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Research Article

Partitioning of bromelain from pineapple peel (*Nang Lae* cultv.) by aqueous two phase system

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Abstract

Pineapple peel is a potential source for the extraction of beneficial bioactive compounds due to the large amount of waste after processing. The extraction of bromelain from pineapple peel by using aqueous two-phase system (ATPs) and its application in muscle models were investigated. Bromelain predominantly partitions to the polyethylene glycol (PEG) rich top phase. The highest enzyme activity and recovery were presented in the top phase of 15% PEG-3000-20%$MgSO_4$. Protein pattern and activity staining showed the molecular weight (MW) of bromelain was 29 kDa. The extracted bromelain showed the highest activity when incubated with buffer pH 9.0. Its activity decreased by 50% after 5 min incubation at 90°C and dropped drastically after 10 min incubation. Its activity also decreased continuously with increasing of NaCl concentration. Crude bromelain extract was applied to the muscles of beef, giant catfish and squid at the levels 0.05-1.0 grams per two grams of muscle. Higher muscle protein breakdown was observed to occur more in the beef and the giant catfish muscles than that of the squid sample. The myosin heavy chain (MHC) of beef and giant catfish muscles was extensively degraded into degradation proteins with lower MW.

Keywords: aqueous two phase, bromelain, meat tenderization, protease, enzyme, Thailand

Introduction

Plant proteases have received special attention in the field of medicine and biotechnology due to their properties of being active at a very wide range of temperature and pH. Latex of the milkweed family contains proteolytic enzymes; the latex of *Ervatamia coronaria, Colotropis procera* [1]. In addition, the best known plant proteases with commercial value are papain

from *Carica papaya*, ficin from *Ficus spp*. and bromelain from *Ananas comosus* [2]. Bromelain is a proteolytic enzyme normally present in pineapple fruit and stem. It has been used for meat tenderization, solubilization of grain proteins, stabilization of beer, baking cookies, production of protein hydrolysates, softening skins in leather and textiles [3]. Bromelain had also been reported to be present in pineapple wastes such as core, peel and leaves [4]. One potential alternative use for value addition of these wastes is the isolation of bromelain. However, practically no reports on the extraction of bromelain from the above wastes are available, especially the breeds normally grown in Thailand. Hence, in the present study an attempt has been made to isolate the bromelain from pineapple waste by using aqueous two phase system (ATPs).

Recently, new strategies for the production of biomolecules have been developed**.** An effective and economically viable method like ATPs is attractive for the separation and purification of a mixture of proteins/enzymes [5]. It can remove undesirable byproducts present in the system such as unidentified polysaccharides, pigments and interfering proteins that lower the activity of enzymes. Compared to other separation and purification methods, extraction using ATPs has many advantages including low cost, ease of scale-up, scope for continuous operation and is environment friendly, thus making it an attractive alternative for separation and purification of biomolecules [6].

The objectives of the present study are (i) extraction and characterization of bromelain from pineapple peel by using ATPs and (ii) application of crude extract bromelain in muscle models.

Materials and Methods

Chemicals and raw materials

Polyethylene glycols (PEG-1000, 2000, and 3000), casein and bovine serum albumin (BSA) were purchased from Fluka (Buchs, Switzerland). Ammonium sulphate ($(NH_4)_2SO_4$), magnesium sulphate ($MgSO_4$), potassium hydrogen phosphate (K_2HPO_4) and trichloroacetic acid (TCA) were procured from Merck (Darmstadt, Germany). Beta-mercaptoethanol (βME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA.).

The pineapple (*Nang lae* cultv.) was obtained from Nang Lae sub district in Chiang Rai, Thailand during April-June. The fruit was washed and air-dried. The pineapple peel was manually separated from the fruit by using a stainless steel knife and then stored at 4°C for further experiments.

Crude enzyme preparation

The pineapple peel was blended in a cold extraction buffer (0.01M sodium phosphate buffer, pH 7) at 1:1 ratio for 10 min and then filtered through a cheese cloth. The filtrate was centrifuged at 10,000xg for 20 min at 4°C. The obtained supernatant was referred to as “crude bromelain extract” and used for further experiments.

