficult and also very expensive to produce in large scale. In order to overcome mentioned drawbacks and explore its benefits three-phase partitioning (TPP) was used as an alternative extraction process for the first time to purify α-glucosidase from corn (*Zea mays indurata*). This knowledge may lead to develop new TPP strategies for the purification of other α-glucosidases.

TPP is a simple, quick, efficient and often one-step process for the separation and purification of proteins from complex mixtures. The process is quite complex and is a combination of salting-out, isoionic and co-solvent precipitation of proteins. The principle of this technique consists in mixing the crude protein extract with solid salt (generally ammonium sulfate) and a water miscible aliphatic alcohol (usually t-butanol) in order to obtain three phases. The upper organic phase which is containing nonpolar compounds (pigments, lipids etc.) is separated from the lower aqueous phase that containing polar compounds (proteins, saccharides etc.) by an interfacial protein precipitate[8-10]. The partitioning process is affected by the hydrophilicity, the molecular weight and pI of protein and also by the physical conditions of the phase system[10,11]. TPP is easily scalable and can be used directly with crude suspensions [10,12,13]. It has been used to purify a number of proteins and enzymes with high recovery and purity levels[14-18]. We have also an experience with TPP systems for the purification of various enzymes from different enzyme sources like; invertase from Baker's yeast[19] and tomato [20], α-galactosidase from tomato [21], pepino [22] and watermelon [23]. Our trials showed that, TPP is an attractive process for primary purification of enzymes compared to conventional chromatographic methods.

Hence in the present study, we have first focused on the partial purification of α-glucosidase from corn by using TPP and second on biochemical characterization of the purified enzyme. Physiological conditions affect partitioning of proteins during TPP[10]. Therefore, in order to achieve a maximum purity and yield of α-glucosidase the effect of various process parameters (ammonium sulfate saturation, crude enzyme to t-butanol ratio and pH) were evaluated. All TPP experiments were carried out using the crude corn extract. The purified α-glucosidase was also characterized with respect to its activity and stability at various pH and temperature ranges. A wide variety of metal ions and other chemicals were also examined for their effects on α-glucosidase activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and determination of kinetic parameters (K_M and V_{max}) were also carried out.

MATERIALS and METHODS

Materials

tert-Butanol, ammonium sulfate, sodium acetate, sodium carbonate and p-nitrophenol were pure grade and were procured from E. Merck (Darmstad, Germany). p-Nitrophenyl-α-D-glucopyranoside (PNPG) and Coomassie Brilliant Blue R-250 were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Corn (*Zea mays indurata*) was purchased

from a local market, Turkey. All other chemicals and reagents were of the highest available purity and used as purchased.

Methods

Preparation of Crude α-Glucosidase Extract

The crude α-glucosidase extract was prepared from corn (*Zea mays indurata)* according to the procedure described below. Dry corn (500 g) was first grinded (Retsch-RM100) and then allowed to stand overnight at 4°C in 0.05 M sodium acetate buffer (pH 4.5) with continuous stirring. The suspension was homogenized (Silverson STL2) and then the obtained homogenate was filtered from two layers of cheese-cloth. The filtrate was centrifuged at 11,000 g for 30 min at 4°C (Hettich Universal 30 RF). The activity, specific activity and protein amount of supernatant were assayed as; 0.024 U/ml, 0.006 U/mg and 4.21 mg/ml, respectively. The supernatant was subjected to 85% (w/v) ammonium sulfate precipitation and then allowed to stand overnight at 4°C with continuous stirring. The precipitate was collected by centrifugation at 11,000 g for 30 min at 4°C. The pellet was dissolved in sodium acetate buffer (0.05 M, pH 4.5) and then dialyzed against the same buffer at 4°C for overnight. The dialysate represented as "crude corn α-glucosidase extract" and used for further three-phase partitioning studies. The protein content, activity and specific activity of the crude enzyme extract were determined as 11.3 mg/ml, 0.6 U/ml, 0.05 U/mg, respectively.

Three-Phase Partitioning of α-Glucosidase

The crude corn α-glucosidase extract (2 ml containing 0.05 U and 1.0 mg protein) was saturated with 50% (w/v) ammonium sulfate at 25°C and then vortexed gently to dissolve the salt. The pH of the system was adjusted to pH 4.5. After the addition of t-butanol with the ratio of 1:0.75 (v/v), the mixture was vortexed gently for 1 min and then allowed to stand for 1 h at 25°C. Afterwards, the system was centrifuged at 1700 g for 10 min at 4°C to facilitate the separation of phases. Three distinct phases were observed. The upper t-butanol layer was removed carefully with a pasteur pipette. The lower aqueous layer (bottom phase) was collected by piercing the interfacial precipitate layer (middle phase) using a pipette. The interfacial protein precipitate containing α-glucosidase was collected and then dissolved in 1 ml of 0.05 M sodium acetate buffer (pH 4.5). The lower aqueous layer was also collected and dialyzed against same buffer. Both phases were then analyzed for α-glucosidase activity and total protein content.

