Separation and Purification Technology 70 (2009) 123–127

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Purification of tomato (*Lycopersicon esculentum*) α-galactosidase by three-phase partitioning and its characterization

Esin Çalci, Tuğba Demir, Evran Biçak Çelem, Seçil Önal*

Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova-İZMİR, Turkey

ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 3 September 2009
Accepted 4 September 2009

Keywords:
Three-phase partitioning
α-Galactosidase
Tomato
Lycopersicon esculentum
Protein purification

ABSTRACT

Three-phase partitioning (TPP) was used to purify α-galactosidase from tomato (*Lycopersicon esculentum*). The technique is a novel separation process used for the extraction and purification of biomolecules. It involves the addition of salt (generally ammonium sulfate) to the crude extract followed by the addition of an organic solvent (generally butanol). The protein appears as an interfacial precipitate between upper organic solvent and lower aqueous phases. The various conditions required for attaining efficient purification of the α-galactosidase fractions were optimized. Under optimized conditions, it was seen that, 50% of ammonium sulfate saturation with 1:1 ratio of crude extract to t-butanol at pH 4.5 gave 4.3-fold purification with 80% activity yield of α-galactosidase. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed substantial purification and the molecular weight of α-galactosidase was nearly found to be as 34 kDa. The purified enzyme was characterized with respect to its activity and stability at various pH and temperature ranges. Optimum pH and temperature were determined at pH 4 and 37 °C, respectively. The enzyme was stable in the range of pH 3–5 and more than 60% of its initial activity was recovered. The α-galactosidase completely retained nearly about 70% of its initial activity at 40 °C. The kinetic constants; K_m and V_{max} using p-nitrophenyl-α-D-galactopyranoside (PNPG) as substrate were 1.07 mM and 0.01 U/mg, respectively. TPP is an attractive process for the purification of α-galactosidase.

1. Introduction

α-Galactosidase (α-D-galactoside galacto-hydrolase, EC 3.2.1.22) catalyzes the hydrolysis of terminal α-1,6-linked D-galactose residues present in galacto-oligosaccharides of sugars of the raffinose family and galactomannan polysaccharides [1–3]. They are widely distributed in nature and found in microorganisms, plants and mammals. There are several reports available in the literature for the purification and characterization of α-galactosidase from different sources [4–6]. α-Galactosidases have many potential biotechnological and medicinal applications such as in food processing, animal feed processing, sugar producing industry, pulp and paper industry, enzymatic synthesis, structural analysis, conversion of blood type and treatment of Fabry's disease [7–13].

Three-phase partitioning (TPP) is a quick and efficient procedure for the purification of proteins from complex mixtures. It has been used both for upstream and downstream protein purification processes and some times been used for direct one-step purification [14]. TPP is a simple technique in which a salt (generally ammonium sulfate) and an organic solvent (generally t-butanol) are added to an aqueous solution of proteins. In less than an hour, three phases are formed. The upper t-butanol rich phase generally removes any lipid or hydrophobic material. The interfacial phase is the protein precipitate. The lower aqueous phase contains remaining proteins, cell debris, etc. [14,15]. It is believed that the extraction process is a combination of salting out, kosmotropic, isoionic and co-solvent precipitation of proteins [16,17]. Proteins show different behaviour in these conditions, depending upon their source, molecular weight, p*I* and temperature [18]. This technique is easily scaleable and can be used directly with crude suspensions [19,20]. TPP has been used to purify a number of biomolecules such as; green florescent protein from *E. coli* [21], pectinase from tomato [22], protease inhibitor from ragi [16], phospholipase D from *Docus carota* [23], exo-polygalacturonase from *Aspergillus sojae* [24], peroxidase from turnip [20], alkaline phosphatase from chicken intestine [25] and α-galactosidase and invertase from *Aspergillus oryzae* [19].

In this work, we have employed three-phase partitioning for direct one-step separation and purification of α-galactosidase from tomato. Different parameters (ammonium sulfate concentration, the ratio of enzyme amount to t-butanol and pH) required for the efficient purification of enzyme were optimized to get highest purification fold and yield. The purified enzyme was also further

* Corresponding author. Tel.: +90 232 3438624; fax: +90 232 3438624.
E-mail address: secil.onal@ege.edu.tr (S. Önal).

1383-5866/$ – see front matter Crown Copyright © 2009 Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.seppur.2009.09.004

characterized. Some parameters affecting the enzyme activity and stability were analyzed. To our knowledge, this is the first report on purification of α-galactosidase by TPP from tomato (*Lycopersicon esculentum*).

2. Materials and methods

2.1. Materials

Fresh ripe tomatoes were purchased from a local market, Turkey. t-Butanol and ammonium sulfate were pure grade and were procured from E. Merck (Germany). All other chemicals and reagents were of the highest available purity and used as purchased.

