รายงานวิจัยฉบับสมบูรณ์

การแยก การศึกษาคุณลักษณะ และการใช้ประโยชน์เอนไซม์ย่อยโปรตีนจากยางรัก ระยะที่ ๒

Isolation, Characterization and Application of Proteolytic Enzymes from *Calotropis procera* Latex, Phase II

โดย

ผู้ช่วยศาสตราจารย์ ดร. สาโรจน์ รอดคืน

งานวิจัยนี้ได้รับเงินอุดหนุนการวิจัยจากงบประมาณแผ่นดิน

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EXECUTIVE SUMMARY

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology because their properties and functions. Among of them, those from plant latex were also widely used to reduce meat toughness for a long time. These proteases include papain from *Carica papaya*, ficin from *Ficus carica*, and cysteine protease from *Funastrum clausam*. *Calotropis procera* is a plant found in tropical and sub-tropical regions. Various parts of *C. procera* have been used in traditional medicine. However, the biochemical properties of enzymes contained in the latex of *C. procera* is limited. In addition, applications of this resource for muscle foods tenderization have not been reported. So, this investigation aims to study the effect of proteases from *Calotropis procera* latex on muscle proteins degradation. Efficiency of hydrolysis on muscle protein when compared with other plant proteases was also investigated.

The degradation of myofibrillar proteins observed by SDS-PAGE of muscle samples was clearly observed when enzyme crude extract or ATPS fraction was added. The muscle samples were marinated with distilled water (control) and 0.05, 0.1, 0.2, 0.3 and 0.5% (w/w) of crude enzyme extract powder for 60 min at 4°C before subjected to quality monitoring. A decrease in moisture content was observed when the crude enzyme extract was added. Firmness and toughness of the muscle samples significantly decreased with the increased addition of crude enzyme extract . The water holding capacity and cooking yield of the treated samples showed no significant difference throughout the crude enzyme extract addition ($p>0.05$). An increasing in protein solubility and TCA-soluble peptides content was observed in all of the treated samples.

The samples were treated with 4 proteases; *C. procera* latex, papaya latex, papain and bromelain. Changes of muscle properties were monitored after 24 h of 4°C aging. Significantly increases of collagen solubility were also found in all muscle samples. An increase in TCA-soluble peptides, and myofibrillar protein degradation were observed in all enzyme treated samples. At the microstructural level, tissue fibers were broken, and the connections between the sarcolemma and the myofibrils were loosen. Comparing to all proteolytic enzymes used papaya latex proteases showed the highest hydrolysis activity to all muscle types.

The results show that the proteolytic enzymes from *Calotropis procera* latex could be used as an effective meat tenderizer. Caution must be taken when high level of proteolytic enzyme was applied cause of negative results will be obtained. For making sure of using the enzyme extract as a food ingredient, cytotoxicity needs to be confirmed.

บทคัดย่อ

งานวิจัยนี้เป็นการแยกเอนไซม์โปรตีเอสจากยางรักด้วยระบบสารละลายน้ำสองวัฏภาค (ATPS; โพลีเอธิลีนไกลคอล 1000 ร้อยละ 18 ในเกลือแมกนีเซียมซัลเฟตร้อยละ 14) ก่อนที่จะนำไปใช้สำหรับการย่อยโปรตีนกล้ามเนื้อ ติดตามรูปแบบการย่อยโปรตีนไมโอไฟบริลโดยเจลอิเล็คโตรโฟเรซิส (โปรตีนไมโอซินเส้นหลักและ แอกติน) พบว่าโปรตีนไมโอไฟบริลของปลาบึกสูงกว่าโปรตีนกล้ามเนื้อจากเนื้อวัวและปลาหมึกโดยสังเกตได้จากรูปแบบการย่อยโปรตีนเป็นสายเล็ก ๆ นอกจากนั้นสารสกัดหยาบจากยางรักยังใช้ในการศึกษาการทำเนื้อนุ่ม (เนื้อหมู เนื้อวัว และเนื้อไก่) อีกด้วย ตัวอย่างเนื้อเหล่านี้จะถูกหมักด้วยสารสกัดหยาบเอนไซม์ในความเข้มข้นต่าง ๆ คือ 0.05 0.1 0.2 0.3 และ 0.5 (ร้อยละโดยน้ำหนัก) นาน 60 นาทีที่อุณหภูมิ 4 องศาเซลเซียส โดยใช้น้ำเป็นชุดควบคุม ก่อนทีจะนำตัวอย่างไปตรวจติดตามคุณภาพ จากผลการทดลองพบว่ามีการลดลงของความชื้นในตัวอย่างเมื่อมีการเติมสารสกัดหยาบเอนไซม์ลงไป นอกจากนั้นยังมีการลดลงของค่าความแน่นเนื้อและความเหนียวของตัวอย่างเนื้ออย่างมีนัยสำคัญภายหลังการบ่มด้วยสารสกัดหบายเอนไซม์ในปริมาณที่เพิ่มขึ้น อย่างไรก็ตามพบว่าความสามารถในการอุ้มน้ำและร้อยละการกลับคืนจากความร้อนไม่มีความแตกต่างระหว่างตัวอย่างที่ผ่านการบ่มที่สภาวะแตกต่างกัน แต่พบการเพิ่มขึ้นของการละลายของโปรตีนและปริมาณเปปไทด์ที่ละลายได้ในกรดในทุกตัวอย่างที่มีการบ่มด้วยสารสกัดหยาบเอนไซม์ ซึ่งสามารถสังเกตได้อย่างชัดเจนจากรูปแบบการย่อยโปรตีนภายใต้เจลอิเล็คโตรโฟเรซิส

สำหรับการทดลองสุดท้ายเป็นการศึกษาเปรียบเทียบประสิทธิภาพของโปรตีเอสแต่ละชนิดกับโปรตีเอสจากยางรัก โดยโปรตีเอส 4 ชนิดที่นำมาใช้ในการบ่มตัวอย่างได้แก่ โปรตีเอสจากยางรัก ยางมะละกอ เอนไซม์ปาเปน และ เอนไซม์โบรมิเลน หลังจากบ่มตัวอย่างนาน 24 ชั่วโมงที่อุณหภูมิ 4 องศาเซลเซียสทำการตรวจติดตามการเปลี่ยนแปลงสมบัติของกล้ามเนื้อ โดยพบว่า พีเอชในเนื้อไก่ ปลาบึกและ หมู มีค่าลดลงหลังการบ่มด้วยสารสกัดหยาบเอนไซม์ นอกจากนั้นพบว่าคอลลาเจนมีการละลายเพิ่มขึ้นอย่างมีนัยสำคัญในทุกตัวอย่างที่ทำการทดสอบ เช่นเดียวกันกับการเพิ่มขึ้นของเปปไทด์ที่ละลายได้ในกรดและการย่อยสลายของโปรตีนไมโอไฟบริล สำหรับการติดตามการเปลี่ยนแปลงในระดับโครงสร้างกล้ามเนื้อขนาดเล็ก พบว่ามีการทำลายของกล้ามเนื้อในระดับเนื้อเยื่อ และการเชื่อมกันของเส้นใยกล้ามเนื้อระหว่างซาร์โคเลมมาและไมโอไฟบริลถูกทำลายเมื่อมีการบ่มด้วยเอนไซม์ เมื่อเปรียบเทียบเอนไซม์โปรตีเอสทุกชนิดที่ทดสอบพบว่า โปรตีเอสจากยางมะละกอให้ผลการย่อยกล้ามเนื้อทุกชนิดดีที่สุด รองลงมาคือโปรตีเอสจากยางรัก เอนไซม์ปาเปน และ โบรมิเลน ตามลำดับ จากผลการทดลองดังกล่าวแสดงให้เห็นว่าเอนไซม์โปรตีเอสจากยางรักสามารถใช้เป็นสารทางเลือกจากธรรมชาติในการทำเนื้อนุ่ม

คำสำคัญ: ระบบสารละลายน้ำสองวัฏภาค รัก ยาง โปรตีเอส โปรตีนกล้ามเนื้อ การทำเนื้อนุ่ม ความเหนียว คอลลาเจน

ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS; 18% PEG 1000 containing 14% $MgSO_4$) and then applied to hydrolyze muscle proteins. The degradation of myofibrillar proteins observed by SDS-PAGE (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. In addition, crude enzyme extract from *C. procera* latex was also conducted in order to tenderize muscle foods (pork, beef, and chicken). The muscle samples were marinated with distilled water (control) and 0.05, 0.1, 0.2, 0.3 and 0.5% (w/w) of crude enzyme extract powder for 60 min at 4°C before subjected to quality monitoring. A decrease in moisture content was observed when the crude enzyme extract was added. Firmness and toughness of the muscle samples significantly decreased with the increased addition of crude enzyme extract ($p<0.05$). The water holding capacity and cooking yield of the treated samples showed no significant difference throughout the crude enzyme extract addition ($p>0.05$). An increasing in protein solubility and TCA-soluble peptides content was observed in all of the treated samples. The electrophoresis pattern of the muscle treated samples also revealed extensive proteolysis occurring in each muscle type.

The last investigation was compared the efficiency of different proteases to *C. procera* latex protease. The samples were treated with 4 proteases; *C. procera* latex, papaya latex, papain and bromelain. Changes of muscle properties were monitored after 24 h of 4°C aging. Decrease of muscle pH on chicken, giant catfish and pork was observed when enzymes was added ($p<0.05$). Significantly increases of collagen solubility were also found in all muscle samples. An increase in TCA-soluble peptides, and myofibrillar protein degradation were observed in all enzyme treated samples ($p<0.05$). At the microstructural level, tissue fibers were broken, and the connections between the sarcolemma and the myofibrils were loosen when each enzyme was applied. Comparing to all proteolytic enzymes used papaya latex proteases showed the highest hydrolysis activity to all muscle types followed by *C. procera* latex proteases, commercial papain and bromelain. The results show that the proteolytic enzymes from *Calotropis procera* latex could be used as an effective meat tenderizer.

Keywords: aqueous two phase, *Calotropis procera*, latex, proteases, muscle protein, tenderization, toughness, collagen

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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). Proteases from plant sources have received special attention from the pharmaceutical industry 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, squid and in coarse dry sausage. Protease from ginger rhizome is a new interesting hydrolytic enzyme that has also been increasingly used for meat tenderization. Proteases from plant latex were also widely used to reduce meat toughness for a long time. These proteases include papain from *Carica papaya*, ficin from *Ficus carica*, and cysteine protease from *Funastrum clausam*. 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. However, the biochemical properties of enzymes contained in the latex of *C. procera* is limited. In addition, applications of this resource for muscle foods tenderization have not been reported.

Objectives

1. To study the effect of proteases from *Calotropis procera* latex on muscle proteins degradation.
2. To compare the efficiency of protease from *C. procera* latex with other plant proteases in terms of muscle hydrolysis.

Scope of research work

Both crude enzyme extract and fraction from ATPS of *Calotropis procera* latex proteases were apply to tenderize different types of muscle sample including beef, pork, squid, chicken and farmed giant catfish. Quality attributes especially biochemical and physico-chemical properties of muscle samples were monitored during treatment and storage. For comparative study, protease from *Calotropis procera* latex was compared with other plant proteases in terms of muscle hydrolytic activity.

LITERATURE REVIEW

The tenderness of fresh meat

The tenderness of meat ranks as the second most important criterion to ensure repeat customer purchase of meat, the first being attractive colour and appearance. A lack of tenderness in meat is caused by a combination of toughness in the fibre structure of meat and toughness in the connective tissue (Figure 1). The degree of toughness of meat can predominantly be linked to the age of the animal and to a small degree to the species of animal. Muscle tissue of older animals shows signs of an increased number of cross-links between actin and myosin as well as increased numbers of cross-links within collagen.

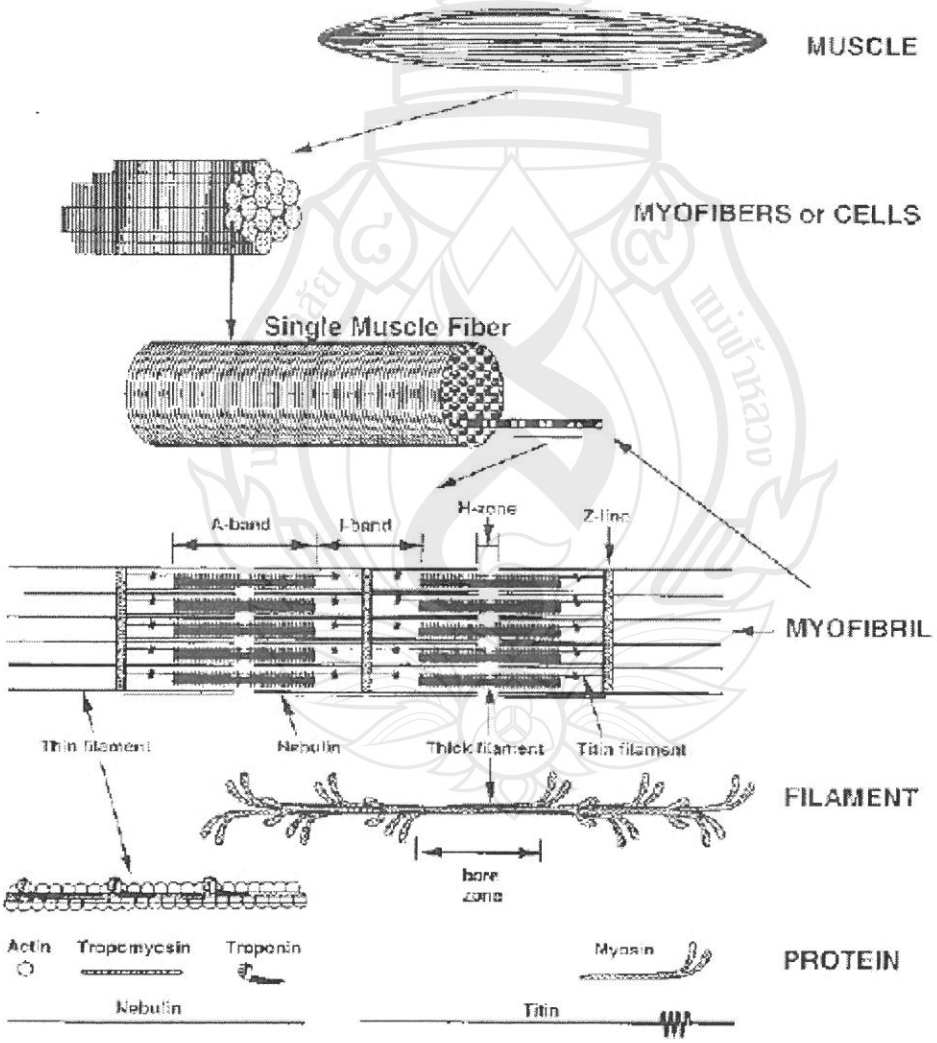

Figure 1. Diagram showing the levels of organization of muscle.

Source: Hui et al. (2001)

To obtain tender meat, a certain temperature–pH relationship should be adhered to post-slaughter; the pH value must be at or below 5.7 once a temperature of 7C is present within meat. If the meat or carcass cools too quickly and the temperature is below 7C, once a pH value of 5.7 is reached, there is a risk of cold shortening. To avoid cold shortening, another temperature–pH relationship to be observed is that the internal temperature of meat must not be below 14 ∞C if the pH is still at 6.2 or above. Of course, a carcass must also not be cooled too slowly for microbiological reasons; microbial growth must be kept under control.

Ageing of meat for enhancing tenderness

Tenderness of meat is related to a combination of breakdown within muscle fibres, predominantly due to the activity of enzymes, and loosening of connective tissue, specifically collagen (Figure 2). Collagen in raw meat is usually loosened by the enzyme collagenase over a prolonged period of time but this process takes place only to a small degree as the action of collagenase is very slow and meat would be microbiologically spoiled before collagen would be significantly softened. Another method of softening collagen in raw meat is to place meat in a sour (acidic) soaking solution containing wine and/or vinegar under chilled conditions for 24–48 h as practised during the preparation of meat dishes in countries such as Germany (Sauerbraten). Collagen exposed to a sour environment starts to swell, thus taking up moisture and loosening the collagen structure is the result. The toughness in meat is the combination of toughness in muscle and the toughness in connective tissue.

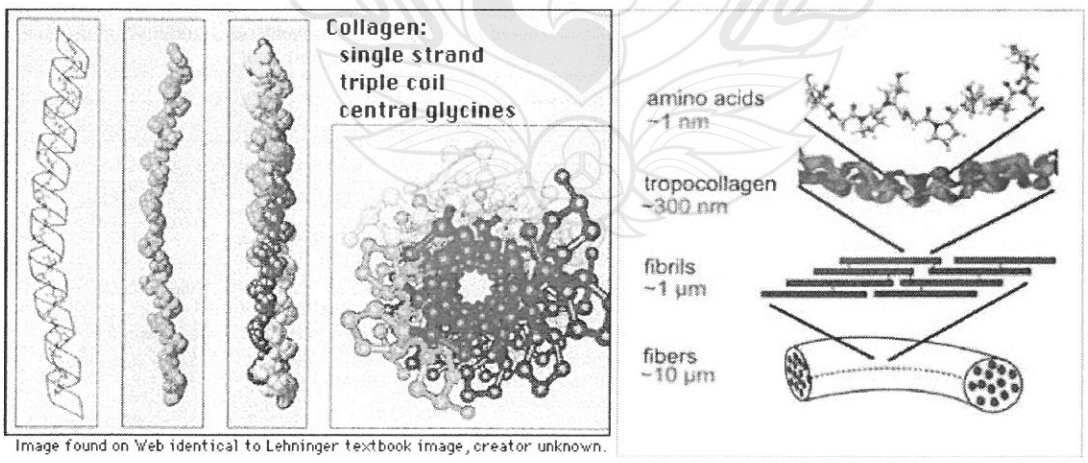

Figure 2. Molecular structure of collagen

Source: http://www.foodnetworksolution.com; http://images.sciencedaily.com

Naturally occurring enzymes in meat, predominantly cathepsins (which are cysteine proteases) tenderize meat by slowly breaking down the muscle fibres (Figure 3). Cathepsins are found in the lysosomes of a muscle cell, protected by a wall of fat, and over 30 different enzymes are involved. During rigor mortis, as a result of the formation of lactic acid and therefore the decrease in pH value, the walls of fat are destroyed by the impact of lactic acid, and cathepsins are released. Cathepsins work on the bonds between actin and myosin and contribute greatly to the tenderness of meat. Calpains (calcium-activated proteases), which are present in the sarcoplasm is another type of enzyme naturally present in meat, contribute to the tenderization of meat in a different way; these enzymes cut along the Z lines and long fibres are 'cut' into smaller units. The level of enzyme activity is largely determined by the temperature that meat is stored under as well as the level of enzymes naturally present in meat.

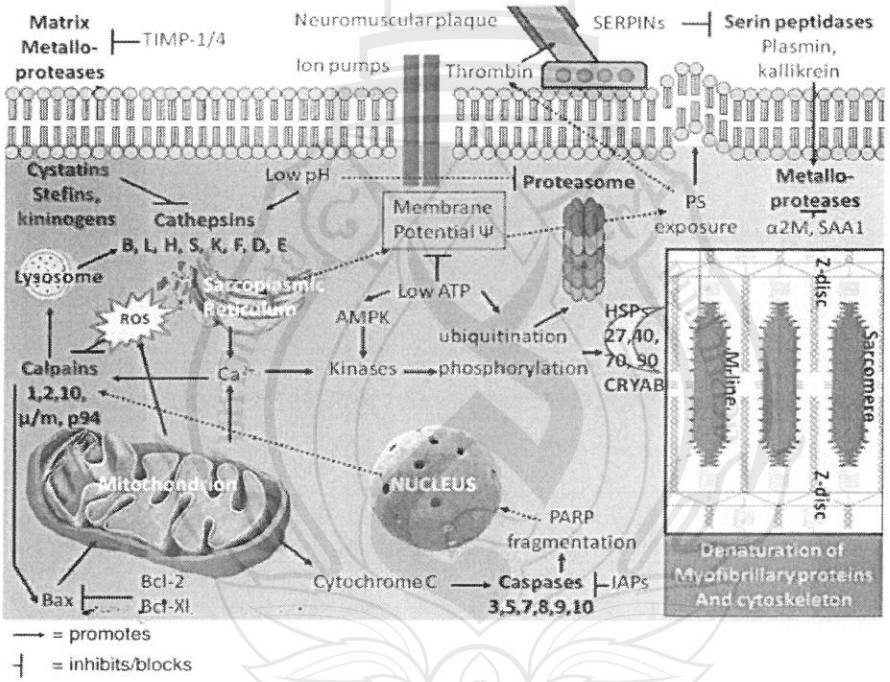

Figure 3. An overview of the interplay among muscle proteases (in bold font) in the frame of meat tenderization. While activation of calpains is triggered by calcium ions and sustained at the transcriptional level by PARP-mediated pro-apoptotic signaling, calpains themselves trigger release of cathepsins from the lysosome. In the frame of the apoptotic process, caspase are activated via the intrinsic mitochondrial pathway of apoptosis. In addition, a role is proposed for the proteasome (inhibited by acidic pH of longer stored meat), metalloproteinases (activated by extracellular serine peptidases) and matrix metalloproteinases. Each one of these proteases ends up attacking the structure of myofibrils, especially at the Z-disk level, promoting tenderization.
Source: Alessandro and Zolla (2013)

Calpains are known to work more effectively at higher pH values in meat such as 6.2–7.0, while cathepsins prefer a lower pH value around 5.4–5.9. This leads to the assumption that calpains are more important during the early stage of post-mortem glycolysis than are cathepsins, which seem to act later in the process. It is not fully understood yet the degree to which each enzyme ultimately contributes to overall tenderness, but it is thought that both types of enzyme, namely calpains and cathepsins, act synergistically towards enhancing the tenderness of meat. Another contributing factor to the tenderness of meat is the loosening of the actomyosin complex. During maturing of meat, actin and myosin remain to a large degree cross-linked together as the actomyosin complex and are therefore responsible only to a small degree for the tenderness of meat. The impact of enzymes such as cathepsins and calpains on the toughness of collagen is insignificant.

During ageing (Figure 4), the visual appearance of meat does not change, given that breakdown of muscle fibres takes place on a microscopic level. Meat can age without being packed for up to 14 days depending on the microbiological status of the meat and the storage temperature. Vacuum-packed meat can age for up to 3 months as aerobic spoilage bacteria are kept away effectively. The speed of tenderization varies dramatically between different species and ageing of beef requires a significantly longer time than pork and chicken in order to achieve a comparable degree of tenderness. The reason for this is that pork and chicken contain a significantly higher level of proteolytic enzymes such as cathepsins and tenderization occurs in pork and chicken at a significantly faster rate as a result. In order to obtain a tender piece of beef, stored between 0 and 3C, at least 2 weeks are needed. A similar tenderizing effect can be seen in pork within 2–3 days at the same storage temperature. On the other hand, a comparable effect on tenderness in poultry can be seen within 1–2 days. Lamb lies somewhat in between beef and pork and generally requires around 7–10 days in order to become tender. The 'speed of tenderization' between 4 and 45C is 85 times faster; meat exposed for 1 h to a temperature of 45C would need to tenderize for 85 h at a temperature of 4C in order to demonstrate similar tenderizing enzyme activity. This fact is often utilized in restaurants where chefs place raw meat, which is shortly to be prepared, in the oven at the above-mentioned temperatures to speed up tenderization.

Figure 4. Inside a dry aging room of meat.

