SEPARATION, CHARACTERIZATION AND APPLICATION OF BROMELAIN FROM PINEAPPLE WASTES IN CHIANG RAI

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MASTER OF SCIENCE

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MAE FAH LUANG UNIVERSITY

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Sunantha Ketnawa

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ABSTRACT

This study investigated the extraction, isolation, characterization and application of bromelain extract from wastes of *Nang Lae* and *Phu Lae* pineapple cultivars. The waste portions such as the peel, core, stem and crown were 29-40, 9-10, 2-5 and 2-4% (w/w), respectively. The extract of crown from both cultivars gave the highest proteolytic activity and protein contents. The peel was considered as the source with the highest potential for bromelain extraction. The best extractant for bromelain extraction was sodium phosphate buffer pH 7.0 containing cysteine and EDTA (PB-CE) due to the highest bromelain activity obtained (867 and 1,032 units for *Nang Lae* and *Phu Lae* cultvivar, respectively). TCA-soluble peptides content of all the treated muscles (beef, chicken and squid) with bromelain extract increased when the amount of bromelain extract was increased ($P<0.05$). Reduction in myosin heavy chains (MHC) and actin (AC) was observed in the entire muscle types when bromelain extract was applied.

Bromelain from pineapple peel (*Nang Lae* and *Phu Lae* cultivars) was predominantly partitioned to the polyethylene glycol (PEG) rich phase in aqueous two-phase system (ATPS). For *Nang Lae* cultivar, the highest enzyme activity recovery (113.54%) with purification fold of 2.23 was observed in the top phase of 15% PEG2000-14% $MgSO_4$. The bromelain extract showed

the highest activity at pH 7.0 and 55°C. Its activity decreased continuously when concentration of NaCl was increased (up to 1.5%, w/v) ($P<0.05$). The β, α_1, α_2 of giant catfish skin collagen extensively degraded into lower MW proteins when treated with 0.02 units of the bromelain extract. For *Phu Lae* cultivar, the best ATPS condition for bromelain partitioning was 18% PEG6000-17% $MgSO_4$, which increased the purity by 3.44-fold and the activity recovery to 205.78%. The obtained bromelain showed the highest relative activity at pH 8.0. The highest activity of bromelain was found at 60°C and then decreased 70% after 5 min of incubation at 90°C. SDS-PAGE and activity staining showed that bromelain from crude extract of both cultivars had the MW approximately 28 kDa.

According to the activity recovery, TCA soluble peptides content and hydrolysis of muscle proteins, *Phu Lae* was selected for application study. The tenderizing effect of bromelain extract powder (0, 3, 7, and 20%, w/w) obtained from the top phase of ATPS comprising of 18% PEG 6000–17% $MgSO_4$ on muscle foods (beef, chicken and squid) was investigated. Lower of pH and moisture content were observed in the samples treated with bromelain extract, but the TCA-soluble peptides content significantly increased ($P<0.05$). Reduction of meat firmness, toughness, water holding capacity and cooking yields were observed when the concentration of bromelain extract was increased ($P<0.05$). Electrophoretic patterns also revealed extensive proteolysis of the treated samples. At the microstructural level, tissue fibers were broken; cell membranes were much more degraded and the generation of numerous gaps was clearly observed when 20% (w/w) of bromelain extract was added. From the results, the bromelain extract derived from two-phase extraction of pineapple peel could be used as an effective meat tenderizer. Results from this study suggests that local pineapple wastes are rich sources of bromelain and application of bromelain as meat tenderizer helps add value to such sources.

Keywords: Bromelain / *Nang Lae* / *Phu Lae* / Pineapple wastes / Aqueous two phase system / Meat tenderization

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Thailand is one of the world largest pineapple producer (~ 2.0 million tons in 2009) (Food and Agriculture Organization of the United Nations, 2009) and the biggest exporter of fresh pineapple (2,700 tons), frozen pineapple (646 tons) and cannery of pineapple (500,000 tons) to around the world which provided the income around 15 billion baht per year (Ministry of Agriculture and Cooperatives, 2009). The central part of Thailand is the main area of pineapple cultivation (~ 1.6 million tons), followed by the northern part (~ 200,000 tons), especially Lampang and Chiang Rai province. In Chaing Rai, *Nang Lae* and *Phu Lae* are economically well known cultivars. Average pineapple production in Chiang Rai during 2009 was 12,000 tons (Department of Agricultural Extension, 2009). Although the season for pineapple runs from May through August, they are available year-round in local markets.

During pineapple processing, the crown and stem are cut off before peeling. The core is then removed for further processing. These wastes (peel, core, stem, crown and leaves) generally account for 50% of total pineapple weight (MOAC, 2009). Pineapple wastes were generated in the large amount after fresh cut processing in Chiang Rai (~ 4,800 tons per year) (DOAE, 2009). Therefore, with increasing pineapple production, the wastes are also proportionally increasing. Waste disposal represents a growing problem since it is usually prone to microbial spoilage and it causes serious environmental problems. The utilization of waste would be an innovation to handle the great deal of waste from processing.

Pineapple wastes are found to have potential uses as raw materials that can be converted into value-added products. The peel is a rich source of cellulose, hemicelluloses and other carbohydrates (Bardiya, Somayaji & Sunil, 1996; Nigam, 1999). It has been used to produce

paper, banknotes, and cloth (Bartholomew, Paull & Rohrbach, 2003). The core could be used for the production of frozen pineapple juice concentrates or extracted juice for alcoholic beverages or for vinegar (Pukrushpan, 1985). In addition, the juice from pineapple wastes has been used as a nutrient substance in culture broth (Nigam, 1998) and cellulase production (Omojasola, Folakemi, Omowumi & Ibiyemi, 2008). Moreover, the pineapple wastes have also been used as substrates for the production of ethanol (Tanaka, Hilary & Ishizaki, 1999; Nigam, 1999), citric acid (Chau & David, 1995; Kumar, Jain, Shanker & Srivastava, 2003; Imandi, Bandaru, Somalanka, Bandaru & Garapati, 2008), antioxidant compounds (Schieber, Stintzing & Carle, 2001), bioprotein (Jamal, Fahururrazi & Zagangir, 2009), and proteolytic enzymes (Rolle, 1998). Although the pineapple wastes in Chiang Rai Province are occasionally utilized as fertilizer or animal feed particularly in the summer when forage is scarce, it is still very low in value. The utilization of pineapple wastes as sources of bioactive compounds, especially proteolytic enzymes, is an alternative means. Many researches reported that bromelain and other cysteine proteases could be found in different parts of pineapple (Rowan, Buttle & Barrett, 1990; Sriwatanapongse, Balaban & Teixeira, 2000; Umesh, Sumana & Raghavarao, 2008)

Bromelain, a proteolytic enzyme, had been reported to be present in pineapple wastes such as core, peel, and leaves, besides in fruit and stem (Umesh et al, 2008). It has been used for meat tenderization (Melendo, Beltrin & Roncales, 1997; Lizuka & Aishima, 1999; Wada, Suzuki, Yaguti & Hasegawa, 2002), beer clarification (Walsh, 2002), protein hydrolysate production in fish sauce, and oyster sauce (Chuapoehuk & Raksakulthai, 1992; Walsh, 2002; Aehle, 2007), skins pre-tanning, softening and bating in leather industries (Walsh, 2002), improve the dyeing properties of protein fibers (Koh, Kang , Kim , Cha & Kwon, 2006), decomposing or partially solubilizing protein fiber from silk and wool (Singh, 2003) and preventing of apple juice browning (Tochi, Wang, Xu & Zhang, 2009). Furthermore, there was report of novel use of bromelain as hydrolyzing agent for release of antimicrobial peptides of leatherjacket insoluble proteins (Salampessy, Phillips, Seneweera & Kailasapathy, 2010). In addition, it is known for clinical and therapeutic applications, particularly for modulation of tumor growth, third degree burns, improvement of antibiotic action and as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases (Maurer, 2001). In cosmetics,

bromelain has been used as active ingredient in skin care products to provide gentle peeling effect (removing stratum corneum cell) (Aehle, 2007).

