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Research Article

Aqueous two phase partitioning of protease from *Calotropis procera* latex

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Abstract

The protease from the latex of *Calotropis procera* was isolated by aqueous two-phase system (ATPs). Two phases formation was obtained when the concentration of polyethylene glycol (PEG) was 8-10% (w/w) with the ammonium sulphate ($(NH_4)_2SO_4$) concentration of 15 and 20% (w/w). The highest protease recovery (70%) was found in the bottom phase of the system comprising of 10% PEG and 20% $(NH_4)_2SO_4$. The isolated protease had the optimum temperature and pH at 60°C and 8.0, respectively. The activity decreased continuously when the salt concentration increased. The degradation of muscle proteins of buffalo and farmed giant catfish was observed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Degradation of myofibrillar proteins (myosin heavy chain and actin) of farmed giant catfish was higher than that of buffalo meat, especially at the temperature of 60°C compared to the room temperature. The protease present in *C. procera* latex was quite heat stable enzymes which corresponded to the hydrolysis behaviour on muscle proteins.

Keywords: aqueous two phase, *Calotropis procera*, latex, protease, meat tenderizer, Thailand

Introduction

Protein separation based on partition in aqueous two-phase system (ATPs) has attracted interest for many years. It offers many advantages such as a biocompatible environment, low interfacial tension, low energy, ease of scale-up, and continuous operation [1]. This approach has been shown to be powerful for separating and purifying the mixtures of proteins [2]. ATPs forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration [3]. It could be used to combine several features of the early processing steps in only one or two partitioning operations. Thus, the technique has been developed as a primary purification step in which overall recovery, together with the removal of insoluble and major classes of contaminant [4].

Protease is the digestive enzymes that have been exploited commercially in food industry such as the papain for meat tenderizing (to separate partially connective tissues), ficin and bromelain for brewing (to solubilize grain proteins and stabilizer beer), and α- amylase for cookie (to improve crispness) [5]. Proteases from plant sources have received special attention in the pharmaceutical industry and biotechnology due to their properties of being active over wide ranges of temperature and pH. The proteases are present in the plant's latex for example: papain from *Carica papaya*, ficin from *Ficus carica*, and cysteine protease from *Funastrum clausam*. Presence of proteases in the latex of plants has been believed as a defense mechanism function. *Calotropis procera*, is a plant found in tropical and sub-tropical regions. This plant is well-known for its great capacity of producing latex which exudates from damage parts. Various parts of *C. procera* including latex have been used in traditional medicine [6]. However biochemical property of enzyme containing in the latex of *C. procera* is limitation. In addition, extraction of the protease from this plant by aqueous two phase system (ATPs) had not been studied. Therefore, the objective of this research was to isolate the proteolytic enzyme from *C. precera* latex by using aqueous two phase system. The other aim of this work was to characterize and apply isolated enzyme in a muscle system.

Materials and Methods

Chemicals and raw materials

Polyethylene glycol (PEG-1000), Sodium dodecyl sulfate (SDS) and bovine serum albumin 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: $(NH_4)_2SO_4$, hydrochloric acid, tris (hydroxymethyl) aminomethane and other chemicals with the analytical grade were obtained from Merck (Darmstadt, Germany).

Latex of *C. procera* was collected from Bandoo district, Chiang Rai. Buffalo meat was purchased from Bandoo Market, Chiang Rai. Farmed giant catfish was obtained from a farm in Phan, Chiang Rai.

Isolation of protease from latex of C. procera

Crude extract preparation

The latex was collected in a clean tube by breaking the stems of the plant. This latex was diluted with distilled water (1:1), mixed well and then subjected to centrifuge at 15,000xg for 10 min at 4ºC. The supernatant obtained was filtered through the Whatman paper No. 1. This sample referred to as "crude extract" and used for further study.