The partitioning and purification of enzymes in TPP systems is influenced by several factors. Therefore, the effect of percent saturations of ammonium sulfate (20%, 30%, 40%, 50% and 60%, w/v), crude enzyme extract to t-butanol ratios (1:0.5, 1:0.75, 1:1, 1:1.5 and 1:2, v/v) and pH of the medium (4.0, 4.5, 5.0 and 5.5) on the partition behavior of α-glucosidase were investigated. The optimized best condition which resulted into maximum recovery was used as standard purification procedure for α-glucosidase. The activity of the crude extract initially added (0.05 U) was taken as 100%. The blank system was prepared containing ammonium sulfate, distilled water and t-butanol in the similar manner as for the crude extract. All the experiments were run in duplicate and the difference in the readings in triplicates was less than ±5%. The purified enzyme obtained from optimized TPP was used for further characterization studies in order to determine the general biochemical properties of the enzyme.

Assay of α-Glucosidase Activity

α-Glucosidase activity was determined spectrophotometrically by using p-nitrophenyl-α-D-glucopyranoside(PNPG) as substrate. A typical reaction mixture consisted of 50 µl of enzyme solution, 75 µl of 2 mM PNPG and 125 µl of 0.05 M sodium acetate buffer (pH 4.5) was incubated at 37°C for 10 min. The reaction was stopped by adding of 2 ml of sodium carbonate (1 M). The quantity of liberated p-nitrophenol was measured at 405 nm [24].

One unit (1 U) of enzyme activity was defined as the amount of enzyme which released 1 µmol of p-nitrophenol from PNPG per minute at pH 4.5 and 37°C. The data presented for all ˙ -glucosidase activity determinations are mean values of triplicate

assay in which the standard deviations were always smaller than 10%.

Protein Determination

The protein concentrations were quantified according to the Coomassie Blue G-250 dye binding assay using bovine serum albumin as the standard protein [25]. Specific activity was expressed as units per milligram of protein with PNPG as substrate.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS–PAGE of the protein samples with 12% gel was performed according to the method of Laemmli [26] with slight modification using Biorad Mini Protean II electrophoresis unit. For protein staining, the gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained with 40% (v/v) methanol and 10% (v/v) acetic acid for 2–3 h. Standard proteins were used as contained Sigma Molecular Weight Markers Calibration Kit for SDS-PAGE (Molecular Weight Marker Range 14 -66 kDa).

Effect of Temperature on the Activity and Stability of Partitioned α-Glucosidase

The temperature profile of the ˙ -glucosidase was conducted from 25 to 80°C by performing the standard assay procedure at the given temperature. The purified enzyme was exposed to different temperatures (25-80°C) for 30 minutes followed by testing the enzyme solution for residual activity. The relative activities as percentages were expressed as the ratio of the α-glucosidase activity obtained at a certain temperature to the maximum activity obtained at the given temperature range.

The thermal stability experiments were conducted without any additives. The termal stability of α-glucosidase was determined by measuring the residual activity of the enzyme exposed to different temperatures (25-60°C) in sodium acetate buffer (0.05 M, pH 4.5) for 30 minutes with continuous shaking. After desired incubation periods enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual α-glucosidase activity. Each set of experiment for the effect of temperature on the activity and stability of α-glucosidase were carried out in triplicates.

Effect of pH on Activity and Stability of Partitioned α-Glucosidase

The effect of pH on the activity of α-glucosidase was investigated by incubating enzymes with PNPG (2 mM) in 0.05 M sodium acetate buffer of different pHs ranging from 3.5 to 5.5 at 37°C. Activity of the enzyme was determined according to standard enzyme assay procedure at different pHs.

In order to determine the pH-stability, α-glucosidase was incubated in above buffers for 3 h at 4°C and then the residual activity(%) with respect to control was assayed under standard activity assay conditions. The relative activities at each pH were calculated as the percentage ratio of activity at a given temperature to the activity at 37°C. Each set of experiment for pH-activity and pH-stability were carried out in triplicates.

Determination of Kinetic Constants (K_M and V_{max})

The influence of substrate concentration on α-glucosidase activity was carried out with the initial concentration of PNPG ranging from 0.1 to 2.5 mM at 0.05 M sodium acetate buffer (pH 4.5) and 37°C. The maximum velocity of reaction (V_{max}) and the Michaelis–Menten constant (K_M) for α-glucosidase were calculated from Lineweaver–Burk plot which is a plot of 1/V against 1/[S] for systems obeying the Michaelis–Menten equation. Each set of experiment for the effect of subtrate concentration on the activity of α-glucosidase was carried out in triplicates.