There are a number researches involved in the extraction and purification of bromelain from several parts of pineapples such as flesh, core, stem and crown. Normally, pineapple fruit, core and stem were cut into small pieces, triturated, filtered to obtain filtered juice and then subjected to further purification. Bromelain has been isolated by microfiltration or ultrafiltration (Doko, Bassani, Casadebaig, Cavailles & Jacob 1991; Liang, Huang & Kwok, 1999), precipitation with acetone (Rowan et al., 1990; Pukrushpan & Vongmekiat, 1987) or ammonium sulfate (Doko et al., 1991; Shian, Aishah, Yu, Taher, Majid & Adiba, 2005; Devakate, Patil, Waje & Thorat, 2009). Chromatography techniques were generally used such as gel-filtration (Wharton, 1974; Arroyo-Reyna & Hermindez-Arana, 1995), ion-exchange (Devakate et al., 2008), affinity chromatography (Rowan et al., 1990) and expanded bed adsorption (Silveira, Souza, Santana, Chaves, Porto & Tambourgi, 2009). Lyophilization process to obtain the purified bromelain powder in the final step was also investigated (Doko et al., 1991). The other methods for bromelain purification related with reversed micelles system (Umesh et al., 2008) and aqueous two- phase system (Rabelo, Tambourgi & Pessoa, 2004; Babu, Rastogi & Raghavarao, 2008). Therefore, it is anticipated that the juice from the waste material can be utilized for a bromelain extraction.

An effective and economically viable method like aqueous two phase system (ATPS) is attractive for the separation and purification of mixture of proteins/enzymes (Walsh, 2002; Raghavarao, Ranganathan, Srinivas & Barhate, 2003). It can remove undesirable byproducts present in the system such as unidentified polysaccharides, pigments and interfering proteins that lower the activity of enzyme. Compared to other separation and purification methods, extraction using ATPS has many advantages including low cost, ease of scale-up, scope for continuous operation, and environment friendly thus, making it an attractive alternative for isolation of biomolecules (Walsh, 2002; Raghavarao et al., 2003; Nitsawang, Hatti-Kaul & Kanasawuda, 2006). The other advantages of ATPS compared to other purification methods lie in volume reduction, high capacity and short processing times (Rabelo et al., 2004). ATPS has been used for the partitioning and recovery of various proteases such as bromelain (Rabelo et al., 2004; Babu et al., 2008), papain (Nitsawang et al., 2006), trypsin (Klomklao, Benjakul,Visessanguan &

Kishimura, 2005), chymosin and pepsin (Spelzini, Farruggia & Pico, 2005; Nalinanon, Benjakul, Visessanguan & Kishimura, 2009), protease from fermentation broth (Chouyyok, Wongmongkol, Siwarungson, & Prichanont, 2005; Porto, Monteiro, Moreira, Lima-Filho, Silva & Porto, 2005) and protease from *Calotropis porcera* latex (Rawdkuen, Pintathong, Chaiwut & Benjakul, 2010).

Applications of enzymes extracted from natural sources have been interested by consumer. Marination of muscle foods by any proteolytic enzyme is one of the popular methods for meat tenderization. Proteolytic enzymes derived from plants, such as papain, bromelain and ficin have been widely used as meat tenderizers in most parts of the world (Naveena, Mendiratta & Anjaneyulu, 2004) when compared to bacterial derived enzyme mainly because of safety problems, such as pathogenicity, or other disadvantageous effects (Qihe, Guoqing, Yingchun & Hui, 2006). Many previous researches reported the use of proteolytic enzyme for meat tenderization (Melendo et al., 1997; Ashie, Sorensen & Nielsen, 2002, Wada et al., 2002; Naveena & Mendiratta, 2001; Naveena, 2004; Naveena et al., 2004; Pawar, Mule & Machewad, 2007).

Therefore, regarding to the utilization of pineapple wastes, bromelain extraction and characterization could be used as an alternative solution to success the target.

1.2 Objectives

1.2.1 To determine the potential source of pineapple wastes for bromelain extraction

1.2.2 To extract and isolate the bromelain from pineapple wastes by using aqueous two phase system

1.2.3 To determine the biochemical characteristics of bromelain

1.2.4 To study the effect of the bromelain extract in different types of muscle food samples

1.3 Scope of the research

The experiment covered with pineapple wastes selection, bromelain extraction, isolation and characterization. Application of bromelain extract for muscle food tenderization also determined.

For pineapple waste selection, different parts of pineapple wastes (peel, core, stem and crown) from *Nang Lae* and *Phu Lae* cultivars were used as staring material for bromelain extraction. The waste part that gave the largest waste portion and an acceptable bromelain activity was selected for the next investigation. Extractants including distilled water, distilled water with 15mM cysteine and 2mM EDTA, 100mM sodium phosphate buffer pH 7.0 and 100 mM sodium phosphate buffer pH 7.0 with 15mM cysteine and 2mM EDTA were used as a medium for extract bromelain from the selected waste. The extractant that provided the highest bromelain activity was selected for next step investigation. Aqueous two phase system comprising of different molecular weight and concentrations of polyethylene glycol (PEG: MW 2000, 4000, 6000; 12%, 15%, 18%, w/w) and types and concentrations of salts (ammonium sulfate, magnesium sulphate, potassium hydrogen phosphate; 14%, 17%, 20%,w/w) on bromelain partitioning were investigated. Biochemical properties (protein content, bromelain activity, pH profile and stability, thermal profile and stability, salt stability, protein patterns, activity staining and collagen hydrolysis) of bromelain extract were also determined. Application of bromelain extract in different muscle samples (beef, chicken, and squid) was done for testing the potential use of the bromelain extract. Physico-chemical properties and some consuming qualities properties of treated samples were determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Pineapple

Pineapple was originally native to South America where it was cultivated before the discovery of America. Columbus discovered the pineapple on the Island of Guadeloupe in 1493 and on other islands of the West Indies later on. The Spaniards spread the pineapple to nearly all tropical countries, including the Philippines in the 16th century, from where it was re-introduced into other Southeast and South Asian countries. It quickly became established in these countries and so widespread that it was considered indigenous. It was introduced into Thailand probably by the Portuguese during the reign of King Narai the Great of Ayutthaya during 1670-1700. Although the fruit is best eaten fresh, more pineapples are being processed for export in most pineapple-producing countries. It was only at the end of the 19th century, however, that pineapple was first put into cans. Since then it became a major international export commodity of these countries since it is among the few fruits most appropriate for industrial processing. The fruits are canned as whole, slices, spears, tidbits, chunks, chips, cubes or crushed. The flesh adhering to the skin after peeling is scraped and made into crush or juice. The latter can also be made by crushing the peel, core and pieces of flesh which cannot be used as specified cuts. Pieces may be stuffed inside de-seeded rambutan fruit and canned in light syrup. Together they provide a unique blend of tastes and also a unique product of Thailand.

At present, with the 2 million tons of fresh pineapple produced each year, Thailand is the World's largest producer of fresh pineapple as well as canned pineapple and pineapple juice (Tables 2.1 and 2.2).

Annually, Thailand exports 3,000 tons of fresh pineapple, 510,000 tons of canned pineapple, and 151,000 tons of pineapple juice with the value of 31, 15,000 and 6,500 million baht, respectively. Export of other processed products accounts for 690,000 tons with the value of Baht 23,100 million. As such, Thailand is the World largest exporter of canned pineapple and pineapple juice, satisfying 40-45% of the World demand (MOAC, 2009).

Table 2.1 World Pineapple Production in 2007

Country	Production (tons)	Production ($1000)
Thailand	2,815,275	544,446
Brazil	2,676,417	517,592
Indonesia	2,237,858	432,779
Philippines	2,016,462	389,963
Costa Rica	1,968,000	380,591
China	381,901	268,195
India	1,308,000	252,954
Nigeria	900,000	174,051
Mexico	671,131	129,790
Viet Nam	470,000	90,893

From Food and Agriculture Organization of the United Nations (2007)

Table 2.2 Export of Pineapple and Its Products

Items	2007		2008		2009	
	Quantity	**Value**	**Quantity**	**Value**	**Quantity**	**Value**
Fresh pineapple	2,826	51	3,613	45	2,740	31
Frozen pineapple	3,170	137	900	39	646	34
Dried pineapple	988	154	421	65	273	25
Sweeten pineapple	25,076	1,435	27,341	1,604	26,556	1,519
Canned pineapple	568,047	14,496	618,526	18,740	508,970	15,013
Pineapple juice	135,719	4,250	152,793	5,497	151,414	6,523
Total	**735,826**	**20,523**	**803,593**	**25,990**	**690,600**	**23,147**

Note. Units: Quality in tons, value in million Baht

From MOAC (2009)

2.1.1 Main cultivars in Thailand

There are 6 cultivars of pineapple available in around Thailand. Difference for each cultivar is presented in Figure 2.1.