Aqueous two-phase system (ATPs)

ATPs was performed in 50 ml centrifuge tubes according to the method of Nitsawang *et al.* [6] with slight modification. To study the effect of salts on partitioning of bromelain, different amounts (14, 17, 20%, w/w) of $(NH_4)_2SO_4$), $MgSO_4$ and K_2HPO_4 were used with 18% (w/w) of PEG-1000. To study the effect of PEG concentration (12, 15 and 18%, w/w) and molecular

weights (1000, 2000, and 3000 Da) on bromelain partitioning, the PEG was added into the system with 20% $MgSO_4$. Polymer and salt were added into the crude bromelain extract (12.5 g), and then the total mixture was made up to 25 g with distilled water. The mixture was mixed continuously for 3 min using a Vortex mixer. Phase separation was achieved by centrifugation at 7000x*g* for 20 min at 4ºC. The obtained fraction was dialyzed overnight against distilled water before determination of protein and protease activity. To study the efficiency of bromelain partitioning, these parameters were calculated; specific activity (SA), purification factor (PF), partitioning coefficient of protein (Kp), volume ratio (VR) and bromelain recovery (%yield). The phase giving the highest specific activity or bromelain recovery was chosen for characterization.

Characterization of isolated bromelain

Determination of protease activity

The protease activity of bromelain was determined according to the casein digestion unit (CDU) method and tyrosine was used as a standard. The reaction mixture contained 0.1 mL enzyme solution, 0.8 mL of phosphate buffer pH 7.0 and 0.2 mL of 0.03M cysteine-0.006M EDTA. The mixture was incubated at 37°C for 10 min before starting the reaction by adding 1 mL of 1.5% casein solution. After exactly 5 min, the reaction was stopped by adding 3.0 mL 5% TCA. Precipitated protein was removed by centrifugation at 8000 rpm for 20 min. The absorbance of the clear supernatant was measured at 275 nm. One unit of protease activity is defined as the amount of enzyme releasing product equivalent to 1 µg of tyrosine min^{-1} mL^{-1} under the assay conditions.

Protein determination

Protein concentration of the sample was determined by using the Bradford method [7]. The protein content was calculated by using BSA as a standard.

SDS-PAGE

The molecular weight distribution of the extracted samples was determined by using SDS-PAGE according to the method of Laemmli [8]. The sample was mixed at the ratio of 1:1 with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS and 20% glycerol). For reducing condition, 10% of βME was added to the sample buffer. The samples (20 µg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gels) and subjected to electrophoresis at a constant current of 15 mA/gel. After separation, the protein was stained with staining solution (0.02% Coomassie Brilliant Blue R-250) and destained with a mixture of acetic-methanol solution.

Activity staining

The protein separated by electrophoresis was verified for bromelain by using activity staining as per the method of Garcia Carreno *et al.* [9]. The gel was immersed in 50 mL of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 7.5 for 45 min with constant agitation at 4ºC. The gel was then incubated at 50°C for 30 min with constant agitation. Development of a clear zone on the blue background indicated the activity of bromelain.

Determination of pH stability

The pH stability of isolated bromelain was determined by incubating the sample at different pH buffers for 20 min at room temperature. The buffers of 0.05 M Glycine-HCl (2.0-3.0), 0.05 M Na-acetate (4.0-5.0), 0.05 M Na-phosphate (6.0-7.0), 0.05 M Tris-HCl (8.0-10.0) and 0.05 M Na-carbonate (11.0-12.0) were used. After incubation, the proteolytic activity of the enzymes was assayed as mentioned in 2.4.1.

Determination of thermal stability

The effect of temperature on the activity of the isolated bromelain was investigated by incubating the enzyme sample (200µl) at the temperature of 90ºC for 0-120 min. The residual activity of bromelain was determined by the method as mentioned in 2.4.1

Measurement of salt stability

The isolated bromelain was incubated at room temperature for 20 min in the presence of NaCl ranging from 0-3.0% (w/v) with the ratio of 1:1 (v/v). The residual activity of bromelain was measured as previously described (2.4.1).

