J Ind Microbiol Biotechnol (2009) 36:123–128
DOI 10.1007/s10295-008-0479-6

ORIGINAL PAPER

Three-phase partitioning of α-galactosidase from fermented media of *Aspergillus oryzae* and comparison with conventional purification techniques

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Received: 12 May 2008 / Accepted: 17 September 2008 / Published online: 10 October 2008

Abstract Simple, attractive and versatile technique, three-phase partitioning (TPP) was used to purify α-galactosidase from fermented media of *Aspergillus oryzae*. The various conditions required for attaining efficient purification of the α-galactosidase fractions were optimized. The addition of *n*-butanol, *t*-butanol, and isopropanol in the presence of ammonium sulfate pushes the protein out of the solution to form an interfacial precipitate layer between the lower aqueous and upper organic layers. The single step of three-phase partitioning, by saturating final concentration of ammonium sulfate (60%) with 1:1 *t*-butanol, gave activity recovery of 92% with 12-fold purification at second phase of TPP. The final purified enzyme after TPP showed considerable purification on SDS-PAGE with a molecular weight of 64 kDa. The enzyme after TPP showed improved activity in organic solvents. Results are compared with conventional established processes for the purification of α-galactosidase produced by *Aspergillus oryzae* and overall the proposed TPP technique resulted in 70% reduction of purification cost compared to conventional chromatographic protocols.

Keywords Three-phase partitioning · *t*-Butanol · Conventional purification · α-Galactosidase · Non aqueous enzymology

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Introduction

In recent years, the trend has been to develop efficient, economical and scalable approach for separation and purification of enzymes [1, 2]. Three-phase partitioning (TPP) is an emerging technique for protein separation in recent years [3]. TPP is the accumulation of precipitated material at the interface between an organic liquid phase and aqueous solution of salt. TPP is a concentrating or dewatering step and some enzymes have enhanced catalytic activities in these conditions. TPP employs collective operation of principles involved in numerous techniques like salting out, isoionic precipitation, cosolvent precipitation, osmolytic, and kosmotropic precipitation of proteins [3]. It is easily scalable and can be used directly with crude suspensions. It has been used both for upstream and downstream protein purification processes and has same times been used as a one-step purification protocol [4, 5].

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is widely distributed in microorganisms, plants, and animals. It hydrolyzes a variety of simple α-D-galactoside as well as more complex molecules such as oligosaccharides and polysaccharides [6]. α-Galactosidase has a number of biotechnological applications like production of sugar, processing of soymilk, conversion of blood type, and treatment of Fabry's disease. In the past few years, α-galactosidase has been considered as effective feed additive to remove non-digestible oligosaccharides, which occur in soybean meal-containing diet [7–9].

The effect of organic solvents and different salts on TPP has never been extensively studied. Moreover, not much recent information is available on TPP in organic solvents beside *t*-butanol, except Morton's *n*-butanol extraction methods like isoionic precipitation cold solvent precipitation, osmolytic, and kosmotropic precipitation [10]. But

different enzymes show different behavior in these conditions, depending upon their molecular weight, pI, and temperature [11]. The present work involves separation and purification of α-galactosidase from fermented media of *Aspergillus oryzae* and different parameters required for the efficient purification of enzyme were optimized, and finally results were compared with the established conventional purification techniques with TPP.

Materials and methods

Chemicals

t-Butanol and other organic solvents were obtained from Qualigens and S.D fine Chemicals Mumbai, India. Ammonium sulfate and other salts were procured from Ranbaxy, India. PNPG, (P-Nitrophenyl-α-D-galactopyranoside) was procured from Sigma Chemicals, USA. All solvents, other reagents and chemicals used were of analytical grade.

Microorganism and α-galactosidase production

Aspergillus oryzae capable of producing extracellular α-galactosidase was isolated in our laboratory from soil sample [12] and produced in defined medium as described earlier [13].