Influence of Various Effectors on α-Glucosidase Activity

The activity of α-glucosidase is known to be affected by number of monovalent and divalent cations and compounds. To test the effect of various chemicals on enzyme activity, assays were performed in the presence of different concentrations (5-50 mM) of compounds (NaCl, $CaCl_2$, $MgCl_2$, KCl, SDS, Urea, EDTA). The effectors were added to the reaction mixture separately and after 30 min preincubation at

room temperature the activity of α-glucosidase was measured by standard enzyme assay procedure. Relative activity(%) of α-glucosidase was calculated by considering the activity of the control (without compound) as 100% activity. Each set of experiment for effectors was carried out in triplicates.

RESULTS AND DISCUSSION

Three-Phase Partitioning of α-Glucosidase

In the present work, we have employed three-phase partitioning (TPP) for direct one-step purification of α-glucosidase from corn (*Zea mays indurata*). We have first focused on the development of a suitable TPP process in order to achieve a maximum purity and yield of α-glucosidase. In addition, our second goal is to characterize and determine the enzymatic properties of the enzyme which are very important for its different applications. The purification and characterization of α-glucosidases from different sources is still very important because of their useful applications in industry, especially in food technology. They have a number of potential applications because of their wide range of substrate specificity. Various purification protocols containing ammonium sulfate fractionation and chromatographical steps were reported for α-glucosidases [4-7]. However, TPP has several advantages in comparison to this conventional separation and purification methods like; ease of operation, cost-effectiveness and scalability. Therefore, the process is calling the researchers' attention and gaining much popularity.

Effect of Ammonium Sulfate Concentration on TPP

TPP is affected by certain physico-chemical properties of proteins (hydrophilicity, molecular weight and pI) and also by physical conditions (ammonium sulfate concentration, ratio of aqueous phase to organic solvent and pH) [8-10]. Therefore, in order to efficiently separate and purify α-glucosidase from complex media and get perfect results, the influence of several parameters on the partitioning of the enzyme was investigated. For this aim, different partitioning experiments as a function of ammonium sulfate saturations (20%, 30%, 40%, 50% and 60%, w/v), crude enzyme extract to t-butanol ratios (1:0.5, 1:0,75, 1:1, 1: 1.5, 1:2, v/v) and pHs (4.0, 4.5, 5.0 and 5.5) were realized. The starting protein concentration was 11.3 mg/ml. The effects of these parameters on the degree of purification and on the activity recovery of ˙ -glucosidase from crude corn extract are presented in Figures 1-3, respectively. The results showed that, all these three process parameters are very important and effective on the partitioning of the corn α-glucosidase.

Different salts like; ammonium sulfate, sodium sulfate, sodium phosphate and potassium phosphate as cosmotropic salt, magnesium sulfate as chaotropic salt and sodium chloride as neutral salt were used in TPP systems. In the present study, ammonium sulfate was used as phase forming salt that is often provided the most effective salt on the protein partitioning [14,15,23,27]. Salting out of proteins with ammonium sulfate purify the proteins and also concentrate them into one of the phases according to net charge of the proteins and ammonium sulfate concentration of the system. Therefore, ammonium sulfate saturation is very critical parameter in TPP as it is responsible for protein-protein interaction and precipitation. So it has to be optimized for the efficient purification of proteins in TPP. We have studied the influence of ammonium sulfate saturation by maintaining the ratio of crude enzyme extract to t-butanol ratio (1:0.75, w/v) constant and varying the ammonium sulfate saturation in the ranges of 20%, 30%, 40%, 50% and 60% (w/v). The results are shown in Figure 1.

As seen from the Figure 1, the best partitioning results were obtained at 50% ammonium sulfate saturation(w/v) with a maximum purification fold of 3.5 along with 54% activity recovery of α-glucosidase in middle phase of the system. With an increase in ammonium sulfate concentration from 20% to 50%(w/v) the purification fold degree and activity recovery values were increased. Higher concentration of ammonium sulfate causes a reduction in the selectivity of extraction and also degree of purification. Fifty percentages (w/v) salt saturation was sufficient to concentrate the α-glucosidase in the middle phase. This finding is in agreement and comparable with several studies

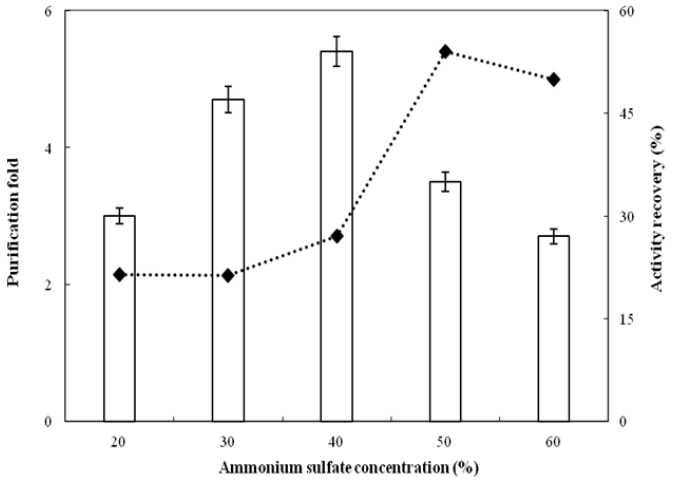

Figure 1. Effect of varying saturations of ammonium sulfate on the degree of purification and activity recovery of corn α-glucosidase [the crude extract (2 ml containing 0.05 U) was brought to different levels of saturation w.r.t. ammonium sulfate (20%, 30%, 40%, 50% and 60%, w/v) and t-butanol was added in the ratio of 1:0.75 (v/v) with respect to the volumes of the aqueous extract] (♦ Activity recovery; □ Purification fold).

