Biochemical Engineering Journal 50 (2010) 145–149

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Short communication

Three-phase partitioning of protease from *Calotropis procera* latex

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ARTICLE INFO

Article history:
Received 12 March 2010
Received in revised form 7 April 2010
Accepted 17 April 2010

Keywords:
Calotropis procera
Latex
Protease
Three-phase partitioning
t-Butanol

ABSTRACT

Three-phase partitioning (TPP) was used to partially purify protease from the latex of *Calotropis procera* (*C. procera*). To optimize the TPP for protease isolation a ratio of crude extract to *t*-butanol, percent saturation of $(NH_4)_2SO_4$, and the cycle of TPP was required. The highest proteolytic recovery (first cycle) of 182% with a purification of 0.95 folds was obtained at the interphase of the system comprising the ratio of the crude extract to *t*-butanol of 1.0:0.5 with the presence of 50% $(NH_4)_2SO_4$. The second cycle of TPP was prepared by adding of $(NH_4)_2SO_4$ up to 65% (w/v) to the bottom phase obtained from 30% $(NH_4)_2SO_4$–1.0:0.5 system of the first TPP. A purification of 6.92-fold was achieved with about 132% activity recovery. SDS-PAGE and zymography profiles revealed the substantial isolation of protease from *C. procera* latex by the TPP. The molecular weight of major protease was found to be around 28 kDa. The present study shows high interesting outcomes and could be used as a primary purification process in comparison with existing literature's values.

1. Introduction

Three-phase partitioning (TPP) is a relatively recent bioseparation technique, which employs collective operation of principles involved in numerous techniques for protein precipitation. The TPP has widely been used for the extraction and purification of various proteins [1–5]. It uses $(NH_4)_2SO_4$ with certain saturation to precipitate the protein, and *t*-butanol was added to make three-phase layers and to remove some small molecular weight compounds such as lipids, phenolics and some detergents [6]. In general, biomolecules are recovered in a purified form at the interphase, while the contaminants mostly partition in *t*-butanol (top phase) and aqueous phase (bottom phase) [3]. This method was scalable and could be used directly with the crude suspensions [4].

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology because their properties of activity over wide range of temperature and pHs. They have been exploited commercially in the food industry such as papain for meat tenderizing and ficin and bromelain for brewing [7]. Although recently enzymes from microorganism have been widely commercially produced, consumer still aware for used it as an edible ingredients into their meals. Therefore, natural sources from both plant and animal tissues still desired.

Calotropis procera is a plant found in tropical and sub-tropical regions. It is well-known for its great capacity of producing latex which exudates from the green damaged parts. Scientific reports have mentioned various medicinal activities of *C. procera* latex, such as insecticidal [8], anti-fungal [9] and wound healing [10]. Some biochemical properties of the enzyme containing in the latex of *C. procera* have been documented and named as procerain [11]. It has been reported that *C. procera* latex is a potential material for enzyme purification [12,13]. Consequently, scientific researches of protease extraction from *C. procera* have recently been reported. Protease activity recovery of 74.6% with 4.08-fold of purification was obtained from *C. procera* latex by using aqueous two-phase system [13]. However, there is no report of using TPP as the single method to separate the protease from this potential source. Therefore, the aim of this study was to optimize the separation process of protease from the latex of *C. procera* by using TPP technique.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), casein and L-cysteine were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β-ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate ($(NH_4)_2SO_4$),

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1369-703X/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.bej.2010.04.007

magnesium sulfate ($MgSO_4$), trichloroacetic acid (TCA), tert-butanol (*t*-butanol) and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Latex of *C. procera* was collected from Nayong, Trang Province, Thailand.

2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems and then diluted with distilled water (1:1, v/v). After mixing, it was centrifuged at 8000 × *g* at 4 °C for 10 min. The obtained supernatant was referred to as the "crude extract" [protein content: 3.49 mg/ml, activity: 1138 unit/ml, specific activity: 326 unit/mg protein] and used for further study.

2.3. Three-phase partitioning

2.3.1. Effect of crude extract to t-butanol ratio on protease partitioning

The TPP was carried out as described by Roy and Gupta [3]. The effect of the ratio of crude extract to *t*-butanol was studied. Firstly, *t*-butanol was added to the crude extract at the ratios of crude extract to *t*-butanol of 1.0:0.5, 1.0:1.0, 1.0:1.5, and 1.0:2.0 (v/v) with a constant $(NH_4)_2SO_4$ saturation of 30%. The mixture was mixed thoroughly and then allowed to stand for 60 min before subjecting to centrifuge at 5000 × *g* for 10 min to facilitate the separation of phases. The lower aqueous layer and the interfacial precipitate were collected and dialyzed against water overnight at 4 °C. After dialysis, the samples were analyzed for protease activity and total protein content. The ratio employing the highest enzyme recovery was chosen for further study.