Application of bromelain in muscle models

Crude bromelain preperation

The pineapple peel was cut into small pieces and then pressed with an hydraulic press machine. The fluid obtained was filtered through a cheese cloth. The filtrate was centrifuged at 10,000xg for 20 min at 4°C and then subjected to freeze drying (Drywinner, Helo FD 8-55, Denmark). The powder of crude bromelain was kept in polyethylene bags at -18ºC for further use.

Effect of crude bromelain on muscle protein degradation

Two grams of muscle (beef, giant catfish and squid) were marinated with 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0 grams of crude bromelain powder and distilled water (control). The mixture was then incubated at room temperature for 1 hour. The marinated samples were solubilized with 18 mL of 5%SDS and then heated at 85ºC for an hour before subjecting to electrophoresis as previously described (2.4.3).

Statistical analysis

Analysis of Variance (ANOVA) was used to analyze the data from triplicate measurements. Differences between means were evaluated by Duncan's Multiple Range Test by using the SPSS statistics program (Version 11.5).

Results and Discussion

Extraction of bromelain by aqueous two-phase system

Effect of salts on bromelain partitioning by ATPs

The partitioning of bromelain from *Nang lae* pineapple peel was carried out in several biphasis systems of 18%PEG-1000 with different salts ($(NH_4)_2SO_4$, $MgSO_4$ and K_2HPO_4) at different concentrations (14, 17 and 20%). Salts are frequently used in ATPs to improve partitioning of the target molecules between the phases [10]. After phase separation, PEG-rich top phase and salt-rich lower phase were obtained. For all ATPs studied, the bromelain was partitioned prominently in the polymer phase, principally those with hydrophobic characteristic of enzymes [11]. In PEG–salt systems, partitioning of biomolecules depends on volume exclusion effect of the polymer in the polymer rich phase and salting out in the salt rich phase [12]. The effect of salt and concentration on the partitioning factors (V_R, Kp, SA, PF and Yield are shown in Table 1. The V_R of the systems tested generally decreased when salt concentration increased, except in the K_2HPO_4. The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around PEG molecules due to their water structure breaking effects [13]. The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecule.

Table 1. Effect of salts on partitioning of bromelain from pineapple peel.

Phase composition	V_R	K_p	SA	PF	Yield (%)
18% PEG1000–14% $(NH_4)_2SO_4$	2.21	7.50	2.21	11.96	66.34
18% PEG1000–17% $(NH_4)_2SO_4$	1.54	8.33	2.30	12.45	71.99
18% PEG1000–20% $(NH_4)_2SO_4$	1.34	8.58	2.51	13.58	80.31
18% PEG1000–14% K_2HPO_4	1.39	7.19	2.58	13.95	72.96
18% PEG1000–17% K_2HPO_4	0.95	6.40	2.67	14.42	20.64
18% PEG1000–20% K_2HPO_4	1.08	10.77	2.80	15.13	77.52
18% PEG1000–14% $MgSO_4$	4.27	8.38	2.87	15.52	52.00
18% PEG1000–17% $MgSO_4$	3.06	8.53	3.66	19.78	59.01
18% PEG1000–20% $MgSO_4$	1.44	4.90	4.56	24.68	74.14

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; SA: specific activity (unit/mg protein); PF: purification factor.

The distribution of the protein in ATPs is reported by Kp. From the results, the Kp of all ATP systems were in the rages of 4.9 to 10.77 with the average around 8. The highest Kp (10.77) was found in the system of 18%PEG-1000-20%K_2HPO_4. High Kp values indicates that most of the protein in the sample partitioned in only the bottom phase or the top phase. The phase composition of 18%PEG-1000-20%$MgSO_4$ showed the lowest K_p (4.90). This indicated that it caused a shift of contaminant protein nucleic acid and other undesirable components to the lower phase [13]. Thus, the extraction conditions employed, resulted in the enrichment of SA and PA.

