Purification, recovery, and characterization of chick pea (*Cicer arietinum*) β-galactosidase in single step by three phase partitioning as a rapid and easy technique

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In this study chick pea β-galactosidase was first time purified and recovered in single step by three phase partitioning (TPP). Optimal purification parameters for TPP were 60% ammonium sulfate saturation (w/v) with 1:0.5 (v/v) ratio of crude extract:t-butanol at pH 6.8, which gave 10.1 purification fold with 133% recovery of β-galactosidase. SDS–PAGE analysis showed that protein has two subunits with molecular masses of 48 and 38 kDa, respectively. Characterization of enzyme showed that optimal pH of purified enzyme was 2.8 and optimal temperature was 50 °C. Enzyme was further characterized by the Arrhenius activation energy and Michael–Menten kinetic constants. Activation energy (*Ea*) was calculated by using Arhenius equation and determined to be 15.52 kcal mol \(^{-1}\). *Km* value of purified enzyme was estimated for the o-nitrophenyl β-D-galactopyranoside (ONPG) substrate as 1.09 mM, while its maximum velocity, *Vmax* was 0.90 U/mL/min at 37 °C. TPP improved substrate affinity of enzyme by the increased flexibility during the partitioning. TPP is simple, easy and economic technique for purification and recovery of β-galactosidase from chick pea, and has a big potential use for industrial applications.

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**Introduction**

β-α-Galactosidase (EC 3.2.1.23, β-α galactoside, galactohydrolase, lactase) catalysis the non-reducing β-α-galactosyl residues from polysaccharides. They are widely distributed in plants, microorganisms and animals [1–3]. This enzyme has many industrial and medicinal applications like cleavage of blood group A and B glycotopes, biosensors for lactose determination and enzymatic hydrolysis of lactose [4]. Especially enzymatic hydrolysis of lactose has many advantages in food industry. Lactose hydrolyzed products decrease the lactose intolerance problem [5]. Lactase hydrolyses the lactose to glucose and galactose and nutrition value of milk treated by the lactase, does not decrease, because glucose and galactose are not removed [5,6]. Furthermore, compared to lactose, glucose and galactose more increase the sweet taste of products and result in fewer calories [6–8]. β-Galactosidase has been purified from different plants like kiwifruit, mango, kidney beans, pea and also chick pea [9–13]. Purification methods of β-galactosidase include various traditional methods such as salting out, acid fractionation and different chromatographic assays. These methods are time consuming, involve expensive reagents and need various equipment. Three phase partitioning (TPP) is a simple technique in which ammonium sulfate and t-butanol are added to crude extract [14,15]. In less than 1 h, three phases are formed. The upper phase is organic phase and contains non-polar compounds, the lower phase is aqueous phase and contains polar compounds, and the middle phase is protein rich phase [16,17]. The addition of t-butanol in presence of ammonium sulfate pushes the protein out of solution [18]. Furthermore, kosmotropy, salting out, isionic precipitation and hydration shifts of proteins are collective effects for precipitation of protein at the interphase [15].

As TPP is easy and can be used directly to crude extract [18–20], it is an alternative method for purification of industrial important enzyme [15,21].

In some cases, precipitation of proteins by TPP results higher catalytically efficiency. TPP causes high flexibility of protein and increasing of conformational flexibility shows higher catalytic activity of protein [15,22,23].

There have been a number of reports about purification and recovery of enzyme such α-galactosidase from different sources [21,24,25], protease form papaya peels [22], viscara [26], *Calotropis procera* latex [27] and peroxidase from *Ipomoea* [28]. In this study, we tried to purify and recover β-galactosidase from chick pea by TPP. According to our knowledge, this enzyme has not been purified from chick pea by this technique before. Partitioned and recov-
ered enzyme was also characterized with optimal pH and temperature values and kinetic constant ($k_{\text{m}}$ and $V_{\text{m}}$). Molecular weight of enzyme was determined by the SDS–PAGE electrophoresis system. This method has an important potential use in various industries.

**Materials and methods**

**Materials**

Dry seeds of C. arctium were purchased from local market (Turkey). All the chemicals for buffers and t-butanol were of analytical or electrophoresis grade from Merck (Eurolab GmbH Darmstadt, Germany). Ammonium sulfate, Coomassie Brilliant Blue R-250 and substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) and Bradford reagent and molecular markers for electrophoresis were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were used of analytical grade.

