Separation and Purification Technology 129 (2014) 57–63

Contents lists available at ScienceDirect

Separation and Purification Technology

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

Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp

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ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 24 March 2014
Accepted 25 March 2014
Available online 4 April 2014

Keywords:
Three-phase partitioning system
Aqueous two-phase system
Back extraction
Pacific white shrimp
Protease
Hepatopancreas

ABSTRACT

Recovery of proteases from hepatopancreas of Pacific white shrimp using three-phase partitioning (TPP) system in combination with aqueous two-phase system (ATPS) was investigated. TPP was performed using *t*-butanol with different crude protease extract (CPE)/*t*-butanol ratios. The interphase of system comprising CPE and *t*-butanol with a ratio of 1:1 in the presence of 30% ammonium sulphate yielded the highest purification fold (PF) (2.6-fold) with the recovery of 76.0%. Subsequently, TPP fraction was subjected to ATPS. Effects of phase compositions including PEG molecular weight (MW) and concentration as well as types and concentration of salts on partitioning of proteases were studied. ATPS comprising PEG1000 (15%, w/w) and magnesium sulphate (25%, w/w) provided the best condition for the maximal partitioning of proteases into the top phase and gave the highest PF (8.6-fold). The yield of 65.5% was obtained. ATPS fraction was further mixed with PEG8000 and several salts for back extraction (BE). BE using 25% PEG8000 and 10% $MgSO_4$ gave the highest PF (9.9-fold) with the yield of 46.2%. Based on electrophoresis and activity staining, the fractionated proteases had the MW of 36 and 26 kDa. Therefore, the combined partitioning systems, TPP–ATPS–BE, could be effectively used to recover and purify proteases from hepatopancreas of Pacific white shrimp.

1. Introduction

Pacific white shrimp and its products have become economically important for Thailand. By the year 2010, frozen Pacific white shrimp and products were manufactured and exported totally for 407,978 metric tons, particularly to USA and Japan [1]. During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. is generated [2]. Whole shrimp without hepatopancreas is another product with increasing demand. Practically, hepatopancreas is removed by vacuum sucking machine and can serve as the potential source of protease [1]. Fish proteases e.g. trypsin and chymotrypsin may have some unique properties for industrial applications, e.g. in the detergent, food, pharmaceutical, leather and silk industries [3,4]. Serine proteases especially, trypsins, have been reported as the dominant proteases in hepatopancreas of freshwater prawn [5], kuruma prawn (*Penaeus japonicus*) [6], Indian prawn [7], Northern shrimp (*Pandalus borealis*) [8] and white shrimp (*Penaeus vannamei*) [9].

TPP is a bioseparation technique which may be used for fractionation of enzymes and proteins from aqueous solutions [10]. $(NH_4)_2SO_4$ with certain saturation is generally added to precipitate the protein, and *t*-butanol is added to make three-phase layers [11]. In general, biomolecules are recovered in a purified form at the interphase, while the contaminants such as lipids mostly partition in *t*-butanol (top phase) and aqueous phase (bottom phase) [12].

ATPS has been used successfully for the separation and purification of proteins or enzymes [3]. ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold. Phase separation occurs over certain concentrations of phase component concentration [4,13]. ATPS has been applied for partitioning and recovery of various proteases such as pepsin [4] and chymosin [14]. In general, ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for selectively partitioning of the enzyme to one of the phases [4]. Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system [15]. These systems have good resolution, low material cost, high yield, less energy consumption, and less process time. In general, the single system has been implemented for enzyme recovery. The use of combined partitioning systems, e.g. TTP and ATPS could be an advantageous separation method to increase the purity of target enzyme as well as to increase the recovery yield. Our objective was to investigate the

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http://dx.doi.org/10.1016/j.seppur.2014.03.025
1383-5866/

use of combined TPP and ATPS for partitioning and recovery of proteases from hepatopancreas of Pacific white shrimp.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol (PEG) 1000, PEG2000, PEG4000, PEG8000, *tert*-butanol (*t*-butanol) and tris (hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), sodium caseinate, bovine serum albumin (BSA), wide range molecular weight markers and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was obtained from Fluka (Buchs, Switzerland). *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

2.2. Preparation of crude protease extract

Hepatopancreas of Pacific white shrimp was powdered in liquid nitrogen using a blender (Philips, Guangzhou, China) until the fine powder was obtained. The powder was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) in three volumes of acetone (−20 °C) for 2 min, followed by stirring for 30 min according to the method of Kishimura and Hayashi (2002). The homogenate was filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then stirred in two volumes of acetone (−20 °C) for 30 min. The residue was left at room temperature until dried and free of acetone odour.