Three-phase partitioning (TPP)

TPP was carried out by saturating the crude enzyme solutions (5.5 U) with 30% (w/v) salts like ammonium sulfate, sodium sulfate, magnesium sulfate, potassium phosphate, sodium phosphate, and sodium chloride in a series of tubes. The solutions in the tubes were mixed vigorously to dissolve the salt, followed by adding various organic solvents (*t*-butanol, isopropanol, *n*-propanol, *n*-butanol, *t*-amyl alcohol, tetrahydrofuran, dimethylformamide, 1, 4 dioxane and dimethylsulphoxide) in the ratio of 1:1 (v/v) (ratio of crude extract to organic solvents). Then, the tubes were kept at 37°C for 1 h for complete phase formation. The mixtures were then centrifuged at 2,000 g for 10 min. Three-phase formed were collected separately; since the enzyme was not precipitated completely in the middle layer in the first stage, the lower aqueous layer was used to carry out for the second stage of TPP. The aqueous layer obtained in the first stage was again saturated with 30% salt (such that final concentration was 60% w/v) followed by addition of an equal volume of organic solvent [14]. The solutions were incubated for 1 h at 37°C followed by centrifugation at 3,000 g for 10 min; the interfacial precipitate was collected and enzyme activity was determined in the interfacial precipitate.

Assays of α-galactosidase

Assay of α-galactosidase was carried out by the method of Dey and Pridham [6] using PNPG as substrate. Reaction mixture containing 0.1 ml of suitably diluted enzyme + 0.8 ml of 0.02 M acetate buffer (pH 4.8) + 0.1 ml of 2.0 mM PNPG was added and incubated at 37°C for 15 min. The reaction was arrested by adding 0.2 M Na_2CO_3 solution and the absorbance was read at 405 nm in a spectrophotometer (Elico Ltd., India).

One unit of enzyme activity is defined as the amount of enzyme preparation required to liberate one μmol of *p*-nitrophenol from PNPG per minute under the standard assay conditions.

Protein estimation

Protein was estimated according to the method of Lowry et al. [15] using BSA as standard protein.

SDS-PAGE

SDS-Polyacrylamide gel electrophoresis was performed following the procedure of Laemmli [16] using Genei electrophoresis unit (Bangalore Genei Pvt. Ltd., Bangalore, India). The gel was stained with coomassie brilliant blue R-250 for 45 min and then destained in 40% methanol and 10% acetic acid.

Results and discussion

Traditionally, various methods were used in past decades to purify α-galactosidase by conventional chromatographic methods such as gel filtration, ion-exchange, and hydrophobic interaction etc. But none of them could be applied generally because several of these separation techniques are expensive and need pretreatment of samples [17–19]. Hence, the trend has been to develop protocols with limited number of steps for protein purification to make the process economical. TPP is a non-chromatographic separation technique, which need to be tested for its wider range of enzymes for its practical applications. Moreover, nearly all development of TPP so far has been dependent on *t*-butanol and ammonium sulfate, a C_4 alcohol which is a differentiating solvent and ammonium sulfate as effective kosmotropic agent. It has been reported that, it might not be necessary to use *t*-butanol always, other C_4, C_5 and cosolvents may also serve because they too act as a differentiating cosolvents [3]. Therefore, in present study various process parameters on partitioning of α-galactosidase were determined.

Effect of different organic solvents on partitioning of α-galactosidase

Figure 1 shows the results of TPP with fermented media of *Aspergillus oryzae* α-galactosidase using various solvents. Of all the solvents tried *t*-butanol gave the best results with 12-fold purification and 92% activity recovery. Although *n*-butanol is less miscible in water than *t*-butanol it gave three fold purification with 69% recovery. Whereas, tetrahydrofuran gave higher purification, the recovery activity was much lower (39%). It appears that both higher deactivation of the enzyme as well as lower interfacial precipitates are responsible for the low activity yield [20]. Other solvents tried were *t*-amylalcohol, dimethylformamide (DMF), dimethylesulphoxide (DMFSO). In all these solvents, the formation of third phase was not observed even altering the phase system with salts and varying solvent ratios. *t*-Butanol appears to be kosmotropic and crowding agent at room temperature, resulted in "α-galactosidase-*t*-butanol co-precipitate" which float above denser aqueous salt layer. It is believed that because of its size and branched structure, *t*-butanol does not easily permeate inside the folded protein molecules and hence does not cause denaturation [21]. In this TPP based protocol for protein separation, *t*-butanol has been found to consistently perform better than all other organic solvents [3, 14].We have also tried acetone, ethanol, but they did not behave as *t*-butanol except at low temperature. The amount of protein precipitated in to the third phase and the solubility of protein has no effect on TPP by temperature. However, it was observed that, after TPP at 37°C (room temperature) the protein redissolved less quickly than after TPP at low temperature using *t*-butanol and its analog.