**Methods**

**Enzyme assay**

β-Galactosidase activity was routinely measured by using ONPG substrate [13] with some modifications. The assay mixture for activity measurement consisted of 450 μL 20 mM ONPG substrate (in 50 mM PH 2.8 glycine–HCl buffer) and 50 μL appropriately diluted enzyme was incubated at 37 °C for 10 min. Liberated o-nitrophenol was measured spectrophotometrically at 405 nm after stopping the reaction with the addition of 500 μL sodium carbonate (1 M in pure water). One unit of β-galactosidase is defined as the amount required for releasing 1 μmol of o-nitrophenol per min, under standard conditions (extinction coefficient of o-nitrophenol equals 4.05 × 103 M⁻¹ cm⁻¹). The data presented for all β-galactosidase activity determinations are mean values of duplicate assay.

**Protein determination**

Concentration of the protein was determined by Bradford method [29] using Coomassie brilliant blue G-250 dye as a reagent and bovine serum albumin (BSA) as standard, by measuring the absorbance at 595 nm at 25 °C. Assays were performed in duplicate and the averages were used in calculations. Specific activity was expressed as units/milligram protein.

**Electrophoretic analysis of crude and purified enzyme**

Molecular weight of β-galactosidase was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [30] on a Biorad Mini Protean dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Bradford reagent and molecular markers for electrophoresis were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were used of analytical grade.

**Preparation of crude extract of β-galactosidase**

30 g dry seeds were sterilized with 2% hydrogen peroxide for 10 min at +4 °C, then thoroughly washed with distilled water and soaked in extraction buffer (25 mM PH 6.8 phosphate buffer included in 1 mM EDTA, 1 mM mercaptoethanol, 1 mM PMSF) for 24 h at +4 °C. 10 g of soaked seeds were homogenized using simple blender in 40 mL 25 mM PH 6.8 phosphate buffer for 2 min at +4 °C. The homogenate was filtered from five layers of cheese cloth and then centrifuged at 14,000 rpm for 30 min at +4 °C. The supernatant was collected and pellet containing cell debris was discarded. The pH of supernatant was reduced to 5.0 by adding of 0.5 M HCl at +4 °C with continuous stirring. After one hour the mixture was centrifuged at 14,000 rpm for 30 min at +4 °C. The pellet was discarded and the clear supernatant was collected and pH was adjusted to 6.8 with the 0.5 M NaOH at +4 °C [13]. This extract was named as crude β-galactosidase extract and used for further TPP process.

**Three phase partitioning**

TPP experiments were carried out employing various t-butanol ratios (crude extract:t-butanol; 1:0.25, 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) with a constant ammonium sulfate saturation at 30%. The mixture was mixed gently and then allowed to stand for 30 min at +4 °C. Afterward the mixture was centrifuged at 5000 rpm for 10 min at +4 °C to facilitate the separation of phases. The upper t-butanol phase was removed by the Pasteur pipette. The lower aqueous phase and the interfacial phase were separated carefully and each of phases was analyzed for enzyme activity and protein content. The interfacial phases containing β-galactosidase were collected and dissolved 2 mL of 25 mM PH 6.8 phosphate buffer. Different ammonium sulfate saturation effects (20%, 30%, 40%, 50%, 60%, 70%, 80%) (w/v) were also investigated at the best recovery activity crude enzyme:t-butanol ratio that is 1:0.1.05. The bottom phase and interfacial phase were analyzed for enzyme activity and protein content. Several parameters affected TPP studies. After, the t-butanol and ammonium sulfate effects with different pH values of study medium were studied. Crude extract was saturated with 60% ammonium sulfate and pH was adjusted to 2, 3, 4, 5, 6, 7, 8, 9 with the 0.5 M HCl or 0.5 M NaOH, then 1:0.05 t-butanol was added and the best pH value on the partition behavior of β-galactosidase was investigated.

**Kinetic properties of the purified β-galactosidase**

**Effect of pH on β-galactosidase activity**

The influence of pH on the enzymatic activity at 37 °C was determined by assaying the activity at different pH values ranging from 2.0 to 10.0, using 25 mM concentrations of the following buffer systems: glycine–HCl (pH 2.0, 3.0), sodium acetate (pH 4.0, 5.0, 6.0), sodium phosphate (pH 7.0, 8.0) and glycine–NaOH (pH 9.0, 10.0). The relative activities (as percentage) were expressed as the ratio of the β-galactosidase activity obtained at a certain pH to the maximum activity obtained in the given pH range.