2.1.1.1 Pattawia (*Smooth Cayenne* group)

Leaves are dark green with purple patches along the base of the upper surface; no spine along the margin. Large plant is bearing large cylindrical fruit (2-6 kg), with shallow ‘eyes’. Peel dark green or reddish yellow when ripe with orange yellow flesh. Core is large but not firm. This cultivar is sweet and lightly sour taste and produces 2-3 suckers but no slip. Pattawia is most popular both for fresh consumption and/or canning. This variety has a unique quality when grown in Thailand.

2.1.1.2 Nang Lae (*Smooth Cayenne* group)

Leaves are dark green with numerous spines along the margin. Medium-sized plant with obovate, medium-sized fruit and somewhat exposed ‘eyes’. Peel is dark green or yellowish yellow color. Flesh is yellow color, sweet and slightly sour taste. It is cultivated only in Chiang Rai province in northern Thailand for fresh fruit consumption.

2.1.1.3 Phuket (Queen group)

Leaves are narrower but longer than cultivars of the Cayenne group, light green with red stripes along the midrib. Along the margin are regularly-arranged red spines. Fruit is small (0.5-1.0 kg), ovate, with characteristic bulging ‘eyes’ with long fruit stalk. Peel is brownish yellow. Flesh is bright yellow, sweet and slightly sour taste, with mild aroma, and crunchy texture. This cultivars produces few slips, but lots of suckers. It is only use for fresh consumption and mainly grown in Phuket province, especially as intercrop in rubber plantation during the first few years of their growth.

2.1.1.4 Phetchaburi (Queen group)

A clonal selection of an introduced cultivar, ‘Tainan 41’ from Taiwan, it is typical of the Queen group, similar to ‘Phuket’ and ‘Sawi’, having bulging ‘eyes’. The characteristic feature is having spines along the rim of the leaf. The plant is compact, producing ground sucker, aerial sucker and slip. It produces more slip than ‘Phuket’ and ‘Sawi’ cultivars. Fruit is oblong, 1 kg in weight. Fruit is rind and flesh turn orange yellowish. It can be harvested 17 days earlier than ‘Phuket’ and ‘Sawi’ cultivars, having the period from flower induction to fruit harvest of 126 days, with higher yield than both varieties. The flesh is sweet and slightly sour taste, crispy, and the fruitlets can be removed easily, making eating easier. It is commended to grow in all parts of the country, and can grow well even in rather dry condition, but it cannot withstand water-logging.

2.1.1.5 Sawi (Queen group)

Sawi is very similar to ‘Phuket’, except for having smaller fruit. It is grown mainly in Sawi district of Chumphon province, especially as intercrop in coconut and other fruit orchards.

2.1.1.6 Trat Si Thong (Queen group)

A local cultivar is grown in Trat province, mainly for fresh consumption. It has yellow flesh with sweet and a little sour taste. It has 15.6-17.4°Brix and acid content of 0.6-0.8%. It can be Harvested 146 days after flower induction. Its fruit weight is 1.0 -1.5 kg.

Pattawia

Nang Lae

Phuket

Phetchaburi

Sawi

Trat Sri Thong

From Horticultural Research Institute, Department of Agriculture and Horticultural Science Society of Thailand (2006)

Figure 2.1 Morphology of Thai Pineapples

2.1.2 Main cultivars in Chiang Rai province

2.1.2.1 Nang Lae

Nang Lae was cultivated only in Chiang Rai province in northern Thailand for fresh fruit consumption. Morphology is similar to Pattawia but more circle fruit, thinner peel and sweeter (Figure 2.2).

2.1.2.2 Phu Lae

Phu Lae Pineapple is Phuket cultivars that cultivated in the area of Nang Lae sub district and the name of Phu Lae derived from 'Phu' from Phuket and 'Lae' from Nanglae. The size of the pineapple is around 3-4 fruit per a kilogram, smaller and more convenient to carry than

Phuket and Nang Lae varieties (Figure 2.2). Phu Lae is yellow with sweet taste and crispy texture. Most important, it is now highly marketable. It has also been added to the Provincial (One Tumbon One Product; OTOP) list of Chiang Rai, and is served on Thai Airways flights.

Figure 2.2 Nang Lae and Phu Lae Pineapples Morphology

Table 2.3 Nutrient of 100 grams of Raw Pineapple

Nutrient	Value	Units	Nutrient	Value	Units
Proximates			Minerals		
Water	86.00	g	Calcium, Ca	13.00	mg
Energy	50.00	kcal	Iron, Fe	0.29	mg
Energy	209.00	kJ	Magnesium, Mg	12.00	mg
Protein	0.54	g	Phosphorus, P	8.00	mg
Total lipid (fat)	0.12	g	Potassium, K	109.00	mg
Ash	0.22	g	Sodium, Na	1.00	mg
Carbohydrate (by difference)	13.12	g	Zinc, Zn	0.120	mg
Fiber, total dietary	1.40	g	Copper, Cu	0.110	mg
Sugars, total	9.85	g	Manganese, Mn	0.927	mg
Lactose	0.00	g	Thiamin	0.079	mg
Sucrose	5.99	g	Selenium, Se	0.100	µg

Table 2.3 (continued)

Nutrient	Value	Units	Nutrient	Value	Units
Glucose (dextrose)	1.73	g	Vitamins		
Fructose	2.12	g	Vitamin C, total ascorbic acid	47.80	mg
Lactose	0.00	g	Thiamin	0.079	mg
Maltose	0.00	g	Riboflavin	0.032	mg
Galactose	0.00	g	Niacin	0.500	mg
Starch	0.00	g	Thiamin	0.079	mg
Lipids			Pantothenic acid	0.213	mg
Fatty acids, total saturated	0.009	g	Vitamin B-6	0.112	mg
4:0	0.000	g	Folate, total	18.00	µg
6:0	0.000	g	Folic acid	0.00	µg
8:0	0.000	g	Folate, food	18.00	µg
10:0	0.000	g	Folate, DFE	18.00	µg
12:0	0.000	g	Choline, total	5.50	mg
14:0	0.000	g	Betaine	0.10	mg
16:0	0.005	g	Vitamin B-12	0.00	µg
18:0	0.003	g	Vitamin B-12, added	0.00	µg
Fatty acids, total monounsaturated	0.013	g	Vitamin A, RAE	3.00	µg
16:1 undifferentiated	0.001	g	Retinol	0.00	µg
18:1 undifferentiated	0.012	g	Carotene, beta	35.00	µg
20:1	0.000	g	Carotene, alpha	0.00	µg
22:1 undifferentiated	0.000	g	Cryptoxanthin, beta	0.00	µg
Fatty acids, total Polyunsaturated	0.040	g	Vitamin A, IU	58.00	IU
18:2 undifferentiated	0.023	g	Lycopene	0.00	µg
18:3 undifferentiated	0.017	g	Lutein + zeaxanthin	0.00	µg

Table 2.3 (continued)

Nutrient	Value	Units	Nutrient	Value	Units
18:4	0.000	g	Vitamin E (alpha tocopherol)	0.02	mg
20:4 undifferentiated	0.000	g	Vitamin E, added	0.00	mg
20:5 n-3 (EPA)	0.000	g	Tocopherol, beta	0.00	mg
22:5 n-3 (DPA)	0.000	g	Tocopherol, gamma	0.00	mg
22:6 n-3 (DHA)	0.000	g	Tocopherol, delta	0.00	mg
Cholesterol	0.000	mg	Vitamin D (D2 + D3)	0.00	µg
Amino acids			Vitamin D	0.00	IU
Tryptophan	0.005	g	Vitamin K (phylloquinone)	0.70	µg
Threonine	0.019	g	Theobromine	0.00	mg
Isoleucine	0.019	g	Amino acids		
Leucine	0.024	g	Alanine	0.033	g
Lysine	0.026	g	Aspartic acid	0.121	g
Methionine	0.012	g	Glutamic acid	0.079	g
Cystine	0.014	g	Glycine	0.024	g
Phenylalanine	0.021	g	Proline	0.017	g
Tyrosine	0.019	g	Other		
Valine	0.024	g	Alcohol, ethyl	0.00	g
Arginine	0.019	g	Caffeine	0.00	mg
Histidine	0.010	g	Theobromine	0.00	mg
Serine	0.035	g			