Effect of different salts on partitioning of α-galactosidase

One of the critical parameter in TPP is the concentration of salt used for precipitating the protein in the interfacial phase. The concentration should be less than one which causes 'salting out' of any protein. Salts like magnesium sulfate, (chaotropic salt); sodium sulfate, potassium phosphate, ammonium sulfate (kosmotropic salt) and sodium chloride (neutral slat) were tested for partitioning of alpha-galactosidase by TPP (Fig. 2). Kosmotropic salt showed effective partitioning, particularly ammonium sulfate, and no evidence was found that other salts were superior or even equal to ammonium sulfate. Potassium nitrate can also mount equal ionic strengths; however, this cannot approach ammonium sulfate performance in TPP (pulling and pushing effect combined by *t*-butanol) and hence indicating that ionic strength alone (%) is not a major player in this technique [3]. Generally other salts, apart from ammonium sulfate have also been tried, but ammonium sulfate has been found to work best in most of the cases [3, 22].

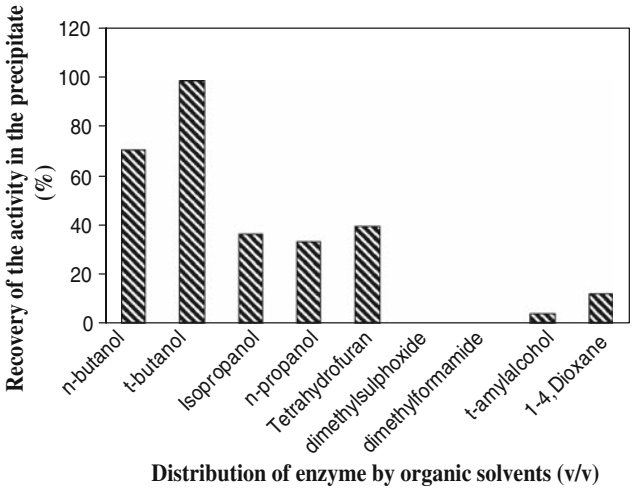

Fig. 1 Effect of different organic solvents on purification of α-galactosidase by TPP. Organic solvents were added to series of tube containing 5 ml of fermented media from *Aspergillus oryzae,* saturated with 30% w/v ammonium sulfate and incubated for 1 h at room temperature. Three phase formed were colleted separately, the lower aqueous layer was subjected to second round of TPP

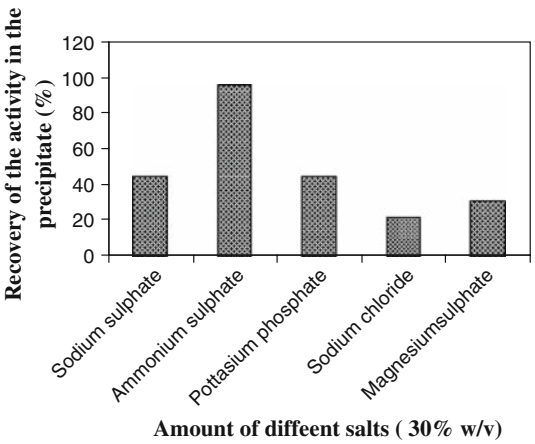

Fig. 2 Effect of different salts on partitioning of α-galactosidase by TPP. Different salts were added to series of tube containing fermented media of α-galactosidase (5 ml), followed addition of 1:1 *t*-butanol and rest of the procedure was same as in materials and methods section

Three-phase partitioning (TPP)

Efforts were also made to optimize the ratio of the volume of organic solvent to fermented media. The best results were obtained when the ratio was 1:1 (data not shown). Thus the optimum condition for TPP was, to use of 30% ammonium sulfate along with 1:1 ratio of *t*-butanol to aqueous crude extract. The optimum concentration determined here seems to be, in general the best concentration to operate TPP with various systems [11, 20, 23]. Figure 3 shows the TPP of α-galactosidase under optimum conditions. The interfacial layer obtained after first TPP, was collection of