[14,15,17,23,27]. The salting out of a protein in TPP system by sulfate ions is depended to ionic strength effects, kosmotropy, osmotic stressor, exclusion crowding agent and binding of sulfate ion to cationic sites of protein [28].

Effect of t-Butanol on TPP

The second important step in TPP systems is the choice of the organic solvent. Various C_4 alcohols can be used as phase forming organic solvent in TPP systems. In the present study, we prefered to use t-butanol due to its several advantages and the reference of earlier published reports on TPP[17,23,29,30]. t-Butanol can make three-phase layers and remove lipids, phenolics, pigments and enzyme inhibitors efficiently[30]. It does not easily permeate inside the folded protein molecules and hence does not cause denaturation due to its size and branched structure. t-Butanol also show significiant cosmotropic and crowding effects at 20-30°C that enhances the partitioning of enzyme[8]. Different TPP experiments with various crude enzyme extract to t-butanol ratios (1:0.5, 1:0,75, 1:1, 1: 1.5, 1:2, v/v) at 50%(w/v) ammonium sulfate saturation were performed. The effect of this ratio for α-glucosidase partitioning is shown in Figure 2.

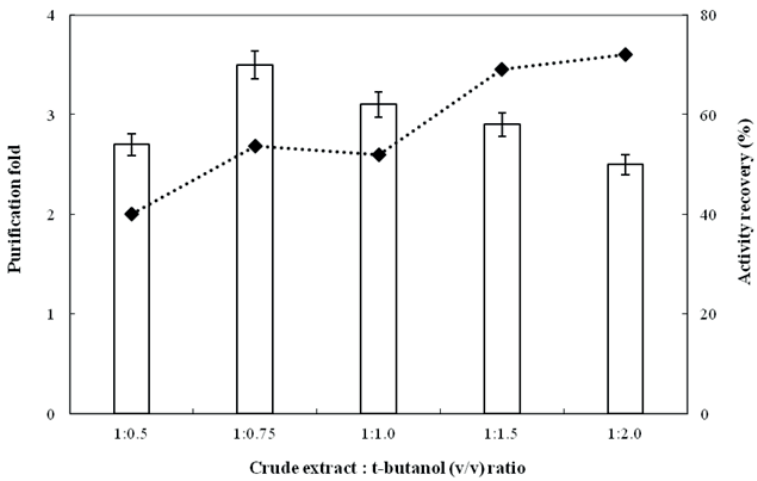

Figure 2. Optimization of crude extract to t-butanol ratio for the recovery of corn α-glucosidase [various amount of t-butanol was added to crude extract (2 ml containing 0.05 U and saturated with 50% (w/v) ammonium sulfate) in the following volumetric ratios viz. 1:0.5, 1:0.75, 1:1, 1:1.5 and 1:2] (♦ Activity recovery; □ Purification fold).

The best partitioning results were obtained with 1:0.75(v/v) ratio as 3.5-purification fold and 54% activity recovery. As is seen from the figure, with an increase in t-butanol volume the purification fold at the middle phase of the system was decreased. As the amount of t-butanol is increased, more water is taken up in the organic phase, thereby increasing the salt concentration of the aqueous phase and eventually resulting in protein precipitation at the interphase [17]. When the amount of t-butanol is less, it does not adequately synergize with ammonium sulfate [8,30]. From these findings, the ammonium sulfate concentration and crude enzyme extract to t-butanol ratio were selected as 50%(w/v) and 1:0.75 (v/v), respectively, to investigate the effect of pH value on the TPP system.

Effect of pH on TPP

The pH of the medium is very significant parameter in TPP systems that affects the ionization of amino acids present in the protein. Therefore, TPP systems usually sharply change around the isoelectric point (pI) of the proteins. The distribution and partitioning of proteins in TPP systems change with pH due to electrostatic interactions between phases and charged proteins. The basic mechanism is based on binding of sulfate anion on the cationic sites in the proteins[8,30]. In order to investigate the effect of pH on the partitioning of α-glucosidase in TPP system, the crude α-glucosidase extract was first saturated with 50%(w/v) ammonium sulfate and then the pH was adjusted to desired pH's in the range of 4.0-5.5. This was followed by addition of t-butanol in the ratio of 1:0.75 (v/v). TPP behavior of corn α-glucosidase with respect to pH is illustrated in Figure 3.

