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# Extraction of protease from *Calotropis procera* latex by polyethylene glycol–salts biphasic system

Phanuphong Chaiwut[a], Saroat Rawdkuen[b,*], Soottawat Benjakul[c]

[a] School of Cosmetic Science, Mae Fah Luang University, Muang, Chiang Rai 57100, Thailand
[b] Food Technology Program, School of Agro-Industry, Mae Fah Luang University, Chiang Rai 57100, Thailand
[c] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

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ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS). The systems consist of polyethylene glycol (PEG 4000, 6000 and 8000) at concentrations of 9, 12 and 15% (w/w) with salts (Na-citrate, $MgSO_4$, $K_2HPO_4$, and $(NH_4)_2SO_4$) at concentrations of 11, 14 and 17% (w/w) were investigated. The highest protease recovery was found in the PEG-rich phase of the system, comprising of 12% PEG 4000–17% $MgSO_4$. For optimization of the system to obtain the higher yield of protease, the system pH (4, 7 and 10) or NaCl addition (2, 4 and 6%, w/w) was studied. At acidic (pH 4.0) and alkaline (9.0) conditions of the systems the reduction of $K_E$ and protease recovery was clearly observed compared to that of the neutral pH (7.0). The addition of NaCl up to a final concentration of 6% (w/w) significantly increased the yield to 107% of the control. Molecular weight distribution and activity staining showed that the isolated protease had the molecular weight of ~38 kDa. However, the isolated protease had no activity under reducing condition ($\beta$ME). Under cathodic electrophoresis, protease from *C. procera* showed the same protein pattern to purified papain.

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## 1. Introduction

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology because of their properties and functions [1,2]. Among them, those from plant latex were introduced for meat tenderization for several years [3,4]. The presence of proteolytic enzymes in the latex of plants is believed to be a defense mechanism function. *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 [5], anti-fungal [6] and wound healing [7]. Some biochemical properties of the enzyme containing in the latex of *C. procera* have been documented and named as procerain [8]. It has been reported that *C. procera* latex is a potential material for enzyme purification [9]. However, the enzyme extraction involved several steps such as precipitation and chromatography. Nitsawang et al. [10] purified papain from *Carica papaya* latex by using two-step salt precipitation. Ammonium sulfate precipitation and CM-Sepharose column were used for procerain purification from the latex of *C. procera* [8]. These processes are time and cost consuming, inducing loss of enzyme activity.

In this respect, partitioning in an aqueous two-phase system (ATPS) has shown to be powerful for separating and purifying the mixtures of proteins [11]. It could be used to combine several features of the early processing steps in only one or two partitioning operations. It offers many advantages: a biocompatible environment, low interfacial tension, low energy, ease of scale-up, and continuous operations [12]. Coincidentally, ATPS can remove undesirable proteins, unidentified polysaccharides and pigments that are present in the system [8]. Together with the removal of insoluble and major classes of contaminants this technique has been developed as a primary purification step in the overall recovery [11]. The basis for separation by ATPS is the selective distribution of biomolecules between the phases quantified by the partition coefficient. It is important to maximize the differences in partition coefficient between target protein and impurities. The coefficient value depends on various parameters from biomolecule (size, concentration and surface property), phase compositions (type, concentration and molecular weight of polymers or salts), pH and temperature of the system [12]. The pH value and the presence of electrolytes in the system have a pronounced effect on the partitioning of proteins between the two phases [13]. The phase behavior is also influenced by the presence of salts, the effect depending on both their type and concentration [14]. A favorable interaction between polyethylene glycol (PEG) and pro-

* Corresponding author. Tel.: +66 5391 6752; fax: +66 5391 6739.
*E-mail address:* saroat@mfu.ac.th (S. Rawdkuen).

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tein molecule decreased when the molecular weight (MW) of PEG increased due to its exclusion from the protein molecule [15,16]. ATPS has been applied for recovery of various proteases including, bromelain [17], papain [10], trypsin [15] and pepsin [16]. Nonetheless, extraction of the protease from *C. procera* latex by ATPS had not been reported.

Hence, in view of this potential application and scale-up, it would be interesting to look into extraction of proteolytic enzyme from *C. procera* latex by using ATPS. The present paper will also report the optimization of the ATPS and the verification of the extracted protein by gel electrophoresis.

## 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 ($\beta$ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), potassium phosphate ($K_2HPO_4$), trichloroacetic acid (TCA) 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. The obtained latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at 15,000 × *g* at 4 °C for 10 min. The supernatant was filtered through a Whatman paper no. 1. This sample was referred to as the "crude enzyme extract" and was used for further study.

### 2.3. 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 incubating 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 peptide in supernatant was measured at 280 nm. One of caseinolytic activity unit is defined as the amount of enzyme needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition [18].

### 2.4. Protein determination

The protein concentration in the sample was measured by the Bradford method [19] using BSA as a protein standard.

### 2.5. Aqueous two-phase partitioning

The ATPS was prepared in 50-ml centrifuge tubes according to the method in Klomklao et al. [15] with a modification. Various amounts and molecular weights of PEG (PEG 4000, 6000 and 8000) as well as salts (Na-citrate, $MgSO_4$, $K_2HPO_4$, and $(NH_4)_2SO_4$) were added to the 30% (w/w) of crude extract from *C. procera* to generate the biphasic system.