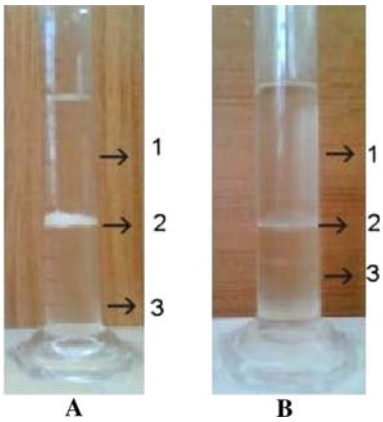

Fig. 3 TPP of α-galactosidase from fermented media of *Aspergillus oryzae*. TPP was carried out as described in the text. The initial activity present in fermented media is taken as 100%. **a** Precipitate-1 (First TPP): *1* organic layer, *2* unwanted low molecular weight proteins and cell mass, *3* aqueous layer. **b** Precipitate-2 (Second TPP): *1* organic layer, *2* α-galactosidase, *3* aqueous layer

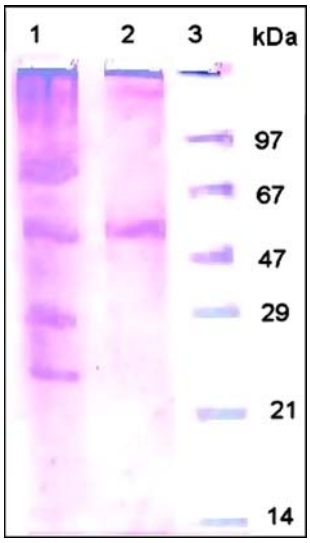

Fig. 4 SDS-PAGE analysis of α-galactosidase. *Lane 1* protein precipitation obtained after first cycle of TPP, *Lane 2* purified α-galactosidase after second cycle of TPP, *Lane 3* molecular weight marker proteins

high molecular weight contaminants, cell debris and it precipitates only 6% of α-galactosidase activity (Fig. 3a). Therefore aqueous layer was subjected to second stage of TPP under same conditions. When it was subjected to a second round of TPP, 92% recovery of α-galactosidase activity was observed in middle layer (Fig. 3b). TPP enzyme after second stage showed electrophoretic homogeneity with molecular weight of 64 kDa (Fig. 4). But when gel was stained with silver staining few low molecular weight protein bands were observed (Data not shown). The overall result in terms of purification of α-galactosidase by TPP is summarized in Table 1.

Purified α-galactosidase in non aqueous medium

In this view, considerable amount of effort is being directed towards enhancing the catalytic efficiency of enzymes in organic solvents [24]. We have studied the effect of different organic solvents on purified α-galactosidase. α-Galactosidase which has comparatively less activity in 1,4, dioxane, improved 30% of its activity after TPP. Dimethylformamide and dimethylsulphoxide, which decreases the enzyme activity in aqueous phase, but after TPP recovered its full activity in the same concentration and gave 60 and 45% of its activity. This study shows that enzyme improved its activity after TPP. It has been shown that proteinase K, when subjected to TPP, led to 2.1 times higher caseinolytic activity in aqueous medium. It was also found that substilisin Carlsberg, after being subjected to TPP showed four times higher activity in both *n*-octane and *t*-amyl alcohol. X-ray diffraction studies with proteinase K subjected to TPP, resulted in higher overall flexibility of the enzyme conformation. This was the structural basis for the higher activity of enzyme molecule. Presumably, the higher activity of subtilisin Carlsberg originates in similar structural changes. The increased flexibility of the molecule probably is mostly responsible for higher activity of α-galactosidase [25, 26].

Comparison of TPP with other conventional purification techniques

It may be interesting to compare this TPP based separation strategy with the one based upon the conventional purification techniques. In our laboratory, an overall yield of 11.5% and a purification factor of 4.1 were obtained when the α-galactosidase crude extract was applied to the conventional purification (crude-extract–filtration- precipitation-gel filtration followed by ion-exchange chromatography) of totally 6 unit operations [17]. In contrast, the TPP process that includes addition of ammonium sulfate and *t*-butanol, one step operation produces an overall α-galactosidase recovery of 92% with purification factor 12 with much lower salt concentration. However, TPP produce a favorable increase in the purification of (12-fold) α-galactosidase compared with that produced after conventional purification techniques (4.1 fold). A further comparison between the processes highlights the superiority of the TPP approach (Table 2). Implementation of the TPP extraction after filtration resulted in a reduction of the number of unit operation involved in the chromatography protocols. Consequently there was significant economical benefit in the reduction in cost of unit operations. Such analysis supports the fact that TPP process for the recovery of α-galactosidase is superior to conventional chromatographic methods. In addition, a notable advantage compared to C_2 frigid