Source: http://www.seriouseats.com

The pH value of meat rises significantly during ageing owing to the formation of alkaline metabolic by-products of enzyme activity, which eventually lead to spoilage. Once the pH value exceeds 6.4–6.5, meat is spoiled from a sensorial point of view as high levels of ammonia (NH_3) and other metabolic by-products are produced. Formation of slime is observed commonly at this stage and discolouration also takes place at this point. If deboned meat is aged whilst vacuum packed, this has the benefit that anaerobic bacteria, such as *Lactobacillus* spp., have an advantage over aerobic spoilage bacteria. Because of the degree of acidity obtained within the vacuum packed meat, the collagen swells, resulting in softening of the connective tissue. When meat is vacuum packed, contamination via handling is also avoided, extending the shelf life of the product. Research has been carried out to investigate whether injecting a calcium chloride or enzyme solution into living animals (e.g. cattle) shortly before slaughter increases the tenderness of meat after slaughter. The results, however, were not convincing and the meat even exhibited a slight bitter taste after this treatment.

Beef can be tenderized by the application of the tender-stretch method: hanging the halves of carcasses straight after slaughter by the hip or pelvic bone (Figure 5). Gravity pulls down on both ends of the carcass and therefore the muscles cannot contract as severely as they would if the carcass were hung the conventional way, vertically from the Achilles tendon. Because of this counter-force, a reduced number of cross-links between actin are myosin are formed and the degree of toughness is reduced. However, the technique of hanging the carcass on the pelvic bone is not

used as often as the technique of hanging on the Achilles tendon, given that the space needed per half of a carcass is significantly more than if the carcass is hung on the Achilles tendon. Cutting up a carcass hanging on its pelvic bone into a forequarter and a hindquarter is also more difficult compared with cutting up a carcass hanging on its Achilles tendon. Another occasionally practised method is to hang half of a carcass (cattle) on the pelvic bone first for several hours for the above-mentioned reasons and then to hang it on its Achilles tendon during further cooling and maturing. Another means of tenderizing meat is steaking or scoring. Fine needles introduced into the muscle meat literally 'cut' fibres into smaller units. Such smaller units of muscle fibres require less shearing or chewing forces and the meat appears tender. Yet another means of tenderizing meat is to thaw frozen meat, as ice crystals are formed during freezing of meat. Ice crystals have sharp edges and also 'cut' fibres into smaller units, which enhances tenderness slightly.

Figure 5. Tenderization of meat by the application of the tender-stretch method.
Source: http://www.harrismeats.co.nz

Enzymes used for enhancing the tenderness of meat

Enzymes have been used to tenderize meat for hundreds of years. Recently, too many commercial enzymes are available in the market for helping the housewife to cook the tender meat (Figure 6). One of the most common enzymes applied is papain and the origin of this enzyme is papaya, a fruit grown in tropical countries. Papain is usually produced as a crude dried material by

collecting the latex from the fruit of the papaya tree. This latex is further dried and purified and papain is sold in liquid and powdered form. The enzyme is totally inactivated at a temperature of 82C and the optimum pH of papain is similar to that of meat itself. The application of papain has to be tightly controlled. If papain is left to tenderize meat for a long period of time, the result will be a structureless piece of meat with no texture and bite at all. Other enzymes occasionally applied are ficin, from the milky juice of the fig tree, and bromelain. Bromelain originates from the stump or root of the pineapple plant after harvest of the fruit. The stump is peeled and crushed to extract the juice, which contains the enzyme. Bromelain is sold in powdered form. Contrary to papain, bromelain and ficin also enhance the tenderness of connective tissue, while papain focuses almost solely on the tenderness of meat. However, bromelain or ficin are hardly used. Ginger root also tenderizes meat but is not used commercially. The major disadvantage of all these 'natural' tenderizers is that, if they are applied in a slight overdose, or if the tenderization process is too lengthy, the tenderizing effect becomes very strong and the entire fibre structure of the meat is destroyed. Meat exhibiting a mushy soft texture, with no or little bite, is the result of such excessive enzyme activity.

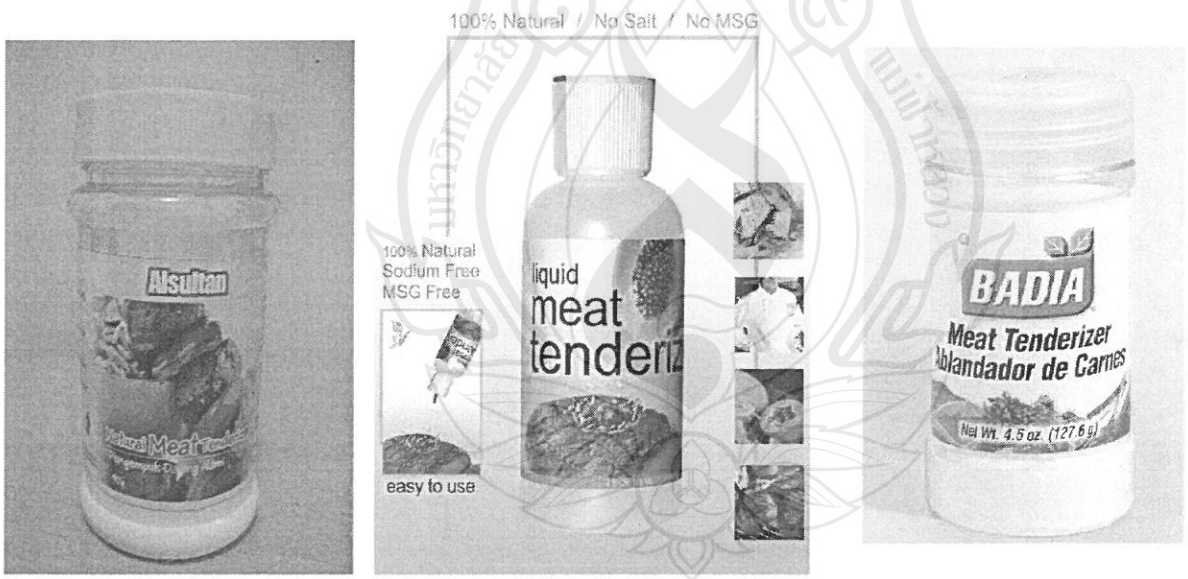

Figure 6. Commercial meat tenderizers

Source: http://www.tenderizemeat.com

Meat tenderization researches

Meat tenderness is generally considered one of the most important attributes of meat quality. Meat tenderness depends on the amount of intramuscular connective tissue, the length of the sarcomere, and also the proteolytic potential of the muscle (Kemp and Parr, 2012). On the other hand, meat toughness is one of the most undesirable meat qualities for the consumers (Kemp et al., 2010). Actomyosin and background toughness are 2 main types of classified meat toughness. The former is attributable to changes in myofibrillar proteins, whereas the latter is due to connective tissues or stromal proteins (Chen et al., 2006). Myofibrillar toughness is considered to be affected by the development of rigor-mortis and tenderization caused by enzymatic breakdown of the contractile proteins (Naveena et al., 2011). The collagen in connective tissue becomes more complex and stronger when the chronological age of the animal advances. In addition, as the collagen accounts for about 80% of the connective tissue (Gelse et al., 2003), therefore, any proteolytic enzyme that could hydrolyze collagen have been sought for meat tenderization.

A number of attempts have been made to improve meat tenderness and the overall quality of muscle foods. Normally, chemicals and physical treatments are used to do this. All of these methods focus on reduction or disrupting the myofibrillar proteins and connective tissues. Marinating muscle foods in acidic solutions such as acetic or lactic acid has been traditionally applied as a means of softening and flavoring meats (Berge et al., 2001). However, an extended period of time is needed for full marination because of the slow penetration of exogenous acids and also because of the specificity of the chemical compounds used by the target protein components, which may hinder the efficiency of this treatment (Seuss and Martin, 1993). Alternative methods for meat tenderization are still needed.

Treatment by exogenous proteases is one of the most progressive methods used for meat tenderization. Proteases derived from plants such as papain, bromelain and ficin have been widely used as meat tenderizers (Naveena et al., 2004; Sullivan and Calkins, 2010; Koak et al., 2011; Ketnawa and Rawdkuen, 2011; Ha et al., 2012). Ketnawa et al. (2010) also confirmed that bromelain from pineapple peel can extensively degrade the collagen from beef and giant catfish skin. While, protease from *C. procera* latex can degrade the muscles of beef, squid, and farmed giant catfish effectively (Rawdkuen et al., 2011). Plant proteases are superior to bacterial derived enzymes mainly because of safety problems such as pathogenicity or other disadvantageous effects (Chen et al., 2006). These enzymes can digest muscle protein when they were mixed with meat. They also can hydrolyze the proteins of collagen and elastin, which lessens the toughness of meat.

However, the proper quantity of enzymes needs to be considered because an excessive amount would result in meat decomposition.

For meat tenderness measurement, physical and chemical methods have been developed including measuring of forces (shearing, penetrating, biting, mincing, and compressing) or determination of connective tissue and its solubility, as well as enzymatic digestion. Most research on the digestion mechanism of meat proteins has been carried out using homogenized meat and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Besides, many sophisticated techniques have been widely used to assess the tenderness of meat, such as enzyme activity estimation, myofibrillar fragmentation index, hydroxyproline measurement and scanning electron microscopic studies (Maiti et al., 2008). The present study investigated the application of plant proteases from different sources to meat samples to determine improvement in tenderness via biochemical and microstructural changes.

Plant Proteases

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 (Table 1). They have been exploited commercially in the food industry such as papain for meat tenderizing and ficin and bromelain for brewing (Walsh, 2002). 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.

Table 1. Characteristics of plant cysteine proteases

Plant source	Enzyme	Optimum pH	pH stability
Carica papaya (papaya)	Papain	4-10	To pH 4
	Chymopapain	3-10	To pH <1.2
	Caricain	3-10	To pH 4
	Glycyl endopeptidease.	3-10	To pH 3
Ficus carica (Mediterranean fig)	Ficin	4-8.5	To pH 4
Ficua glabrata	Ficin	4-8.5	To pH 3.3
Ananas comosus (Pineapple)	Stem bromelain	5.5-8	NA
	Fruit bromelain	5.5-8	NA

Plant source	Enzyme	Optimum pH	pH stability
	Ananain	5.5-8	NA
	Comosain	5.5-8	NA
Actinidia chinensis (Kiwi fruit)	Actinidain	4-10	NA
Calotropis gigantean (Madar plant)	Calotropin	4-8	NA
Asclepias spp. (Milkweed)	Asclepain	6-10	NA

Source: Stepek et al. (2004)

Calotropis procera

C. procera is a plant found in tropical and sub-tropical regions (Figure 7). 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 (Ramos et al., 2007), anti-fungal (Sehgal et al., 2005) and wound healing (Rasik et al., 1999). Some biochemical properties of the enzyme containing in the latex of *C. procera* have been documented and named as procerain (Dubey and Jagannadham, 2003). It has been reported that *C. procera* latex is a potential material for enzyme purification (Kareem et al., 2003; Rawdkuen et al., 2011). 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 (Rawdkuen et al., 2011). 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.

Figure 7. *Calotropis procera*

Source: http://www.westafricanplants.senckenberg.de

METHODOLOGY

Chemicals and raw materials

Polyethylene glycol (PEG), L-Cysteine, sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (βME), Coomassie Brilliant Blue G-250, Chloramine T hydrate, 4-dimethylamino-benzaldehyde, hydroxyproline, and casien were purchased from Sigma-Aldrich Co., LLC (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, Folin phenol reagent and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Papain from papaya latex (1.5-10 units/mg solid and bromelain from pineapple stem (3-7 units/mg protein) were obtained from Sigma-Aldrich Co., LLC (St. Louis, MO, USA). Latex of *Calotropis procera* and papaya was collected from Nayong, Trang Province, Thailand. Beef, pork, and chicken muscles were purchased from Ban Du Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from Charan Farm, Chiang Rai Province, Thailand.

Part I. Effect of ATPS fraction on muscle hydrolysis

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 15,000xg at 4°C for 10 min according to the method previously reported by Rawdkuen et al (2011). The obtained supernatant was filtered through a Whatman paper No. 1 and then freeze dried. This sample was thereafter referred to as the "crude enzyme extract" and was used for further study.

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 (PEG 1000, 2000 and 3000) 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. From the protease recovery, the protease from the ATPS fraction that rendered the maximal yield (18% PEG 1000-14% $MgSO_4$) was chosen for further study.

Caseinolytic activity assay

An enzyme sample of 0.1 ml was mixed with 1.1 ml of 1% (w/v) casein in 0.1 M Tris-HCl, pH 8.0, containing 12 mM cysteine. The mixture was incubated at 37°C 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 measured at 280 nm. One caseinolytic unit is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance units per minute in an assayed condition (Rawdkuen et al., 2011).

Protein determination

The protein concentration in the sample was measured by the Bradford method (Bradford, 1976) using BSA as a protein standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of crude extract and the extracted protease from ATPS fractions was performed according to the Laemmli method (Laemmli, 1970). 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 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). 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. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

Effect of selected ATPS fraction on hydrolytic pattern of muscle proteins

Two grams of muscles (beef, giant catfish and squid) were marinated with 0, 3, 5, 10, 20, 30, and 50 units of enzymes from the top phase of the ATPS system (18% PEG 1000-14% $MgSO_4$) and then incubated at a refrigerated temperature (~4°C) for 60 min. Twenty units of the crude extract was also used as the control. Three grams of a marinated sample were added with 27 ml of 5% SDS (85°C). The mixtures were homogenized at a speed of 11,000 rpm for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was incubated at 85°C for 60 min, followed by centrifugation at 8,000xg for 5 min at room temperature using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were mixed at a 1:1 (v/v)

ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (15 μg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gel) and subjected to electrophoresis as previously described.

Part II Effect of crude enzyme extract on muscle tenderization

Marination of muscle samples with crude enzyme extract

Meat samples were purchased from Bandoo market and packed in low-density polyethylene (LDPE) bags, and then cleaned and stored in a refrigerator at 4±1°C for 24 h. The samples were then taken out from the refrigerator and cut into small 3 cm^3 sized chunks.

The prepared samples were weighed and then marinated with the powder of *Calotropis procera* latex at concentrations of 0, 0.05, 0.1, 0.2, 0.3, and 0.5 % (w/w). After mixing the samples by hand, the chunks were placed in a bowl and covered with polyethylene bags and kept at 4°C for 60 min according to the method previously reported by Naveena et al. (2004). After incubation, the meat chunks were evaluated for both their chemical and physical properties.

Physical properties determinations

Shear Force Value

Texture was analyzed by using a TA.XT2 texture analyzer (Stable Micro Systems, Surrey, UK), equipped with a Warner-Bratzler blade (square shape) in accordance with the method used in Ketnawa and Rawdkuen (2011). Seven rectangular shaped samples of raw beef, raw chicken, and raw pork were prepared. Each sample was cut perpendicular to the longitudinal orientation of the muscle fibers. The blade was pressed and applied at a constant speed of 2 $mm \cdot s^{-1}$ through the sample. The maximum shear force (Firmness: N) and total work (Toughness: N*Sec) were recorded.

Water-holding capacities (WHC)

WHC was determined according to the method used in Wardlaw et al. (1973). Minced meat (20 g) was placed in a centrifuge tube containing 30 ml of 0.6 M NaCl and was stirred with a glass rod for 1 min. The tube was then kept at 4 ± 1°C for 15 min, stirred again, and then centrifuged at 3000 g (PLC-05, Industrial Corp., Taipei, Taiwan) for 25 min. The supernatant was measured, and the WHC was expressed in percentage of initial volume.

Cooking yield

The treated samples (10 g) were steamed for 1 min and then cooled at room temperature. The cooked sample was surface-dried with a filter paper and re-weighed using an analytical balance

(Sartorius, ED224S Sartorius AG, Goettingen, Germany). The cooking yield was calculated by the difference in weight when raw and then when cooked as described in Ketnawa and Rawdkuen (2011).

Chemical properties determinations

Moisture content

The moisture content of the samples was determined according to the Association of Official Analytical Chemists (AOAC) method No. 950.46 (2000).

pH

To determine pH, 10 g of the sample were homogenized with 50 ml of chilled distilled water. The pH values were measured with a digital pH meter (Model pH 510, Eutech Instrument, Ayer Rajah Crescent, Singapore).

Protein solubility

Protein solubility was determined according to the procedures of Naveena et al. (2004). The proteins in the treated samples were extracted from 2 g of minced meat by using 40 ml of ice-cold 1.1 M potassium iodize in a 0.1 M potassium phosphate buffer (pH 7.2). The samples were homogenized and kept overnight at 4°C with frequent shaking. The samples were then centrifuged at 1,500g for 20 min and the protein concentration in the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940). The solubility was expressed as the percentage of total protein in meat samples solubilized directly in 0.5 M NaOH.

TCA-soluble peptides

The TCA-soluble peptides content of the samples was measured by the method used in Ketnawa and Rawdkuen (2011). Two grams of the samples were weighed and then homogenized with 18 ml of 5% (w/v) TCA for 1 min and kept at 4°C for 1 hr before they were centrifuged at 8000 × g for 5 min. The soluble peptides in the supernatant were measured by using the Lowry method (1951). The TCA soluble peptides content was calculated as the μmol of tyrosine/g of the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method in Laemmli et al. (1970). Samples (2 g) subjected to different treatment conditions were all mixed with 18 ml of 5% (w/v) SDS solution (85°C). The mixture was then homogenized with a homogenizer (IKA Ultra Turrax, T25D, Germany). The homogenate was incubated at 85°C in a water bath for 1 h to dissolve the protein. It was then centrifuged at 8000 × *g* for 5 min at room temperature using a centrifuge (PLC-05, Industrial Corp., Taipei, Taiwan) to remove the un-dissolved debris. The supernatants were mixed

at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and then boiled for 3min. The samples (20 µg protein) were loaded into a poly-acrylamide gel (10% running and 4% stacking gels). Then, they were subjected to an electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were then 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. The protein patterns were then made visible after de-staining the gel until a clear background was achieved.

Part III Comparative study of plant proteases on muscle tenderization

Enzyme treatment to meat samples

Meat samples were packed in low-density polyethylene bags, and store in refrigerator at 4±1°C for 24 h. After reached the chilling time, samples were taken out of refrigerator and cut into small pieces, then subjected to mix with different types and concentrations of enzymes. Stock solution of each enzyme was prepared to get the same starting enzyme activity units (standardize). The meat samples were then thoroughly mix with the enzyme solution to get the final concentrations of 2 x 10^3, 4 x 10^3 and 6 x 10^3 units/100 g sample. After mixing, chunks were placed in bowl and covered with polyethylene bags and keep at 4°C for 24 h. After reached the treatment time, the samples were evaluated for both biochemical and microstructural properties.

Biochemical properties determinations

pH

To determine pH, 10 g of the treated samples were homogenized with 50 ml of chilled distilled water. The pH values were measured with a digital pH meter (Model pH 510, Eutech Instrument, Ayer Rajah Crescent, Singapore).

Collagen solubility

Soluble collagen for the treated samples was extracted according to the method of Wattanachant et al. (2004). Two g muscle samples were homogenized with 8 mL of 25% Ringer's solution (32.8 mmol/L NaCl, 1.5 mmol/L KCl, and 0.5 mmol/L $CaCl_2$). The homogenate was heated at 77°C for 70 min and then centrifuged for 30 min at 4°C. The extraction was repeated twice, with supernatants combined. The sediment and supernatants were then hydrolyzed with 6 M HCl at 110°C for 24 h in an oil bath. The hydrolyzates were allowed to equilibrate to room

temperature, neutralized with an equal volume of 6 M NaOH, filtered through filter paper Whatman No. 1, and diluted 10 times with distilled water. The hydroxyproline concentrations of the diluted samples were determined by measuring the absorbance at 570 nm against a standard curve of hydroxyproline. The collagen content (hydroxyproline x 7.25) of the sediments and supernatants were determined separately (Bergman and Loxley, 1963), with total collagen content as the sum of the collagen content in the sediment plus that in the supernatant. The amount of heat-soluble collagen (collagen solubility) was expressed as a percentage of the total collagen.

TCA-soluble peptides

The TCA-soluble peptides content of the samples was measured by the method used in Ketnawa and Rawdkuen (2011). Two grams of the samples were weighed and then homogenized with 18 ml of 5% (w/v) TCA for 1 min and kept at 4°C for 1 hr before they were centrifuged at 8000 × g for 5 min. Soluble peptides in the supernatant were measured by using the Lowry method (1951). The TCA soluble peptides content was calculated as the μmole of tyrosine/g of the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method in Laemmli et al. (1970). Samples (2 g) subjected to different treatment conditions were mixed with 18 ml of 5% (w/v) SDS solution (85°C). The mixture was then homogenized using a homogenizer (IKA Ultra Turrax, T25D, Germany). The homogenate was incubated at 85°C in a water bath for 1 h to dissolve the protein. It was then centrifuged at 8000 × *g* for 5 min at room temperature using a centrifuge (PLC-05, Industrial Corp., Taipei, Taiwan) to remove the un-dissolved debris. The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and then boiled for 3min. The samples (20 μg protein) were loaded into a poly-acrylamide gel (10% running and 4% stacking gels). Then, they were subjected to an electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were stained over-night with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. The protein patterns were then visualized after de-staining the gel until a clear background was achieved.

Microstructure determination

Microstructures of the samples were determined by using a scanning electron microscope (LE01450VP, Cam-bridge, UK). Muscle specimens for SEM were prepared from inside of meat with a thickness of 2 - 3 mm were fixed with 2.5% (v/v) glutaraldehyde in an 0.2 M phos-phate buffer (pH 7.2) for 2 h. The samples were then rinsed for 1 h with distilled water before being

dehy-drated in ethanol with a serial concentration of 50%, 70%, 80%, 90% and 100% (v/v). They were then criti-cal-point-dried (Balzers mod CPD 030, Blazers Process Systems, Vaduz, Liechtenstein) by using CO_2 as a transi-tion fluid. The dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater, SC7620, Polaron, UK). The specimens were observed with a SEM with a magnification of 250× at an acceleration voltage of 10 kV.

Statistical analysis

The experiment was carried out in triplicate using different three lots of samples. Completely randomized design was used throughout the study. The obtained data was statistically analyzed with the SPSS program for Windows (SPSS version 11.5, SPSS Inc., Chicago, IL, USA). Duncan's multiple-range test was used to compare the difference between means. The accepted level of significance for all comparisons was $p<0.05$. Experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Part I. Effect of ATPS fraction on muscle hydrolysis

Protein and activity staining of protease from *C. procera* latex and their ATPS fractions

Among all ATPS tested, systems comprising of 18% PEG 1000 and 14% $MgSO_4$ effectively partitioned the protease from *C. procera* latex to the PEG-rich top phase. Under this condition, the highest proteolytic yield of 74.6% was recovered in the top phase, providing PF of 4.08 fold. Though the highest PF was observed in the system of 15% PEG 2000 and 14% $MgSO_4$, it provided only 45.80% yield. Therefore, the fraction of the ATPS system containing 18% PEG 1000-14% $MgSO_4$ with the highest yield was selected for biochemical properties characterization.

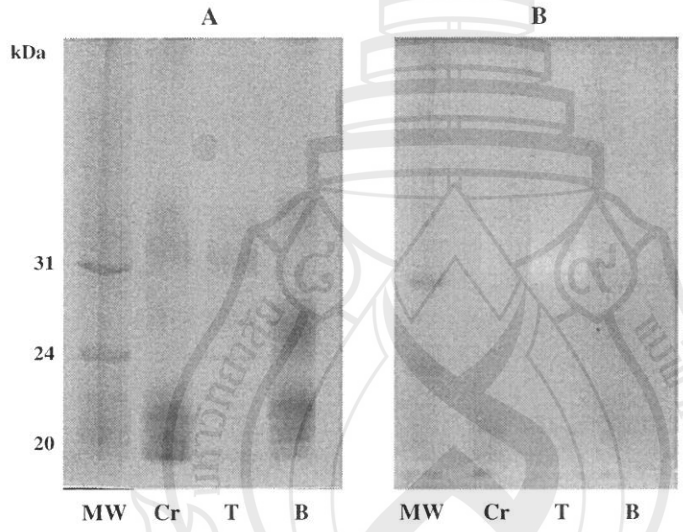

Figure 8. SDS-PAGE patterns (A) and activity staining (B) of *Calotropis procera* latex extract and its ATPS fractions of 18% PEG 1000-14% $MgSO_4$; MW: molecular weight marker; Cr: crude enzymes extract; T: top phase and B: bottom phase.