**Effect of temperature on β-galactosidase activity**

In order to define the optimal temperature of enzyme against ONPG, different temperatures ranging from 25 to 60 °C (25, 30, 35, 40, 45, 50, 55, 60) were assayed. After incubation periods, enzyme was assayed at standard activity conditions for determining the relative activity. The relative activity (as percentage) was expressed as the ratio of the β-galactosidase activity obtained at a certain temperature to the maximum activity obtained in the given temperature range. The activation energy of β-galactosidase was determined by measuring the maximum velocity (V) at the same temperatures (25–60 °C), and plotting $\log_{10}V$ against 1/T.
effect of temperature on enzyme stability was also assayed by measuring residual activity of enzyme in the matter of different temperatures for 30 min. After desired incubation time, enzyme was assayed at standard activity conditions for determining the residual activity.

Result and discussion

Purification and recovery of β-galactosidase by TPP

One of the major applications of β-galactosidase in industry is the preparation of lactose-hydrolyzed milk and whey. Especially in the cheese industry lactose is a big trouble because, it has uncertain solubility and it is associated with the high biochemical and chemical oxygen demand. Lactose hydrolysis can be achieved by hard conventional method which is acid treatment at higher temperature, but enzymatic catalysis of lactose carries out milder operating conditions. But, bioseparation of proteins are expensive and time consuming protocols. Hence researchers try to develop new methods which have limited number of steps and have general applicability for reducing of separation and purification costs of wide range of enzymes. TPP is quietly appropriate for this aim because it is economic, cheap and in one or two steps enzyme can be purified with high recovery. However, there are no reports on purification of β-galactosidase from chick pea by using of TPP. In this study, we optimized purification and recovery of β-galactosidase at single step by using TPP from chick pea.

TPP is affected by various process conditions, such as salt concentration, pH and solvent ratio. Therefore, different ratios of t-butanol, different concentrations of salt and various pH values were performed in this study. The effects of these parameters on purification and recovery of β-galactosidase from chick pea by using TPP are given in Figs. 1–3, respectively.

Effect of crude extract:t-butanol ratio on TPP

Different extract:t-butanol ratio effect for the partitioning of β-galactosidase at the constant saturation of ammonium sulfate (30%, w/v) is shown in Fig. 1. The highest enzyme recovery (126%) and purification fold (2.24) were obtained at 1:0.5 crude extract:t-butanol ratio in aqueous phase. An increasing t-butanol quantity caused decreasing of the purification fold for each phase. This may be attributed to synergistic effects of the increase in concentration of t-butanol and decrease in saturation of ammonium sulfate. For the precipitating of protein in interfacial phase usually 0.2–0.5 mL of butanol is required for per milliliter of beginning crude sample. In our study, the best result was obtained at the ratio 1:0.5 (v/v) of crude enzyme:t-butanol too. Salt effects on partitioning of β-galactosidase were examined under the 1:0.5 (v/v) ratio of crude enzyme:t-butanol for aqueous and interfacial phases. Although, t-butanol is branched alcohol and does not permeate into folded structure protein molecules and does not cause denaturation on protein molecule, when the ratio of sample:t-butanol is higher than 1, the denaturation of protein is more possible.

Effect of ammonium sulfate saturation on TPP

Crude extract t-butanol ratio was chosen as 1:0.5 (v/v). According to Fig. 1 results; aqueous and interfacial phases were collected and analyzed for protein content and activity.

Although TPP is carried out at about 40% saturation of ammonium sulfate; in our study we decided to use high concentrations of salts in order to see the effect of salt on partitioning of enzyme. As seen in Fig. 2, activity of β-galactosidase was completely decreased at 60% ammonium sulfate saturation in aqueous phase, in the same conditions, the best results of recovery and the purification fold of β-galactosidase (133% and 10.1, respectively) was obtained in the interfacial phase. Probably, this saturation was caused by the precipitation of whole target protein molecule in the interfacial phase. Generally, researchers start minimum salt concentration of 20% (w/v) to optimize the partitioning conditions. In this study we also started at minimum salt saturations for the beginning and the increasing of salt concentrations resulted with the increasing of recovery and purification fold in the interphase until the 60% (w/v) saturations. Higher salt concentrations from this point were resulted with reducing of recovery and purification fold, which may be due to irreversible denaturation of protein. Fig. 2 also showed that TPP strongly depend on saturation of ammonium sulfate. According to Figs. 1 and 2; during the TPP, activation of enzyme is also possible. This may be due to the phenomenon that the increased flexibility of enzyme can lead to higher catalytically activity in TPP process.