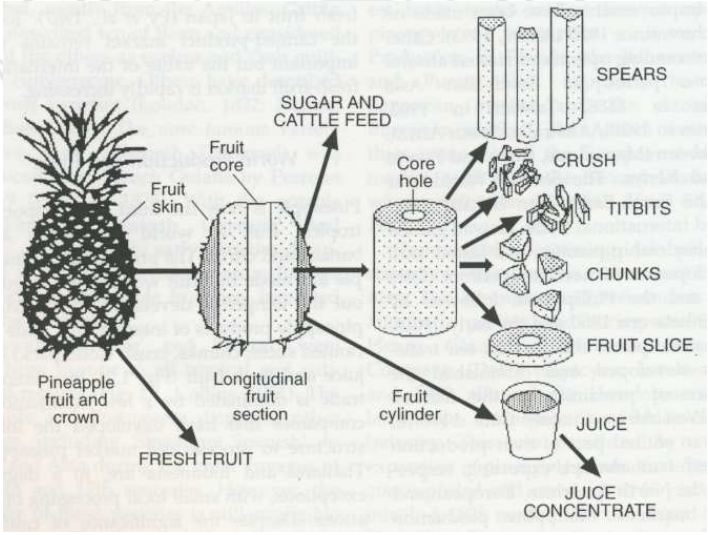
From USDA's National Nutrient Database for Standard Reference (2009)

2.2 Pineapple processing and products

Various forms of processed pineapple are produced in Thailand, ranging from canned products (in light/heavy syrup), juice (ready-to-drink and concentrated), frozen, dried (dehydrated and sweetened by sugar or honey, salted), jam, puree, candy, fruit paste, boiled in syrup, expressed juice from whole fruits, pineapple wine and sweets. Some pineapple products were shown in Figure 2.3. For large factories, especially canneries (a total of 26 exist in Thailand), fruits are obtained mostly through contract growers, either directly or through middlemen. Post-harvest operations of freshly harvested fruits which are carried out in the field consist of removing the crown and fruit stalk then loaded onto trucks (mostly pick-up trucks owned by growers). Upon arrival at the factory, the fruits are randomly inspected for nitrate residue, not exceeding the allowable limits. They are then graded according to size and quality. In the normal season, a large portion of about 5-7% is rejected since they are too small, too large, infected with marbling, bruised or spoiled. Selected fruits are passed through conveyor belts to be cleaned by water spray to remove dust and soil attached to fruits. The top and bottom ends are cut off by automatic cutting machine, then passed through automatic peeling machine which also remove the core in the same operation. The outer peel is removed twice, the flesh attached to the peel is of the highest quality and used as crushed pineapple. The cores are crushed to extract juice. Peeled and de-cored, the whole fruits with hollow center are passed through the conveyor for workers to select and provide the final touches, e.g. cut off the parts to which the eyes and peel still remain or remove those that are bruised or spoiled. The fruits are then passed through automatic slicing machine. The slices are graded according to the color and flesh quality into fancy, choice and standard grades. The rest may be used as half-slices, quarter slices, triangular pieces, cubes and tidbits as shown in Figure 2.4. Small pieces are crushed and canned as crunch. Different grades are packed into cans of different sizes and filled with syrup, sterilized and sealed. Finally, after cooling and dried, the cans are labeled and packed in cartons ready for shipment. Canned pineapple processing was shown in Figure 2.5.

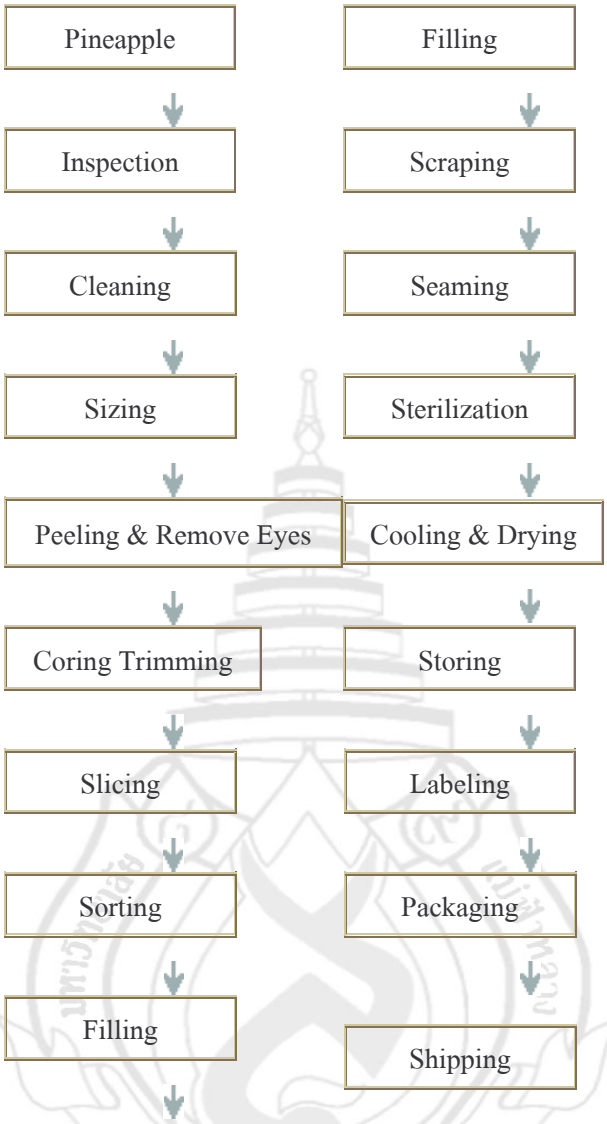
From HRI, DOA and HHST (2006)

Figure 2.3 Pineapple Products in Thailand



From Bartholomew et al. (2002)

Figure 2.4 Products from Pineapple



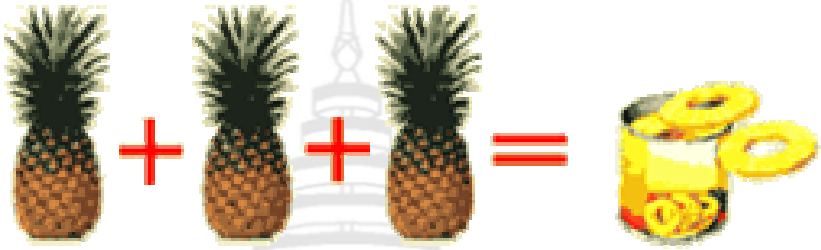
From MOAC (2009)

Figure 2.5 Production Process of Canned Pineapple

2.3 Wastes generation and utilization

Due to increasing demand by consumers, pineapple wastes generated from processing is proportionally increasing. In pineapple industry, it was found that around three pineapple fruits are required in the production of only a can of canned pineapple (Figure 2.6) (MOAC, 2007). Besides, pineapple wastes namely peel and core account for about 40% of total pineapple weight were generated in the large amount after fresh cut processing in Chiang Rai (~ 24 tons per year)

(DOAE, 2009). Hence, disposal represents a growing problem since the plant material is usually prone to microbial spoilage, thus limiting further exploitation. Wastes of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of compounds which may be used because of their favourable technological or nutritional properties (Table 2.4, 2.5).