Aqueous two-phase system (ATPs)

Different concentrations of PEG-1000 (8, 10, 12%, w/w) and $(NH_4)_2SO_4$ (10, 15, 20%, w/w) were used for the system forming. Polymer and salt were added to the crude extract (15g), and the total mixture was made up to 25g with distilled water. The mixture was gently shaken for 15 min. The two phases was separated by centrifugation at 9,000x*g* for 30 min at 4ºC. Aliquots of each phase were taken for protein analysis and protease activity assay after removing the polymer and salt by dialysis. Specific activity, protease recovery, purity as well as the partition coefficient of proteolytic enzyme from each phase were determined and calculated.

Caseinolytic activity assay

The enzyme sample of 0.1 ml was mix with 1.1 ml of 1% (w/v) casein in 12 mM cysteine, 0.1M Tris-HCl pH 8.0. The mixture was incubated at 37ºC for 20 min. After that the reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at 3,000xg for 15 min, the absorbance of the supernatant was measure at 280 nm. One caseinolytic unit defined as the amount of enzyme that produces increment of 0.001 absorbance unit per minute in the assay conditions.

Protein determination

Protein concentration was determined by the Biuret method using BSA as a standard.

Characterization of isolated protease from the latex of C. procera

From ATPs result, the phase providing the highest recovery/purity was chosen for biochemical characterization. This sample referred to as "isolated protease".

pH profile

The activity of the isolated protease was determined as 2.2.3 but the pH assayed was altered to pHs 3 to 12. Buffers of 0.05M Glycine-HCl (pH 2.0-3.0); 0.05M Na-acetate (pH 4.0–5.0); 0.05M Na-phosphate (pH 6.0-7.0); 0.05M Tris-HCl (pH 8.0-10.0) and 0.05M carbonate (pH 11.0-12.0) were used.

Thermal profile

The optimum temperature of the isolated protease was measured as 2.2.3 but the temperature was changed to 30-100ºC.

Salt stability

The isolated protease was incubated at room temperature for 20 min in the present of NaCl ranging from 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v). The caseinolytic activity of the reaction mixture was determined as described in 2.2.3.

Effect of isolated protease on muscle proteins

Treatment sample

Two grams of buffalo or farmed giant catfish muscles were mixed with the different concentrations of isolated protease (T8:20: 0, 10, 20, 50, 90, 130, 180 units). The mixers were then subjected to incubate at room temperature (25-27°C) or 60°C for 1 hour. The treated samples were solubilized with 5% SDS before electrophoresis analysis.

SDS-PAGE

Solubilized protein was mixed at the ratio of 1:1 with sample buffer (0.5M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% βME) and then boiled for 3 min. The treated sample (20µg proteins) was loaded into the gel made of 4% stacking and 10% separating gels, and then subjected to electrophoresis at a constant current of 15 mA/gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 in the mixture of methanol-acetic solution.

Results and Discussion

ATPs of proteases from the latex of C. procera

The partitioning of protease from *C. procera* latex was performed in the biphasic system of 8, 10, and 12% PEG-1000 with $(NH_4)_2SO_4$ at the concentration of 10, 15 and 20% (w/w). In general either PEG-1000 or $(NH_4)_2SO_4$ alone, no phase separation was found indicating that the combination of both PEG and salt was necessary for partitioning process. However, in the system contained low concentration of both salt and polymer also showed no phase separation occurred (Table 1). The formation of two phases typically requires total system concentrations of polymer in the range 8-16% (w/w) and salt concentration must be as high as 10% (w/w) [1]. As the concentration of polymer or salt increased above a certain critical value (binodal curve) phase separation occurred. From the result, the system comprised with 8-12% PEG and $(NH_4)_2SO_4$ at the concentration of 15 and 20% showed the separation of the system into two phases. The lower the molecular weight, the higher is the concentration of the polymer required for phase formation and vice-versa [1]. However, in the system with high polymer concentration resulted in a highly viscous, whereas the system consisting of higher salt provoked the precipitation of protein from the mixture. The mechanism of phase separation in PEG-salt systems is dependent on balancing enthalpic and entropic forces involved in the aqueous hydration of the solutes [4].