Figure 8A shows the protein patterns of the crude enzyme and extracted protease partition from 18% PEG 1000-14% $MgSO_4$. For the migration of protein composition in the crude enzyme, the top phase and bottom phase of their fractions were quite different. The three major protein bands of crude *C. procera* latex were observed with a MW of around 20 kDa, ~31 kDa and above 31 kDa. This corresponds with the investigation of Freitas et al. (2007), but the protein band at around 31 kDa of that study was the main component instead of that below 20 kDa in the present research. Using ATPS with 18% PEG 1000-14% $MgSO_4$, the effective protein separation was

obtained (as shown in the Figure 8A) by most of the proteins partitioning to the salt-rich bottom phase. Only the protein band with the MW of around 31 kDa was separated to the PEG-rich top phase. It was reported that the purified protease from *C. procera* appeared as a single band at around 29 kDa in a reducing condition of SDS-PAGE (Dubey and Jagannadham, 2003).

To verify the band of proteolytic enzymes, activity staining was performed by using substrate (casein) gel electrophoresis. The clear zone by proteolytic activity on the dark background resulted only at the protein band around 31 kDa (Figure 8B). This clear zone was more apparent in the band of the top phase of ATPS than that of the crude latex. There are previous reports that the maximum proteolytic activity of *C. procera* protein found a protein band possessing around 30 kDa (Freitas et al., 2007). Low detectable clear zones were observed in both the crude extract and in the bottom phase of their ATPS fractions. This is probably due to a rather low specific activity of protease content in both fractions. In contrast, the top phase fraction is mainly composed of activated protease, which results in detectable activity of clearer zones. For this result, the protein component of *C. procera* latex with the MW of ~31 kDa was the predominant protease.

Effect of the protease fraction on the hydrolytic pattern of muscle proteins

Proteolytic patterns of the muscle food treated with ATPS isolated *C. procera* latex protease are depicted in Figure 9. The PEG-rich phase of 18% PEG 1000-14% $MgSO_4$ system of was used as the isolated protease fraction in this study. Different protease activity units (0-50 units) were added to three kinds of muscle samples; beef, farmed giant catfish, and squid muscles. The treated samples were incubated at a refrigerated temperature (4°C) for 60 min. Increasing protease activity of isolated protease fractions on the treated muscle samples were observed, evidenced by gradually decreasing numbers of protein bands as well as the protein band intensity as compared with the control (without the fraction addition). The degradation of protein increased by increasing the enzyme concentration. Naveena et al. (2004) 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 a reduction in the level of higher MW fractions due to the degradation of myosin, thus increasing the meat tenderness (Jorgova et al., 1989).

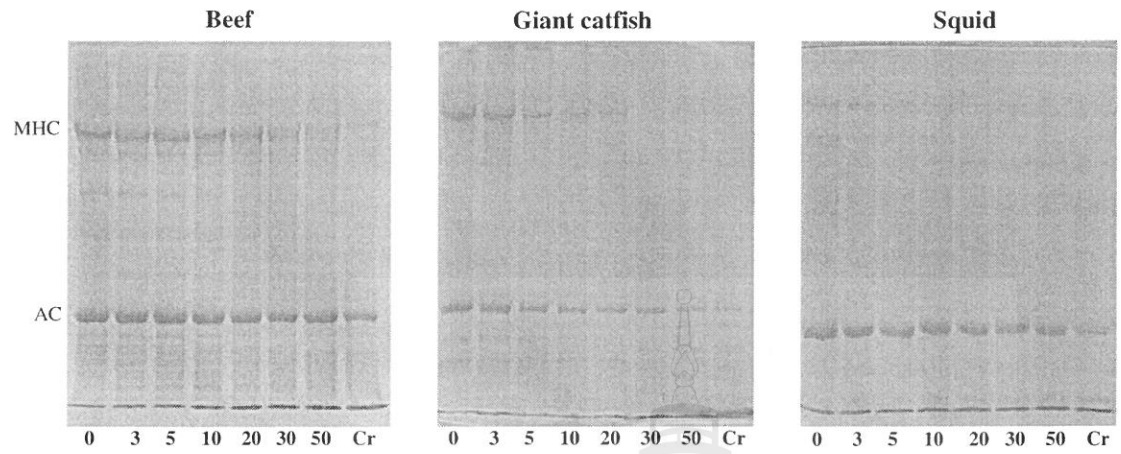

Figure 9. SDS–PAGE patterns of muscle samples incubated with enzyme from ATPS fraction (top phase of system 18% PEG 1000-14% $MgSO_4$) at refrigerated temperature. Numbers indicated enzyme activity (unit) the fraction used. MHC: myosin heavy chains; AC: actin; Cr: crude enzymes extract (20 units).

From the figure, it is also evident that protein breakdown in all of the enzyme treated samples were generated. High amounts of protein decomposition were more visible in the squid and giant catfish muscles than that of the beef sample. Not only was the myosin heavy chain (MHC) degraded by the ATPS enzyme fraction, but also by the actin (AC), especially in the giant catfish muscle. The squid and giant catfish muscles, MHC band was extensively degraded into lower MW when the concentration of the enzyme fraction was increased. Furthermore, high breakdowns of protein were markedly clearer in the giant catfish than in the beef for both MHC and AC bands. This is probably due to the co-hydrolysis from the endogenous enzymes contained in the giant catfish. The result also showed that there was a slight change in degradation of the AC band in squid and beef muscles, even with increased enzyme fraction concentrations when compared to those of the giant catfish muscle. It can be explained by the difference in protein structure and composition 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 (Melody et al., 2004). Myosin was degraded very intensely throughout the meat, and totally in the presence of high concentrations of protease fractions. AC, on the other hand, was only slightly hydrolyzed unless a high amount of enzymes were added. Nonetheless, differences between the controls and the treated meat with lower enzyme concentrations were unclear in all samples.

Part II Effect of crude enzyme extract on muscle tenderization

Effect of crude enzyme extract on meat firmness and toughness

The texture properties of the muscle samples treated with the powder of crude enzyme extract at different concentrations are presented in Figure 10. The firmness and toughness values significantly decreased in all of the treated samples when compared to the control (without crude enzyme extract) ($p<0.05$). It was observed that the shear force values continuously decreased in all of the treated samples when the level of crude enzyme extract from *C. procera* latex increased, especially when the concentration was >0.10% (w/w) (Figure 10A). The lowest firmness value was found in all of the meat samples treated with 0.5% (w/w) of the crude enzyme extract, especially in the chicken sample. The firmness for the starting pork, beef, and chicken, were 535, 650, and 302 N, respectively. The addition 0.5% (w/w) crude enzyme extract to the muscle reduced the firmness of the samples by about 59, 65, and 52% when compared to the control for pork, beef, and chicken, respectively. The reduction of meat firmness came by the action of the proteolytic enzymes on the myofibrillar proteins or the disruption of the connective tissues. Post-mortem proteolysis by endogenous proteases causes a weakening of myofibril structures and associated proteins, which results in tenderization (Kemp and Parr, 2012). Both myofibrillar proteins and collagen tissues fragment when treated with ammonium hydroxide, resulting in the tenderization of buffalo meat (Naveena et al., 2011). When the breakdown of myofibrillar protein occurred, small peptides with low molecular weight were generated, and thereby reduced the firmness of the meat samples.

The toughness of the treated meat samples are presented in Figure 10B. Beef muscle showed the highest toughness value while the chicken sample provided the lowest. Slightly decreased toughness was observed when the crude enzyme extract was added ($p>0.05$) at the level of 0.05 to 0.20% (w/w). However, at the highest addition level, the lowest toughness was observed in all of the samples. The lowest toughness value was found in chicken for both the treated and untreated samples with crude enzyme extract. According to Koak et al. (2011), using kiwi fruit content (containing proteolytic enzymes), beef texture became softer when the kiwi was added. In the beef sample, adding crude enzyme extract at the level of 0.50% (w/w), a decrease was observed of about 50%. The toughness is determined by the amount of intramuscular connective tissue, intramuscular fat, and the length of the sarcomere (Kemp and Parr, 2012). In addition, older animals normally produce the tougher meat than young ones. The tougher the meat, the more connective tissues present. Proteolytic enzymes, especially plant proteases, are widely used for meat tenderization (Sullivan and Calkins, 2010; Ketnawa and Rawdkuen, 2011; Ha et al., 2012). Naveena et al. (2004) also observed that in buffalo meat with extensive muscle fiber and connective

tissue degradation, shear force values decreased when ginger rhizome extract was added. Ha et al. (2012) reported that the actinidin protease was the most effective for hydrolyzing beef myofibril proteins, while zingibain protease is most effective for degrading connective tissue.

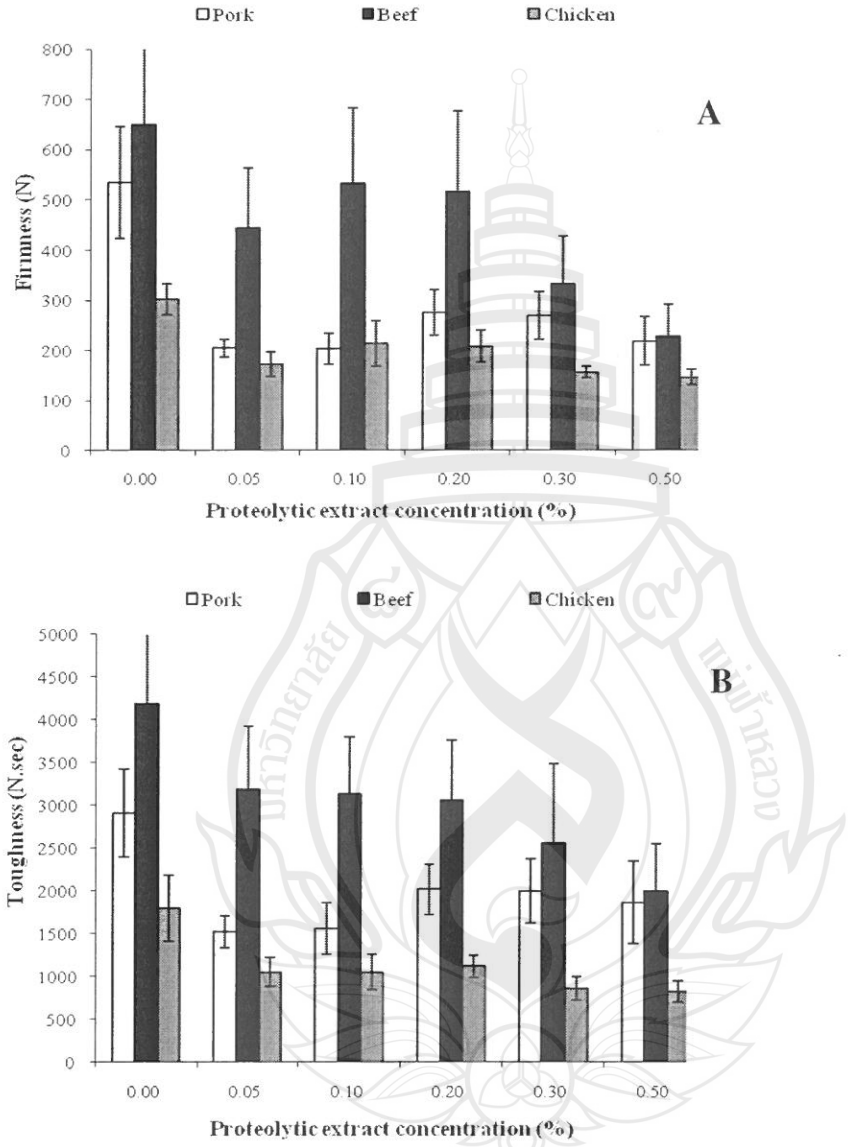

Figure 10. Firmness (A) and toughness (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Effect of crude enzyme extract on water holding capacity and cooking yield

The water holding capacity of the meat samples when treated with different concentrations of crude enzyme extract is shown in Figure 11A. A slightly decreased WHC was observed when the crude enzyme extract was added (P>0.05). Pork showed the highest WHC while the lowest value was found in the treated chicken muscle. The higher WHC in the control sample may be due to the overall reduction in the protein reactive group, which is available for water binding (Forrest et al., 1994). Slight denaturation of sarcoplasmic proteins, which is important role for determining WHC, could be the reason for decreased WHC (Joo et al.,1999). Reduced WHC is a result of myofibrillar shrinkage, as well as of the movement of water from the myofilament space to the extra-cellular space. One of the main factors involved in the shrinkage and/or swelling of the myofibrils is protein fragmentation (Huff-Lonergan and Lonergan, 1999). The WHC of meat is very important since many physical properties such as color, texture, and firmness are partially dependent on the WHC.

The cooking yield of the meat samples treated with different concentrations of crude enzyme extract is shown in Figure 11B. Decreased cooking yield was observed in all samples when the *C. procera* latex powder was added. The control beef sample showed the highest cooking yield while the chicken gave the lowest value. At the level of 0.5% (w/w) addition, pork muscles provided the highest cooking yield and chicken muscles gave the lowest. The addition of more crude enzyme extract led to decreased cooking yield for all of the meat muscles. This result indicates that thermal treatment could remove more water from treated chicken samples than that of pork muscles. It can be implied that the enzymes in crude *C. procera* latex could hydrolyze protein in chicken more than in beef and pork. Kumar and Berwal (1998) reported that spent hen meat treated with sulphate extracted Cucumis powder produced a significant reduction in cooking yield value. Degradation of sarcoplasmic and myofibrillar proteins may be caused by the reduction of cooking yield in the enzyme treated sample (Pawar et al., 2007).

3.3 Effect of crude enzyme extract on moisture content

No changes in the moisture content of the meat samples treated with 0.5% (w/w) of crude enzyme extract were observed, compared with the control (p>0.05) (Table 2). However, increased crude enzyme extract concentration could reduce some amount of moisture content, especially in pork muscle. In general, the hydroscopic nature of the powder likely contributed to the moisture reduction when it was attached to the wet surface of the sample. The low moisture content of the

treated meat samples is normally related to consumer preference. It also affects the yield of the final product. Naveena and Mendiratta (2001) reported that moisture retention in spent-hen meats treated

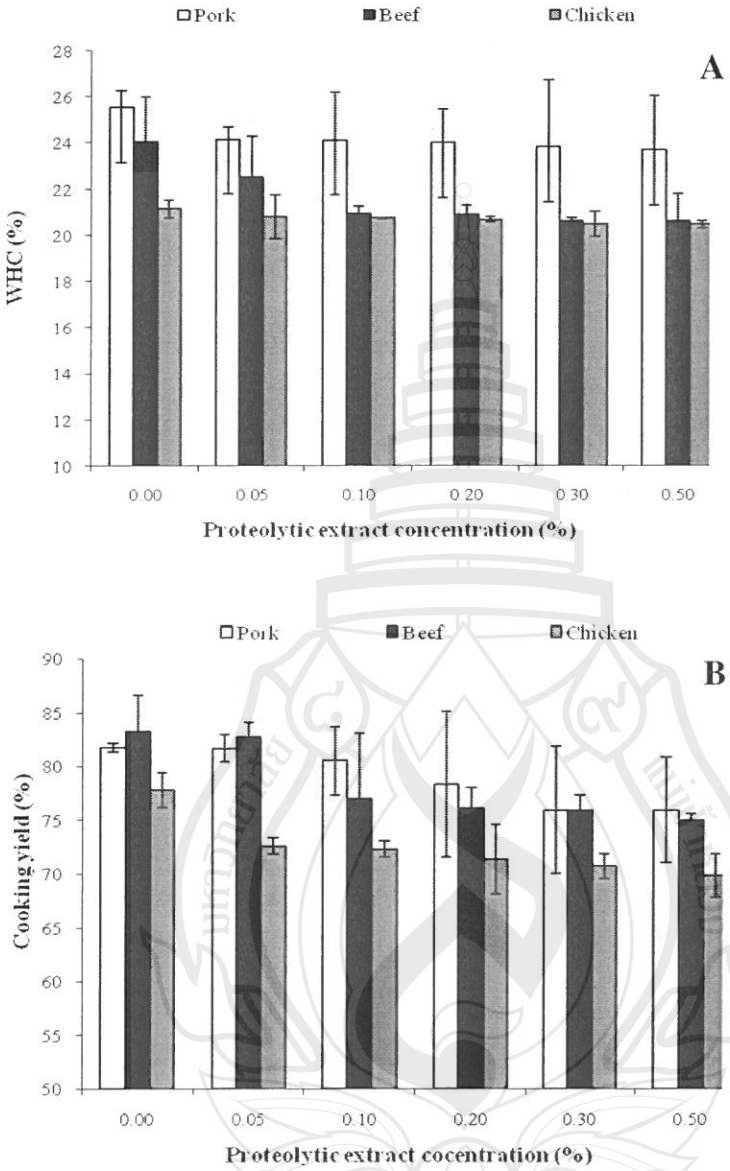

Figure 11. Water holding capacity (A) and cooking yield (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

with ginger rhizome extract as compared with the untreated sample. Similar observations were found: 77.18% moisture in ginger rhizome extract was found in a treated buffalo meat sample as compared with 76.51% in an untreated one.

Table 2. Moisture content of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Crude enzyme extract (%)	Moisture Content (%)*		
	pork	beef	chicken
control	84.51±1.78^{ab**}	80.18±4.70^{a**}	82.28±0.67^{b}
0.05	87.63±0.29^{c}	85.24±4.13^{a}	81.14±0.68^{ab}
0.10	85.08±2.97^{bc}	82.16±0.58^{a}	82.53±0.38^{b}
0.20	84.76±1.61^{abc}	81.13±1.00^{a}	80.93±1.32^{a}
0.30	81.83±1.15^{ab}	80.48±0.01^{a}	80.35±1.30^{a}
0.50	81.00±2.90^{a}	81.23±0.58^{a}	81.46±0.83^{ab}

* Values are given as mean ± SD from triplicate determinations.

**Different superscripts in the same column indicate significant differences ($p<0.05$)

Effect of crude enzyme extract on muscle pH

The pH value of the meat samples treated with different concentrations of crude enzyme extract slightly increased when the concentration of crude enzyme extract increased (Table 3). Significantly increased pH value was found in beef and chicken muscles when compared with the control ($p<0.05$). However, the pH value in the pork sample was not significantly different ($p>0.05$), except at the concentration of 0.50% (w/w) addition. The high pH of the crude enzyme extract (6.3) was probably caused by the higher pH of the treated samples, especially when a high level of crude enzyme extract was applied. Moreover, enzymatic hydrolysis of the muscle may result in releasing amino acids that can increase the pH of the system. The increases in pH of the treated sample (0.5%, w/w) ranged from 5.42-5.51, 5.59-5.67, and 5.50-5.60 when compared to the control for pork, beef, and chicken, respectively. The pH value in meat products is highly important because it has a major influence on other physico-chemical and quality properties such as WHC, tenderness, and juiciness. Changes in pH are caused by postmortem metabolism and also by the application of any added substances to the meat during technological processes.

Table 3. pH values of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Proteolytic	**pH***		
Extract (%)	**Pork**	**Beef**	**Chicken**
control	5.42±0.01^{a**}	5.59±0.01^{a}	5.50±0.01^{a}
0.05	5.41±0.02^{a}	5.61±0.01^{b}	5.52±0.01^{ab}
0.10	5.43±0.02^{a}	5.63±0.01^{c}	5.53±0.01^{bc}
0.20	5.44±0.02^{a}	5.66±0.01^{cd}	5.54±001^{cd}
0.30	5.46±0.02^{a}	5.64±0.01^{c}	5.55±0.01^{d}
0.50	5.51±0.02^{b}	5.67±0.01^{d}	5.60±0.10^{e}

* Values are given as mean ± SD from triplicate determinations.

**Different superscripts in the same column indicate significant differences ($p<0.05$)

Effect of crude enzyme extract on protein solubility and TCA-soluble peptides content

The protein solubility of meat samples treated with different concentrations of crude enzyme extract is shown in Figure 12A. The protein solubility was significantly affected by the crude enzyme extract from *C. procera* latex. Significantly higher protein solubility values were observed in all enzyme treated samples compared to the control ($p<0.05$). The control chicken meat (without added crude enzyme) showed the highest protein solubility, while the lowest value was found in the control beef sample. The regularly aligned filament of myofibrils in the control sample may have helped to prevent crude enzyme extract penetration, thus making the action seemingly resistant to extraction (Davey and Gilbert, 1968). Continuously increased protein solubility was clearly observed in the beef and pork muscles. At the level of 0.5% (w/w) crude enzyme extract addition, the solubility of protein in pork, chicken, and beef was 42, 43 and 52%, respectively. More than a 50%

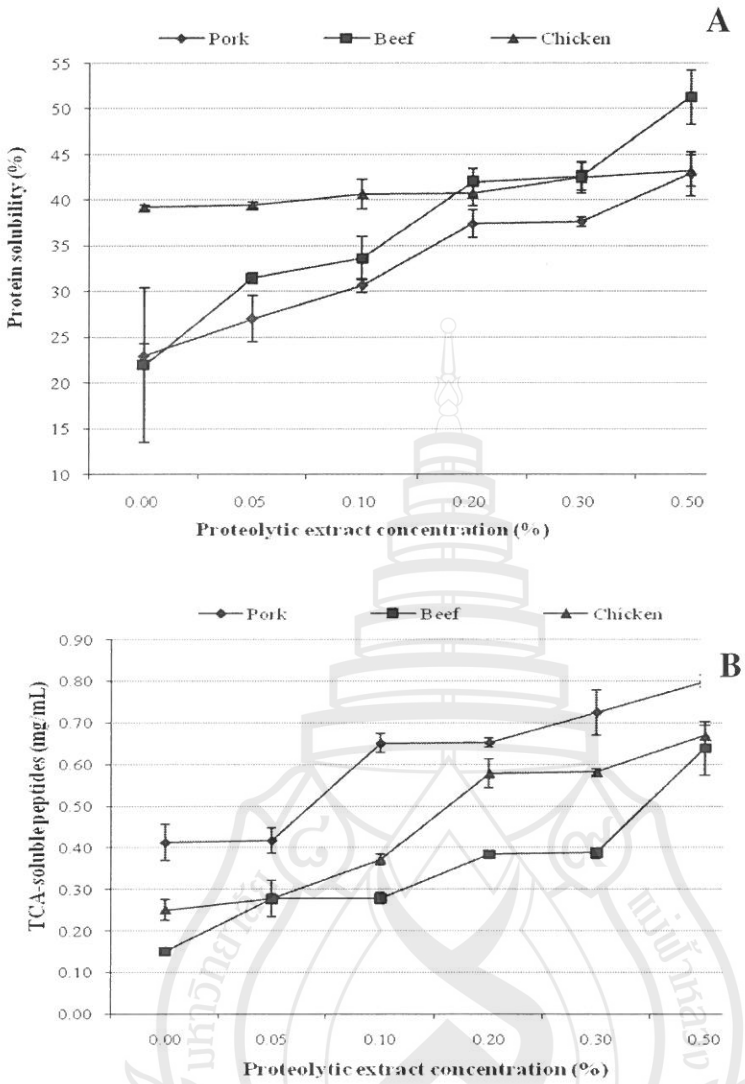

Figure 12. Protein solubility (A) and TCA-soluble peptides content (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

percent increase of protein solubility was found in the pork and beef muscle when compared with the control. These results suggest that the protein solubility changes were due to myofibrillar protein degradation. Increase in solubility of enzyme-treated samples might be due to an increase in permeability of myofibrils, which will disintegrate easily. Differences in protein solubility may be caused by the difference in structure of the meat muscle. An increase in protein solubility with ginger and papain treatment was also reported by Naveena and Mendiratta (2001) in spent hen meat.