From MOAC (2007)

Figure 2.6 Pineapple Usages in Canned Pineapple Production

Table 2.4 Mean Chemical Composition of Pineapple Cannery Wastes

Chemical constituent	Concentration (g/L)	Chemical constituent	Concentration (g/L)
Total sugars	82.53 ± 0.78	Protein	6.40 ± 0.33
Reducing sugars	39.46 ± 0.60	Fat	1.20 ± 0.17
Glucose	22.70 ± 0.85	Kjeldahl nitrogen	2.32 ± 0.15
Sucrose	38.70 ± 1.12	Total solids	50 – 60
Fructose	15.81 ± 0.83	Microbial count	$10^2 – 10^4$ ml^{-1}
Raffinose	2.62 ± 0.27	pH	4.0 ± 0.08
Galactose	2.85 ± 0.33		

From Nigam (1999)

The waste is occasionally utilized as fertilizer or animal feed particularly in the summer when forage is scarce however, it is still very low in value. Pineapple wastes are found to have potential uses as raw materials that can be converted into value-added products. The peel is a rich source of cellulose, hemicelluloses and other carbohydrates. It has been used to produce paper, banknotes, and cloth (Bartholomew et al., 2003). The peel and core wastes could be used for the production biogas (methane) (Bardiya, Somayaji & Khanna, 1996; Rani & Nand, 2004). Correia, McCue, Magalhães, Macedo & Shetty (2004) studied phenolic antioxidants production by the solid-state bioconversion of pineapple waste mixed with soy flour using *Rhizopus oligosporu.* Pineapple core used for the production of frozen pineapple juice concentrates or extracted juice for alcoholic beverages or for vinegar (Pukrushpan, 1985). In addition, the juice from pineapple wastes has been used as a nutrient substance in culture broth (Nigam, 1998) and cellulose production (Omojasola et al., 2008).

Table 2.5 Characteristics of Pineapple Wastes

Chemical composition	**Concentration (g/100 g)**	**Chemical composition**	**Concentration (g/100 g)**
Total soluble solid °Brix	7.80	Cellulose	19.80
Volatile solids	89.40	Hemicellulose	11.70
Ash	10.60	Total solubles	30.00
Organic carbon	51.85	Total nitrogen	0.95
Total carbohydrates	35.00	C/N ratio	55:1

From Bardiya et al. (1996)

Moreover, the pineapple wastes have also been used as substrates for the production of ethanol (Tanaka et al., 1999; Nigam, 1999), citric acid (Kumar et al., 2003), antioxidant compounds (Schieber et al., 2001), bioprotein (Jamal et al., 2009) Furthermore, pineapple wastes can be used for proteolytic enzyme extraction for utilization in food cosmetic medical and

pharmaceutical industries (Rolle, 1998; Maurer, 2001; Umesh et al., 2008). Among of them, pineapple peel also seemed to be a potential source for bromelain extraction due to the large amount of waste after processing.

2.4 Proteolytic enzyme

Proteolytic enzymes, also called proteases, are the enzymes that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins. The term 'protease' refers to all enzymes that hydrolyse peptide bonds.These enzymes are widely distributed in nearly all plants, animals and microorganisms. Other names include peptidase and peptide hydrolase. This group of enzymes can be subdivided into exopeptidases and endopeptidase for exo-acting and endo-acting patterns. Endopeptidase is used synonymously with protenase (Belitz & Grosch, 2000; Naz, 2002).

2.4.1 Classification of proteases

The standard method of classification proposed by the Enzyme Commision (EC) of the International Union of Biochemists (IUB), is based on the mode of catalysis. Proteases are classified into four groups according to the catalytic residues involved in the nucleophilic attack at the carbonyl carbon of the scissile bond (Table 2.6).

Table 2.6 Protease Group

Group	Example
Serine protease	Trypsin, Chymotrypsin, elastase, subtilin. Protenase K
Cysteine protease	Papain. Ficin, bromelain, procerain
Aspartic protease	pepsin, rennin (chymosin), aspartic protease from bacterial protease
Metallo protease	Collagenase, elasetase, thermolysin

From Walsh (2002)

The four groups are (1) the serine protease (EC. 3.4.21), 2) the sulhydryl protease (the name thiol proteases and cysteine protease are also used), (EC. 3.4.22), 3) the aspartic proteases (the name carboxyl protease and acidic proteases are also used) (EC. 3.4.23), and 4) the metal-containing protease (metallo proteases) (EC 3.4.24). The names indicate one of the key catalytic groups in the active site (Belitz & Grosch, 2009; Naz, 2002).

2.4.1.1 Serine proteases

The serine proteases include the chymotrypsin family, trysin family and the subtilisin family. Chymotrysin and trypsin are formed in the digestive tract by autocatalytic reactions in which trypsinogen is hydrolyzed to trypsin by action of trypsin itself. Unlike trypsin and chymotrypsin, which are mammalian enzymes, subtilisin is a group of alkaline serine proteases secreted by species of *Bacillus.* Alcalase is produced by *Bacillus licheniformis* and is the protease that is produced in the largest amounts. Since all of these enzymes consist in common of catalytic triad of Ser-His-Asp, therefore the general features of catalysis by this group of enzymes are identical; only the specific groups involved in binding of substrate are different which results in the different specificities. Chymotrypsin catalyses the hydrolysis of peptide bonds in which the peptide carbonyl group is contributed by aromatic residues like phenylalanine, tyrosine or tryptophan while trypsin catalyses the hydrolysis of peptide bonds in which the peptide carbonyl group is contributed by lysine or arginine. Alcalase has a broad specificity and cleaves many types of peptide bonds, preferentially those with a hydrophobic side chain on the carbonyl side. (Naz, 2002)

These enzymes have in common the presence of a serine and a histidine residue in their active sites. Typical representatives from animal sources are trypsin, chymotrypsin, elastase, plasmin and thrombin. Serine proteinases are produces by a great number of bacteria and fungi. Dahlmann et al. (1985) reported serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11.

2.4.1.2 Cysteine protease

The sulfhydryl proteases (also called the thiol protease and cysteine protease) have in common the ability to hydrolyze the peptide bonds and their inhibition by sulfhydryl reagents. The group of enzymes includes the higher plant enzymes, papain (EC. 3.4.4.10) from papaya, ficin (EC. 3.4.4.12) from fig, and bromelain (EC.3.4.4.24) from pineapple, the microbial enzymes

Streptococcus protease (*Streptococcus* cysteine proteinases, EC 3.4.22.10), several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. The enzyme is exceptionally stable to high temperatures at neutral pH, but denatured at acidic pH< 4. The optimum pH of papain is 5.5-7.0 and its pI is 9.6. It is also resistant to denaturaturaion in concentrated (8M) urea solution at neutral pH range. Papain retains its activity in various organic media. In fact, the enzyme is extensively investigated for organicsysthesis of peptides and other chemical compounds. The high stability of papain toward various extreme conditions and its availability at affordable cost has made it widely used as a meat tenderizer and in chill haze prevention in the brewery industry (Whitaker, 1994; Naz, 2002).

2.4.1.3 Aspartic proteases

The group name aspartic protease indicates that carboxyl groups of two aspartic acid residues are the catalytic groups in the active sites. This group of enzymes is also referred to as carboxyl protease and acid protease since the pH optima around pH 2-to 4. The best studied of this group are animal origin enzymes such as pepsin and rennin active in pH range of 2-4. Pepsin is formed by an autocatalytic reaction from pepsinogen, which is found in the stomach mucosa of animals. Pepsin is a composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35,000. Its tertiary structure is stabilized in part by three disulphide bridges and phosphate linkage. The phosphate group, attached to hydroxyl group of seryl residue, can be removed without loss of enzymatic activity. Rennin is the other most useful enzyme commercially because of its wide use in cheese production. cathepsin D which has a pH optimum between 3 and 5 depending on the substrate and on the source of the enzyme. Chymosin (previous name rennin) is the most useful commercially because of its wide use in cheese production. A large number of microbial proteases also belong in this group Among this group are three fungal protease from *Endothia parasitica*, *Mucor pusillus* and *M. miehei*, which are good chymosin substitutes. This group of protease is inhibited by pepstatin (Whitaker, 1994; Naz, 2002).

2.4.1.4 Metallo proteases

The metallo proteases include enzymes from a variety of origins, such as collagenases from higher organisms, toxins from snake venoms, and thermolysin from bacteria. They differ widely in sequence and structure, but the great majority contain a zinc atom which is catalytically active (Barret, 1995). Thermolysin, which is produced by *Bacillus stearothermophillus*,

is the most hat stable of the commercially available proteases. In the presence if Ca^{2+} it is only slowly inactivated, even at temperature near 80 °C. Its pH optimum is slightly alkaline (pH 7-9). Like other metalloproteases, thermolysin is inhibited by chelating agents such as EGTA, citrate and phosphate, which is of concern in food uses. With respect to peptide bond specificity, thermolysin preferentially hydrolyzes peptide bonds with a hydrophobic side chain on the amino side. The molecular mass of thermolysin is 34 kDa. Astacin is one example of metallo protease found the freshwater crasyfish *Astacus astacus* (Whitaker, 1994; Naz, 2002).