After phase separation, the PEG is mainly enriched in the top and $(NH_4)_2SO_4$ is mainly enriched in the bottom phase. From all ATPs studied, the protease from *C. procera* latex was partitioned predominantly in the salt-rich lower phase, principally those with salting out effect or hydrophobic characteristic. The highest protease recovery (70.17%) was observed in the bottom phase from the system of 10%PEG-20%$(NH_4)_2SO_4$ with the purity of 1.24 fold. However, the recovery of the protease from *C. procera* by using ATPs was found in the rages of 64-86% from both phases. The highest specific activity (87.9) and the purity (1.57) of protease from *C. procera* latex was obtained in the top phase of the system of 8%PEG-20% $(NH_4)_2SO_4$. The enzyme recovery from the systems of 12%PEG with different salts showed no different in partitioning. In general, positively charged proteins normally partition selectively to the bottom phase, while negatively charged proteins prefer the upper phase in PEG/salt systems. Hence, moth of latex protease from *C. procera* partitioned in the bottom phase might be positively charged. Dubey & Jagannadham [7] reported that pI of procerain isolated from *C. procera* latex was 9.32. This leads the enzyme possessed the positive net charge at the neutral pH of the two phase system of PEG-$(NH_4)_2SO_4$.

The distribution of the proteins in ATPs is characterized by partitioning coefficient of protein (K_P). From the result, ATPs with the system of 8%PEG-20% $(NH_4)_2SO_4$ showed the lowest K_P (0.81) indicating that it caused a shift of contaminant proteins, nucleic acid and other undesirable components to the salt rich phase. Thus, the employed isolation condition resulted in the enrichment of specific protease activity, which was due to the differential partitioning of the desired protease and the contaminating enzymes and proteins to the opposite phases. The distribution of protein in ATPS of protease from *C. procera* latex likely depended on the differences in surface charge of protease and others protein contaminants, which were governed by different salts.

From this result, the bottom phase of the system containing 10%PEG-20% $(NH_4)_2SO_4$ was selected for further characterization and referred to as “isolated protease”.

Table 1. Aqueous two phase separation of protease from *C. procera* latex.

Phase composition	Phase	Total protein (mg)	Total activity (unit)	Specific activity (unit /mg)	K_P	Yield (%)	Purity fold
Crude latex	*NS*	228.5	7,665	55.9	-	100	1.00
8 % PEG - 10% $(NH_4)_2SO_4$	*NS*						
8 % PEG - 15% $(NH_4)_2SO_4$	*NS*						
8 % PEG - 20% $(NH_4)_2SO_4$	T	14.8	1,301	87.9	0.81	16.98	1.57
	B	83.7	3,707	44.3	-	48.37	0.79
10 % PEG - 10% $(NH_4)_2SO_4$	*NS*						
10 % PEG -15% $(NH_4)_2SO_4$	*NS*						
10 % PEG - 20% $(NH_4)_2SO_4$	T	19.7	1,235	62.7	0.25	16.12	1.12
	B	77.9	5,378	69.0	-	70.17	1.24
12 % PEG - 15% $(NH_4)_2SO_4$	*NS*						
12 % PEG - 15% $(NH_4)_2SO_4$	T	29.6	1,924	65.0	0.51	25.10	1.14
	B	57.6	3,589	62.3	-	46.83	1.12
12 % PEG - 20% $(NH_4)_2SO_4$	T	28.3	1,857	65.6	0.50	24.23	1.18
	B	56.3	3,474	61.7	-	45.33	1.10

NS: no phase separation, *T*: top phase, *B*: bottom phase, K_P: partition coefficient of protein.