It is seen from Figure 3 that, α-glucosidase had partitioned selectively to the middle phase and the system gave a maximum 3.8-fold purification and 60% activity recovery of α-glucosidase at pH 4.5. It has been reported by Dennison[28] that, a change in pH causes the net charge of the target protein and this influences the partitioning of protein. In TPP systems, when the system pH is above the pI of protein, it will attain net negative charge and will be pushed to the bottom aqueous phase. If the system pH is below the pI of the protein, it will be precipitated at the middle phase of TPP [8].

Overall Purification of Enzyme

The overall purification of α-glucosidase from corn by TPP is summarized in Table 1. The enzyme has tendency to concentrate in the middle phase of the TPP system which is related to the structure of protein. Optimization of TPP results showed that, ammonium sulfate saturation, enzyme to

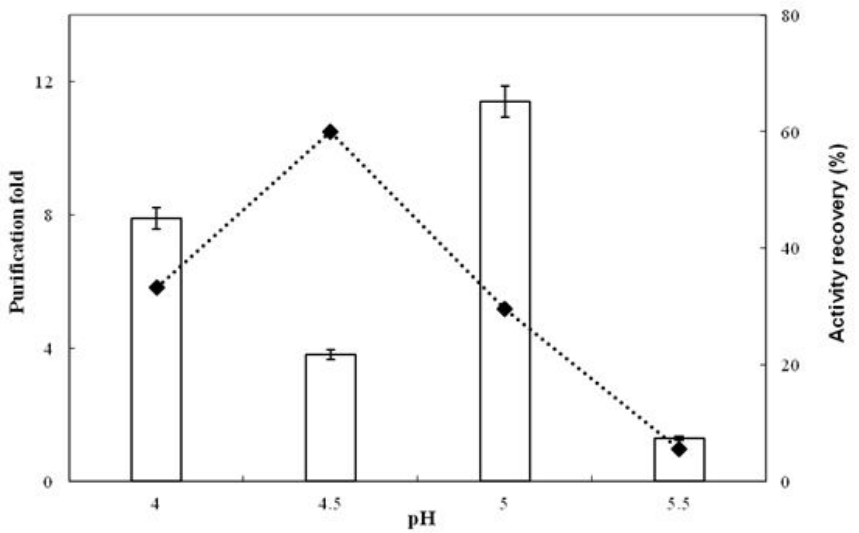

Figure 3. Influence of pH on the degree of purification and activity recovery of corn α-glucosidase [ammonium sulfate (50%, w/v) was added to the crude extract of α-glucosidase (2 ml containing 0.05 U). The pH of the medium was adjusted to different pH values. This was followed by addition of t-butanol in a ratio of 1:0.75 (v/v) (crude extract to t-butanol)] (♦ Activity recovery; □ Purification fold).

Table 1. Three-phase partitioning and purification of α-glucosidase from corn.[a]

	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Activity Recovery (%)
Crude extract -85%, w/v, ammonium sulfate fraction	0.05	1	0.05	1	100
TPP-interfacial precipitate	0.03	0.16	0.19	3.8	60
TPP-aqueous phase	0.012	0.32	0.04	0.8	24

[a] The ammonium sulfate (50%, w/v) was added to the crude extract of corn α-glucosidase (2 ml containing 0.05 U) and then pH was adjusted to pH 4.5. This was followed by addition of t-butanol in a ratio of 1:0.75 (v/v) (crude extract: t-butanol). Three phases formed were collected separately. The upper phase was removed and then the lower aqueous phase and interfacial precipitate were assayed for enzyme activity and protein amount. Each experiment was carried out in triplicate and the difference in the readings was less than ±5%.

t-butanol ratio and pH are very affective on the partitioning and purification of corn α-glucosidase. The optimized system consisting of 1:0.75(v/v) of enzyme extract to t-butanol, 50%(w/v) ammonium sulfate saturation and pH 4.5 resulted in the highest α-glucosidase recovery at 60% and a 3.8-fold purification at the interfacial precipitate. Several reports are available on the partitioning and purification of different enzymes from various sources with TPP such as; laccase from *Ganoderma* sp. WR-1 [17], β-galactosidase from *Lactobacillus acidophilus* [14], protease from papaya peels [31], α-galactosidase from *Aspergillus oryzae*[30], xylanase from *Aspergillus niger* [32] etc.