A phase containing 18%PEG-1000 and 20%$MgSO_4$ gave the highest SA (4.56 unit/µg protein) and PF (24.68). The recovery of bromelain by using ATPs in the study was in the ranges of 52 to 80%. The maximum yield of 80.31% was obtained from the system containing 18%PEG-1000-20%$(NH_4)_2SO_4$. This implies that not only the most bromelain partitioned to the top phase, but also other proteins. Babu *et al.* [12] reported that the system of 18%PEG-1500 and 20% K_2HPO_4 provided the highest activity recovery. Klomklao *et al.* [11] reported that using 20%PEG-1000 with 20%$MgSO_4$ gave the highest SA and PF in partitioning of protease from tuna spleen. In addition, Nalinanon *et al.* [13] reported the same result in partitioning of protease from the stomach of albacore tuna.

The results also showed that increasing of salt concentration (14 to 20%) resulted in an increase in Kp, SA, as well as the bromelain recovery of the system. The solubility of biomolecules in the salt phase decreases with an increase of salt concentration, which results in reduced partitioning of these molecules to the top phase. This could be due to “salting out effect”. From the result, it can be observed that the specific activity increased with an increase of salt concentration. However, based on the highest SA and PF of 20%$MgSO_4$, this condition was chosen for investigation of the effect of PEG on bromelain partitioning.

Effect of PEG on bromelain partitioning by ATPs

The bromelain partitioning in ATPs with different molecular weight (MW) and concentrations of PEG at 20%$MgSO_4$ was studied. As shown in Table 2, partitioning of bromelain was strongly dependent on the MW and the concentration of PEG. The V_R of the system ranged from 0.7 to 1.7. However, most of the system gave the value of V_R close to 1.0, indicating the volume fraction was distributed in both phases. The Kp obtained from all systems were higher than 1, indicating that bromelain preferentially partitioned to the top

phase. The preferential interaction between PEG molecule and protein domain decreased when the MW of PEG increased due to its exclusion from the protein domain [11, 13, 14]. This might lead to the movement of bromelain to the salt lower phase. The increase in concentration of bromelain is due to the more dominant effect of volume exclusion over salting out. Among all ATPs tested, system comprising 15%PEG-3000 and 20%MgSO4 was effectively partitioned the bromelain to the top PEG-rich phase and undesired proteins to the bottom salt phase. Under this condition, 108.45% of the enzyme was recovered in the top phase, providing an approximate PF of 28.28 fold of bromelain from *Nang lae* pineapple peel. The enzyme activity recovery of bromelain increased with an increase in the MW of PEG. The increase in PF was due to relatively less partitioning of bromelain to the bottom phase when compared to that of other proteins. From these results, MW of PEG-3000 (15%) with high concentration of $MgSO_4$ (20%) provided the highest purity and recovery of bromelain from pineapple peel. However, it has been reported that the system comprising polymer with a high concentration or high MW polymer and high salt concentration resulted in partitioning of biomolecules at the interphase due to the influence of both volume exclusion and salting out effect [13].

Table 2. Effect of PEG on partitioning of bromelain from pineapple peel.

Phase composition	V_R	K_p	SA	PF	Yield (%)
12% PEG1000–20% $MgSO_4$	0.76	2.04	1.40	7.59	43.63
15% PEG1000–20% $MgSO_4$	1.13	2.97	1.01	5.45	30.04
18% PEG1000–20% $MgSO_4$	1.44	4.90	4.56	24.68	74.14
12% PEG2000–20% $MgSO_4$	1.02	1.96	2.67	14.46	86.03
15% PEG2000–20% $MgSO_4$	1.08	2.44	2.83	15.29	91.52
18% PEG2000–20% $MgSO_4$	1.70	2.78	1.90	10.26	50.17
12% PEG3000–20% $MgSO_4$	1.06	2.11	2.76	14.90	94.84
15% PEG3000–20% $MgSO_4$	1.62	2.93	5.23	28.28	108.45
18% PEG3000–20% $MgSO_4$	0.84	1.25	4.79	25.92	45.20

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; SA: specific activity (unit/mg protein); PF: purification factor.