Characterization of isolated protease by ATPs

Optimum pH

The isolated protease from *C. procera* latex was measured the optimum pH towards casein at the pH rages of 3-12. The pH activity profile is shown in Figure 1. The highest caseinolytic activity of the isolated protease from latex of *C. procera* was found at the pH of 8.0, while at higher (alkaline condition) and lower pH (acid condition) slight decrease of the caseinolytic activity was observed. This indicates that the protease from *C. procera* is a neutral protease. It has been reported that the procerain purified by CM-sepharose column from *C. procera* showed the optimum pH over a broad ranges of 7.0-9.0, whereas it was no detectable at pH below 4.5 [7]. The sharp decease of the enzyme activity at strong acidic condition or alkaline condition was probably due to the denaturation of the enzyme. Under the very acidic and alkaline pHs, the charge repulsion, which associate with decrease in electrostatic bonds [8-9]. However, optimum pH can be varied, depending upon protein substrates. The optimum pH value of this protease was similar to the protease from the latex of *Funastrum clausum* was pH of 8.0-10.0 [10].

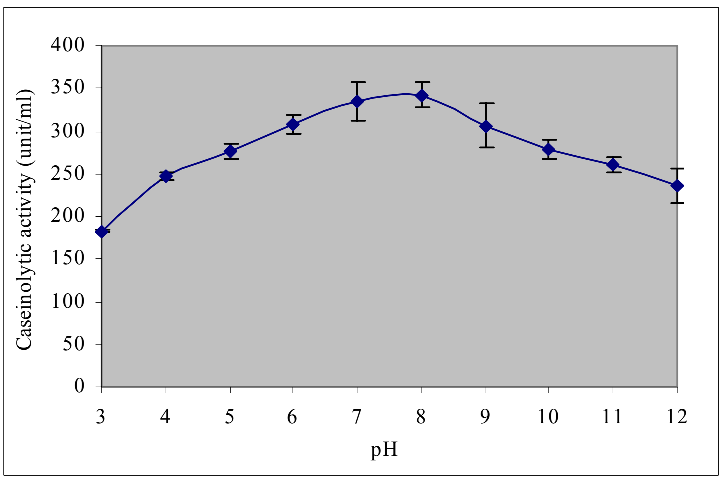

Figure 1. pH profile of isolated protease from *C. procera* latex by ATPs.

Optimum temperature

The effect of various temperatures on the activity of isolated protease from the latex of *C. procera* is shown in Figure 2. The highest activity of enzyme towards casein was found at temperature of 60^{o}C. A sharp decrease in activity was observed when temperature was higher than 60^{o}C, indicating that the enzymes possibly underwent irreversible denaturation and lost their activity. In addition, protease activity assay at 100^{o}C resulted in almost totally lost of the enzyme activity. Protein or enzymes are inactivated due to the partial unfolding of molecular level of the enzyme or proteins [11].

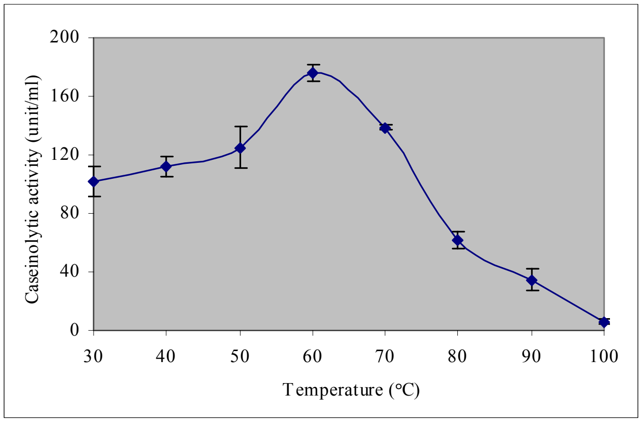

Figure 2. Temperature profile of isolated protease from *C. procera* latex by ATPs.

The optimum temperature of *C. procera* protease is similar to the case of most of plant proteases. It has been reported that the procerain from *C. procera* showed the optimum temperature ranges of 55-60°C [7]. The protease from latex of *Synadenium grantii* exhibited the optimum temperature at 60°C [12]. The purified protease from the latex of *Ficus racemosa* and *Funastrum clausum* exhibited the optimum temperatures at around 60 and 70°C, respectively [10, 13].