2.3.2. Effect of ammonium sulfate saturation on protease partitioning

The $(NH_4)_2SO_4$ saturations at 20, 30, 40, and 50% affecting the partitioning of protease was also investigated by using the ratio of crude extract to *t*-butanol (with the highest enzyme recovery) obtained from 2.3.1. The lower aqueous layer and the interfacial precipitate were collected and dialyzed against water overnight at 4 °C. After dialysis, the samples were analyzed for protease activity and total protein content. The system providing the highest protease recovery was chosen for further study.

2.3.3. Optimization of TPP for proteases recovery

The phase obtained from the first TPP that gave the highest activity recovery was chosen as the starting material for optimization in the second TPP. The selected phase (without dialysis) was mixed with the *t*-butanol in ratio of 1.0:0.5 and $(NH_4)_2SO_4$ was added to the mixture to obtain the final saturations of 50, 55, 60, and 65%. After the complete phase separation, the phases were collected as previously mentioned. The dialyzed phases from the second TPP were subjected to protease activity and total protein content analysis.

2.4. Caseinolytic activity assay

An enzyme sample of 0.10 ml was mixed with 1.10 ml of 1% (w/v) casein in 0.10 M Tris–HCl (pH 8.0) containing 12 mM cysteine. The reaction was started by incubation the mixture at 37 °C for 20 min. The reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at 3000 × *g* for 15 min, the absorption of the soluble peptides in supernatant was measured at 280 nm. One of caseinolytic activity units is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition [13].

2.5. Protein determination

Protein concentrations were measured by Bradford method using BSA as a protein standard [14].

2.6. Electrophoresis

2.6.1. SDS-PAGE

SDS-PAGE of the samples was performed according to the method of Laemmli [15] with slight modification. Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (10 and 2 μg protein for protein and activity staining, respectively) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 20 mA/gel. For protein staining, the gel obtained after electrophoresis was stained overnight with a solution of 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Gels were destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 40 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 20 min. Pierce blue prestained protein molecular weight marker mix consists of (kDa) myosin (215), phosphorylase B (120), BSA (84), ovalbumin (60), carbonic anhydrase (39.2), trypsin inhibitor (28) and lysozyme (18.3) was used.

2.6.2. Protease activity staining

The protease separated on the gel was verified by using activity staining as done in Garcia-Carreno et al. [16]. The gel was immersed in 50 ml of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0 containing 12 mM cysteine for 30 min with constant agitation at 4 °C. The reaction was generated by incubation the gel at 37 °C for 15 min. The treated gel was then stained and destained as described above. The development of a clear band on the dark background indicated the caseinolytic activity of protease from *C. procera* latex.

3. Results and discussion

3.1. Effect of the ratio of crude extract to t-butanol on protease partitioning

The effect of crude extract to *t*-butanol ratio for protease partitioning in the first TPP was firstly investigated. Different TPP experiments with various extract to *t*-butanol ratios (1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0) at 30% (w/v) ammonium sulfate saturation were performed. The highest protease recovery (99.3%) and purification fold (1.13-fold) were obtained from the bottom phase of a TPP system with the ratio of 1.0:0.5. An increase in *t*-butanol volume resulted in a decrease in activity recovery and purification fold values of the bottom phase. This may be attributed to the synergistic effects of the increase in concentration of *t*-butanol and decrease in saturation of ammonium sulfate [5]. Dennison and Lovrien [6] reported that only 0.2–0.5 ml of *t*-butanol usually is required per milliliter of beginning aqueous sample to precipitate out protein. The best results were obtained when the ratio was 1.0:1.0 used for partitioning of α-galactosidase from fermented media of *Aspergillus oryzae* with 60% $(NH_4)_2SO_4$ saturation [4]. The ratio of sample to *t*-butanol content is higher than one, the denaturation of the protein is more likely [4]. The partitioning of protease in the interphase was contrary with the bottom phase. However, the yield obtained was still lower than 30% in all ratios tested.

As the results, most protease was still remained in the aqueous phase rather than in the interphase. Therefore, the ratio of crude extract to *t*-butanol of 1.0:0.5 which provided the highest recovery