The TCA-soluble peptides content of the muscle samples treated with different concentrations of crude enzyme extract is shown in Figure 12B. The highest content of TCA-soluble peptides was found in the pork, followed by the chicken and beef muscles treated with 0.5% (w/w) crude enzyme extract. When the concentration of crude enzyme extract was increased, the TCA-soluble peptides content in the entire treated sample increased ($p<0.05$). The TCA-soluble peptides content was the lowest in the beef muscle. More than a 50% increase of TCA-soluble peptides content was found in the sample treated with 0.5% (w/w) crude enzyme extract as compared to the control. This result suggests that the enzyme that exists in the latex had hydrolytic activity, and thereby degrading the protein. The TCA-soluble peptides content indicated that the endogenous oligopeptides and/or free amino acids, as well as degradation products, all accumulated after being marinated with the crude enzyme extract. From these results, it is found that for high TCA-soluble peptide content, a greater muscle protein hydrolysis is generated by the proteolytic enzymes present in *C. procera* latex.

Effect of crude enzyme extract electrophoretic patterns

A representative protein pattern by SDS-PAGE for the muscle sample treated with different concentrations of crude enzyme extract can be seen in Figure 13. Similar protein patterns in original beef, chicken, and pork were observed (lane 1). The myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle types. There was increased proteolysis of the muscle proteins in all treated samples as evidenced by the reduction in number and intensity of the protein bands when the crude enzyme extract was added. The breakdown of proteins in high amounts was more visible in the pork sample than in the others. When comparing the treatment (crude enzyme treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. In addition, when compared between beef and chicken muscles, the MHC band of the former was markedly degraded into lower molecular weight than that found in the latter. Hydrolysis of these proteins has been shown to disrupt muscle fiber structures with an associated decrease in shear force and an improvement in meat tenderness (Kemp et al., 2010). Jorgova et al., (1989) reported that bacterial proteolytic enzyme treatment of muscle protein reduced the level of higher molecular weight fractions due to degradation of myosin, thus increasing meat tenderness. The degradation of muscle

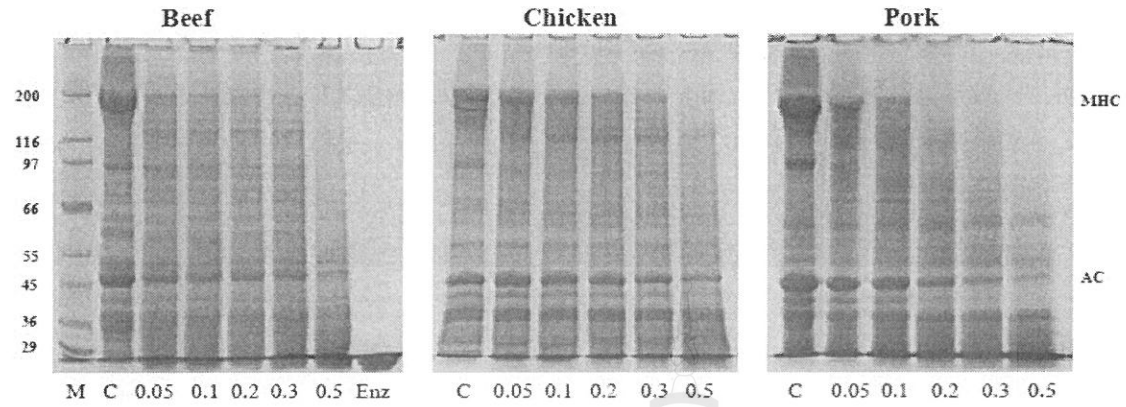

Figure 13. Effect of crude enzyme extract from *Calotropis porcera* latex on meat sample degradation. MHC: myosin heavy chain, AC: actin. M: Molecular weight marker, C: control, Enz: crude enzyme extract. Numbers represent the concentration of proteolytic extract (%, w/w).

protein plays a major role in determining the tenderness and WHC of meat during the postmortem storage (Melody et al., 2004). Naveena et al. (2004) revealed that protease from *Cucumis trigonus Roxb* and *Zingiber officinale Roscoe* improved the tenderness and the overall qualities of tough buffalo meat. Ketnawa and Rawdkuen (2011) also concluded that bromelain extract from pineapple peels could be used as an effective meat tenderizer for beef, chicken, and squid muscles. The bromelain extract applied to the meats plays a role in collagen hydrolysis into small peptides (Ketnawa et al., 2010). Moreover, degradation of the AC from the treated sample was also observed on the SDS–PAGE, especially in chicken and pork muscles. According to Wada et al. (2002), plant thiol proteases affect the structure of the MHC and the AC filaments of myofibrillar proteins. Furthermore, these enzymes have very broad specificities; and therefore, indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderization and a mushy-textured product. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during the postmortem storage. Based on these results, it is determined that crude enzyme extract from *C. procera* latex potentially has high proteolytic activity, making meat more tender.

Part III Comparative study of plant proteases on muscle tenderization

Effect of plant proteases on muscle pH

The pH value of the meat samples treated with different sources of proteolytic enzyme at the level of 6 KU/100 g sample are presented in Table 4. Without enzyme addition, beef showed the lowest in pH (5.58), followed by pork (5.99), chicken (6.19) and giant catfish (6.31). Decrease in pH was observed in the treated chicken, and giant catfish when compared with the control ($p<0.05$). However, not significantly decreased of pH was found in pork and beef for all types of enzyme applied ($p>0.05$). In beef sample, addition of *C. procera* latex proteases, papaya latex proteases or bromelain was not affected to the pH. The low pH of enzyme extracts was probably caused by the lower pH of the treated samples. Pawar et al. (2007) reported that lower pH value of treated chevon compared with the control could be due to the restructured and nature of chevon and the effect of the additives on the ionic strength and could be due to the acidity of ascorbic acid. Moreover, enzymatic hydrolysis of the muscle may result in releasing amino acids that can increase the pH of the system.

Table 4. pH of meat samples treated with different sources of proteolytic enzyme.

Sample	Chicken	Giant catfish	Pork	Beef
Control	6.19 ± 0.010d	6.31 ± 0.010c	5.99 ± 0.006c	5.58 ± 0.010a
Calotropis	5.88 ± 0.015b	6.18 ± 0.010a	5.97 ± 0.006b	5.52 ± 0.010a
Papain	5.92 ± 0.015c	6.25 ± 0.010b	5.99 ± 0.006c	5.67 ± 0.162b
Papaya	5.83 ± 0.015a	6.18 ± 0.015a	5.98 ± 0.006bc	5.57 ± 0.010ab
Bromelain	5.87 ± 0.010b	6.29 ± 0.010c	5.91 ± 0.010a	5.58 ± 0.006ab

Different a,b,c,... in the same column indicated significant differences ($p < 0.05$).
The activity unit of enzyme is 6 KU/100 g of sample.

The pH value in meat product is highly important because it has a major influence on other physico-chemical and quality properties such as water holding capacity (WHC), tenderness, and juiciness. Changes in pH are caused by postmortem metabolism and also by the application of any added substances to the meat during technological processes. Naveena et al. (2004) reported that drop in pH of meat sample may be responsible for on overall reduction in reactive groups of proteins available for water holding, resulting in the reduction of WHC. Increasing and decreasing the meat pH improves the WHC by moving the meat pH further from the isoelectric point. As the

pH moves further from the isoelectric point, the WHC increases due to an increase in the amount of negative charges on the meat protein that can bind water (Lawrie and Ledward, 2006).

Effect of plant proteases on collagen solubility

The collagen solubility of meat samples treated with different sources of plant proteases is shown in Table 5. The collagen solubility was significantly affected by both of enzyme type and type of meat sample. Significantly higher collagen solubility values were observed in all enzyme treated samples compared to the control ($p<0.05$). The lowest collagen solubility was found in the control sample for all meat types. Addition of plant proteolytic enzymes the collagen solubility was increased, especially when the activity enzyme unit increased ($p<0.05$).

Table 5. Collagen solubility of samples treated with different sources of proteolytic enzyme.

Sample	Enz. Act. (Unit)	Collagen **solubility** (%)			
		Chicken	**Giant catfish**	**Pork**	**Beef**
Control	-	37.64 ± 5.47a	52.82 ± 2.34a	14.34 ± 4.59a	26.02 ± 2.22a
Calotropis	**2kU**	69.85 ± 1.90d	80.42 ± 2.09de	59.84 ± 2.63d	63.87 ±1.50e
	4kU	70.36 ± 2.09de	82.17 ± 1.92ef	65.34 ± 1.17ef	70.79 ±1.53f
	6kU	81.30 ± 1.17f	82.09 ± 0.97ef	82.53 ± 3.55gh	81.80 ±1.42g
Papain	**2kU**	56.67 ± 1.61c	77.93 ± 1.57d	42.05 ± 4.09c	59.34 ±2.05d
	4kU	60.19 ± 3.50c	79.96 ± 1.01de	56.02 ± 4.99d	60.67 ±0.65de
	6kU	69.28 ± 2.26d	80.04 ± 0.55de	60.84 ± 2.93de	63.33 ±2.50e
Papaya	**2kU**	71.42 ± 3.29de	81.81 ± 0.48ef	69.49 ± 2.16f	83.91 ±2.07gh
	4kU	75.36 ± 1.32e	82.17 ± 1.47ef	81.26 ± 0.10g	85.92 ±1.79h
	6kU	83.59 ± 3.48f	84.14 ± 1.12f	86.78 ± 1.59h	86.18 ±1.97h
Bromelain	**2kU**	44.09 ± 3.29b	60.74 ± 3.87b	28.01 ± 1.52b	26.17 ±4.53a
	4kU	59.40 ± 3.61c	72.65 ± 2.20c	38.03 ± 1.91c	37.20 ±2.29b
	6kU	61.09 ± 1.01c	78.43 ± 0.19d	42.29 ± 3.55c	47.32 ±1.01c

Different a,b,c,... in the same column indicated significant differences ($p < 0.05$).

The control pork meat showed the lowest collagen solubility (14.34%), while the highest value was found in the control giant catfish sample (52.82%). Markedly increased of collagen solubility was clearly observed in all muscle types when papaya latex proteases was added. While

bromelain showed low efficiency for providing collagen solubility of all muscle types. However, when compared between papain and papaya latex proteases the former was less effect to collagen solubility than the latter. This result may caused by the content of proteolytic enzymes exist in the latex of papaya. Sullivan and Calkins (2010) also reported that papain-treated samples had significantly greater percent collagen solubility than the other treatments (bromelain, ficin, and *B. subtilis* proteases). Takagi et al. (1992) also reported significantly higher collagen solubility in beef meat treated with papain compared to water-treated control and alkaline elastase-treated sample. More than 50% increasing of collagen solubility were found in all muscle types compared with the control when about 4KU of papin, proteases from *C. procera* and papaya latex was applied. These results suggested that the collagen solubility changes were due to the degradation of these stromal proteins.

Increase in collagen solubility of enzyme treated samples might be due to increase in permeability of connective tissue, which will disintegrate easily. In addition, proteases may also promote structural alterations through action on intermolecular cross-links and solubilization of collagen. Solubilization of collagen to gelatin is an indicator of tender meat. The role of collagen is of particular interest, as it has been proposed that collagen is actually the determining factor in the textural differences among various muscles. The solubility of connective tissue rather than total amount of connective tissue is more highly associated with sensory characteristics (Naveena et al., 2011). Naveena et al. (2004) observed that in buffalo meat with extensive muscle fiber and connective tissue degradation, shear force values decreased when ginger rhizome extract was added. Ha et al. (2012) reported that the zingibain protease most effective for the connective tissue degradation, while the actinidin protease was the most effective at hydrolyzing beef myofibril proteins. However, they also reported that bromelain showed more capacity with collagen hydrolysis compared with papain. Bromelain increased tenderness and degraded collagen more than the contractile proteins, while ficin gave the most balanced degradation of both myofibrillar and collagen proteins (Sullivan and Calkins, 2010).

Table 6. TCA-soluble peptide of samples treated with different concentrations of plant proteases.

Sample	Enz. Act. (Unit)	TCA-soluble peptide content (μmole Tyr/g sample)			
		Chicken	**Giant catfish**	**Pork**	**Beef**
Control	-	4.13 ± 0.07a	0.90 ± 0.07a	2.90 ± 0.03a	2.09 ± 0.04a
Calotropis	**2kU**	11.64 ± 0.22g	7.83 ± 0.15h	9.35 ± 0.09h	12.50 ± 0.15h
	4kU	13.64 ± 0.07h	9.62 ± 0.14i	11.90 ± 0.13i	15.31 ± 0.22i
	6kU	14.25 ± 0.25i	12.04 ± 0.03k	13.66 ± 0.12j	17.44 ± 0.16k
Papain	**2kU**	6.51 ± 0.04e	2.33 ± 0.07c	5.32 ± 0.11e	5.53 ± 0.09e
	4kU	8.01 ± 0.05f	4.26 ± 0.09f	6.66 ± 0.02f	6.78 ± 0.08f
	6kU	8.08 ± 0.06f	5.64 ± 0.14g	7.87 ± 0.59g	8.43 ± 0.11g
Papaya	**2kU**	13.49 ± 0.10h	9.89 ± 0.08j	12.18 ± 0.15i	16.75 ± 0.12j
	4kU	15.62 ± 0.08j	13.03 ± 0.23l	14.02 ± 0.18k	18.53 ± 0.24l
	6kU	16.96 ± 0.21k	15.25 ± 0.22m	15.15 ± 0.15i	18.32 ± 0.09l
Bromelain	**2kU**	4.94 ± 0.06b	1.71 ± 0.06b	3.41 ± 0.04b	3.65 ± 0.03b
	4kU	5.80 ± 0.06c	2.56 ± 0.07d	4.05 ± 0.03c	4.64 ± 0.06c
	6kU	6.30 ± 0.02d	3.48 ± 0.12e	4.76 ± 0.14d	5.00 ± 0.04d

Different a,b,c,... in the same column indicate significant differences ($p < 0.05$)

Effect of plant proteases on TCA-soluble peptides content

The TCA-soluble peptides content of muscle samples treated with different sources of plant proteases is shown in Table 6. The lowest content of TCA-soluble peptides was found in the control sample of giant catfish (0.90), followed by beef (2.09), pork (2.90), and chicken (4.13), respectively. When the enzyme activity unit addition increased, the TCA-soluble peptides content in the entire treated sample increased ($p<0.05$), In addition, increasing of activity unit resulting in high amount of TCA-soluble peptides content was obtained. Increase in TCA-soluble peptides content of treated samples might be due to increase in permeability of myofibrillar proteins, resulting in easy to disintegrate and the peptide was released. Ketnawa and Rawdkuen (2011) showed that high TCA-soluble peptides content of bromelain treated samples indicated a greater muscle protein hydrolysis. The bromelain extract applied to the meats play a role in collagen hydrolysis into small peptides (Ketnawa et al., 2010). According to the results, beef showed the highest content of TCA-soluble peptides when added with papaya latex proteases, while the lowest of treated meat sample was found in bromelain addition for all muscle samples. Papaya latex

proteases showed the most effective to produced peptides from all meat samples, followed by *C.procera* latex proteases, papain, and bromelain. More than 3-times increase of TCA-soluble peptides content was found in the sample treated with 2KU/100 g sample of plant proteases, especially with papaya and *C. procera* latex proteases. This result suggested that the proteolytic enzymes exist in the latex had hydrolytic activity towards degradation of protein. The tyrosine level indicated that the oligopeptides and/or free amino acids, as well as degradation products, all accumulated after being mixed with proteolytic enzymes. From these results, high TCA-soluble peptide content indicated a greater muscle protein hydrolysis generated by proteolytic enzymes.

Effect of plant proteases on protein patterns

A representative protein patterns by SDS-PAGE for the meat sample mixed with different sources and concentrations of plant proteases are presented in Figure 14. The myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle types. There was increased proteolysis of the muscle proteins in the entire treated samples as evidenced by the reduction in the number and intensity of the protein bands when each enzyme was added. At higher enzyme activity units, these proteins were totally degraded and disappeared, especially at 4 to 6 KU/100 g sample. The breakdown of proteins in high amount was more visible in the chicken sample than others. When comparing the treatments (enzyme treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. Jorgova et al. (1989) reported that bacterial proteases treatment of muscle protein showed reduction in the level of higher molecular weight fractions due to degradation of myosin. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during postmortem storage (Melody et al., 2004). Moreover, degradation of the AC (band disappearing) from the treated sample was also observed on the SDS-PAGE, especially in chicken, pork, and giant catfish muscles added with enzymes activity >4KU/100g sample. Papaya latex proteases showed the highest hydrolysis activity against all muscle types, followed by proteases from *C. procera* latex, papain, and bromelain. Ha et al. (2012) reported that papain, bromelain and zingibian preparations appeared to have a similar hydrolysis profile to both beef connective tissue and topside myofibril extracts. According to Wada et al. (2002), plant thiol proteases affect the structure of MHC and the AC filaments of myofibrillar proteins. Furthermore, these enzymes have very broad specificities, and therefore; indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderization and a mushy-textured product. Zhao et al. (2012) reported that almost all the myofibrillar proteins (including MHC and AC) were degraded into fragments with

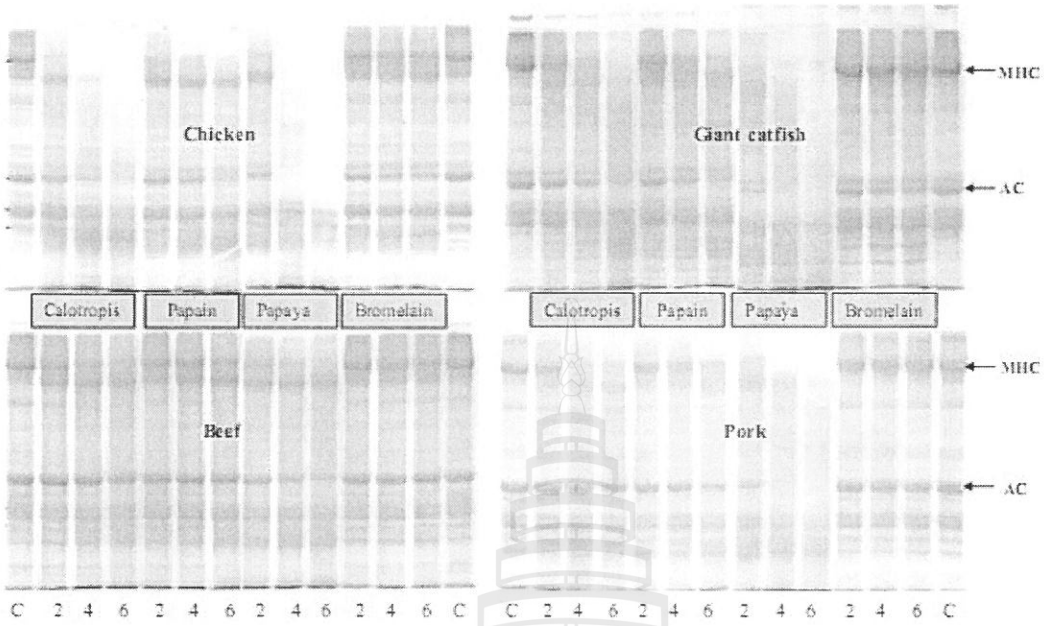

Figure 14. Protein patterns of meat samples treated with different sources of proteolytic enzymes. Numbers represent the enzyme activity unit (KU), C: control, MHC: myosin heavy chain, AC: actin.

molecular weight lower than 20 kDa when beef meat were treated with bromelain or papain at 37°C for 1h. Moreover, they also said that papain and bromelain are all thiol proteases, and their strong activity towards all the myofibrillar proteins may result in extensive degradation of myofibrillar proteins and meat structure. Fragmentation of both myofibrillar proteins and collagen tissue when treated with ammonium hydroxide resulted in tenderization of buffalo meat (Naveena et al., 2011). When the breakdown of myofibrillar protein occurred, small peptides with low molecular weight were generated and resulted in reducing the firmness of the meat samples. However, the ultimate tenderness of meat is dependent on the degree of alteration and weakening of myofibriallar structures (Kemp et al., 2010).

Effect of plant proteases on muscle microstructure

Scanning electron micrographs of the meat samples treated with different plant proteases are shown in Figure 15. Based on the above results, the level of enzyme activity at 4 KU/ 100 g sample and the control (without enzyme) was considered for microstructural studies. The control samples of all the muscle fibers had well organized structure and were closely bound to each other. High compact structure was found in giant catfish when compared to others. Transverse sections of

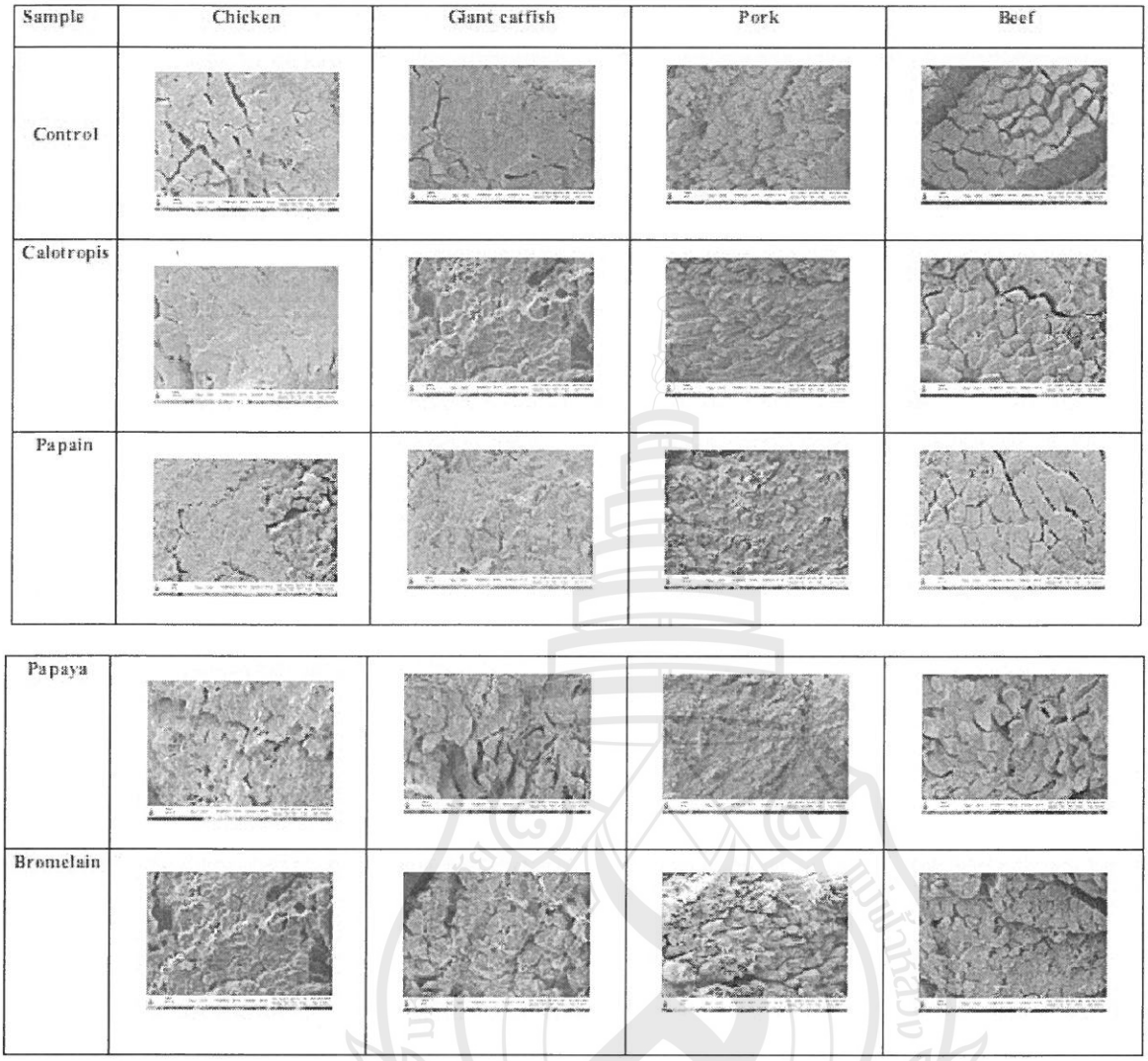

Figure 15. Microstructure of meat treated with different sources of proteolytic enzymes at the level of 4 KU/100 g sample.