Salt stability

The relative activity of isolated protease from the bottom phase of ATPs of *C. procera* latex against the different salt concentration (0.0-3.0%, w/w) was also determined. In the present of NaCl, the protease activity was continuously decreased in proportion to the salt concentration increased. More than 50% of protease activity was lost when the presence of salt concentration higher than 2.0% (w/w) (Figure 3.). High salt content in the enzyme sample could reduce or increase the enzyme activity. Loss of enzyme activity might be due to the denaturation of protein caused by the salting out effect [11]. NaCl at higher concentration possibly competed with the enzyme for binding to the water, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation that can not play a role to the substrate [8].

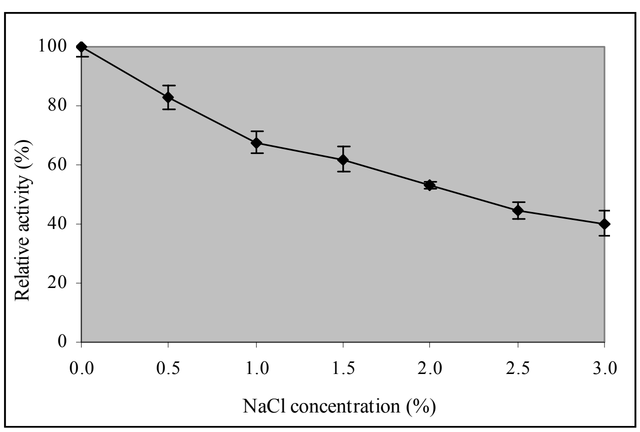

Figure 3. Effect of salt on the activity of isolated protease from *C. procera* latex by ATPs.

Effect of isolated protease on proteolysis of muscle proteins
Proteolytic patterns of the muscle foods treated with isolated protease from *C. procera* latex by ATPs are depicted in Figures 4-5. Different concentrations (10-180 units) of isolated protease were added into 2 kinds of muscle samples; buffalo meat (Figure 4) and farmed giant catfish (Figure 5). The treated samples were incubated at room temperature (25-28°C) or 60°C (generally optimum temperature of proteolytic enzymes). Increasing in activity of isolated protease on the treated muscle samples were observed as evidenced by gradually decreased in the number of protein bands and the protein band intensity. Incubation temperatures of the treated samples clearly showed the different results. High proteolytic activity was found in the sample incubated at 60°C as indicated by disappearance of protein bands. Completely disappeared of MHC was observed when 20 units of isolated protease was added to the muscle sample, while actin band was still mostly presented. Degradation products of muscle sample as a low molecular weight protein were apparently observed when the isolated enzyme was added to perform hydrolysis. Therefore, protease from *C. procera* latex extracted by using ATPs could be useful for muscle food tenderization, especially in mammalian meat. Naveena *et al.* [14] reported that higher range of protein breakdown was clear in cucumis-treated sample than ginger and papain-treated samples, indicating more pronounced proteolysis. Melendo *et al.* [15] reported that the MHC was degraded very intensely throughout the ripening of control sausages, and totally degraded in the presence of 60 U bromelain.