Biochemical Properties of α-Glucosidase

Biochemical characteristics of an enzyme are very important for evaluation of its potential use especially in biotechnological applications. Since the applications of α-glucosidases in various fields are broadening, it is important to discover and purify new α-glucosidases by using various purification protocols and also to understand the nature and biochemical properties of these enzymes for their efficient and effective usage in production processes. Hence, we have an attempt to determine the biochemical characteristics of α-glucosidase partially purified from corn (*Zea mays indurata*) by using TPP. The enzyme was partially characterized with respect to a range biochemical properties, including; optimum pH and temperature, stability at different pH and temperatures, kinetic parameters, effect of effectors and moleculer weight. The results of characterization studies are given below in proper sections, also discussed and compared with the similar studies.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of α-glucosidase was nearly homogenous with a molecular weight corresponding to 29 kDa (Figure 4). In general, molecular weight of α-glucosidases are varying according to their source and the applied method and also found between 26-130 kDa. As an example; Matsui et al. [33] have reported the molecular weight of α-glucosidase from sweet corn (*Zea mays varsaccharata*) as 96 kDa by SDS-disc electrophoresis. The relative molecular weight of 58 kDa obtained for α-glucosidase from *Trichoderma longibrachiatu* [7] by SDS-PAGE. According to SDS-PAGE results of rice α-glucosidase the molecular weight was estimated as 60 kDa [6]. SDS-PAGE gels of α-glucosidase purified from *Entamoeba histolytica* revealed a single polypeptide of 55 kDa [34].

Effect of Temperature on Enzyme Activity and Stability

Temperature dependence of the enzymatic reaction of α-glucosidase was studied in the temperature range of 25-80°C. The corn α-glucosidase was very active over a broad temperature range of 45-75°C with on optimum temperature of 55°C (Figure 5a). As it can be observed from Figure 5(a), α-glucosidase was retained more than 60% of its initial activity at 75°C. The enzyme was not lost rapidly its activity at temperatures higher than 55°C. As is known, the reaction rate of enzymes increases with

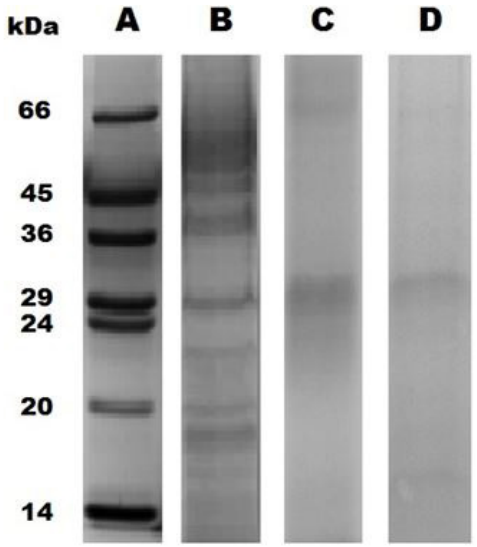

Figure 4. SDS-PAGE pattern of corn α-glucosidase (lane A; molecular weight markers (14-66 kDa), lane B; crude α-glucosidase extract, lane C; TPP purified α-glucosidase (middle phase) lane D; TPP of α-glucosidase (bottom phase).

increasing temperature, but higher temperatures can result in breaking of the ionic and hydrogen bonds between the amino acid residues of the enzyme. This causes denaturation of the enzyme thus rendering the enzyme inactive [35]. The results obtained for corn α-glucosidase were in correlation with the previous reports. Most of α-glucosidases have an optimum temperature between 35-65ºC [7,14,33,36,37]. Similar results have been reported by Iwata et al. [6]. They have determined optimum temperature for rice α-glucosidase as 55ºC.

Thermostability is an ability of enzyme to resist against thermal unfolding in the absence of substrate and is very important for its biotechnological applications. So, in the present study the effect of temperature on the stability of α-glucosidase was determined by incubating the enzyme at various temperatures (25-60ºC) for 30 min and the results are shown in Figure 5(b). Investigations on thermostability revealed that, the corn α-glucosidase was very stable between the temperature range of 25-60ºC and enzyme still retained more than 56% of its initial activity at 60ºC. Various temperature-stability results of α-glucosidases have been obtained depending the source of enzyme, temperature, incubation time and reaction conditions. Cihan et al. [37] have found that both intracellular and extracellular α-glucosidases from *G. toebii* strain were very stable between temperature ranges of 35-70ºC. Marin et al. [38] have also found that α-glucosidase from *X. dendrorhous* was very stable to thermal treatment up to 50ºC.

Effect of pH on Enzyme Activity and Stability

The influence of pH on the activity of α-glucosidase was studied by changing the pH of the medium from 3.5 to 5.5 at 37ºC and the results are presented in Figure 6(a). pH optima of α-glucosidase was found to be as pH 4.5. As is seen from the figure, more than 85% of its initial activity was recovered between the pH range of 3.5-5.5. α-Glucosidases from various enzyme sources exhibits pH optima ranging from 4.5 to 7.0 [39]. The optimum pH of rice α-glucosidase has also been found pH 4.5 by Iwata et al. [6].