SEM photographs clearly indicate a loss of muscle fibers interaction and reduction in diameter of muscle fibers in enzyme treated meats compared to control. Extensive breakdown of endomysial connective tissue layers surrounding muscle fibers without gap between fibers is well evident in papaya and *C. procera* latex proteases treated sample indicating action of proteolytic enzymes on connective tissue and/or myofibrillar proteins. It showed that strong muscle fibers were broken and the cell membranes were severely degraded. In addition, transverse section of beef treated SEM photograph also showed the wide gap between fiber indicating complete breakdown and solubilization of collagen layers (endothelium) surrounding individual muscle fibers. Similar findings were observed in the previously study of Naveena and Mendiratta (2004) on buffalo meat

tenderization using ginger extract. Disruption of the intramuscular connective tissue structure is another cause for meat tenderization. The microstructure of the enzyme treated samples showed a strong correlation with the previous results in term of collagen solubility, TCA-soluble peptides content, and SDS-PAGE patterns. Naveena (2004) studied the effect of ginger extract on the microstructure of buffalo muscle by using SEM, and found that the ginger extract treatment broke muscle fibers into different bundles and also increased the space between the bundles. In addition, there was marked deformation and disruption of honey-like structure observed in the beef treated with elastase from the *Bacillus* strain (Chen et al., 2006).

CONCLUSION AND SUGGESTION

The results obtained in this experiment clearly indicate that the tenderness and other physicochemical properties of the meat samples were improved by using crude enzyme extract and ATPS fraction (18% PEG 1000-14% $MgSO_4$) from *Calotropis procera* latex. By adding more crude enzyme extract, the quality characteristics of the treated meat samples were improved.

Papaya latex proteases provided the greatest ability to improve tenderness in all tested samples. Bromelain used in this experiment gave the lowest capacity when compared with others. Increasing in enzyme activity units resulted in markedly changes in all quality characteristics of the treated samples. Technology for applying this enzyme is easily and cheaply available and can be exploited at the household or industrial level for tenderizing tough meat, and it can be used as a better alternative to chemical tenderizers or other plant proteases.

Caution must be taken when high level of proteolytic enzyme was applied cause of negative results will be obtained. For making sure of using the enzyme extract as a food ingredient, cytotoxicity needs to be confirmed.

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ADDRESS
Mae Fah Luang University
333 Moo 1, Thasud,
Muang, Chiang Rai
57100 Thailand

TELEPHONE
(+66) 053-91-6752
(+66) 081-599-5960

FACSIMILE
(+66) 053-91-6739

E-MAIL
saroat@mfu.ac.th

DATE OF BIRTH
March 8, 1975

MARITAL STATUS
Single

NATIONALITIY
THAI-3920100570514

POSITION
Lecturer
Assistant Professor

INTERESTs
- Biochemistry of Food Protein & Enzymes
- Technology of Meat, Poultry & Fish Products
- Agricultural by-Products Utilization

CURRICULUM VITAE SAROAT RAWDKUEN

EDUCATION

2001-2005 Prince of Songkla University THAILAND
Doctor of Philosophy in Food Technology (emphasis in Food Chemistry and Biochemistry)
GPA. 3.95

1995-1999 Prince of Songkla University THAILAND
Bachelor of Science in Agro-Industry (First Class Hons.) (emphasis in Food Science and Technology)
GPA. 3.51

PROFESSIONAL EXPERIENCE

Oct 2008- **Assistant Professor**
School of Agro-Industry, Mae Fah Luang University, Chiang Rai. Thailand

Mar 2006- Oct 2008 **Lecturer**
School of Agro-Industry, Mae Fah Luang University, Chiang Rai. Thailand

Aug 2004- Dec 2004 **Research Supervisor**
Thai Foods and Drinks Development Project (OTOP & SMEs for Southern Part) Faculty of Agro-Industry, Prince of Songkla University, Songkhla, Thailand

Jul 2000- Mar 2001 **Assistant Quality Control Manager**
Pan Asia (1981) Co., Ltd. Suratthani, Thailand

Apr 1999- Jun 2000 **Production Supervisor**
Pan Asia (1981) Co., Ltd. Suratthani, Thailand

Mar- Apr 1997 **Trainee**
South East Asian Packaging and Canning Co., Ltd. Pattani, Thailand

SUPPORTING EXPERIENCE/TRAINING

Apr 2013 **Application of Hydrocolloids in Foods**
National Food Institute, IMPACT, Bangkok, Thailand

May 2012 **Techniques in the Analysis of Free Radicals, Antioxidants, and Oxidative Stress Markers**
Society for free radical research Thai and School of medical science, University of Phayao, Phayao, Thailand

Apr 2011 **Meat Products Processing Workshop**
Research and Development of Meat Products, Department of Livestock, Chiang Mai, Thailand

Oct 2010	**Improved Utilization of Fishery by Products as Potential Nutraceuticals and Functional Foods** Faculty of Fisheries, Kasetsart University, Bangkok, Thailand
Aug 2009	**Thai Qualifications Framework for Higher Education (TQF: HEd)** Office of the Higher Education Commission, Ayudhya, Thailand
Apr 2009	**Research Project Management** National Research Council of Thailand, Bangkok, Thailand

TEACHING EXPERIENCE

Undergraduate (BSc.)	Food Chemistry Food Analysis Process Operation Food Processing Dairy Products Technology Technology of Meat, Poultry and Egg Products Food Quality Assurance Quality Characteristics of Agricultural Materials Quality System and Management in Agro-Industry Research Project in Food Technology
Graduate (MSc. & Ph.D)	Advanced Food Chemistry Advanced Food Processing Advanced Food Enzyme Technology Functional Properties of Food Selected Topics in Food Chemistry Quality Control Design in Agro-Industry Research Methodology for Agro-Industry

AD HOC REVIEWER FOR

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SpringerLink	Food and Bioprocess Technology International Aquatic Research
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Taylor & Francis Online	Journal of Aquatic Food Product Technology Separation Science and Technology

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Others-Open Access	International Journal of Fisheries and Aquaculture International Journal of Nutrition and Metabolism ISABB Journal of Food and Agricultural Sciences Journal of Agricultural Science and Technology Journal of De La Soiete Chimique De Tunisie Journal of Food Science and Engineering Journal of Scientific Research and Reports Scientific Research and Essays The Natural Product Journal

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APPENDIX

Publication

1. Rawdkuen, S., Pintathong, P., Chaiwut, P., and Benjakul, S. 2011. The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins. Food Bioprod. Process. 89: 73-80.
2. Rawdkuen, S., and Benjakul, S. 2012. Biochemical and microstructural characteristics of meat samples treated with different plant proteases. Afr. J. Biotechnol. 11(76): 14088-14095.
3. Rawdkuen, S., Jaimakreu, M., and Benjakul, S. 2013. Physicochemical properties and tenderness of meat samples using proteolytic extract from *Calotropis procera* latex. Food Chem. 136: 909-916.

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

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

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

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,

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

The protease activity recovery (yield) was defined as:

$$\text{yield}\,(\%) = \frac{A_T}{A_I} \times 100 \quad (6)$$

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

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

2.4. Caseinolytic activity assay

An enzyme sample of 0.1 ml was mixed with 1.1 ml of 1% (w/v) casein in 0.1 M Tris–HCl, pH 8.0 containing 12 mM of cysteine. The mixture was incubated at 37 °C for 20 min. After that, the reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at 3,000 × *g* for 15 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition (Vallés et al., 2007).

2.5. Protein determination

The protein concentration in the sample was measured by the Bradford method (Bradford, 1976) using BSA as a protein standard.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1. Protein staining

SDS-PAGE of crude extract and the extracted protease from ATPS fractions was performed according to the Laemmli method (Laemmli, 1970). 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 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). 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. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

2.6.2. Protease activity staining

The protease separated on the gel was verified by using activity staining as the method described in Garcia-Carreno et al. (1993). 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 45 min, and agitated constantly at 4 °C. The gel was then incubated at 37 °C for 30 min with constant agitation to generate the reaction. The treated gel was then stained and destained as described above. The appearance of a clear zone on the dark background indicated the *C. procera* latex protease activity.

2.6.3. Glycoprotein staining

Glycoprotein staining was conducted by using a GelCode® Glycoprotein Staining Kit (PIERCE Biotechnology, IL, USA). The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min, followed by washing the gel by gently agitating it with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with 3% (v/v) acetic acid for 10 min before treating it with 15 ml of GelCode® Glycoprotein Stain for 15 min. The gel was incubated with 25 ml of reducing solution for 10 min and then washed by 3% (v/v) acetic acid. The magenta band could be observed in the presence of glycoprotein.

2.7. Effect of selected ATPS fraction on hydrolytic pattern of muscle proteins

Two grams of muscles (beef, giant catfish and squid) were marinated with 0, 3, 5, 10, 20, 30, and 50 units of isolated protease from the top phase of the ATPS (18% PEG 1000–14% $MgSO_4$) and then incubated at a refrigerated temperature (~4 C) for 60 min. Twenty units of the crude extract were also used as the control. Three grams of a marinated sample were added with 27 ml of 5% SDS (85 °C). The mixtures were homogenized at a speed of 11,000 rpm for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was incubated at 85 C for 60 min, followed by centrifugation at 8,000 × *g* for 5 min at room temperature using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (15 μg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gel) and subjected to electrophoresis as previously described.

2.8. Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's multiple range test (DMRT). Analysis was performed using a SPSS package (SPSS 10.0 for windows, SPSS, Inc. Chicago, IL).

3. Results and discussion

3.1. Effect of salts on partitioning of protease from C. procera latex

The partitioning was carried out in several biphasis compositions of 18% PEG 1000 with different salts ($(NH_4)_2SO_4$, $MgSO_4$ and K_2HPO_4) and their concentrations (14, 17 and 20%, w/w). Salts are frequently used in ATPS to improve partitioning target molecules between the phases. In general, no phase separation was found in either PEG 1000 or salts on their own. This indicates that the combination of both PEG and salt was necessary for the partitioning process. Phase separation will occur if the concentrations of polymers or salts are above a certain critical value of the binodal curve. Raghavarao et al. (1998) reported that two phases are formed when the polymer concentration is in the range of 8–16% (w/w), and salt concentration must be as high as 10% (w/w). In addition, the lower the molecular weight, the higher the polymer concentration is required for phase formation. From the result, it was shown that the two-phase separations of PEG-rich top phase and salt-rich bottom phase were obtained in all systems comprising of 18% PEG and 14–20% salts. 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 (Huddleston et al., 1991).

Table 1 – Effect of phase composition in PEG 1000-salts ATPS on partitioning of protease from *Calotropis procera* latex.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield (%)
18%PEG 1000–14% $(NH_4)_2SO_4$	2.96 ± 0.16f	0.13 ± 0.00a	0.57 ± 0.02e	293 ± 7.80e	5.82 ± 0.16e	65.7 ± 2.71g
18%PEG 1000–17% $(NH_4)_2SO_4$	1.94 ± 0.11cd	0.18 ± 0.01a	0.52 ± 0.02d	266 ± 7.71d	5.29 ± 0.15d	62.2 ± 1.51f
18%PEG 1000–20% $(NH_4)_2SO_4$	0.93 ± 0.03a	0.16 ± 0.01a	0.34 ± 0.01c	308 ± 1.73e	6.12 ± 0.03e	11.9 ± 0.69c
18%PEG 1000–14% K_2HPO_4	2.31 ± 0.06de	0.33 ± 0.01ab	1.06 ± 0.02f	163 ± 0.60b	3.23 ± 0.01b	68.0 ± 3.39g
18%PEG 1000–17% K_2HPO_4	1.62 ± 0.02bc	0.60 ± 0.03b	1.49 ± 0.01g	265 ± 6.46d	5.27 ± 0.13d	56.6 ± 1.90e
18%PEG 1000–20% K_2HPO_4	1.22 ± 0.15ab	4.00 ± 0.57c	2.59 ± 0.02h	144 ± 0.57b	2.87 ± 0.01b	15.7 ± 1.28d
18%PEG 1000–14% $MgSO_4$	7.41 ± 0.76g	0.16 ± 0.01a	0.53 ± 0.01d	205 ± 9.92c	4.08 ± 0.20c	74.6 ± 1.00h
18%PEG 1000–17% $MgSO_4$	2.48 ± 0.25ef	0.03 ± 0.01a	0.05 ± 0.01b	154 ± 32.6b	3.01 ± 0.65b	6.93 ± 1.63b
18%PEG 1000–20% $MgSO_4$	1.77 ± 0.21c	0.03 ± 0.00a	0.03 ± 0.00a	79.8 ± 1.97a	1.58 ± 0.04a	2.61 ± 0.08a

V_R: volume ratio (top/bottom); K_P: partition coefficient of protein in the top phase; K_E: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery. Different letters in the same column indicate significant differences ($p < 0.05$).

The basis for separation by ATPS is the selective distribution of biomolecules between the phases, quantified by the partition coefficient (Raghavarao et al., 1998). The protease was partitioned prominently in the polymer phase, principally those with hydrophobic characteristics the enzyme. Dubey and Jagannadham (2003) reported that most amino acids in procerain, a cysteine protease in *C. procera* latex, were hydrophobic amino acids, with especially high amounts of tyrosine and tryptophan. Glycine and valine were predominant non-polar amino acids present in *C. gigantean*. In PEG-salt systems, proteolytic enzyme partitioning depends on a "volume exclusion effect" of the polymer and a "salting-out effect" of the salts. The effect of salts and their concentrations on the partitioning (V_R, K_P, SA, PF and yield) are shown in Table 1. The V_R of the assayed systems generally decreased when salt concentration increased. The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecules. This was due to their water structure breaking effects (Farruggia et al., 2004). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecules. This led to the decrease in volume of the PEG-rich top phase.

The distribution of the protein and proteases in ATPS are reported by K_P and K_E, respectively. High K_P values indicate that most of the proteins from the crude extract were partitioning more to the top phase, while the high K_E was the only protease to favor the top phase. From the results, the highest K_P (4.00) and the highest K_E (2.59) were found in the system of 18% PEG 1000–20% K_2HPO_4. In contrast, the phase composition of 18% PEG 1000–20% $MgSO_4$ showed the lowest K_P (0.03), indicating that both desired proteases and contaminant proteins would shift to the lower phase.

The phase containing 18% PEG 1000 and 14% $(NH_4)_2SO_4$ gave the highest SA (293 unit mg protein^{-1}) and PF (5.82) ($p < 0.05$), whereas the maximal proteolytic yield of 74.6% was obtained from the system containing 18% PEG 1000 and 14% $MgSO_4$. Babu et al. (2008) reported that the system of 18% PEG 1500 and 20% K_2HPO_4 provided the highest activity recovery of bromelain from pineapple. It was reported that using 20% PEG 1000 and 20% $MgSO_4$ gave the highest SA and PF in the partitioning of protease from tuna spleen (Klomklao et al., 2007). The same result was also found in the extraction of protease from the stomach of an albacore tuna (Nalinanon et al., 2009). Johansson (1998) reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of the protein. The presence of salt may affect the partitioning in two ways: (1) the weakening or strengthening of the interactions, or (2) the interaction between ionized groups with the opposite net charge of proteins. The effectiveness of the salt is mainly determined by the nature of the anion. Multi-charged anions are the most effective in the order of $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^-$. The order of cations is usually given as $NH^{4+} > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$ (Roe, 2000). Huddleston et al. (1991) concluded that the effectiveness of various salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out ability).

Based on the highest proteolytic yield obtained, the 14% $MgSO_4$ was chosen for further experiment on the effect of PEG on the partitioning of protease from *C. procera* latex.

Table 2 – Effect of phase composition in PEG–14% $MgSO_4$ ATPS on partitioning of protease from *Calotropis procera* latex.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield (%)
12%PEG 1000–14% $MgSO_4$	ns	ns	ns	ns	ns	ns
15%PEG 1000–14% $MgSO_4$	ns	ns	ns	ns	ns	ns
18%PEG 1000–14% $MgSO_4$	7.41 ± 08c	0.16 ± 0.01ab	0.53 ± 0.01f	205 ± 9.92a	4.08 ± 0.2a	74.6 ± 1.00f
12%PEG 2000–14% $MgSO_4$	ns	ns	ns	ns	ns	ns
15%PEG 2000–14% $MgSO_4$	2.84 ± 0.39ab	0.01 ± 0.01a	0.18 ± 0.01d	1279 ± 213b	25.6 ± 4.2b	45.80 ± 0.16d
18%PEG 2000–14% $MgSO_4$	3.32 ± 0.44b	na	0.02 ± 0.00b	na	na	5.71 ± 0.36b
12%PEG 3000–14% $MgSO_4$	2.62 ± 0.15ab	0.26 ± 0.03b	0.32 ± 0.00e	361 ± 2a	7.17 ± 0.05a	65.2 ± 1.53e
15%PEG 3000–14% $MgSO_4$	2.24 ± 0.20a	0.03 ± 0.01a	0.05 ± 0.01c	208 ± 10a	4.12 ± 0.20a	10.5 ± 1.67c
18%PEG 3000–14% $MgSO_4$	2.65 ± 0.04ab	0.02 ± 0.01a	0.01 ± 0.00a	24.0 ± 8a	0.47 ± 0.17a	1.10 ± 0.02a

V_R: volume ratio (top/bottom); K_P: partition coefficient of protein in the top phase; K_E: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein); PF: purification factor; yield: activity recovery. ns: no phase separation; na: no protein content. Different letters in the same column indicate significant differences ($p < 0.05$).

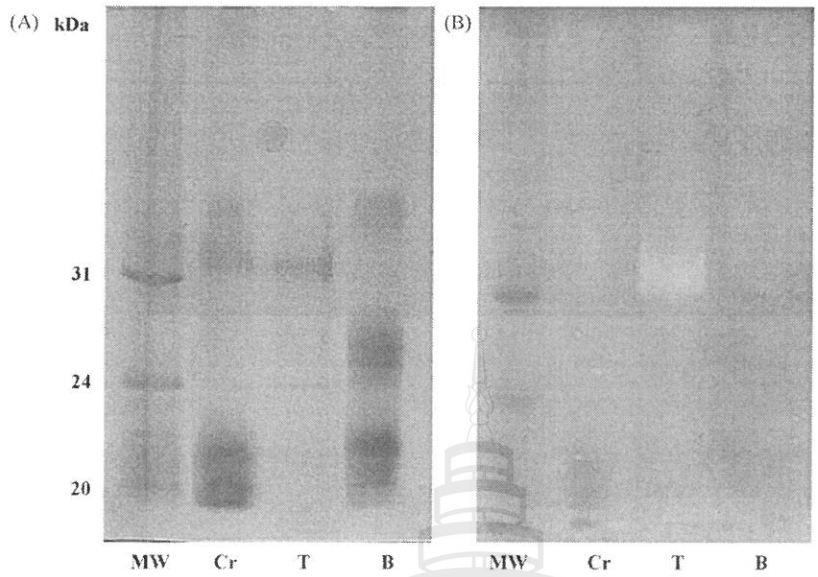

Fig. 1 – SDS-PAGE patterns (A) and activity staining (B) of *Calotropis procera* latex extract and its ATPS fractions of 18% PEG 1000–14% $MgSO_4$; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.

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

This study examined the partitioning of protease from *C. procera* latex in ATPS with different molecular weights (MW) and concentrations of PEG at 14% $MgSO_4$. As shown in Table 2, the protease partitioning was strongly dependent on the MW and the PEG concentration. No phase separation was observed in the 14% $MgSO_4$ system with 12% PEG 1000, 15% PEG 1000, and 12% PEG 2000. As mentioned before, two-phase formation requires high concentrations of low MW polymer, or requires low concentrations with high MW polymer. The V_R of the system ranged from 2.24 to 7.41. Most of the systems produced a value of V_R higher than 1.0, indicating that the volume fraction was distributed in the top phase rather than in the bottom phase. The K_P obtained from all systems was lower than 1, indicating that most of the protein components from *C. procera* latex preferably partitioned to the bottom phase of the ATPS system. A favorable interaction between PEG molecules and protein domain decreased when the MW of PEG increased because of its' exclusion from the protein domain (Reh et al., 2002; Klomklao et al., 2005; Nalinanon et al., 2009). Tubio et al. (2009) reported that the best ATPS separation of trypsin from bovine pancreas was found in the system with a molecular weight of PEG 3350 compared with 600, 1000, 1450, and 8000. However, Hotha and Banik (1997) found that for the production of alkaline protease by ATPS, the lower MW of PEG 4000 produced a higher yield than PEG 6000 and 9000. Marcos et al. (1999) also reported that a system consisting of PEG 1000 for penicillin acylase production achieved a high yield compared with that of PEG 3350 and 8000.

Among all ATPS tested, systems comprising of 18% PEG 1000 and 14% $MgSO_4$ effectively partitioned the protease from *C. procera* latex to the PEG-rich top phase. Under this condition, the highest proteolytic yield of 74.6% was recovered in the top phase, providing PF of 4.08 fold. Though the highest PF was observed in the system of 15% PEG 2000 and 14% $MgSO_4$, it provided only 45.80% yield. Therefore, the fraction of the ATPS system containing 18% PEG 1000-14% $MgSO_4$ with the highest yield was selected for biochemical properties characterization.

3.3. *Protein pattern and activity staining of protease from C. procera latex and its ATPS fractions*

Fig. 1A shows the protein patterns of the crude extract and extracted protease fractions from 18% PEG 1000–14% $MgSO_4$. For the migration of protein composition in the crude extract, the top phase and bottom phase of their fractions were quite different. The three major protein bands of crude *C. procera* latex were observed with a MW of around 20 kDa, ~31 kDa and above 31 kDa. This corresponds with the investigation of Freitas et al. (2007), but the protein band at around 31 kDa of that study was the main component instead of that below 20 kDa in the present research. Using ATPS with 18% PEG 1000–14% $MgSO_4$, the effective protease separation was obtained (as shown in the Fig. 1A) by most of the proteins partitioning to the salt-rich bottom phase. Only the protein band with the MW of around 31 kDa was separated to the PEG-rich top phase. It was reported that the purified protease from *C. procera* appeared as a single band at around 29 kDa in a reducing condition of SDS-PAGE (Dubey and Jagannadham, 2003).

To verify the band of proteolytic enzymes, activity staining was performed by using substrate (casein) gel electrophoresis. The clear zone by proteolytic activity on the dark background resulted only at the protein band around 31 kDa (Fig. 1B). This clear zone was more apparent in the band of the top phase of ATPS than that of the crude latex. There is previous report that the maximum proteolytic activity of *C. procera* protein found a protein band possessing around 30 kDa (Freitas et al., 2007). Low detectable clear zones were observed in both the crude extract and in the bottom phase of their ATPS fractions. This is probably due to a rather low specific activity of protease content in both fractions. In contrast, the top phase fraction is mainly composed of activated protease, which results in detectable activity of clearer zones. For this result, the protein component of *C. procera*

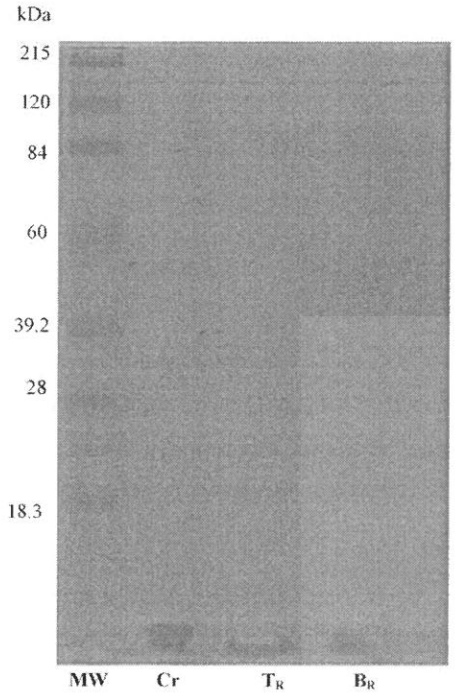

Fig. 2 – Glycoprotein staining of *C. procera* latex extract and its ATPS fractions 18% PEG 1000–14% $MgSO_4$; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.

latex with the MW of ~31 kDa was the predominant protease.