2.5 Proteolytic enzyme applications

Enzymes exhibiting proteolytic activities constitute the single most important group of proteins produced in bulk quantities. Such protease finds application in a multitude of industrial applications, some of which are listed in Table 2.7. Proteolytic rave long been used, often unknowingly, in processes such as brewing, baking and cheese-making. Such enzymes have also found important application in the tanning industry and in medicine (Walsh, 2002).

Many of the proteolytic enzymes traditionally used in industrial processes were obtained from plant or animal sources. The widespread incorporation of microbial proteases in detergent powders now renders microorganisms the major producers of these enzymes. Microbial proteases used industrially are generally extracellular in nature and are produced on a large scale by either semisolid or submerged fermentation. Incorporation into detergent preparations represents by far the single largest application of such “industrial” proteinases (Walsh, 2002).

2.5.1 Proteolytic enzymes of medical significance

A variety of proteolytic enzymes have found important medical applications (Table 2.7). In industrial terms, most such enzymes are produced in small to moderate quantities and are subject to significant downstream processing. Most, therefore, could not truly be described in the traditional sense as bulk industrial enzymes (Walsh, 2002).

2.5.2 Protease enzymes used in the brewing and baking industries

The cooling of beer after brewing often promotes haze formation. The haze is composed largely of protein, carbohydrate and polyphenolic compounds. Haze formation can be arrested by

addition of proteolytic enzymes to the beer. Although various microbial enzymes have been assessed, plant-derived proteases such as papain and bromelain are most commonly used for such purposes.

Fungal proteases also enjoy limited application in the baking industry. Such enzymes, generally source from *Aspergillus species*, are used in order to modify the protein components of flour, and thus alter the texture of dough. Gluten represents a major protein fraction of flour. It is a complex between two protein types: gliadin and glutenin. When flour is wetted during dough preparation gluten binds a portion of the water and expands to form a lattice-like structure. This promotes a resistance to dough stretching. The addition of low levels of a neutral fungal protease derived from *Aspergillus oryzae* results in a limited degradation of the gluten lattice, thereby reducing the dough's resistance to stretching. This better facilitates retention within the dough of CO_2 produced by yeast fermentation. In turn this influence pore structure of leavened bread and allows the dough to rise uniformly during baking (Walsh, 2002).

2.5.3 Protease used in the meat industry

Papain is often used as a meat tenderizing agent. The proteolytic preparation may be applied directly to the meat or it may be injected into the animal immediately prior to slaughter. This practice facilitates even distribution of the enzyme. The proteolytic activity renders meat tenderer by enzymatically degrading connective tissue collagen and elastin. It is primarily these components that render meat tough. This process may also be promoted naturally by storing fresh carcases in cold rooms for several days after slaughter. During this time various degradative enzymes are released as the integrity of cells is disrupted. This process is known aging (Walsh, 2002).

2.5.4 Proteases and leather production

Proteolytic enzymes are used extensively in the dehairing and bating of leather. Hair may be removed from animal hides by treatment with a combination of lime and sodium sulfide. Such chemicals, however, are unpleasant to work with and often give rise to problems of waste disposal. Proteolytic enzymes are often used as alternatives, either alone or (more usually) in combination with reduced concentrations of lime. The alkaline conditions generated by the lime renders it essential to employ alkaline proteases,such as subtilisin, in such a process.

Various proteolytic enzymes are also used in the bating of leather. The bating process renders the leather soft and pliable. Leather employed in the manufacture of gloves is highly bated, whereas shoe sole leather is not bated. Trypsin has traditionally been used in the bating process. More recently, microbial enzymes produced by various species of *Aspergillus* and *Bacillus* have become more popular in this regard. A range of proteases are employed during leather manufacture, including animal pancreatic proteases and microbial proteases (acidic, neutral and alkaline bacterial and fungal proteases). Application of plant-derived proteases (papain and bromelain) has been recorded, as has the use of carbohydrases and lipases, although these latter activities have not gained widespread application in the industry. The enzymes help degrade and hence remove unwanted skin components, but a secondary affect is the consequent reduction in chemical treatments required. This has environmental benefits as less chemical waste is generated (Walsh, 2002).

2.5.5 Protease incorporated into detergent products

The major quantities of proteases produced in industrial quantities are incorporated into detergents. Enzymes were first introduced into detergent preparations at the beginning of the twentieth century. Few such products were successful, as the enzymes chosen, generally from animal sources, were invariably inactivated by other components present in the detergent mix or by the ensuing washing process.

Most clothing becomes soiled by substances such as dyes, biological molecules, soil and miscellaneous particulate matter. Biological “dirt” includes protein, lipid and carbohydrate-based materials. Such dirt components may be derived directly from humans or animals, such as shedding of skin and blood, or may be derived from other sources such as foodstuffs or grass.

Alkaline proteases synthesized by a variety of additional bacteria have been studied in order to assess their potential application in detergent preparations. Activities produced by a variety of alkalophilic species of Bacillus have gained a particular importance in this regard. Like subtilisin, these enzymes are single-chain serine proteases. Many exhibit overall characteristics which render them as suitable as subtilisin Carlsberg in terms of applications in detergents (Walsh, 2002).

2.5.6 Proteases used in cheese manufacture

Rennin, also termed chymosin, represents another proteolytic enzyme subject to considerable industrial demand. This protease finds application in the cheese manufacturing process. The initial step in cheese-making involves the enzymatic coagulation of milk. Rennin catalyses limited proteolytic cleavage of milk kappa casein. This destabilizes casein micelles and promotes their precipitation, thus forming curds. The remaining liquid or whey is removed and the curd is further processed, yielding cheese or other dairy products.

Rennin is obtained from the fourth stomach of suckling calves. Extraction often involves prolonged treatment of dried strips of the calf stomach with a salt solution containing boric acid. The enzymatic extract obtained, rennet, contains a variety of proteolytic and other enzymatic activities. Rennin accounts for no more than 2-3% of such preparations (Walsh, 2002).

2.5.7 Additional protease applications

Proteases are used in smaller quantities for a variety of additional applications. Such uses include the cleaning of contact lenses and the removal of unwanted body hair. During use contact lenses adsorb various solutes present in tear fluid, necessitating their regular cleaning. The major solutes present in tear fluid which adsorb onto contact lenses include proteins (largely the aforementioned antibodies and lysozyme), mucins (high molecular mass glycoproteins which have a lubricant function), and lipid. Standard lens cleaning agents are at best modestly successful in removing such substances, hence deposit build-up can occur on the lens' surface, reducing its transparency and rendering it uncomfortable to wear. Proteases or a combination of proteases and lipases have proven most effective in removing such deposits and such enzyme-containing cleaning solutions are now commercially available. The cleaning process generally entails immersing the contact lenses in the enzyme containing fluid for several hours (preferably overnight), followed by extensive rinsing of the lens with saline. The rinsing step is essential to physically remove the enzyme breakdown products, as well as any active enzyme present on the lens surface before it is placed back in the eye.

The plant protease papain has also found limited application in slowing or preventing hair regrowth on the body and legs. Removal of unwanted body hair can be undertaken chemically, or by waxing, tweezing or shaving. It has been shown that regular application of a papain-containing solution to the cleared area slows or prevents hair regrowth. The papain

enzymatically degrades the growing hair (i.e. α-keratin) and loosens the hair follicle. It also likely helps degrade dead surface skin cells, supposedly leaving the skin softer and smoother. Because of its non-human origin papain can provoke an allergic response in some people, and its initial application to a small area of the skin is encouraged, (to test for sensitivity) before it is applied to large surface areas (Walsh, 2002).