#### 2.5.1. Effect of PEG on the partitioning of protease from C. procera latex

To study the effect of PEG on the protease partitioning from *C. procera* latex using ATPS, different concentrations (9, 12 and 15%, w/w) and MW (4000, 6000 and 8000) of PEG were mixed with 14% w/w $MgSO_4$ in an aqueous system. The distilled water was used to adjust the system obtaining the total weight of 15 g. The mixtures were gently mixed for 15 min before pH measurement. Phase separation was achieved by centrifuging the mixture at 7000 × *g* at 4 °C for 30 min. The top and bottom phases were carefully separated by using Pasteur pipette. Aliquot from each phase was taken for enzyme activity assay and protein determination. The phase composition giving the highest proteolytic yield was chosen for further study.

#### 2.5.2. Effect of salts on the partitioning of protease from C. procera latex

To study the effect of salts on the protease partitioning from *C. procera* latex using ATPS, different salts (Na-citrate, $MgSO_4$, $K_2HPO_4$, and $(NH_4)_2SO_4$) with different concentrations (11, 14 and 17%, w/w) were mixed with the constant 12% PEG 4000. Two-phase separation was performed as previously described.

#### 2.5.3. Effect of pH on the partitioning of protease from C. procera latex

Based on the protease recovery, the ATPS containing 12% PEG 4000–17% $MgSO_4$ and 30% (w/w) crude latex was chosen for optimization of the protease partitioning. The original pH of the system was measured and then adjusted to 4.0, 7.0 and 9.0 with 1 M HCl or 1 M NaOH.

#### 2.5.4. Effect of NaCl on the partitioning of protease from C. procera latex

The selected system of 12% PEG 4000–17% $MgSO_4$ and 30% (w/w) crude latex was also chosen for study the effect of NaCl on protease partitioning. Adjustment of salt content in the system was made by addition of NaCl (solid form) into the system to obtain concentrations of 0, 2, 4, and 6% (w/w). Complete phase separation was achieved by centrifugation after the whole system was filled up to 15 g.

#### 2.5.5. ATPS extraction parameters

ATPS parameters were calculated as follows:

The volume ratio ($V_R$) was defined as:

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

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

The partition coefficient of protein ($K_P$) was defined as:

$$K_P = \frac{P_T}{P_B}$$

where $P_T$ and $P_B$ are concentrations of protein in the top and the bottom phase, respectively.

The partition coefficient of enzyme ($K_E$) was defined as:

$$K_E = \frac{E_T}{E_B}$$

where $E_T$ and $E_B$ are concentrations of enzyme in the top and the bottom phase, respectively.

The specific activity (SA) of extracted protease in each phase of the ATPS was defined as:

$$\mathrm{SA} = \frac{\text{Total protease activity}}{\text{Total protein content}};\quad \text{unit/mg protein}$$

The purification factor (PF) was defined as:

$$\mathrm{PF} = \frac{\mathrm{SA_E}}{\mathrm{SA_I}}$$

where $SA_E$ is the SA of each phase and $SA_I$ is the SA of initial phase (crude latex before partitioning).

The protease activity recovery (Yield) was defined as:

$$\text{Yield}\,(\%) = \frac{A_T}{A_I} \times 100$$

where $A_T$ is total protease activity in top phase and $A_I$ is the initial protease activity (crude latex before partitioning).

From the protease recovery, the protease obtained from the ATPS fraction that rendered the maximal yield was chosen for further study.

### 2.6. Electrophoresis

#### 2.6.1. Cathodic electrophoresis

The experiment was carried out according to the method of Nitsawang et al. [10] using a polyacrylamide gel consisting of 15% (w/v) separating gel (pH 4.3) and 7.5% (w/v) stacking gel (pH 6.7). The electrode buffer consisted of 0.36 M beta-alanine–0.14 M acetic acid, pH 4.3. The protein sample was diluted (1:1, v/v) with stacking buffer containing 10% sucrose and 0.002% basic fuchsin (used as a tracking dye) prior to loading onto the gel. Electrophoresis was run at a constant current of 40 mA/gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). The protein samples migrated toward the cathode during electrophoresis. After electrophoresis, the gel was stained overnight with staining solution (0.02%, w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with a solution of methanol–acetic acid.

#### 2.6.2. SDS-PAGE

SDS-PAGE of *C. procera* crude extract and the protease fractions extracted from ATPS fractions were performed according to the method of Laemmli [20]. 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) with or without $\beta$ME. 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. After electrophoresis, the gel was stained overnight with staining solutions (0.02%, w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, and 7.5% (v/v) acetic acid and destained as described above.

#### 2.6.3. Protease activity staining

The protease separated on the gel was verified by using activity staining as the method of Garcia-Carreno et al. [21]. 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 with constant agitation at 4 °C for 30 min. The reaction was generated by incubating the gel at 37 °C for 15 min. The treated gel was then stained and destained as described above.

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