3.4. *Glycoprotein staining of protease from C. procera latex and its ATPS fractions*

The presence of carbohydrates in the protease from the *C. procera* latex was investigated. The crude extract and its 18% PEG 1000–14% $MgSO_4$ ATPS fractions were subjected to glycoprotein staining. This staining method was reported to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) (Moller and Poulsen, 2002) in which polypeptides covalently bonded to a carbohydrate moiety (Mathews and Holde, 1990). The result (as depicted in Fig. 2) revealed that all of the latex major proteins of *C. procera* latex were not contained in their carbohydrate molecules. Similar results were obtained by Dubey and Jagannadham (2003) in which protease from *C. procera* was devoid of carbohydrate moiety in procerain.

3.5. *Effect of the protease fraction on the hydrolytic pattern of muscle proteins*

Proteolytic patterns of the muscle samples treated with ATPS isolated *C. procera* latex protease are depicted in Fig. 3. The PEG-rich phase of 18% PEG 1000–14% $MgSO_4$ system was used as the isolated protease fraction in this study. Different protease activity units (0–50 units) were added to three kinds of muscle samples; beef, farmed giant catfish, and squid muscles. The treated samples were incubated at a refrigerated temperature (4 °C) for 60 min. Increasing protease activity of isolated protease fractions on the treated muscle samples were observed, evidenced by gradually decreasing numbers of protein bands as well as the protein band intensity as compared with the control (without the fraction addition). The degradation of protein increased by increasing the enzyme concentration. Naveena et al. (2004) 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 a reduction in the level of higher MW fractions due to the degradation of myosin, thus increasing the meat tenderness (Jorgova et al., 1989).

From the Fig. 3, it is also evident that protein breakdown in all of the enzyme treated samples was generated. High amounts of protein decomposition were more visible in the squid and giant catfish muscles than that of the beef sample. Not only was the myosin heavy chain (MHC) degraded by the ATPS enzyme fraction, but also by the actin (AC), especially in the giant catfish muscle. The squid and giant catfish muscles, MHC band was extensively degraded into lower MW when the concentration of the enzyme fraction was increased. Furthermore, high breakdowns of protein were markedly clearer in the giant catfish than in the beef for both MHC and AC bands. This is probably due to the co-hydrolysis from the endogenous enzymes contained in the giant catfish. The result also showed that there was a slight change in degradation of the AC band in squid and beef muscles, even with increased enzyme fraction concentrations when compared to those of the giant catfish muscle. It can be explained by the difference in protein structure and composition among these three muscles.

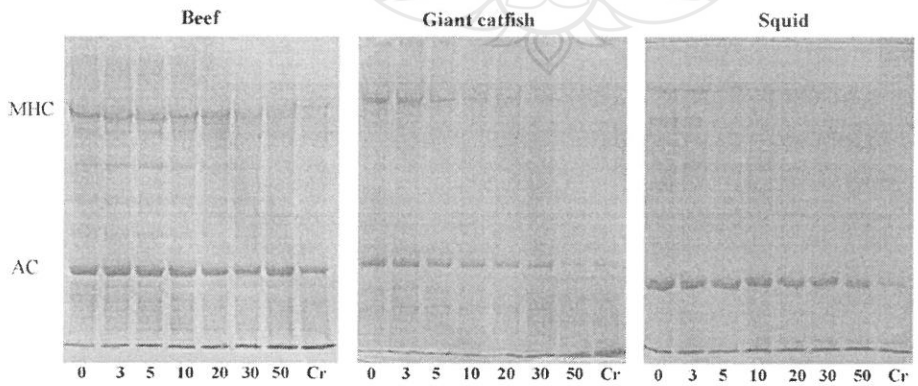

Fig. 3 – SDS-PAGE patterns of muscle samples incubated with enzyme from ATPS fraction (top phase of system 18% PEG 1000–14% $MgSO_4$) at refrigerated temperature. Numbers indicated enzyme activity (unit) the fraction used. MHC: myosin heavy chains; AC: actin; Cr: crude enzymes extract (20 units).

The degradation of muscle protein plays a major role in determining the tenderness and water holding capacity of meat during postmortem storage (Melody et al., 2004). Myosin was degraded very intensely throughout the meat, and totally in the presence of high concentrations of protease fractions. AC, on the other hand, was only slightly hydrolyzed unless a high amount of enzymes were added. Nonetheless, differences between the controls and the treated meat with lower enzyme concentrations were unclear in all samples.

In conclusion, the protease from *C. procera* latex was extracted by the aqueous two- phase system. The top phase from 18% PEG 1000–14% $MgSO_4$ provided the highest protease recovery (74.6%). By using casein substrate gel electrophoresis, the protease from the phase partitioning showed the MW of ~31 kDa with high specificity. The protease obtained had no oligosaccharide in the molecule as determined by the glycoprotein staining. Based on the protein degradation of beef, giant catfish and squid muscles, it is suggested that the protease fraction from *C. procera* latex is suitable for meat tenderization.

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Full Length Research Paper

Biochemical and microstructural characteristics of meat samples treated with different plant proteases

Saroat Rawdkuen[1]* and Soottawat Benjakul[2]

[1]Food Technology Program, School of Agro-Industry, Mae Fah Luang University, Muang, Chiang Rai 57100, Thailand.
[2]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.

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This study was conducted to compare the efficiency of different plant proteases for changing biochemical and microstructural characteristics in muscle foods. The meat samples from chicken, giant catfish, pork and beef were treated with four types of proteolytic enzymes: *Calotropis procera* latex proteases, papaya latex proteases, commercial papain and bromelain at the concentrations of 2 × 10^3 to 6 × 10^3 activity units/100 g of muscle. The pH, collagen solubility, trichloroacetic acid (TCA) soluble peptides, protein patterns and muscle microstructures of the treated samples were evaluated after 24 h at 4°C. A decrease of muscle pH in chicken, giant catfish and pork was observed when the enzymes were added ($p < 0.05$). A significant increase in collagen solubility was also found in all of the muscle samples (chicken increased from 37.64 to 83.59%; giant catfish increased from 52.82 to 84.14%; pork increased from 14.34 to 86.78; and beef increased from 26.02 to 86.18%; $p < 0.05$). An increase in TCA-soluble peptides (from 0.90 to18.53 µmole/g sample), and myofibrillar protein degradation was observed in all of the enzyme treated samples as compared to the control ($p < 0.05$). The electrophoretic pattern of the muscle proteins also revealed extensive proteolysis and reduction of protein bands in all of the treated samples. At the microstructural level, tissue fibers were broken, and the connections between the sarcolemma and the myofibrils were loosened when each enzyme was applied. When comparing all proteolytic enzymes used, papaya latex proteases showed the highest hydrolysis activity in all muscle types, which was followed by *C. procera* latex proteases, commercial papain, and then bromelain. The results show that these proteolytic enzymes could be used as an effective meat tenderizer.

Key words: Proteases, muscle foods, collagen, tenderization, toughness.

INTRODUCTION

Meat tenderness is generally considered one of the most important attributes of meat quality. The toughness of meat depends on the amount of intramuscular connective tissue, the length of sarcomere, and also the activity of endogenous proteolytic enzymes (Kemp and Parr, 2012). Actomyosin toughness is attributable to changes in myofibrillar proteins, whereas background toughness is due to the connective tissue (Chen et al., 2006). Myofibrillar toughness is affected by the development of rigor-mortis and tenderization caused by the enzymatic breakdown of the contractile proteins (Naveena et al., 2011). The collagen in connective tissue becomes more complex and stronger when the animal gets older. Additionally, as collagen accounts for about 80% of the connective tissue (Gelse et al., 2003), any proteolytic enzyme that could hydrolyze collagen has been sought out for meat tenderization. A number of attempts have been made to improve muscle food tenderness. All of the methods focus on disrupting the myofibrillar proteins and/or connective tissues.

Treatment of meat by exogenous proteases is one of

*Corresponding author. E-mail: saroat@mfu.ac.th. Tel: 66-5391-6752. Fax: 66-5391-6739.

the methods used for tenderization. Currently, five exogenous enzymes (papain, bromelain, ficin, *Aspergillus oryzae* protease and *Bacillus subtilis* protease) have been approved as 'generally recognized as safe' (GRAS) by the USDA. Three of these enzymes are derived from plants (Sullivan and Calkins, 2010). These enzymes can digest muscle proteins when they are mixed with meat. They also can hydrolyze collagen and elastin, which helps to tenderize meat. Wada et al. (2002) reported that plant thiol proteases, such as papain, bromelain, and ficin, affect the structure of myosin and actin filaments. Ketnawa et al. (2010) also confirmed that bromelain from pineapple peels can extensively degrade the collagen from beef and giant catfish skin. Protease from *Calotropis procera* latex can degrade the muscles of beef, squid and farmed giant catfish effectively (Rawdkuen et al., 2011). Plant proteases are superior to bacterial derived enzymes mainly because of safety problems such as pathogenicity or other disadvantageous effects (Chen et al., 2006). However, the proper quantity of enzymes needs to be considered because an excessive amount would result in meat decomposition.

For studies on meat tenderness, physical and chemical methods have been developed, which include measuring forces (shearing, penetrating, biting, mincing and compressing) and determining the solubility and enzymatic digestion of connective tissue. Most research on the digestion mechanism of meat proteins has been carried out by using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Besides, many sophisticated techniques have been widely used to assess the tenderness of meat, such as enzyme activity estimation, myofibrillar fragmentation index, hydroxyproline measurement, and scanning electron microscopic studies (Maiti et al., 2008). The present study investigates the application of plant proteases from different sources to determine their potential to improve tenderness through biochemical and microstructural changes.

MATERIALS AND METHODS

Chemicals and raw materials

L-Cysteine, SDS and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (βME), Coomassie Brilliant Blue G-250, Chloramine T hydrate, 4-dimethylamino-benzaldehyde, hydroxyproline, and casein were purchased from Sigma-Aldrich Company, LLC (St. Louis, MO, USA). *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane, Folin phenol reagent, and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Papain from papaya latex [1.5 to 10 units/mg solid and bromelain from pineapple stem (3 to 7 units/mg protein)] was obtained from Sigma-Aldrich Company, LLC (St. Louis, MO, USA). Latex of *C. procera* and papaya were collected from Nayong, Trang Province, Thailand. Beef, pork, and chicken muscles were purchased from Ban Du Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from Charan Farm, Chiang Rai Province, Thailand.

Enzyme preparation

Latex was collected in a clean tube by breaking the *C. procera* stems or from fresh papaya. The collected latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at 15000 × g at 4°C for 10 min. The obtained supernatant was filtered through a Whatman paper No. 1 and then freeze dried. This sample was referred to as the "*C. procera* latex proteases or papaya latex proteases" and was used for further study.

Enzyme activity determination

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% (w/v) TCA. After centrifugation at 3000 × *g* for 15 min, the absorption of the soluble peptides in the supernatant was measured at 280 nm. One unit of caseinolytic activity was defined as the amount of enzymes needed to produce an increment of 0.01 absorbance units per minute at the assayed condition (Rawdkuen et al., 2011).

Enzyme treatment of meat samples

Meat samples were packed in low-density polyethylene bags, and stored in a refrigerator at 4 ± 1°C for 24 h. After, the samples were cut into small pieces, and mixed with different types and concentrations of enzymes. Stock solutions of each enzyme were prepared to get the same starting units of enzyme activity. The meat samples were then thoroughly mixed with the enzyme solution to get the final concentrations of 2×10^3, 4×10^3 and 6×10^3 units/100 g sample. After mixing, the resulting chunks were placed in bowls and covered with polyethylene bags and stored at 4°C for 24 h. After the treatment, the samples were evaluated for both their biochemical and microstructural properties. Two replications were done for each treatment with three measurements performed for each replication.

Biochemical properties determinations

pH

To determine pH, 10 g of the treated samples were homogenized with 50 ml of chilled distilled water. The pH values were measured with a digital pH meter (Model pH 510, Eutech Instrument, Ayer Rajah Crescent, Singapore).

Collagen solubility

Soluble collagen for the treated samples was extracted according to the method of Wattanachant et al. (2004). Muscle samples (2g) were homogenized with 8 ml of 25% Ringer's solution (32.8 mM NaCl, 1.5 mM KCl, and 0.5 mM $CaCl_2$). The homogenate was heated at 77°C for 70 min and then centrifuged for 30 min at 4°C. The extraction was repeated twice with supernatants combined. The sediment and supernatants were then hydrolyzed with 6 M HCl at 110°C for 24 h in an oil bath. The hydrolyzates were allowed to

Table 1. pH of meat samples treated with different sources of proteolytic enzyme.

Sample	Chicken	Giant catfish	Pork	Beef
Control	6.19±0.010^{d}	6.31±0.010^{c}	5.99±0.006^{c}	5.58±0.010^{a}
Calotropis	5.88±0.015^{b}	6.18±0.010^{a}	5.97±0.006^{b}	5.52±0.010^{a}
Papain	5.92±0.015^{c}	6.25±0.010^{b}	5.99±0.006^{c}	5.67±0.162^{b}
Papaya	5.83±0.015^{a}	6.18±0.015^{a}	5.98±0.006^{bc}	5.57±0.010^{ab}
Bromelain	5.87±0.010^{b}	6.29±0.010^{c}	5.91±0.010^{a}	5.58±0.006^{ab}

^{a,b,c}Different letters in the same column indicated significant differences ($p < 0.05$). The activity unit of enzyme is 6 KU/100 g of sample.

equilibrate to room temperature, and they were neutralized with an equal volume of 6 M NaOH. They were then filtered through filter paper Whatman No. 1 and diluted 10 times with distilled water. The hydroxyproline concentrations of the diluted samples were determined by measuring the absorbance at 570 nm against a standard curve of hydroxyproline. The collagen content (hydroxyproline × 7.25) of the sediments and supernatants were determined separately (Bergman and Loxley, 1963), with total collagen content as the sum of the collagen content in the sediment in addition to that in the supernatant. The amount of heat-soluble collagen (collagen solubility) was expressed as a percentage of the total collagen.

TCA-soluble peptides

The TCA-soluble peptides content of the samples was measured by the method used in Ketnawa and Rawdkuen (2011). The samples (2g) were weighed and then homogenized with 18 ml of 5% (w/v) TCA for 1 min and stored at 4°C for 1 h before they were centrifuged at 8000 × g for 5 min. The soluble peptides in the supernatant were measured by using the Lowry assay (Lowry et al., 1951). The TCA soluble peptides content was calculated as the µmole of tyrosine/g of the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method in Laemmli et al. (1970). Samples (2g)subjected to different treatment conditions were mixed with 18 ml of 5% (w/v) SDS solution (85°C). The mixture was then homogenized using a homogenizer (IKA Ultra Turrax, T25D, Germany). The homogenate was incubated at 85°C in a water bath for 1 h to dissolve the protein. It was then centrifuged at 8000 × *g* for 5 min at room temperature to remove the un-dissolved debris. The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol, and 10% βME) and then boiled for 3 min. The samples (20 µg protein) were placed onto a poly-acrylamide gel (10% running and 4% stacking gels). Electrophoresis was performed at a constant current of 15 mA per gel by using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were stained over night with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. The protein patterns were then visualized after de-staining the gel until and a clear background was achieved.

Microstructure determination

The microstructure of the samples was evaluated by using a scanning electron microscope (LE01450VP, Cam-bridge, UK). The muscle specimens were cut with a thickness of 2 to 3 mm and then fixed with 2.5% (v/v) glutaraldehyde in a 0.2 M phosphate buffer (pH 7.2) for 2 h. The samples were then rinsed for 1 h with distilled water before being dehydrated in ethanol with a serial concentration of 50, 70, 80, 90, and 100% (v/v). They were then critical-point-dried (Balzers mod CPD 030, Blazers Process Systems, Vaduz, Liechtenstein) by using CO_2 as a transition fluid. The dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater, SC7620, Polaron, UK). The specimens were observed with a scanning electron microscopy (SEM) with a magnification of 250× at an acceleration voltage of 10 kV.

Statistical analyses

The obtained data was statistically analyzed with the SPSS program for Windows (SPSS version 11.5, SPSS Inc., Chicago, IL, USA). Duncan's multiple-range test was used to compare the difference between means. The accepted level of significance for all comparisons was $p < 0.05$. Experiments were conducted in two replications.

RESULTS AND DISCUSSION

Effect of plant proteases on muscle pH

The pH value of the meat samples treated with different sources of proteolytic enzymes at a level of 6 KU/100 g sample are presented in Table 1. Without enzyme addition, beef was shown to be the lowest in pH (5.58), followed by pork (5.99), chicken (6.19) and giant catfish (6.31). A decrease in pH was observed in the treated chicken and giant catfish when compared to the control ($p < 0.05$). However, no significant decrease in pH was found in pork and beef for all types of enzymes applied ($p > 0.05$). Pawar et al. (2007) reported that a lower pH value of treated chevon when compared with the control could be due to the effect of the additives (acidity of ascorbic acid) on the ionic strength of the system. Moreover, the enzymatic hydrolysis of the muscle may result in releasing acidic amino acids that can increase the pH of the system.

Effect of plant proteases on collagen solubility

The collagen solubility of meat samples treated with

Table 2. Collagen solubility of meat samples treated with different sources of proteolytic enzyme.

Sample	Enzyme account (unit) KU	Collagen solubility (%)			
		Chicken	Giant catfish	Pork	Beef
Control	-	37.64 ± 5.47^{a}	52.82 ± 2.34^{a}	14.34 ± 4.59^{a}	26.02 ± 2.22^{a}
Calotropis	2	69.85 ± 1.90^{d}	80.42 ± 2.09^{de}	59.84 ± 2.63^{d}	63.87 ±1.50^{e}
	4	70.36 ±2.09^{de}	82.17 ± 1.92^{ef}	65.34 ± 1.17^{ef}	70.79 ±1.53^{f}
	6	81.30 ±1.17^{f}	82.09 ± 0.97^{ef}	82.53 ± 3.55^{gh}	81.80 ±1.42^{g}
Papain	2	56.67 ±1.61^{c}	77.93 ± 1.57^{d}	42.05 ± 4.09^{c}	59.34 ±2.05^{d}
	4	60.19 ± 3.50^{c}	79.96 ± 1.01^{de}	56.02 ± 4.99^{d}	60.67 ±0.65^{de}
	6	69.28 ± 2.26^{d}	80.04 ± 0.55^{de}	60.84 ± 2.93^{de}	63.33 ±2.50^{e}
Papaya	2	71.42 ± 3.29^{de}	81.81 ± 0.48^{ef}	69.49 ± 2.16^{f}	83.91 ±2.07^{gh}
	4	75.36 ± 1.32^{e}	82.17 ± 1.47^{ef}	81.26 ± 0.10^{g}	85.92 ±1.79^{h}
	6	83.59 ± 3.48^{f}	84.14 ± 1.12^{f}	86.78 ± 1.59^{h}	86.18 ±1.97^{h}
Bromelain	2	44.09 ± 3.29^{b}	60.74 ± 3.87^{b}	28.01 ± 1.52^{b}	26.17 ±4.53^{a}
	4	59.40 ± 3.61^{c}	72.65 ± 2.20^{c}	38.03 ± 1.91^{c}	37.20 ±2.29^{b}
	6	61.09 ± 1.01^{c}	78.43 ± 0.19^{d}	42.29 ± 3.55^{c}	47.32 ±1.01^{c}

^{a,b,c}Different letters in the same column indicate significant differences ($p < 0.05$).

different sources of plant proteases is shown in Table 2. The collagen solubility was significantly affected by both the type and concentration of enzyme and meat. Significantly higher collagen solubility values were observed in all enzyme treated samples when compared to the control ($p < 0.05$). The control pork showed the lowest collagen solubility (14.34%), while the highest value was found in the control giant catfish sample (52.82%). Markedly, increased collagen solubility was observed in all muscle types when papaya latex proteases were added. Bromelain showed a low efficiency for increasing collagen solubility for all muscle types. However, when comparing papain and papaya latex proteases, the former showed less effect on collagen solubility than the latter. This result may have been caused by the content and numbers of proteolytic enzymes that exist in the latex of papaya. Sullivan and Calkins (2010) also reported that papain-treated samples exhibited greater collagen solubility than the other treatments (bromelain, ficin, and *B. subtilis* proteases). Takagi et al. (1992) also reported significantly higher collagen solubility in beef treated with papain compared to a water-treated control and an alkaline elastase-treated sample. A more than 50% increase in collagen solubility was found in all muscle types when compared with the control where about 4 KU of papain, protease from *C. procera* and papaya latex, was applied.

Increased collagen solubility of enzyme treated samples might be due to an increase in permeability of the connective tissue, which will disintegrate easily. In addition, proteases may also promote structural alterations through action on intermolecular cross-links. The solubilization of collagen to gelatin is an indicator of tender meat. The role of collagen is of particular interest, as it has been proposed that collagen is actually the determining factor in the textural differences among various muscles. The solubility of connective tissue rather than total amount of connective tissue is more highly associated with sensory characteristics (Naveena et al., 2011). Naveena et al. (2004) observed that in buffalo meat, when adding ginger rhizome extract, it resulted in extensive muscle fiber and connective tissue degradation and a decrease in shear force values.

Effect of plant proteases on TCA-soluble peptides content

The TCA-soluble peptides content of muscle samples treated with different sources of plant proteases is shown in Table 3. The lowest content of TCA-soluble peptides was found in the control sample of giant catfish (0.90), followed by beef (2.09), pork (2.90), and chicken (4.13), respectively. When the enzyme activity unit was increased, the TCA-soluble peptides content in the entire treated sample also increased ($p < 0.05$). The increase in TCA-soluble peptides content in treated samples might be due to an increase in permeability of myofibrillar structures, resulting in disintegration and then the release of peptides. Ketnawa and Rawdkuen (2011) showed that the high TCA-soluble peptides content in bromelain treated samples was due to greater muscle protein hydrolysis. The bromelain extract applied to the meats resulted in collagen hydrolysis into small peptides

Table 3. TCA-soluble peptides content of meat samples treated with different concentrations of plant proteases.

Sample	Enzyme account (unit) KU	TCA-soluble peptide content (µmole Tyr/g sample)			
		Chicken	Giant catfish	Pork	Beef
Control	-	4.13 ± 0.07[a]	0.90 ± 0.07[a]	2.90 ± 0.03[a]	2.09 ± 0.04[a]
Calotropis	2	11.64 ± 0.22[g]	7.83 ± 0.15[h]	9.35 ± 0.09[h]	12.50 ± 0.15[h]
	4	13.64 ± 0.07[h]	9.62 ± 0.14[i]	11.90 ± 0.13[i]	15.31 ± 0.22[i]
	6	14.25 ± 0.25[i]	12.04 ± 0.03[k]	13.66 ± 0.12[j]	17.44 ± 0.16[k]
Papain	2	6.51 ± 0.04[e]	2.33 ± 0.07[c]	5.32 ± 0.11[e]	5.53 ± 0.09[e]
	4	8.01 ± 0.05[f]	4.26 ± 0.09[f]	6.66 ± 0.02[f]	6.78 ± 0.08[f]
	6	8.08 ± 0.06[f]	5.64 ± 0.14[g]	7.87 ± 0.59[g]	8.43 ± 0.11[g]
Papaya	2	13.49 ± 0.10[h]	9.89 ± 0.08[j]	12.18 ± 0.15[i]	16.75 ± 0.12[j]
	4	15.62 ± 0.08[j]	13.03 ± 0.23[l]	14.02 ± 0.18[k]	18.53 ± 0.24[l]
	6	16.96 ± 0.21[k]	15.25 ± 0.22[m]	15.15 ± 0.15[i]	18.32 ± 0.09[l]
Bromelain	2	4.94 ± 0.06[b]	1.71 ± 0.06[b]	3.41 ± 0.04[b]	3.65 ± 0.03[b]
	4	5.80 ± 0.06[c]	2.56 ± 0.07[d]	4.05 ± 0.03[c]	4.64 ± 0.06[c]
	6	6.30 ± 0.02[d]	3.48 ± 0.12[e]	4.76 ± 0.14[d]	5.00 ± 0.04[d]

[a,b,c]Different letters in the same column indicate significant differences ($p < 0.05$).