Another important parameter to be considered for applications of enzymes is pH stability. The effect of pH on α-glucosidase stability was determined by incubating the enzyme in sodium acetate buffer (0.05 M) with different pHs (pH 3.4-5.5) for 3 h and then remaining activities were assayed. The enzyme was very active and stable in the pH range from 3.5 to 5.0 and it retained more than 80% of its initial activity (Figure 6b). However, stability was significantly reduced to 45% of pH 5.5. It has been reported that, α-glucosidases are very stable in respect to pH changes[39].

Kinetics of α-Glucosidase

The kinetic parameters K_M (Michaelis-Menten constant) and V_{max} (maximum velocity of reaction) were determined by measuring the activity of

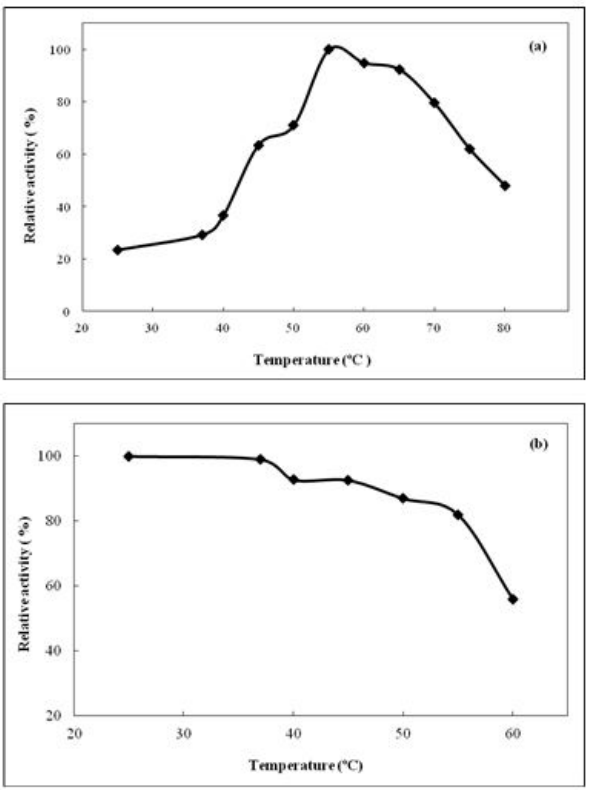

Figure 5. Effect of temperature on the activity (a) and stability (b) of corn α-glucosidase.

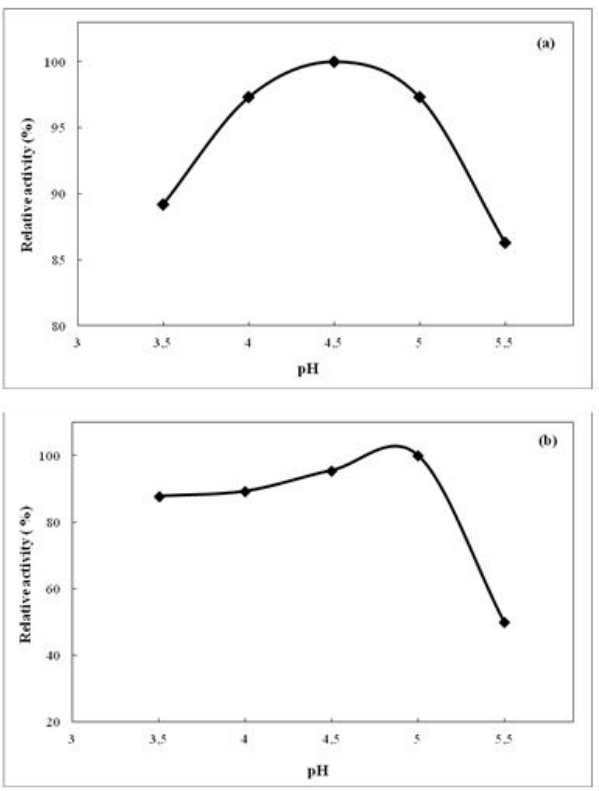

Figure 6. Effect of pH on the activity (a) and stability (b) of corn α-glucosidase.

α-glucosidase at different substrate (PNPG) concentrations (0.1-2.5 mM). The parameters were determined by fitting experimental data to Michaelis–Menten model and were calculated from Lineweaver–Burk graph as 0.64 mM and 0.45 U, respectively (Figure 7). The K_M value of the enzyme is within the range of the majority of other α-glucosidases. The results obtained by several other researchers showed that the kinetic constants had varying values depending on the source of the enzyme and used substrate [1,7,37]. For instance, the K_M values of α-glucosidases from palm weevil [40], *Apis cerena japonica*[41] and *N. aenescens* [42] were found as 0.3, 1.0 and 0.54 mM, respectively.

Effect of Various Metal Ions and Compounds on α-Glucosidase Activity

Various metal ions and compounds were tested for their effects on α-glucosidase activity (Table 2). These effectors had different effects on the activity level of the enzyme. As it can be observed from the table, the effect of effectors on the enzyme activity was dependent to their concentrations. $CaCl_2$ had the most activating effect that by increasing its concentration up to 10 mM the enzyme activity was increased significantly (130%). Similarly, the study of the influence of $MgCl_2$ with 10 mM concentration also showed a significant activation of the enzyme.