2.2. Methods

2.2.1. Enzyme activity assay

α-Galactosidase activity was determined using p-nitrophenyl-α-D-galactopyranoside (PNPG) as substrate. Reaction mixture consisting of 0.25 ml of 2 mM PNPG, 0.5 ml of sodium citrate buffer (pH 6.0) and 0.25 ml of enzyme solution was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 0.2 M sodium borate buffer (pH 9.8). The quantity of liberated p-nitrophenol was measured at 400 nm [26].

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 6.0 and 37 °C.

The data presented for all α-galactosidase activity determinations are mean values of triplicate assay in which the standard deviations were always smaller than 10%.

2.2.2. Protein determination

Protein concentrations were estimated according to the Coomassie Blue G-250 dye-binding assay using bovine serum albumin as a standard [27].

2.2.3. Preparation of α-galactosidase extract from tomato

The crude extract of tomato α-galactosidase was prepared from 500 g fresh tomatoes according to the procedure described below. After removal of the peels and the seeds, the tomato flesh was homogenized with citrate buffer (50 mM, pH 6.0) containing 1 M NaCl. The homogenate was filtered from two layers of cheese-cloth and then the pH of the filtrate was adjusted to 5.7–6.0 with 1 M NaOH. The suspension was stirred for 1 h with magnetic stirrer and then centrifuged at 10 000 rpm for 15 min at 4 °C. The supernatant obtained after centrifugation was subjected to 85% ammonium sulfate precipitation and then allowed to stand overnight at 4 °C with continuous stirring. The precipitate was collected by centrifugation at 10 000 rpm for 30 min at 4 °C. The pellet was dissolved in a minimal volume of citrate buffer (50 mM, pH 6.0) and dialyzed against same buffer for overnight. In order to remove the insoluble matter, dialysate was centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant represented the tomato α-galactosidase extract and used for three-phase partitioning. The protein concentration and specific activity of the enzyme were determined as 1.17 mg/ml and 0.536 U/mg, respectively.

2.2.4. Purification of α-galactosidase by three-phase partitioning

The crude extract of α-galactosidase (2 ml containing 1.25 U and 2.34 mg protein) was saturated with ammonium sulfate to the desired level at 25 °C and vortexed gently to dissolve the salt followed by the addition of t-butanol. The mixture was vortexed gently and allowed to stand for 1 h. After this, the mixture was centrifuged (4000 rpm for 10 min) and the formation of three phases (upper organic phase, middle interfacial precipitate and lower aqueous phase) was observed. The upper t-butanol layer was removed carefully with a pasteur pipette. After this, the lower aqueous layer was collected by piercing the interfacial precipitate layer using a pipette. The interfacial precipitate containing α-galactosidase was collected and then dissolved in 1 ml of citrate buffer (50 mM, pH 6.0). The aqueous layer and interfacial precipitate were then analyzed for α-galactosidase activity and total protein content. In a preliminary study, we have optimized the incubation time for TPP. After 1 h the phases separated completely but lowering the time to 30 min cause no phase separation. It is also determined that 2 h did not cause a significant increase and hence 1 h was applied as a time of treatment. The best conditions which resulted into maximum recovery were used as standard purification procedure. The activity of the crude extract initially added (1.25 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. The purified enzyme obtained under these conditions was used for further characterization. All the experiments were run in duplicate and the difference in the readings in duplicates was less than ±5%.

2.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the samples with 12% gel strength was performed according to Laemmli [28] using Biorad Mini Protean II electrophoresis unit. The gel was stained with Coomassie brilliant blue R-250 for 1 h and then destained in 40% methanol and 10% acetic acid for 2–3 h.

2.2.6. Effect of temperature on the activity and stability of α-galactosidase

The temperature profile of the tomato α-galactosidase was conducted from 25 to 60 °C using the standard α-galactosidase assay at the given temperature. The purified enzyme was exposed to different temperatures viz., 25–60 °C for 30 min followed by testing the enzyme solution for residual activity. Each set of experiment carried out in duplicate.

The thermal stability of the enzyme was determined by measuring the residual activity of the enzyme exposed to different temperatures (4–60 °C) in sodium citrate buffer for 30 min with continuous shaking. After desired incubation periods enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual α-galactosidase activities.

2.2.7. Effect of pH on the activity and stability of α-galactosidase

The pH-activity profile of α-galactosidase was studied by incubating samples with PNPG in citrate-phosphate buffer of different pHs ranging from 2.6 to 7.0 at 37 °C.

In order to determine the pH-stability, α-galactosidase was incubated in above buffers for 3 h at 37 °C and then the residual activity (%) with respect to control was assayed under standard activity assay conditions. Each set of experiment for pH-activity and pH-stability were carried out in duplicates.

2.2.8. Kinetic constants

In order to determine maximum velocity of reaction (V_{max}) and Michaelis–Menten constant (K_m) for tomato α-galactosidase, activity assay was applied for different concentrations (0.05–2.0 mM) of PNPG at 37 °C. These kinetic constants were calculated from Lineweaver–Burk plot which is a plot of $1/V$ against $1/[S]$ for systems obeying the Michaelis–Menten equation.