Effect of pH on TPP

pH value of partitioning medium also affects the recovery and purification fold of proteins. pH changes cause the changing of charge of amino acid residues at the surface and also electrostatic interactions in protein molecule. Changed electrostatic interactions by pH, affect the partitioning property. When pH value of TPP medium is adjusted under pl value, amino acid residues are positively charged and protein molecule will be pre-

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**Fig. 1.** Effect of different crude extract:t-butanol ratio on recovery and purification fold in interfacial phase and aqueous phase of β-galactosidase from chick pea. Saturation amount of ammonium sulfate was fixed to 30% (w/v) and crude extract:t-butanol ratio were varied in the range of 1:0.25–1:2. The each of aqueous phase and interfacial precipitate were collected and analyzed for protein content and activity.
The pl value of β-galactosidase from chick pea was reported to be 8.63 [40]. Fig. 3 shows the partitioning of β-galactosidase into interfacial phase with different pH values. The best combination for providing highest β-galactosidase recovery (10.1-fold purification and 133% recovery) is at 60% ammonium sulfate saturation by using the ratio of crude extract:t-butanol of 1:0.5 with pH 6.8, respectively.

3.2. Purification and recovery results

The purification profile of β-galactosidase from chick pea by TPP is shown in Table 1. According to table, 133% activity recovery and 10.1-fold purification were obtained. As seen in Fig. 4, SDS–PAGE of crude extract has major bands with approximately 96, 60, 45, 22, 16 and 6 kDa, respectively. Most of these bands weren’t observed at purified enzyme. SDS–PAGE analysis of purified enzyme is shown in (Fig. 4), two different polypeptides with non-identical molecular weight 48 and 38 kDa, respectively. This result agrees well with the earlier reported results [40] hence; chick pea β-galactosidase has two different subunits. Native-PAGE and activity staining electrophoretic results showed that the molecular weight of enzyme was almost same with the sum of the subunits molecular weight.
Determination of kinetic constant

The Lineweaver–Burk plot is shown in Fig. 5. The Michaelis–Menten constant $K_m$ for the ONPG substrate was estimated to be 1.09 mM, while its maximum velocity, $V_m$ was 0.90 U/mL/min. $K_m$ value of chick pea $\beta$-galactosidase was observed before as 1.73 mM while substrate was ONPG. Previously it has been reported that TPP leads to improving $K_m$ values of enzyme [15]. X-ray studies of proteinase $K$ showed that, increased flexibility of protein caused increased access to active site of enzyme [41]. Thus, decrease of $K_m$ value of enzyme is not surprising. Similar results were observed by the other works. Pectinase was purified by the two methods: TPP and conventional chromatographic methods. It was reported that the smaller $K_m$ value was obtained by the TPP methods [42].

3.3.2 Effect of temperature on purified $\beta$-galactosidase

The hydrolyzing activity of chick pea $\beta$-galactosidase was monitored at the range of 25–60°C. The optimum temperature of chick pea $\beta$-galactosidase was found to be 50°C (Fig. 6). Although optimal temperature value of chick pea $\beta$-galactosidase was reported as 60°C before [12,13,40] it was found found as 50°C in this study. TPP causes the activation of enzyme via increasing flexibility. But changed flexibility of enzyme by TPP, may cause the reducing of optimal temperature. The enzyme was good thermostable in the range of 25–45°C (Fig. 6). Thermal stability of enzyme is critically important because of industrial application. Similar results were obtained by different researches [12,13,40]. Activation energy ($E_a$) of $\beta$-galactosidase was calculated by using Arrhenius equation and found to be 15.52 kcal mol$^{-1}$. Activation energy for ONPG hydrolysis with different pea $\beta$-galactosidase was found as 11.32 kcal mol$^{-1}$ [40] and 11.68 kcal mol$^{-1}$ [13].

3.3.3 Effect of pH on purified $\beta$-galactosidase

Generally plant $\beta$-galactosidases show pH optima in acidic range [40]. In this study optimal pH value of chick pea $\beta$-galactosidase was found as 2.8 (Fig. 7). This value agrees well with literature results [13,40]. $\beta$-Galactosidases isolated from muskmelon, kiwifruit and papaya also seemed to be optimally active at acidic pH range [43–45].

Conclusions

$\beta$-Galactosidase was purified from the chick pea by using the TPP. The recovery activity of enzyme was found compatibly higher than the other purified plant $\beta$-galactosidase. Purified enzyme was stable for several days at +4°C. Having optimal pH and temperature values and, being especially rapid, one step and cheap purification procedure with highly recovery activity make this technique having good potential for several industrial applications.

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References