Table 2.7 Application of Proteases in Food and Industries Technology

Application	Function	Protease	Source
Detergent manufacturer	To catalytically degrade protein-based stains on clothing	-Microbial protease	Bacteria, fungi
Leather bating	To remove wool, hair, and pigments; to soften skins	-Microbial protease	Bacteria, fungi
Brewing	To solubilize grain protein; to stabilize beer To spilt protein in malt	-Microbial protease -Papain	Bacteria, fungi Plant (papaya fruit)
Cheeses making	To coagulate milk proteins, forming curds, to ripen cheese	-Microbial protease -Rennin	Bacteria, fungi, Animal (stomach of unweaned calves); also recombinant
Debriding agent		Trypsin Papain Collagenase	Animals Plant (papaya fruit) Bacteria
Digestive aid: baby food, animal feed		Trypsin Pepsin Papain Bromelain	Animals Animals Plant (papaya fruit) Plant (pineapple)
Meat tenderization	To partially separate connective tissues	Papain Bromelian Ficin	Plant (papaya fruit) Plant (pineapple)

Table 2.7 (continued)

Application	Function	Protease	Source
Anti-inflammatory agent		Bromelain	Plant (pineapple)
Baking Bread/confectionery Cookies and crackers	To modify gluten elasticity To improve crispness	Fugal protease	Fungi
Protein hydrolysate: Soy protein hydrolysate, milk and whey protein casein hydrolysate, fish protein hydrolysate	To produce protein hydrolysate and enhance flavor	Protease Alcalase and neutrase	Fungi Microbial
Cosmetics	To remove stratum corneum of skin	Papain Bromelain	Papaya Pineapple

From Uhlig (1998); Walsh (2002); Aehle (2007)

2.6 Bromelain

Bromelain, the proteolytic enzyme, is found in the tissues of plant family *Bromeliaceae* of which pineapple (*Ananas comosus* L. Merryl) is the best known source. Cysteine proteinases (bromelain) from pineapples were shown in Table 2.8. The stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) obtained from the pineapple stem and pulp, respectively (Rowan et al., 1990). Bromelain is reported to be present also in pineapple wastes such as core, peel, and leaves in relatively smaller quantities as compared to stem and fruit (Umesh et al., 2008) had been reported to be present in pineapple wastes such as core, peel, and leaves, besides in fruit and stem (Sriwatanapongse et al., 2000; Maurer 2001; Umesh et al., 2008).

Table 2.8 Cysteine Proteinases (Bromelain) from Pineapples (*Ananas comosus*)

Name (EC number)	Molecular mass (Dalton)	Isoelectric point	Sequences	Glycosylation
From pineapple stem:				
Stem bromelain (EC 3.4.22.32)	28,300 (sequence + sugar)	> 10	Completely (sequenced 212 amino acids)	glycosylated
Ananain (EC 3.4.22.31)	23,464 (sequence)	> 10	Completely (sequenced 216 amino acids)	non glycosylated
Comosain	24,509 and 23,569 (esms)	> 10	N term. sequence	glycosylated
	23,550 and 23,560 (esms)	4.8 and 4.9	N term. sequence	highly glycosylated
From pineapple fruits:				
Fruit bromelain (EC 3.4.22.33)	23,000	4.6	N term. sequence	non glycosylated

From Maurer (2001)

2.7 Bromelain Application

Bromelain, a proteolytic enzyme, had been reported to be present in pineapple wastes such as core, peel, and leaves, besides in fruit and stem (Umesh et al., 2008). The application of bromelain was shown in Table 2.9. It has been used for meat tenderization (Melendo, Beltrin, Jaime, Sancho & Roncalks, 1996; Naveena et al, 2004), beer clarification (Walsh, 2002), protein hydrolysates production in fish sauce, and oyster sauce (Uhlig, 1998; Chuapoehuk et al., 1992) and preventing of apple juice browning (Tochi et al., 2009). Furthermore, there was reported of novel use of bromelain as hydrolyzing agent for release of antimicrobial peptides of leatherjacket (*Meuchenia* sp.) insoluble proteins (Salampessy et al., 2010). In addition, it is known for clinical

and therapeutic applications, particularly for modulation of tumor growth, third degree burns, and improvement of antibiotic action and as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases (Maurer, 2002). In cosmetics, bromelain used as active ingredient in western-face and body care products to provide gentle peeling effect (removing stratum corneum cell) (Aehle, 2007). Other industries bromelain used for skins pre-tanning, softening and bating in leather industries, improve the dyeing properties of protein fibers (Koh et al., 2006), decomposing or partially solubilizing protein fiber from silk and wool (Singh, 2003).

Table 2.9 Bromelain Applications

Industry	Application	References
Food	Meat tenderization	
	-Coarse dry sausage, squid	Melendo et al., 1996
	-Beef	Wada et al., 2002
	-Buffalo meat, spent hen meat	Naveena et al, 2004
	-Goat meat (chevon)	Pawar et al., 2007
	Prevention of apple juice browning	Tochi et al., 2009
	Protein hydrolysate production in oyster sauce	Chuapoehuk et al .1992
Cosmetics	Ingredient in active ingredient in western-face and body care products	Aehle, 2007
Medical and pharmaceutical	Anti-inflammatory agent, debriding agent, digestive aid, anti cancer agent	Maurer, 2001 Tochi et al., 2009 Chobotova et al., 2010
Other industry	improve the dyeing properties of protein fibers	Koh et al, 2006
	decomposing or partially solubilizing protein fiber from silk and wool	Singh, 2003

2.8 Protein extraction and purification

In order to isolate intracellular proteins, cells must be disrupted. Several disruption techniques, both mechanical and chemical, are available (Table 2.10). An efficient protocol for cell disruption must be developed to release the protein in a soluble form from its intracellular compartment. The disruption protocol should be as gentle as possible to see protein, as the extraction step is the starting point for all subsequent procedures. The success of cell disruption depends on a number of variables, such as the choice of buffers, the presence of protease inhibitors, and the osmolarity of the resuspension buffer. The condition and the constituent of the extraction buffer depend on the nature of the cell type, the target protein, and its intended application.

Proteins are present within the cell in a reducing environment at a relatively high concentration. After extraction the cellular proteins are disgorged into an oxidizing buffer which also dilutes the protein several-fold. The buffer is present to facilitate successful extraction of the target protein, but at the initial stages of purification the excess water can be viewed as a major contaminant. This concentration step may also have the benefit of removing low-molecular-weight inhibitors from the target protein.

After extraction and clarification the next step in a purification schedule is usually the concentration of the extracted protein. In addition, concentration steps may also be required throughout the purification schedule, for example prior to a chromatographic step or prior to electrophoresis and there are several techniques that can be used as shown in Table 2.11.

There is a wide variety of techniques which can be used in a purification procedure, the most popular of these techniques being covered as following:

1. Charge

The techniques which exploit the charge on protein surface include ion exchange chromatography (JEX), chromatofocusing, hydroxyapatite chromatography (HA), nondenaturing polyacrylamide gel electrophorests (PAGE) and preparative isoelectric focusing (IEP).

2. Hydrophobicity

The techniques which exploit the hydrophobic character of a protein include; hydrophobic interaction chromatography (i-TIC) and reversed phase chromatography.

3. Biospecificity

The techniques which exploit some aspects of biospecificity, post-translational modification or engineering include; affinity chromatography, covalent chromatography, immunoaffinity chromatography and immobilized metal affinity chromatography (IMAC).

4. Molecular mass

The techniques which exploit the molecular mass of a protein include: size exclusion chromatography (SEC), ultrafiltration and denaturing (in the presence of the detergent sodium dodecyl sulfate (SDS)) polyacrylamide gel electrophoresis (SDS-PAGE) (Bonner, 2007).