(Ketnawa et al., 2010). According to the results, beef showed the highest content of TCA-soluble peptides when added with papaya latex proteases, while the lowest content was found in bromelain addition for all of the muscle samples. Papaya latex proteases showed to be the most effective for producing peptides from all of the meat samples, which was followed by *C. procera* latex proteases, papain, and then bromelain. More than three-times of TCA-soluble peptides content were found in the sample treated with 2 KU/100 g sample of plant proteases, especially with papaya and *C. procera* latex proteases. This result suggested that the proteolytic enzymes that exist in the latex had hydrolytic activity towards the degradation of protein. The tyrosine level indicated that the oligopeptides and/or free amino acids, as well as the degradation products, all accumulated after being mixed with proteolytic enzymes. From these results, high TCA-soluble peptides content indicated a greater muscle protein hydrolysis, which was generated by proteolytic enzymes.

Effect of plant proteases on protein patterns

Representative protein patterns by SDS-PAGE for the meat samples mixed with different sources and concentrations of plant proteases are presented in Figure 1. The myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle types. There was increased proteolysis of the muscle proteins in all of the treated samples as evidenced by the reduction in the number and intensity of the protein bands when each enzyme was added. At higher enzyme activity units, these proteins were totally degraded or even disappeared, especially at 4 to 6 KU/100 g sample. The breakdown of proteins in high amounts was more visible in the chicken sample than in the others. When comparing the treatments (enzyme treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. Jorgova et al. (1989) reported that bacterial proteases treatment of muscle protein showed a reduction in the level of higher molecular weight fractions due to the degradation of myosin. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during postmortem storage (Melody et al., 2004). Moreover, degradation of the AC (band disappearance) from the treated sample was also observed on the SDS-PAGE, especially in chicken, pork, and giant catfish muscles added with enzymes activity > 4 KU/100 g sample. Papaya latex proteases showed the highest hydrolysis activity against all muscle types, followed by proteases from *C. procera* latex, papain, and bromelain. Ha et al. (2012) reported that papain, bromelain and zingibian preparations appeared to have a similar hydrolysis profiles to both beef connective tissue and topside myofibril extracts. According to Wada et al. (2002), plant thiol proteases affect the structure of MHC and the AC filaments of myofibrillar proteins. Furthermore, these enzymes have very broad specificities; and therefore, they indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderization to a mushy-textured product.

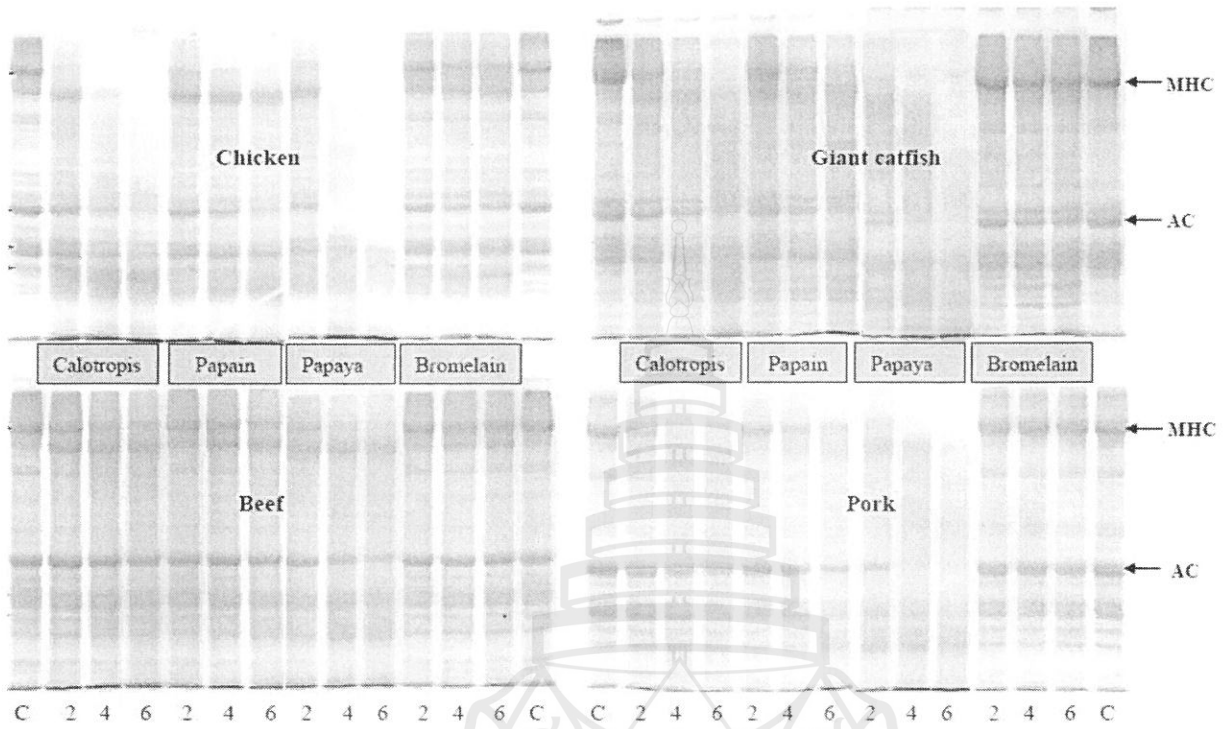

Figure 1. Protein patterns of meat samples treated with different sources of proteolytic enzymes. Numbers represent the enzyme activity unit (KU), C, control; MHC, myosin heavy chain; AC, actin.

Zhao et al. (2012) reported that almost all of the myofibrillar proteins (including MHC and AC) were degraded into fragments with molecular weights lower than 20 kDa when beef was treated with bromelain or papain at 37°C for 1 h. Moreover, they also said that papain and bromelain are all thiol proteases, and their strong activity towards all the myofibrillar proteins may result in extensive degradation of myofibrillar proteins and meat structure. Fragmentation of both myofibrillar proteins and collagen tissue when treated with ammonium hydroxide resulted in the tenderization of buffalo meat (Naveena et al., 2011). When the breakdown of myofibrillar protein occurred, small peptides with low molecular weights were generated and therefore reduced the firmness of the meat samples. However, the ultimate tenderness of meat is dependent on the degree of alteration and weakening of myofibriallar structures (Kemp et al., 2010).

Effect of plant proteases on muscle microstructure

Scanning electron micrographs of the meat samples treated with different plant proteases are shown in Figure 2. Based on the above results, the level of enzyme activity at 4 KU/100 g sample and the control (without enzyme) was considered for microstructural study. The control samples of all the muscle fibers had well organized structures and were closely bound to each other. The most compact structures were found in giant catfish. Transverse sections of SEM photographs clearly indicated a loss of muscle fiber interaction and a reduction in diameter of muscle fibers in enzyme treated meats when compared to the control. Extensive breakdown of endomysial connective tissue layers surrounding muscle fibers without gaps between fibers is clearly evident in papaya and *C. procera* latex proteases treated samples. This indicated proteolytic enzyme activity on connective tissue and/or myofibrillar proteins. Strong muscle fibers were broken and the cell membranes were severely degraded. In addition, for transverse sections of treated beef, SEM photographs showed a wide gap between fibers indicating complete breakdown and solubilization of collagen layers (endothelium) surrounding individual muscle fibers. Similar findings were observed in a previous study by Naveena and Mendiratta (2004) about buffalo meat tenderization using ginger extract. Disruption of the intramuscular connective tissue structure is another cause for meat tenderization. The microstructure of the enzyme treated samples showed a strong correlation with the previous results in terms of collagen solubility, TCA-soluble peptides content, and SDS-PAGE patterns. Naveena (2004) studied the effect of ginger extract on the microstructure of buffalo muscle

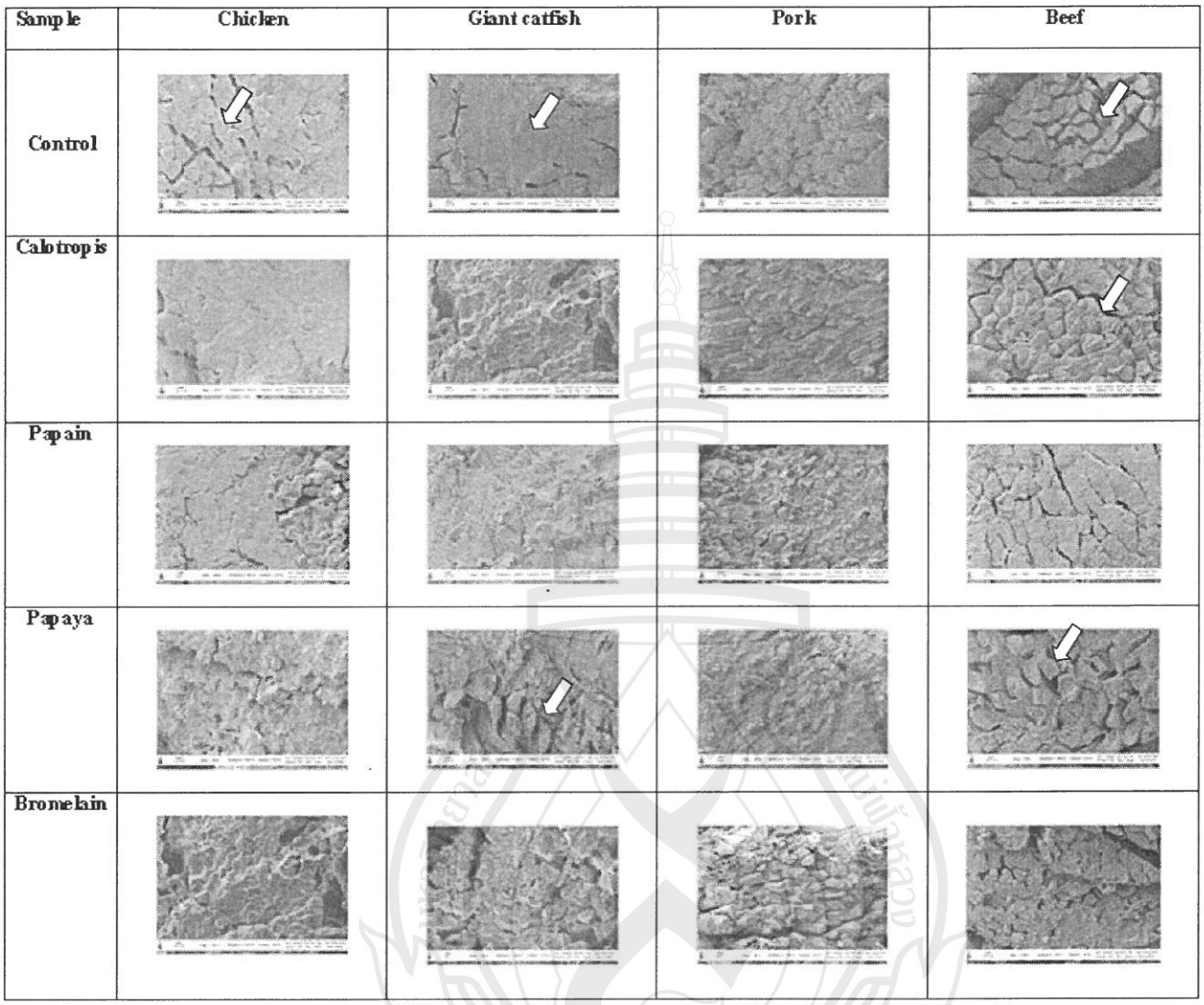

Figure 2. Microstructure of meat treated with different sources of proteolytic enzymes at the level of 4 KU/100 g sample.

by using SEM, and found that the ginger extract treatment broke muscle fibers into different bundles and also increased the space between the bundles. In addition, there was marked deformation and disruption of honey-like structure observed in the beef treated with elastase from the *Bacillus* strain (Chen et al., 2006).

Conclusion

The results obtained in this experiment shows tenderness improvements of proteolytic enzyme treated meats by monitoring biochemical and microstructural changes. Papaya latex proteases provided the greatest ability to improve tenderness in all tested samples. Bromelain used in this experiment showed the lowest capacity when compared with others. Increasing the unit of enzyme activity resulted in marked changes in all characteristics of the treated samples. Caution must be taken when high levels of proteolytic enzymes are applied, because negative results will be obtained.

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Physicochemical properties and tenderness of meat samples using proteolytic extract from *Calotropis procera* latex

Saroat Rawdkuen [a,*], Manon Jaimakreu [a], Soottawat Benjakul [b]

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

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ABSTRACT

This study was conducted in order to tenderise muscle foods (pork, beef and chicken) by using crude enzyme extract from *Calotropis procera* latex. Chunks of knuckle muscle from pork and beef as well as of breast muscle from chicken were marinated with distiled water (control) and 0.05%, 0.1%, 0.2%, 0.3% and 0.5% (w/w) of crude enzyme extract powder for 60 min at 4 °C. The marinated samples were then subjected to various physical and chemical property determinations. A decrease in moisture content was observed when the crude enzyme extract was added. Firmness and toughness of the muscle samples significantly decreased with the increased addition of crude enzyme extract ($p < 0.05$). The water holding capacity and cooking yield of the treated samples showed no significant difference throughout the crude enzyme extract addition ($p > 0.05$). Crude enzyme extract had no effect on the pH of the pork sample, but it slightly increased the pH in the beef and chicken. An increase in protein solubility and TCA-soluble peptides content was observed in all of the treated samples. The electrophoresis pattern of the muscle treated samples also revealed extensive proteolysis occurring in each muscle type. From the results, it is determined that latex from *Calotropis procera* can be used as an alternative source of proteolytic enzymes for the effective tenderising of meat.

1. Introduction

Meat tenderness is generally considered one of the most important determinants of meat quality. Meat tenderness depends on the amount of intramuscular connective tissue, the length of the sarcomere and also the proteolytic potential of the muscle (Kemp & Parr, 2012). On the other hand, meat toughness is one of the most undesirable meat qualities for consumers (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). Actomyosin and background toughness are 2 main types of classified meat toughness. The former is attributable to changes in myofibrillar proteins, whereas the latter is due to connective tissues or stromal proteins (Chen, He, Jiao, & Ni, 2006). A number of attempts have been made to improve meat tenderness and the overall quality of muscle foods. Normally, chemicals and physical treatments are used to do this. All methods focus on reducing or disrupting the myofibrillar proteins and connective tissues. Marinating muscle foods in acidic solutions such as acetic or lactic acid has been traditionally applied as a means of softening and flavouring meats (Berge et al., 2001). However, an extended period of time is needed for full marinaton because of the slow penetration of exogenous acids and also because of the specificity of the chemical compounds used by the target protein components which may hinder the efficiency of this treatment (Seuss & Martin, 1993). Alternative methods for meat tenderisation are still needed.

Treatment by exogenous proteases is one of the most progressive methods used for meat tenderisation. Proteases such as papain, bromelain and ficin, all derived from plants, have been widely used as meat tenderisers (Ha, Dekhit, Carne, & Hopkins, 2012; Ketnawa & Rawdkuen, 2011; Koak, Kim, Choi, Baik, & Kim, 2011; Naveena, Mendiratta, & Anjaneyulu, 2004; Sullivan & Calkins, 2010). Plant proteases are superior to bacterially derived enzymes mainly because of safety problems, such as pathogenicity, or other disadvantageous effects with the latter (Chen et al., 2006). These enzymes can digest muscle protein when they are mixed with meat. They also can hydrolyse the proteins of collagen and elastin, which lessens the toughness of meat. However, the proper quantity of enzymes needs to be considered because an excessive amount would result in meat decomposition.

"*Calotropis procera* latex" is potentially an alternative source for proteolytic enzymes. Protease from *C. procera* latex was extracted by an aqueous two-phase system (18% PEG 1000–14% $MgSO_4$) and provided a recovery of 74.6%. It can degrade the meat samples of beef, squid and farmed giant catfish effectively (Rawdkuen,

* Corresponding author. Tel.: +66 53 916752; fax: +66 53 916739.
E-mail address: saroat@mfu.ac.th (S. Rawdkuen).

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Pintathong, Chaiwut, & Benjakul, 2011). These plants have also been valued as edible and medical resources for some time (Chitme, Chandra, & Kaushik, 2004). However, there is no information describing whether or not the proteases contained in *C. procera* latex have an impact on meat tenderisation. The objective of this investigation was to study the effect of crude enzyme extract from *C. procera* latex on muscle foods tenderisation.

2. Materials and methods

2.1. Materials

L-Cysteine, sodium dodecyl sulphate (SDS) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (βME), Coomassie Brilliant Blue G-250, and casein 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). Trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane, Folin phenol reagent, and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Latex of *C. procera* was collected from Nayong, Trang Province, Thailand. Beef, pork and chicken muscles were purchased from Bandoo Market, 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 distiled water (1:1, v/v). It was mixed well and then centrifuged at 15,000g at 4 °C for 10 min according to the method previously reported by Rawdkuen et al. (2011). The supernatant obtained was filtered through a Whatman paper No. 1 and then freeze dried. This sample was thereafter referred to as the "crude enzyme extract" and was used for further study.

2.3. Caseinolytic activity assay

An enzyme sample of 0.1 ml was mixed with 1.1 ml of 1% (w/v) casein in 0.1 M Tris–HCl, pH 8.0, containing 12 mM cysteine. The mixture was incubated at 37 °C for 20 min. After that, the reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at 3,000g for 15 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit is defined as the amount of enzyme needed to produce an increment of 0.01 absorbance units per minute in an assayed condition (Rawdkuen et al., 2011).

2.4. Marination of muscle samples

Meat samples were purchased from Bandoo market and packed in low-density polyethylene (LDPE) bags, and then cleaned and stored in a refrigerator at 4 ± 1 °C for 24 h. The samples were then removed from the refrigerator and cut into small 3 cm^3 sized chunks.

The prepared samples were weighed and then marinated with the powder of *Calotropis procera* latex at concentrations of 0%, 0.05%, 0.1%, 0.2%, 0.3%, and 0.5% (w/w). After mixing the samples by hand, the chunks were placed in a bowl and covered with polyethylene bags and kept at 4 °C for 60 min according to the method previously reported by Naveena et al. (2004). After incubation, the meat chunks were evaluated for both their chemical and physical properties.

2.5. Physical properties determinations

2.5.1. Shear force value

Texture was analysed using a TA.XT2 texture analyser (Stable Micro Systems, Surrey, UK), equipped with a Warner–Bratzler blade (square shape) in accordance with the method used in Ketnawa and Rawdkuen (2011). Seven rectangular shaped samples of raw beef, raw chicken and raw pork were prepared. Each sample was cut perpendicular to the longitudinal orientation of the muscle fibres. The blade was pressed and applied at a constant speed of 2 mm s^{-1} through the sample. The maximum shear force (Firmness: N) and total work (Toughness: N*Sec) were recorded.

2.5.2. Water-holding capacities (WHC)

WHC was determined according to the method used in Wardlaw, Maccaskill, and Acton (1973). Minced meat (20 g) was placed in a centrifuge tube containing 30 ml of 0.6 M NaCl and was stirred with a glass rod for 1 min. The tube was then kept at 4 ± 1 °C for 15 min, stirred again, and then centrifuged at 3000g (PLC-05, Industrial Corp., Taipei, Taiwan) for 25 min. The supernatant was measured, and the WHC was expressed as a percentage of initial volume.

2.5.3. Cooking yield

The treated samples (10 g) were steamed for 1 min and then cooled to room temperature. The cooked sample was surface-dried with a filter paper and re-weighed using an analytical balance (Sartorius, ED224S Sartorius AG, Goettingen, Germany). The cooking yield was calculated by the difference in weight when raw and then when cooked, as described in Ketnawa and Rawdkuen (2011).

2.6. Chemical properties determinations

2.6.1. Moisture content

The moisture content of the samples was determined according to the Association of Official Analytical Chemists (AOAC) method No. 950.46 (2000).

2.6.2. pH

To determine pH, 10 g of the sample were homogenised with 50 ml of chilled distiled water. The pH values were measured with a digital pH metre (Model pH 510, Eutech Instrument, Ayer Rajah Crescent, Singapore).

2.6.3. Protein solubility

Protein solubility was determined according to the procedures of Naveena et al. (2004). The proteins in the treated samples were extracted from 2 g of minced meat by using 40 ml of ice-cold 1.1 M potassium iodide in a 0.1 M potassium phosphate buffer (pH 7.2). The samples were homogenised and kept overnight at 4 °C with frequent shaking. The samples were then centrifuged at 1,500g for 20 min and the protein concentration in the supernatant was determined by the Biuret method (Robinson & Hodgen, 1940). Solubility was expressed as the percentage of total protein in meat samples solubilised directly in 0.5 M NaOH.

2.6.4. TCA-soluble peptides

The TCA-soluble peptide content of the samples was measured by the method used in Ketnawa and Rawdkuen (2011). Two grams of the samples were weighed and then homogenised with 18 ml of 5% (w/v) TCA for 1 min and kept at 4 °C for 1 h before they were centrifuged at 8000g for 5 min. The soluble peptides in the supernatant were measured by using the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951). The content of TCA soluble peptides was calculated as the μmol of tyrosine/g of the samples.

2.6.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to the method in Laemmli, Beguin, and Gujer-Kellenberger (1970). Samples (2 g) subjected to different treatment conditions were all mixed with 18 ml of 5% (w/v) SDS solution (85 °C). The mixture was then homogenised with an homogeniser (IKA Ultra Turrax, T25D, Germany). The homogenate was incubated at 85 °C in a water bath for 1 h to dissolve the protein. It was then centrifuged at 8000*g* for 5 min at room temperature using a centrifuge (PLC-05, Industrial Corp., Taipei, Taiwan) to remove the un-dissolved debris. The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and then boiled for 3 min. The samples (20 μg protein) were loaded into a poly-acrylamide gel (10% running and 4% stacking gels). Then, they were subjected to electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were 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. The protein patterns were then made visible after de-staining the gel until a clear background was achieved.

2.7. Statistical analyses

The experiment was carried out in triplicate using different three lots of samples. A randomised design was used throughout the study. The data were analysed statistically with the SPSS program for Windows (SPSS version 11.5, SPSS Inc., Chicago, IL, USA). Duncan's multiple-range test was used to compare the difference between means. The accepted level of significance for all comparisons was $p < 0.05$. Experiments were conducted in triplicate.

3. Results and discussion

3.1. Effect of crude enzyme extract on meat firmness and toughness

The texture properties of the muscle samples treated with the powder of crude enzyme extract at different concentrations are presented in Fig 1. The firmness and toughness values significantly decreased in all of the treated samples when compared to the control (without crude enzyme extract) ($p < 0.05$). It was observed that the shear force values continuously decreased in all of the treated samples when the level of crude enzyme extract from *C. procera* latex increased, especially when the concentration was >0.10% (w/w) (Fig. 1A). The lowest firmness value was found in all of the meat samples treated with 0.5% (w/w) of the crude enzyme extract, especially in the chicken sample. The firmness values for the starting pork beef, and chicken, were 535, 650 and 302 N, respectively. The addition of 0.5% (w/w) crude enzyme extract to the muscle reduced the firmness of the samples by about 59, 65, and 52% when compared to the control for pork, beef and chicken, respectively. The reduction in meat firmness came as a result of the action of the proteolytic enzymes on the myofibrillar proteins or by disruption of the connective tissues. Post-mortem proteolysis by endogenous proteases causes a weakening of myofibril structures and associated proteins, which results in tenderisation (Kemp & Parr, 2012). Both myofibrillar proteins and collagen tissues fragment when treated with ammonium hydroxide, resulting in the tenderisation of buffalo meat (Naveena et al., 2011). When the breakdown of myofibrillar protein occurred, small peptides with low molecular weight were generated, thereby reducing the firmness of the meat samples.