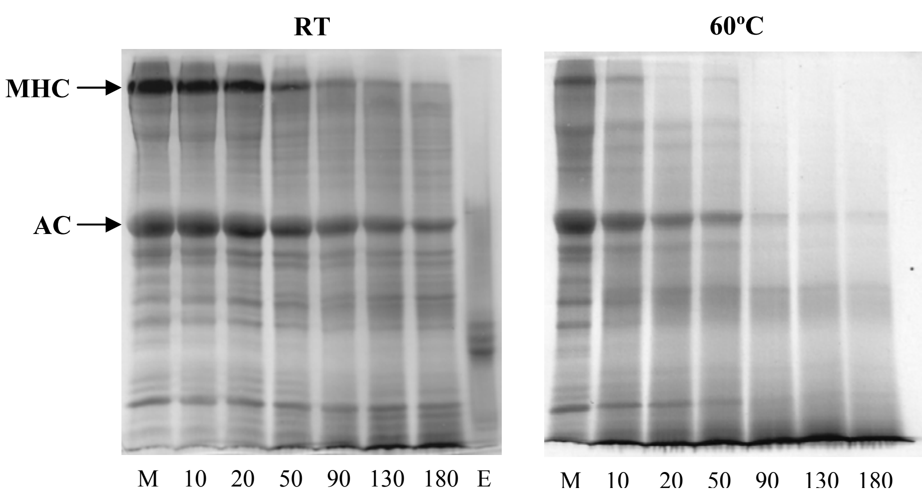

Figure 4. SDS-PAGE pattern buffalo meat treated with isolated protease from ATPs. MHC: myosin heavy chain, AC: actins, M: minced meat, E: isolated protease. Numbers represent the unit of isolated protease (unit).

The hydrolysis of farmed giant catfish by *C. procera* latex protease was also studied. As shown in Figure 5 the same proteolytic pattens with treated buffalo meat were observed through the sample. However, higher hydrolytic activity of isolated protease on muscle degradation was observed via giant catfish meat when compared to that of buffalo meat, especially at 60°C of incubation. The higher incubation temperature, the higher protein breakdown was obtained. Moreover, high level of protein breakdown was markedly clear in the farmed giant catfish muscle than that in the buffalo meat. It was also shown that, in the famed giant catfish muscle, not only MHC was hydrolyzed but also actin and other protein molecules. Those results are probably due to the partial autolysis from the enzyme contained in that fish muscle itself. In addition, structure and composition of protein in the fish are easily digested than that of the buffalo. It is important to notice again that the isolated protease from *C. procera* is efficient as a meat tenderizer.

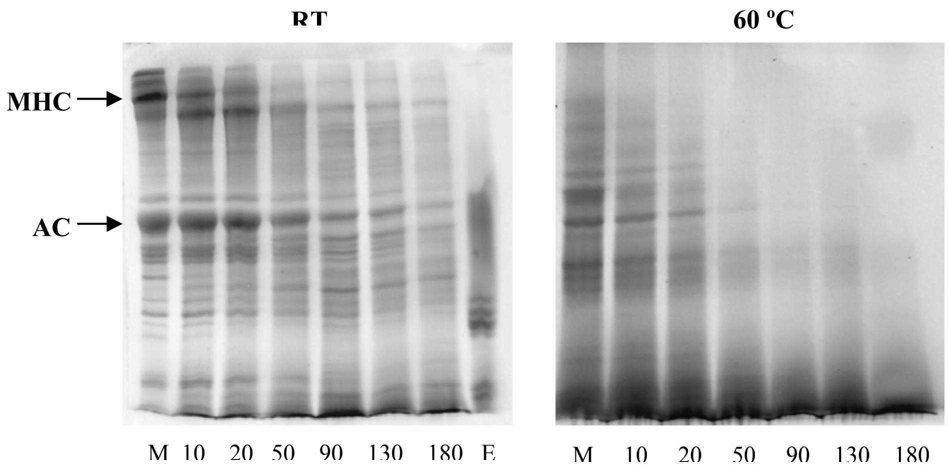

Figure 5. SDS-PAGE pattern of farmed giant catfish muscle treated with isolated protease from ATPs. MHC: myosin heavy chain, AC: actins, M: minced meat, E: isolated protease. Numbers represent the unit of isolated protease (unit).

Conclusion

The protease from the latex of *C. procera* was partitioned by ATPs with 10%PEG-20% $(NH_4)_2SO_4$. The bottom phase of the two phase system gave the highest protease recovery (70%) with the purity of 1.24. The isolated protease possessed the optimum temperature and pH at 60^{o}C and 8.0, respectively. Fifty percentages of its activity was lost when adding more than 2% (w/w) of NaCl. Based on protein degradation of buffalo and giant catfish muscles, it is suggested that protease from *C. procera* latex could be used as the meat tenderizing agent.

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