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The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins

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ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS). Polyethylene glycol (PEG 1000, 2000 and 3000) at a concentration of 12, 15, and 18% (w/w) with salts ($(NH_4)_2SO_4$, K_2HPO_4 and $MgSO_4$) at a concentration of 14, 17, and 20% (w/w) were investigated. The highest protease recovery (74.6%) was found in the PEG-rich phase of the system ($p<0.05$), comprising of 18% PEG 1000 and 14% $MgSO_4$. Protein patterns and activity staining showed that the isolated protease had a molecular weight of ~31 kDa without the oligosaccharide attached to the molecule. Degradation of muscle proteins in beef, farmed giant catfish, and squid was observed by the electrophoresis of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The degradation of myofibrillar proteins (myosin heavy chain: MHC and actin: AC) of farmed giant catfish was higher than that of beef and squid muscles as indicated by the degradation proteins with lower molecular weight.

Keywords: Aqueous two phase; *Calotropis procera*; Latex; Protease; Muscle protein

1. Introduction

Proteases have been exploited commercially in the food industry in processes such as papain for meat tenderizing (to separate partially connective tissues), ficin and bromelain for brewing (to solubilize grain proteins and stabilize beer), and α-amylase for cookies (to improve crispiness) (Walsh, 2002). Proteases from plant sources have received special attention from the pharmaceutical industry, cosmetic, and by food biotechnology because their activity over wide ranges of temperature and acidity. Bromelain from pineapple was used as a tenderizing agent in beef (Lizuka and Aishima, 1999), squid (Melendo et al., 1997) and in coarse dry sausage (Melendo et al., 1996). Protease from ginger rhizome is a new interesting hydrolytic enzyme that has also been increasingly used for meat tenderization (Naveena et al., 2004). Proteases from plant latex were also widely used to reduce meat toughness for a long time (Kang and Warner, 1974; Ashie et al., 2002; Remezani et al., 2003). These proteases include papain from *Carica papaya* (Nitsawang et al., 2006), ficin from *Ficus carica* (Huang et al., 2008), and cysteine protease from *Funastrum clausam* (Morcelle et al., 2004). In general, protease in plant latex plays a role in the defense mechanism of plants. *Calotropis procera* is a plant found in tropical and sub-tropical regions. It is well-known for its great capacity to produce latex, which is exudated from damaged parts. Various parts of *C. procera* including latex, have been used in traditional medicine (Chitme et al., 2004). However, the biochemical properties of enzymes contained in the latex of *C. procera* is limited.

Nowadays, industries demand for efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and purity. Partitioning in an aqueous two-phase system (ATPS) has shown to be powerful for separating and purifying the mixtures of proteins (Huddleston et al., 1991). It offers many advantages: a biocompatible environment, low interfacial tension, low energy,

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ease of scale-up, and continuous operations (Raghavarao et al., 1998). ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration (Tubio et al., 2007). It could be used to combine several features of the early processing steps in only one or two partitioning operations. Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Dubey and Jagannadham, 2003; Morcelle et al., 2004). 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 (Huddleston et al., 1991). ATPS has been applied for the partitioning and recovery of various proteases such as bromelain (Babu et al., 2008), papain (Nitsawang et al., 2006), trypsin (Klomklao et al., 2005), chymosin and pepsin (Spelzini et al., 2005; Nalinanon et al., 2009), amyloglucosidase (Tanuja et al., 1997) and ricin B (Zhang et al., 2005). Dubey and Jagannadham (2003) reported that the latex of *C. procera* contained 4 stable cysteine proteases. However, extraction of the protease from *C. procera* latex by ATPS has not been reported. Therefore, the objective of this research was to isolate the proteolytic enzyme from *C. procera* latex by using an aqueous two-phase system. The other aims of this work were to characterize and apply the isolated enzyme in muscle proteins.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (βME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N,N,N′,N′-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), potassium phosphate (K_2HPO_4), trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Latex of *C. procera* was collected from Nayong, Trang Province, Thailand. Beef and squid muscles were purchased from Bandoo Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from a farm in Phan, Chiang Rai Province, Thailand.

2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems. The collected latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at $15{,}000 \times g$ at 4 °C for 10 min. The obtained supernatant was filtered through a Whatman paper No. 1. This sample was referred to as the "crude extract" and was used for further study.

2.3. Aqueous two phase partitioning

The ATPS was prepared in 10-ml centrifuge tubes according to the method in Nalinanon et al. (2009). Various amounts and molecular weights of PEG (1000, 2000 and 3000 Da) as well as salts ($(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_4$) were added to the crude extract from *C. procera* to generate the biphasic system.

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

To study the effect of salts on the partitioning of protease from *C. procera* latex using ATPS, different salts $(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_4$ at different concentrations (14, 17 and 20%, w/w) were mixed with 18% PEG 1000 in an aqueous system (the concentration of PEG 1000 was fixed to make sure that a two-phase formation would be obtained). Thirty percentages of the crude extract (w/w) were added into the system. The remaining distilled water was used to adjust the system to obtain the final weight of 15 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortex-genie2, G-560E, USA), and then they were gently mixed for 15 min. Phase separation was achieved by centrifuging the mixture at $9{,}000 \times g$ for 30 min. The top phase was carefully separated using a Pasteur pipette. Volumes of the separated top and bottom phases were measured and recorded. Aliquots from each phase were taken for enzyme activity assay and protein determination. The phase composition giving the highest proteolytic yield was chosen for further study.

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

Fourteen percentages of $MgSO_4$ were used in the system to study the effect of the concentrations (12, 15 and 18%, w/w) and molecular weights (1000, 2000 and 3000 Da) of PEG on the partitioning of protease from *C. procera* latex. A two-phase separation was performed as previously described. ATPS parameters were calculated as follows:

The volume ratio (V_R) is as:

$$V_R = \frac{V_T}{V_B} \quad (1)$$

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

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

$$K_P = \frac{P_T}{P_B} \quad (2)$$

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

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

$$K_E = \frac{E_T}{E_B} \quad (3)$$

where E_T and E_B are concentrations of enzymes (unit/ml) in top and bottom phase, respectively.

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

$$SA = \frac{\text{total protease activity}}{\text{total protein content}};\ \text{unit mg protein}^{-1} \quad (4)$$

The purification factor (PF) as:

$$PF = \frac{SA_E}{SA_I} \quad (5)$$

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