It is well known that, glucosidases require calcium ions for their activity which are metalloproteins [24,36] and also they affords stability of enzymes to extreme pH and temperature values. Similar results were also obtained by various authors[4,36]. For example, *R. palmarum* larve α-glucosidase was inhibited with Fe^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2}, Ba^{+2}, DTNB, pCMP, SDS, β-mercaptoethanol and EDTA [40]. α–Glucosidase from millet seeds was only affected with $Hg^{+2}$and Pb^{+2} [43]. Justin et al. [44] have found that, α-glucosidase from the digestive juice of *L. flammea* activated by Mn^{+2} ion and also inhibited by SDS significantly. Our results showed that, α-glucosidase activity was enhanced with increase the concentration of SDS, EDTA and urea up to 20 mM and above this concentration the relative activities were decreased. Ghadamyari et al. (2010) have also obtained similar results. They have reported that the activity of α-glucosidase from lesser mulberry pyralid was enhanced with increasing concentration of EDTA, SDS and urea [36]. Memarizadeh et al. (2014) have also showed that 40 mM of $CaCl_2$ and also EDTA in each concentrations of 10 and 20 mM significantly increased the activity of α-glucosidase [42].

CONCLUSION

To the best of our knowledge, this is the first report for partial purification, concentration and characterization of corn (*Zea mays indurata*)

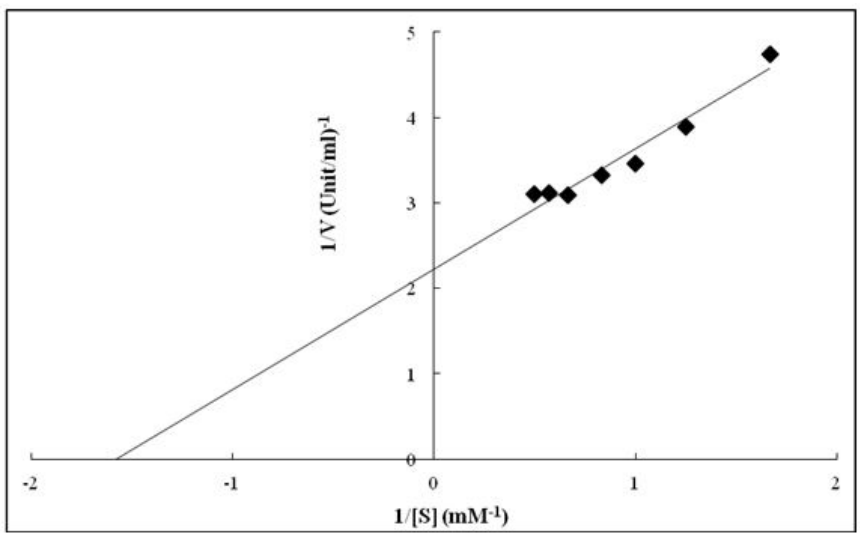

Figure 7. Lineweaver–Burk plot of corn (activities were assayed at 37°C by using different concentrations of PNPG).

Table 2. Effects of various ions and compounds on the activity of corn α-glucosidase.[a]

Compounds	Concentration (mmol/L)	Relative activity (%)
Control	-	100
NaCl	5	78
	10	103
	20	92
	50	87
$CaCl_2$	5	117
	10	130
	20	115
	50	100
$MgCl_2$	5	119
	10	128
	20	125
	50	122
KCl	5	112
	10	114
	20	117
	50	114
SDS	5	95
	10	106
	20	102
	50	100
Urea	5	96
	10	109
	20	113
	50	102
EDTA	5	101
	10	109
	20	119
	50	88

[a] After preincubation of α-glucosidase with different chemicals at various concentrations at 25 °C for 30 min the remaining enzyme activity was measured with standard activity assay procedure. Activity without added chemicals taken as 100% activity. Data present the means of three determinations ±SE.

α-glucosidase by using three-phase partitioning (TPP). TPP does not only purify the enzyme but also concentrate it into one of the phases. In comparison to chromatographic methods, this process is very cheap, simple and efficient. Effect of various process parameters has been evaluated and α-glucosidase was purified to 3.8-fold with 60% activity recovery by optimized single step TPP system (50%, (w/v) ammonium sulfate concentration, 1:0.75 (v/v) ratio of crude extract: t-butanol at pH 4.5). The maximum α-glucosidase retention was in the middle phase of TPP. It can be concluded that, as fast developing non-chromatographic separation technique, TPP could be attractive for the partitioning and purification of α-glucosidases from new enzyme sources. Biochemical characteristics of α-glucosidase have also showed its applicability in biotechnologically important process like; enzymatic synthesis of novel oligosaccharides and hydrolysis of starch to glucose.

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