Characterization of extracted bromelain

SDS-PAGE and activity staining

Protein patterns of crude enzyme and extracted bromelain partitioned from 15% PEG-3000–20% $MgSO_4$ ATPS fraction are shown in Figure 1. Migration of protein composition in crude enzyme, top phase and bottom phase were quite different. To compare with standard protein markers, crude enzyme molecular masses were ranged from 24-30 kDa. Proteins with 20, 29 and 32 kDa were presented in the top phase, while those with 24-29 kDa appeared in the bottom phase. Difference in protein bands among the three fractions are probably due to the difference in amount of interfering proteins and other enzymes in each phase. However, it is important to measure which band of protein belongs to the bromelain. Umesh *et al.* [4] reported that the bromelain extracted from pineapple core was found to be around 26 kDa by SDS-PAGE analysis.

To verify the band of bromelain, activity staining was performed. The clear zone by proteolytic activity surrounded by the blue background was present only at the protein band of 29 kDa. This band was apparently present in the top phase (Figure 1 B). There was no clear zone observation in both crude enzyme and the bottom phase compositions. This is

probably due to rather low bromelain content in both fractions resulting in invisible proteolytic activity. In contrast, the top phase fraction mainly composed of bromelain provided activity sufficient enough for detection by activity staining method. However, molecular mass of bromelain in the top phase was different from previously reported events from crude extract [4]. It can be explained by attachment of PEG molecular mass (3,000 Da) to the bromelain causing its band to appear to be around 29 kDa (Figure 1 A).

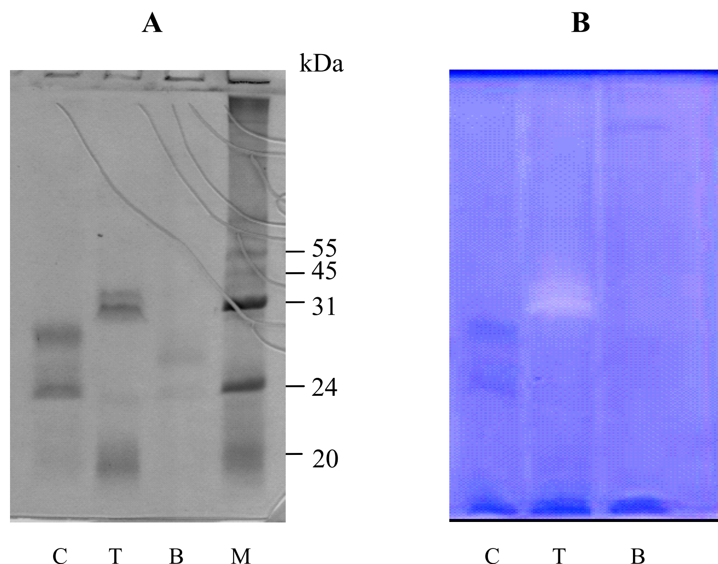

Figure 1. SDS–PAGE patterns (A) and activity staining (B) of protease from *Nang lae* pineapple peel partitioned with 15%PEG-3000–20%$MgSO_4$ ATPs.

C: crude enzyme, T: top phase, B: bottom phase, M: molecular weight protein maker (20 µg protein was loaded in to the gel).

pH stability

The stability of bromelain incubated with various pH buffers is shown in Figure 2. Bromelain was able to retain most of its activity at pH 9.0. The enzyme stability slightly declined in the acidic area, while it was dramatically lost at pH above 9.0. This is supported by the results of Liang *et al.* [15] that the bromelain has a wider pH range for optimum activity at 6.8-9.0. In addition, the pH stability of bromelain was similar to that of the protease from *Funastrum clausum* latex which was 8.0-9.0 [16]. The activity of this enzyme sharply decreased in the very alkaline pH range, possibly due to the denaturation of enzymes. Under the very acidic and alkaline pH, the charge repulsion associates with a decrease in electrostatic bonds [17]. It should be noticed that the enzyme seems to be more stable in acidic than in alkaline conditions.