The toughness values of the treated meat samples are presented in Fig. 1B. Beef muscle showed the highest toughness value while the chicken sample provided the lowest toughness value. Slightly decreased toughness was observed when the crude enzyme extract was added ($p > 0.05$) at the level of 0.05–0.20% (w/w). However, at the highest addition level, the lowest toughness was observed in all of the samples. The lowest toughness value was found in chicken for both the treated and untreated samples with crude enzyme extract. According to Koak et al. (2011), using kiwi fruit content (containing proteolytic enzymes), beef texture became softer when the kiwi was added. In the beef sample, when adding crude enzyme extract at the level of 0.50% (w/w), a decrease in toughness was observed of about 50%. Toughness is determined by the amount of intramuscular connective tissue, intramuscular fat and the length of the sarcomere (Kemp & Parr, 2012). In addition, older animals normally produce tougher meat than is produced by young animals. The tougher the meat, the more connective tissues that are present. Proteolytic enzymes, especially plant proteases, are widely used for meat tenderisation (Ha et al., 2012; Ketnawa & Rawdkuen, 2011; Sullivan & Calkins, 2010). Naveena et al. (2004) also observed that in buffalo meat with extensive muscle fibre and connective tissue degradation, shear force values decreased when ginger rhizome extract was added. Ha et al., 2012 reported that the actinidin protease was the most effective for hydrolysing beef myofibril proteins, while zingibain protease is most effective for degrading connective tissue.

3.2. Effect of crude enzyme extract on water holding capacity and cooking yield

The water holding capacity of the meat samples when treated with different concentrations of crude enzyme extract is shown in Fig. 2A. A slightly decreased WHC was observed when the crude enzyme extract was added ($p > 0.05$). Pork showed the highest WHC while the lowest value was found in the treated chicken muscle. The higher WHC in the control sample may be due to the overall reduction in the protein reactive group, which is available for water binding (Forrest, Aberle, Hedrick, Judge, & Merkel, 1994). Slight denaturation of sarcoplasmic proteins, which has an important role in determining WHC, could be the reason for decreased WHC (Joo, Kauffman, Kim, & Park, 1999). Reduced WHC is a result of myofibrillar shrinkage, as well as of the movement of water from the myofilament space to the extra-cellular space. One of the main factors involved in the shrinkage and/or swelling of the myofibrils is protein fragmentation (Huff-Lonergan & Lonergan, 2005). The WHC of meat is very important since many physical properties such as colour, texture and firmness are partially dependent on the WHC.

The cooking yield of the meat samples treated with different concentrations of crude enzyme extract is shown in Fig. 2B. Decreased cooking yield was observed in all samples when the *C. procera* latex powder was added. The control beef sample showed the highest cooking yield while the chicken gave the lowest value. At the level of 0.5% (w/w) addition, pork muscles provided the highest cooking yield and chicken muscles gave the lowest. The addition of increasing levels of crude enzyme extract led to decreased cooking yield for all of the meat muscles. This result indicates that thermal treatment could remove more water from treated chicken samples than that of treated pork muscles. It can be implied that the enzymes in crude *C. procera* latex could hydrolyse protein in chicken more than in beef and pork. Kumar and Berwal (1998) reported that spent hen meat treated with sulphate extracted Cucumis powder produced a significant reduction in cooking yield value. Degradation of sarcoplasmic and myofibrillar proteins may be caused by the reduction of cooking yield in the enzyme treated sample (Pawar, Mule, & Machewad, 2007).

3.3. Effect of crude enzyme extract on moisture content

No changes in the moisture content of the meat samples treated with 0.5% (w/w) of crude enzyme extract were observed, compared

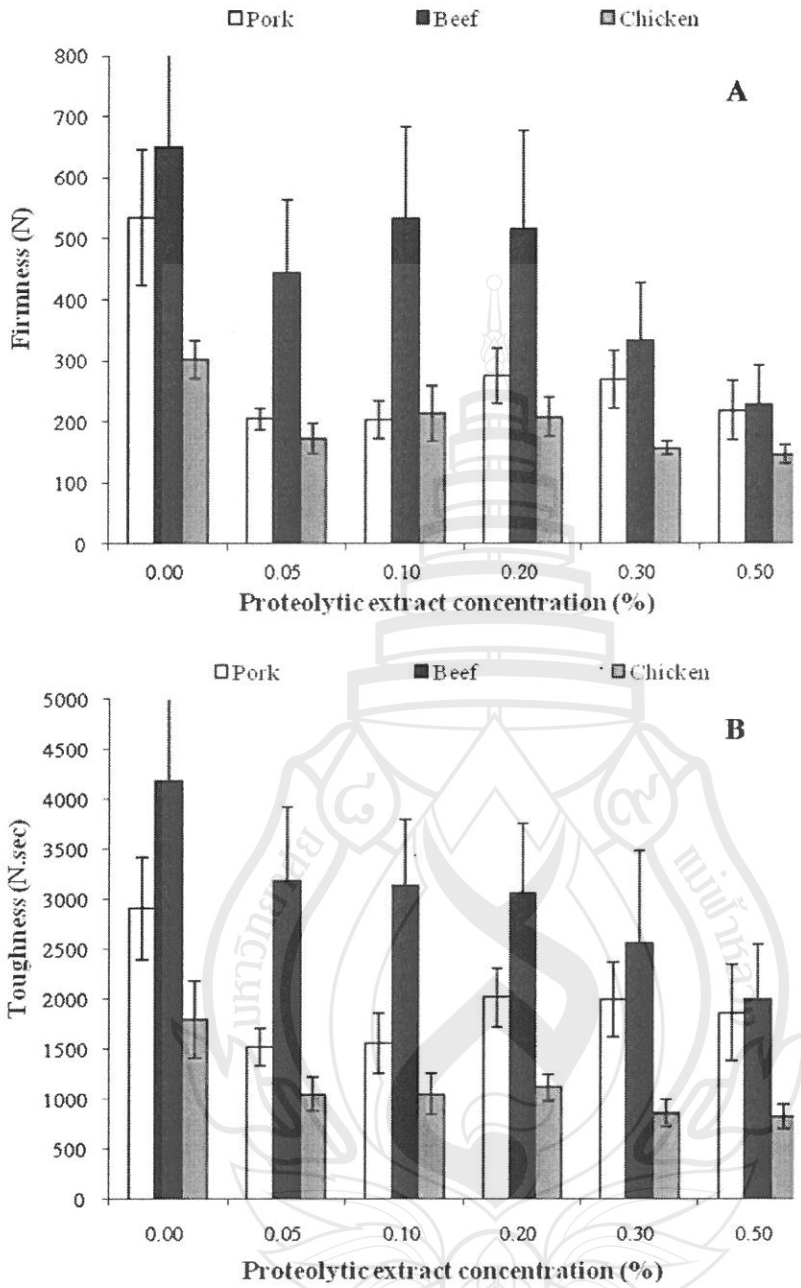

Fig. 1. Firmness (A) and toughness (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

with the control ($p > 0.05$) (Table 1). However, increased crude enzyme extract concentration could reduce some amount of moisture, especially in pork muscle. In general, the hydroscopic nature of the powder probably contributed to the moisture reduction when it was attached to the wet surface of the sample. The low moisture content of the treated meat samples is normally related to consumer preference. It also affects the yield of the final product. Naveena and Mendiratta (2001) reported moisture retention in spent-hen meats treated with ginger rhizome extract as compared with an untreated sample. Similar observations were found: 77.18% moisture in ginger rhizome extract was found in a treated buffalo meat sample as compared with 76.51% in an untreated one.

3.4. Effect of crude enzyme extract on muscle pH

The pH value of the meat samples treated with different concentrations of crude enzyme extract slightly increased when the concentration of crude enzyme extract increased (Table 2). Significantly increased pH value was found in beef and chicken muscles when compared with the control ($p < 0.05$). However, the pH value in the pork sample was not significantly different ($p > 0.05$), except at the 0.50% (w/w) addition level. The high pH of the crude enzyme extract (pH 6.3) was probably caused by the higher pH of the treated samples, especially when a high level of crude enzyme extract was applied. Moreover, enzymatic hydrolysis of the muscle may release amino acids that can increase the pH of the system. The

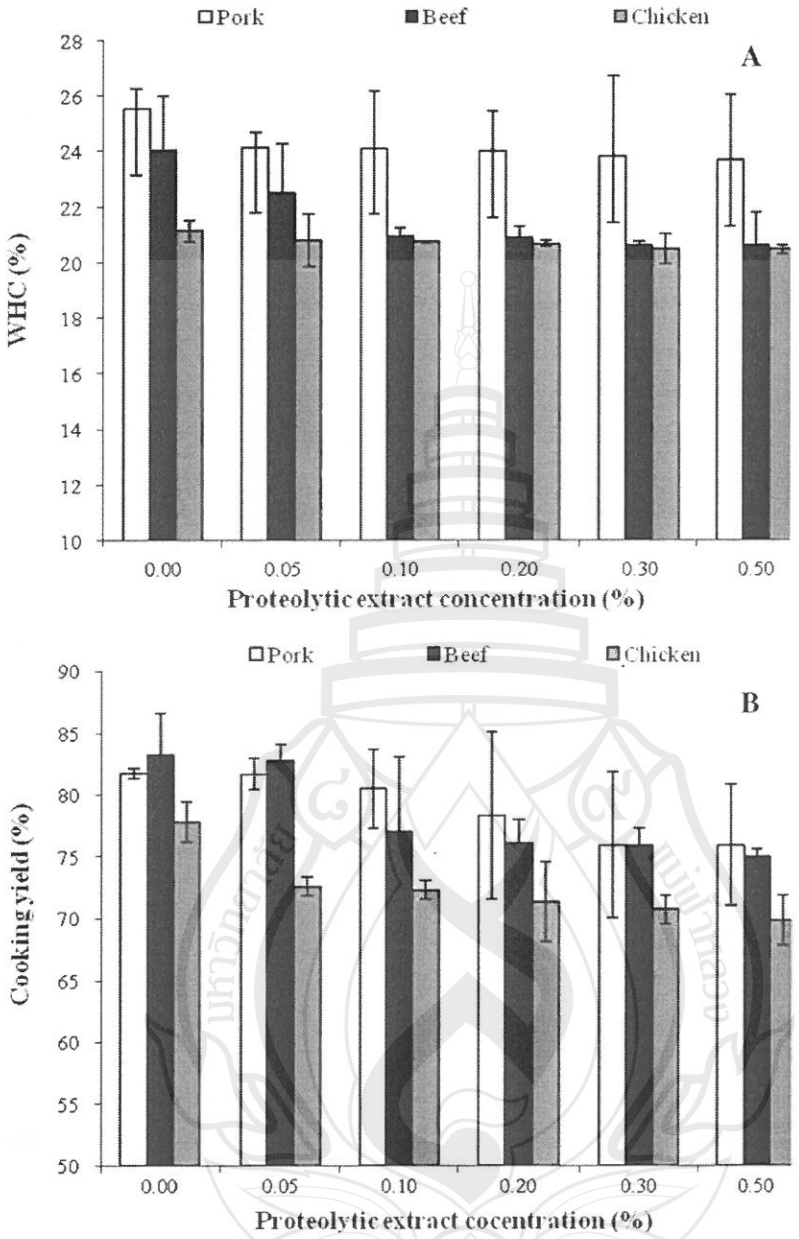

Fig. 2. Water holding capacity (A) and cooking yield (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Table 1
Moisture content of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Crude enzyme extract (%)	Moisture content (%)[A]		
	Pork	Beef	Chicken
Control	84.51 ± 1.78^{ab}	80.18 ± 4.70^{a}	82.28 ± 0.67^{b}
0.05	87.63 ± 0.29^{c}	85.24 ± 4.13^{a}	81.14 ± 0.68^{ab}
0.10	85.08 ± 2.97^{bc}	82.16 ± 0.58^{a}	82.53 ± 0.38^{b}
0.20	84.76 ± 1.61^{abc}	81.13 ± 1.00^{a}	80.93 ± 1.32^{a}
0.30	81.83 ± 1.15^{ab}	80.48 ± 0.01^{a}	80.35 ± 1.30^{a}
0.50	81.00 ± 2.90^{a}	81.23 ± 0.58^{a}	81.46 ± 0.83^{ab}

Different superscripts in the same column indicate significant differences ($p < 0.05$).
[A] Values are given as mean ± SD from triplicate determinations.

Table 2
pH values of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

Proteolytic extract (%)	pH[A]		
	Pork	Beef	Chicken
Control	5.42 ± 0.01^{a}	5.59 ± 0.01^{a}	5.50 ± 0.01^{a}
0.05	5.41 ± 0.02^{a}	5.61 ± 0.01^{b}	5.52 ± 0.01^{ab}
0.10	5.43 ± 0.02^{a}	5.63 ± 0.01^{c}	5.53 ± 0.01^{bc}
0.20	5.44 ± 0.02^{a}	5.66 ± 0.01^{cd}	5.54 ± 001^{cd}
0.30	5.46 ± 0.02^{a}	5.64 ± 0.01^{c}	5.55 ± 0.01^{d}
0.50	5.51 ± 0.02^{b}	5.67 ± 0.01^{d}	5.60 ± 0.10^{e}

Different superscripts in the same column indicate significant differences ($p < 0.05$).
[A] Values are given as mean ± SD from triplicate determinations.

increases in pH of the treated sample (0.5%, w/w) ranged from 5.42 to 5.51, 5.59 to 5.67, and 5.50 to 5.60 when compared to the control for pork, beef and chicken, respectively. The pH value in meat products is highly important because it has a major influence on other physico-chemical and quality properties such as WHC, tenderness and juiciness. Changes in pH are caused by postmortem metabolism and also by the application of any added substances to the meat during the application of any technological processes.

3.5. Effect of crude enzyme extract on protein solubility and TCA-soluble peptides content

The protein solubility of meat samples treated with different concentrations of crude enzyme extract is shown in Fig. 3A. Protein solubility was significantly affected by the crude enzyme extract from *C. procera* latex. Significantly higher protein solubility values were observed in all enzyme treated samples compared to the control ($p < 0.05$). The control chicken meat (without added crude enzyme) showed the highest protein solubility, while the lowest value was found in the control beef sample. The regularly aligned filament of myofibrils in the control sample may have helped to prevent crude enzyme extract penetration, thus making the action seemingly resistant to extraction (Davey & Gilbert, 1968). Continuously increased protein solubility was clearly observed in the beef and pork muscles. At the level of 0.5% (w/w) crude enzyme extract addition, the solubility of protein in pork, chicken, and beef was 42%, 43% and 52%, respectively. More than a 50% percent increase of protein solubility was found in the pork and beef muscle when compared with the control. These results suggest that the protein solubility changes were due to myofibrillar protein degradation. an increase in solubility of enzyme-treated samples might be due to an increase in permeability of myofibrils, which will then disintegrate easily. Differences in protein solubility may be caused by the difference in structure of the meat muscle. An increase in

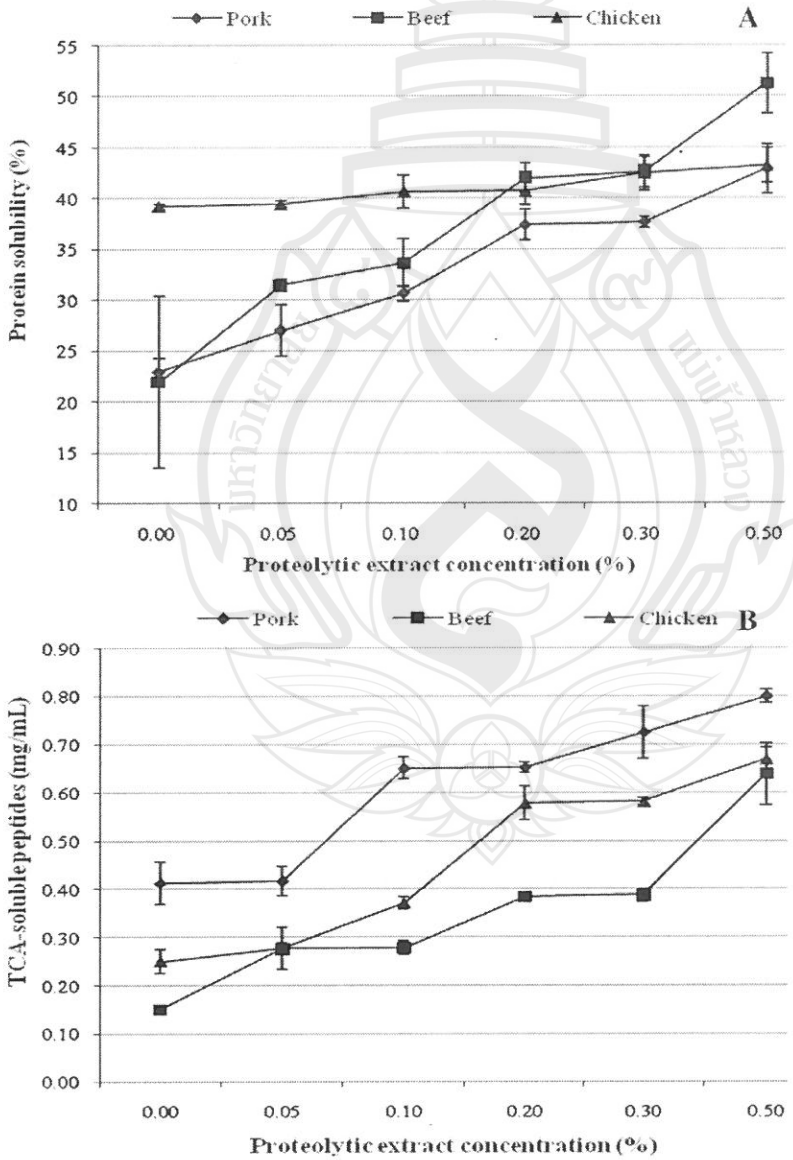

Fig. 3. Protein solubility (A) and TCA-soluble peptides content (B) of meat samples treated with different concentrations of crude enzyme extract from *Calotropis procera* latex.

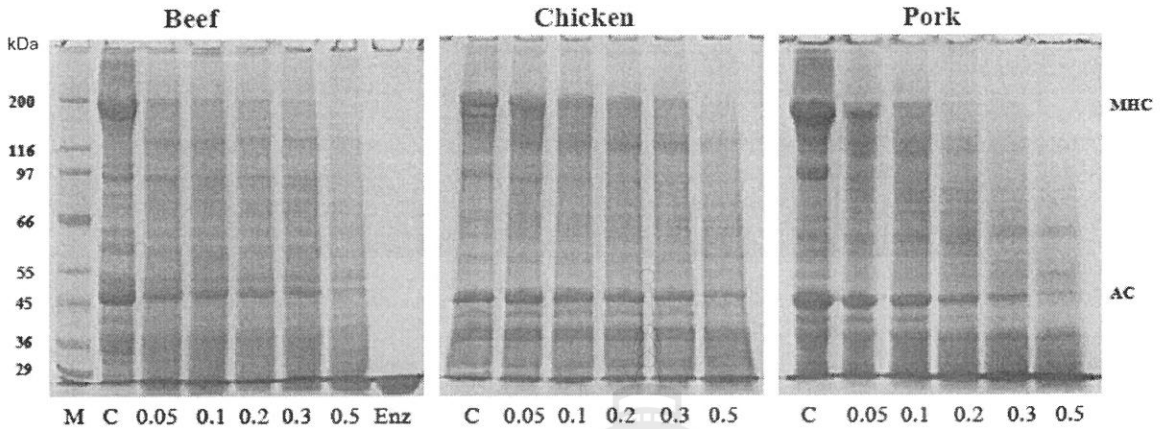

Fig. 4. Effect of crude enzyme extract from *Calotropis porcera* latex on meat samples degradation. MHC, myosin heavy chain; AC, actin; M, Molecular weight marker; C, control; Enz, crude enzyme extract. Numbers represent the concentration of proteolytic extract (%, w/w).

protein solubility with ginger and papain treatment was also reported by Naveena and Mendiratta (2001) in spent hen meat.

The TCA-soluble peptides content of the muscle samples treated with different concentrations of crude enzyme extract is shown in Fig. 3B. The highest content of TCA-soluble peptides was found in the pork, followed by the chicken and beef muscles treated with 0.5% (w/w) crude enzyme extract. When the concentration of crude enzyme extract was increased, the TCA-soluble peptides content in the entire treated sample increased ($p < 0.05$). The TCA-soluble peptides content was the lowest in the beef muscle. More than a 50% increase of TCA-soluble peptides content was found in the sample treated with 0.5% (w/w) crude enzyme extract as compared to the control. This result suggests that the enzyme that exists in the latex had hydrolytic activity, thereby degrading the protein. TCA-soluble peptides content indicated that the endogenous oligopeptides and/or free amino acids, as well as degradation products, all accumulated after being marinated with the crude enzyme extract. From these results, it is found that for high TCA-soluble peptides content, more muscle protein hydrolysis is generated by the proteolytic enzymes present in *C. procera* latex.

3.6. Effect of crude enzyme extract electrophoretic patterns

A representative protein pattern by SDS–PAGE for the muscle sample treated with different concentrations of crude enzyme extract can be seen in Fig. 4. Similar protein patterns in original beef, chicken and pork were observed (lane 1). The myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle types. There was increased proteolysis of the muscle proteins in all treated samples as evidenced by the reduction in number and intensity of the protein bands when the crude enzyme extract was added. The breakdown of proteins in high amounts was more visible in the pork sample than in the other samples. When comparing the treatment (crude enzyme treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. In addition, comparing beef and chicken muscles, the MHC band of the former was markedly degraded into lower molecular weights than that found in the latter. Hydrolysis of these proteins has been shown to disrupt muscle fibre structures with an associated decrease in shear force and an improvement in meat tenderness (Kemp et al., 2010). Jorgova, Danchev, and Kostov (1989) reported that bacterial proteolytic enzyme treatment of muscle protein reduced the level of higher molecular weight fractions due to degradation of myosin, thus increasing meat tenderness. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during the postmortem storage (Melody et al., 2004). Naveena et al. (2004) revealed that protease from *Cucumis trigonus Roxb* and *Zingiber officinale Roscoe* improved the tenderness and the overall quality of tough buffalo meat. Ketnawa and Rawdkuen (2011) also concluded that bromelain extract from pineapple peels could be used as an effective meat tenderiser for beef, chicken and squid muscles. The bromelain extract applied to the meats plays a role in collagen hydrolysis into small peptides (Ketnawa, Rawdkuen, & Chaiwut, 2010). Moreover, degradation of the AC from the treated sample was also observed on the SDS–PAGE, especially in chicken and pork muscles. According to Wada, Suzuki, Yaguti, and Hasegawa (2002), plant thiol proteases affect the structure of the MHC and the AC filaments of myofibrillar proteins. Furthermore, these enzymes have very broad specificities and, therefore, indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderisation and a mushy-textured product. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during postmortem storage. Based on these results, it is determined that a crude enzyme extract from *C. procera* latex potentially has high proteolytic activity and is able to be used to make meat more tender.

4. Conclusion

The results obtained in these experiments clearly indicate that the tenderness and other physicochemical properties of meat samples were improved through the use of a crude enzyme extract from *Calotropis procera* latex. By adding increased amounts of crude enzyme extract, the quality characteristics of the treated meat samples were improved. Technology for applying this enzyme is easily and cheaply available and can be exploited at the household or industrial level for tenderising tough meat, and it can be used as a better alternative to chemical tenderisers or other plant proteases.

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