To prepare the crude extract, acetone powder was suspended in 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ at a ratio of 1:50 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged using a refrigerated centrifuge (Beckman Coulter, Avanti J–E Centrifuge, Fullerton, CA, USA) for 10 min at 4 °C at 10,000*g* to remove the tissue debris, and the supernatant was referred to as "crude protease extract, CPE".

2.3. Three-phase partitioning (TPP) system

TPP was carried out using CPE with protein content of 5.97 mg/mL. Firstly, CPE was added with *t*-butanol at different ratios (1.0:0.5, 1.0:1.0, 1.0:1.5, and 1.0:2.0 (v/v)). Thereafter, ammonium sulphate at 30% saturation was added into the mixture. The mixtures were mixed thoroughly and then allowed to stand for 60 min at 4 °C before subjecting to centrifugation at 5000*g* for 10 min to facilitate the separation of phases. The lower aqueous layer and the interfacial phase were collected and dialysed against 50 volumes of distilled water overnight at 4 °C with 4 changes of distilled water. After dialysis, the samples were determined for protease activity and total protein content. Yield, specific activity (SA) and purification fold (PF) were calculated as follows:

$$\text{Yield}(\%) = \frac{A_t}{A_i} \times 100$$

where A_t is total protease activity in the protease rich phase and A_i is the initial protease activity of the crude extract or fraction before being partitioned.

$$\text{SA}(\text{unit/mg protein}) = \frac{\text{protease activity}}{\text{protein concentration}}$$

$$\text{PF} = \frac{\text{SA}_t}{\text{SA}_i}$$

where SA_t is the SA of the protease rich phase and SA_i is the initial SA of the crude extract or fraction before being partitioned.

The CPE/*t*-butanol ratio yielding the highest enzyme recovery and purity was chosen. The selected TPP fraction was used for ATPS.

2.4. Aqueous two-phase systems (ATPS)

ATPS was prepared in 10-mL centrifuge tubes by adding the different amounts of PEG and salts together with the selected TPP fraction according to the method of Klomklao, Benjakul, Visessanguan, Simpson and Kishimura [3].

2.4.1. Effect of salts on partitioning of protease in TPP fraction

To study the effect of salts on partitioning of the protease in TPP fraction, ATPS containing different salts, ammonium sulphate ($(NH_4)_2SO_4$), sodium citrate ($Na_3C_6H_5O_7$), magnesium sulphate ($MgSO_4$) and dipotassium hydrogen phosphate (K_2HPO_4), at different concentrations (15%, 20% and 25%, w/w) with 20% PEG1000 were used. One mL of TPP fraction (1 mg protein/mL) was added into the system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA). Phase separation was achieved by centrifugation for 5 min at 2000*g*. Top phase was carefully separated using a Pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases, top and bottom phases, were measured. Aliquots from each phase were taken for protease assay and protein determination. Yield, SA and PF were then calculated.

Additionally partition coefficient (K_P) and volume ratio (V_R) were also calculated using the following equations:

$$K_P = \frac{C_T}{C_B}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively.

$$V_R = \frac{V_T}{V_B}$$

where V_T and V_B are the volume of top and bottom phase, respectively.

Based on purity and yield, the appropriate salt in ATPS rendering the most effective partitioning was chosen for further study.

2.4.2. Effect of MW and concentration of PEG on partitioning of protease in TPP fraction

Effect of PEG1000, PEG2000 and PEG4000 at different concentrations (15%, 20% and 25%, w/w) on partitioning of proteases in TPP fraction was studied in the presence of salt with the type and concentration showing the highest yield and purity. Partitioning was performed as described previously. ATPS rendering the most effective partitioning of protease was chosen for further study.

2.5. Back extraction (BE)

BE was used to partition the protease in PEG rich phase to aqueous salt rich phase following the method of Malpiedi, Picó and Nerli [16] with a slight modification. Systems containing 25% PEG4000 or 25% PEG8000 in the presence of $MgSO_4$ at different final concentrations (5%, 10%, 15% and 20%, w/w) were used. One gram of the selected ATPS fraction was added into the prepared systems. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a vortex mixer. Phase separation was achieved by