Table 1 Purification of α-galactosidase from *Aspergillus oryzae* by subjecting to TPP

Enzyme steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	5.5	6.01	0.913	1	100
TPP-precipitate-first	0.39	4.23	0.092	0.10	7
TPP-precipitate-second	5.10	0.47	10.89	12	92

Table 2 Comparison of conventional chromatographic techniques with three-phase partitioning technique

Parameters	Ion-exchange chromatography	TPP
Separating factor	Ionic interaction	Isoionic precipitation cosolvent precipitation salting out etc-
Mode of operation	Semi-continuous	Batch
Required working volume	25–50 ml	0.2–10 ml
Time[a]	40 h[a]	3 h
Required temperature	Low temp (0–4°C)	Room temp
Overall process yield	11.5%	92%
Purification factor	4.1	12
Cost of separating agent[b]	1,270.80 USA$/500gm (DEAE-Sephedex)	19.80 USA$/500 ml *t*-butanol 56.80 USA$/500 gm a.sumplate
Overall process cost[c]	8.48 USA$/column	2.5 USA$/system

[a] The overall time is expressed relative to the starting of unit operations for each process, including precipitation, filtration, centrifugation and chromatographic separation

[b] The cost separating agents (price as per sigma catalog for biochemical and reagents, 2004–2005. price US$)

[c] The overall cost of operation is expressed relative to the starting material for each process and considering chromatographic (DEAE-Sephedex) resin can be reused. In contrast, for the TPP process, the potential saving was low salt content and reuse of *t*-butanol were not considered

co-solvent precipitation is that, TPP via *t*-butanol can be practically used either at room temperature, or even at higher temperature. When compared to other liquid extraction, like two phase-partitioning by dextran and polyethylene glycol (PEG) [27]. TPP is much less expensive, on weight basis, than dextran and PEG. TPP work with microliter to milliliter (Fig. 5), i.e. from 0.5 µl (89% yield with tenfold purification) in eppendroph tube to 2 ml (85% yield with eightfold purification) in test tube and 50 ml (83% yield with eightfold purification) in measuring cylinder. Microlitre volume worked better, because of easy to carry out certification and separation of interfacial precipitate. It requires less butanol and its cost is close to that of reagent grade ethanol. TPP requires somewhat less ammonium sulfate that conventional salt precipitation. A benefit in overall separation is that, TPP-precipitated protein is markedly more concentrated than the other conventional purification techniques within single step.

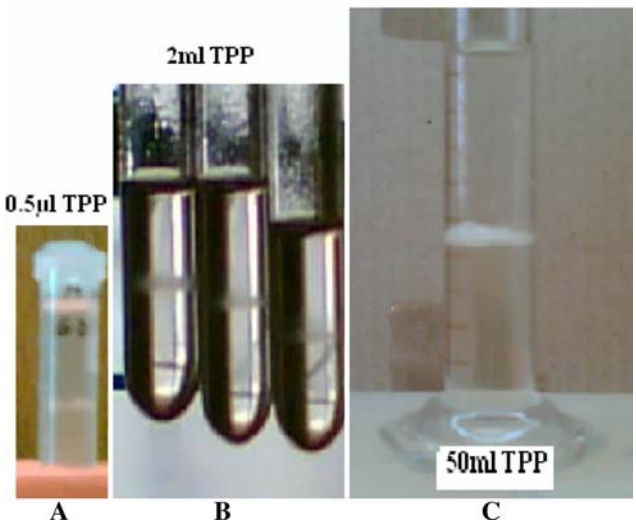

Fig. 5 Three-phase partitioning at microliter to milliliter

Conclusions

Earlier work has shown that purification costs constitute a fairly large percentage of the overall production costs of protein/enzyme [17]. In this work the combination of ammonium sulfate with the organic solvent *t*-butanol was optimum for attaining the best recovery of α-galactosidase with 12-fold purification and recovery of 92%. TPP is quick, economical technique and scalability is convenient. The method described here requires only 3 h to complete the purification process in efficient manner and there was remarkable reduction in the cost of purification. Thus process described here has the potential to carry out as purification protocol for enzymes.

Acknowledgments One the author Dhananjay SK is thankful to Council of Scientific and Industrial Research (CSIR) government of India, for providing financial support in the form of Senior Research Fellowship (SRF).

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