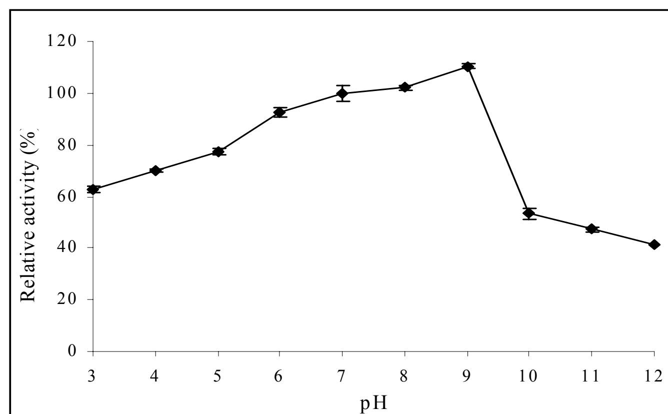

Figure 2. Effect of pH on the activity of protease from *Nang lae* pineapple peel partitioned with 15%PEG-3000–20%$MgSO_4$ ATPs.

Thermal stability

Bromelain extracted from pineapple peel was measured for its thermal stability at 90°C. The results are presented in Figure 3. The activity of bromelain decreased 50% after 5 min of incubation. Moreover, the activity dropped drastically after 10 min. High temperature is found to be an irreversible-denaturant of protein and enzymes. Thus the enzyme is inactivated at high temperature due to the partial unfolding of its molecule [18]. It has been reported that the optimum temperature for the activity of bromelain was found to be at about 55°C by Liang *et al.* [15].

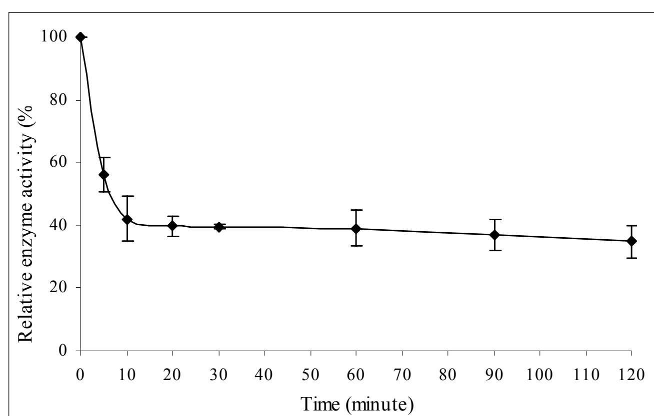

Figure 3. Thermal stability of protease from *Nang lae* pineapple peel partitioned with 15%PEG-3000–20% $MgSO_4$ for 0-120 minute at 90°C.

Salt stability

The ability of fractioned bromelain to retain its activity under growing ionic strength was tested by exposing it to different concentrations of sodium chloride solution 0.5-3.0%. The activity of fractioned enzyme from the top phase of 15%PEG-3000–20% $MgSO_4$ decreased with an increase of NaCl concentration (Figure 4). In the presence of NaCl ranging from 0.5-3.0%, the activity of enzymes was slightly decreased. At 1.5% NaCl, the activity of enzymes decreased to 22% of the control (0% NaCl). At 3.0% NaCl concentration, the proteolytic

activity decreased to 32% of the control. Loss of activity might be due to the denaturation of protease caused by the salting out effect [17]. NaCl at higher concentration possibly competed with the enzyme in water binding, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation [18].

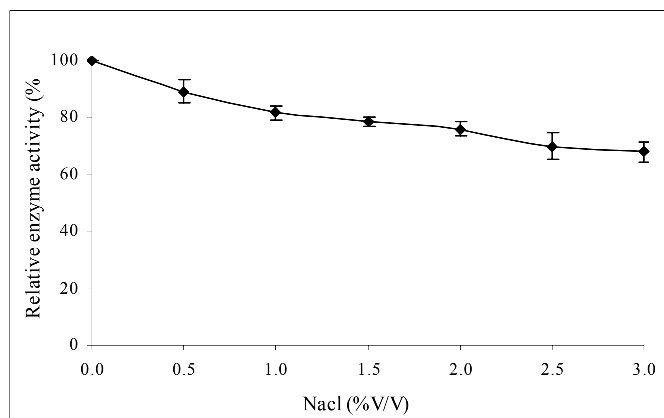

Figure 4. Effect of salt content on the activity of protease from *Nang lae* pineapple peel partitioned with 15%PEG-3000–20% $MgSO_4$.

Effect of enzyme fraction on muscle protein degradation

A representative SDS-PAGE gel for different concentrations of enzyme extract can be seen in Figure 5. There was increased bromelain induced muscle protein hydrolysis in all enzyme-treated samples as evidenced by reduction in the number of protein bands and band intensity. The degradation of protein increased with increasing of enzyme concentration. Naveena *et al.* [21] reported that when using protease from *Cucumis trigonus* in buffalo meat samples the increase in proteolysis can be correlated with significantly higher protein solubility. Bacterial proteolytic enzyme treatment of muscle protein showed reduction in the level of higher molecular weight fractions due to degradation of myosin, thus increasing meat tenderness [22].

From the figure it is also evident that breakdown of protein in all enzyme treated samples was generated. The high breakdown of proteins was more visible in the beef and giant catfish muscle than that of the squid sample. The MHC band of beef and giant catfish muscle was extensively degraded into lower molecular weight. Furthermore, high breakdown of protein was markedly clearer in the giant catfish than in the beef. This is probably due to the co-hydrolysis from the enzyme itself contained in giant catfish. The result also showed that there was a slight change in degradation of actin in squid muscle, even with increased enzyme concentration, when compared to those in beef and giant catfish muscles. It can be explained by the difference in structure and composition of proteins among these three muscles.

The degradation of muscle protein plays a major role in determining the tenderness and water holding capacity of meat during postmortem storage [23]. Myosin was degraded very intensely throughout the meat, and totally in the presence of a high concentration of bromelain. Actin, on the other hand, was only slightly hydrolyzed unless a high amount of enzyme was added. Nonetheless, differences between the controls and the treated meat with lower enzyme concentration were unclear in all samples. Kim & Taub [24] have reported that bromelain hydroyzed myosin faster than actin. This corresponds to myosin band intensity

decrease with increase of low molecular weight peptides as shown in Figure 5. It can be concluded that degradation of myosin could be related to the tenderizing effect of bromelain.

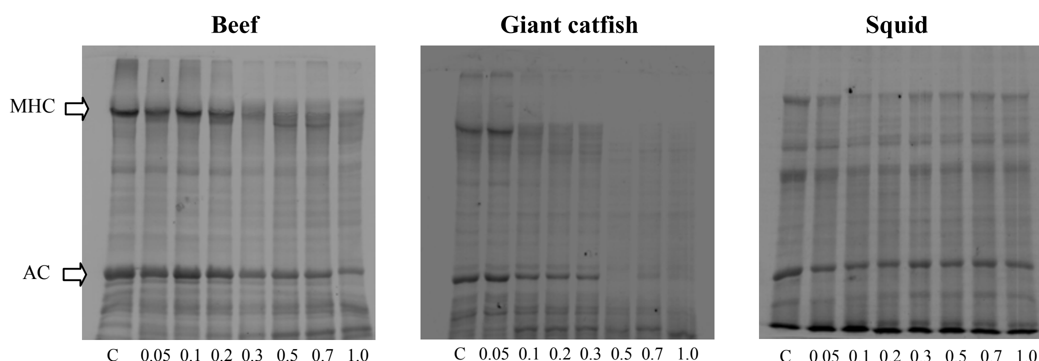

Figure 5. SDS–PAGE patterns of muscle sample incubated with crude enzyme extract from *Nang lae* pineapple peel.
C: control (without fraction); Numbers indicated concentration (g) the fraction used. MHC: myosin heavy chains; AC: actin.

Conclusions

Bromelain from the *Nang lae* pineapple peel was extracted by the aqueous two phase method. The top phase from 15%PEG-3000-20%$MgSO_4$ provided the highest specific activity and bromelain recovery. The bromelain from that phase composition showed very high stability at neutral pH. Its activity decreased 50% when incubated at 90°C for 5 min. 30% of bromelain activity was lost in the presence of 3.0% (w/w) NaCl. Based on protein degradation of beef, giant catfish and squid muscles, it is suggested that bromelain from *Nang lae* pineapple peel is suitable for meat tenderization.

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