Table 2.10 Various Cell Lysis Techniques

Techniques	Principle	Time of lysis	Sample
Enzyme digestion	Digestion of cell wall leading to osmotic disruption of cell	15-30 min	Gram positive bacteria
Osmotic shock lysis	Osmotic disruption of cell membrane	< 5 min	Red blood cells
Hand homogenizer	Cells are forced through narrow gap leading to disruption of cell membrane	10-15 min	Liver tissues
Blade homogenizer	Large cells are broken by chopping action	5-10 min	Muscle tissue, animal tissue, plant tissue
Grinding with aluminum or sand	Cell walls are ripped off by micro roughness	5-15 min	Bacteria
Grinding with glass beads	Cell walls are ripped off by rapid vibration of glass	10-20 min	Bacteria
Fresh press	Cells are forced through small orifice at very high pressure. Shear forces disrupt cells.	10-30 min	Bacteria, plant cell

Table 2.10 (continued)

Techniques	Principle	Time of lysis	Example
Sonication	Cell disruption by shear forces and cavitation caused by high-pressure sound waves	5-10 min	Bacteria

From Ahmed (2005)

Table 2.11 Protein Extraction and Purification

Properties	Method	Size
Solubility	Change in pH	large
	Change in ionic strength	small-large
	Decrease in dielectric constant	large
Charge	Ion-exchange chromatography	small-large
	Electrophoresis	small
	Isoelectric focusing	small
Size and Molecular mass	Centrifugation	small-large
	Gel filtration	small
	Dialysis, Ultrafiltration	small
Biospecificity	Affinity chromatography	small
	Affinity elution	small-large

From Walsh (2002)

2.8.1 Precipitation

Protein precipitation can be promoted by agents such as neutral salts, organic solvents, high molecular mass polymers, or by appropriate pH adjustment. Concentration by precipitation is one of the oldest concentration methods known. Ammonium sulfate is likely the most common protein precipitant utilized. This neutral salt is particularly popular due to its high solubility, inexpensiveness, lack of denaturing properties towards most proteins, and its stabilizing effect on many proteins.

The addition of small quantities of neutral salts to a protein solution often increases protein solubility; the 'salting in' effect. However, increasing salt concentrations above an optimal level leads to destabilization of proteins in solution and eventually promotes their precipitation. This is known as 'salting out'. At high concentrations, such salts effectively complete with the protein molecules for water of hydration. This promotes increased protein-protein interactions, predominantly interaction between hydrophobic patches on the surface of adjacent protein molecules. Such increased protein – protein interactions eventually result in protein precipitation.

Addition of various organic solvents to a protein solution can also promote protein precipitation. Added organic solvents lower the dielectric constant of an aqueous solution. This in turn promotes increased electrostatic attraction between bodies of opposite charge in the solution, in this case proteins. Such increasing interactions between proteins of opposite charges eventually lead to their precipitation. Organic solvents frequently used to promote precipitation include ethanol, isopropanol , acetone, and diethylether. Protein precipitation by utilizing organic solvents must be carried out at temperatures at or below 0°C in order to prevent protein denaturation. As such solvents depress the freezing point of aqueous solution, it is usually feasible to maintain the solution temperature several degrees below 0°C.

Precipitation may also be promoted by the addition of organic polymers such as polyethylene glycol. The addition of such polymers however, often dramatically increases the viscosity of the resultant solution, making recovery of the precipitate more difficult. Recovery is normally achieved by centrifugation or by filtration. The precipitate can subsequently be redissolved in a smaller volume of resuspending liquid and, in this way, it is effectively concentrated. Purification of serum proteins is one example of an industrially important process

which traditionally included several protein precipitation steps. Precipitants normally used include both ethanol and ammonium sulfate. Most modern processes, however, rely on methods other than precipitation to achieve initial concentration of the proteins.

2.8.2 Ultrafiltration

Protein solutions may be quickly and conveniently concentrated by ultrafiltration and this method of concentration is the one most widely applied, both on a laboratory and industrial scale. As previously discussed, the technique of microfiltration is effectively utilized to remove whole cells or cell debris from solution. Membrane filters employed in the microfiltration process generally have pore diameters ranging from 0.1 to 10 μm. Such pores, while retaining whole cells and large particulate matter, fail to retain most macromolecular components such as proteins. In the case of ultrafiltration membranes, pore diameters normally range from 1 to 20 nm. These pores are sufficiently small to retain proteins of low molecular mass. Ultrafiltration membranes with molecular mass cut-off points ranging from 1-300 kDa are commercially available. Membranes with molecular mass cut-off points of 3, 10, 30, 50, and 100 kDa are most commonly used.

Traditionally, ultrafilters have been manufactured from cellulose acetate or cellulose nitrate. Several other materials such as polyvinyl chloride and polycarbonate are now also used in membrane manufacture. Such plastic-type membranes exhibit enhanced chemical and physical stability when compared to cellulose-based ultrafiltration membranes. An important prerequisite in manufacturing ultrafilters is that the material utilized exhibits low protein adsorptive properties. No matter which material is utilized the pore size obtained is not uniform, a point which must be emphasized. A range of pore sizes of wide deviation from the mean pore size are generally observed. The molecular mass cut-off point quoted for any such filter is thus best regarded as being a nominal figure. For protein work it is advisable to use a membrane whose stated molecular mass cut-off point is 5 kDa or more lower than the molecular mass of the protein of interest. It is also important to realize that the molecular mass cut-off point quoted applies to globular proteins. The overall shape of the protein of interest affects its ultrafiltration characteristics. If the protein is somewhat elongated, it may not be retained by an ultrafilter whose cut-off point is significantly lower. Extensive post-translational modifications, in particular glycosylation, may also affect ultrafiltration behavior.

Ultrafiltration is generally carried out on a laboratory scale using a stirred cell system. The flat membrane is placed on a supporting mesh at the bottom of the cell chamber, and the material to be concentrated is then transferred into the cell. Application of pressure, usually nitrogen gas, ensures adequate flow through the ultrafilter. Molecules of lower molecular mass than the filter cut-off pore size (e.g. water, salt and low molecular weight compounds) all pass through the ultrafilter, thus concentrating the molecular species present whose molecular mass is significantly greater than the nominal molecular mass cut-off point. Concentration polarization (the build-up of a concentrated layer of molecules directly over the membrane surface which are unable to pass through the membrane) is minimized by a stirring mechanism operating close to the membrane surface. If unchecked, concentration polarization would result in a lowering of the flow rate. Additional ultrafilter formats used on a laboratory scale include cartridge system, within which the ultrafiltration membrane is present in a highly folded format. In such case the pressure required to maintain a satisfactory flow rate through the membrane is usually generated by a peristaltic pump.

Large-scale ultrafiltration systems invariably employ cartridge-type filters. This allows a large filtration surface area to be accommodated in a compact area. Concentration polarization is avoided by allowing the incoming liquid to flow across the membrane surface at right angles, i.e. tangential flow. The ultrafiltration membrane may be pleated, with subsequent joining of the two ends to form a cylindrical cartridge. Alternatively, the membrane may be laid on a spacer mesh and this may then be wrapped spirally around a central collection tube, into which the filtrate can flow.

2.8.3 Diafiltration

Diafiltration is a process whereby an ultrafiltration system is utilized to reduce or eliminate low molecular mass molecules from a solution. In practice, this normally entails the removal of salt, ethanol and other solvents, buffer components, amino acids, peptides, added protein stabilizers or other molecules, from a protein solution. Diafiltration is generally preceded by an ultrafiltration step to initially reduce process volumes. The actual diafiltration process is identical to that of ultrafiltration except for the fact that the level of reservoir is maintained at a constant volume. This is achieved by the continual addition of solvent lacking the low molecular mass molecules which are to be removed. By recycling the concentrated material and adding

sufficient fresh solvent to the system such that five times the original volume has emerged from the system as permeate, over 99 per cent of all molecules which freely cross the membrane will have been removed from the solution. Removal of low molecular mass contaminants from protein solutions may also be achieved by dialysis or by gel filtration chromatography. Diafiltration, however, is emerging as the method of choice, as it is quick, efficient and utilizes the same equipment as used in ultrafiltration.

2.8.4 Chromatography

All chromatographic systems consist of two phases: stationary phase and mobile phase. The stationary phase may be a solid, gel, liquid, or a mixture of solid and liquid, whereas the mobile phase may be liquid or gaseous and flows through the stationary phase. All chromatographic purifications of proteins are based an equilibrium achieved between the stationary phase and the mobile phase. Most chromatographic systems require some common equipment. These are buffer reservoir, tubing, peristaltic pump, column, UV detector, chart recorder, and fraction collector. This technique can be used in both the early and later stages of a purification protocol (Table 2.12).

2.8.5 Protein/enzyme lyophilization

Lyophilization involves the drying of protein (or other materials) directly from the frozen state. This is achieved by firstly freezing the protein solution in a suitable, unstoppered, container. A vacuum is then applied and the temperature is increased in order to promote sublimation of the ice, which occurs under conditions of reduced pressure. The ice is drawn off directly as water vapour. The containers can be sealed following completion of the freeze drying process. Many freeze-dried proteins may be stored at room temperature for prolonged periods with little or no loss of biological activity. Some freeze-dried products, however, exhibit significant loss of activity if stored under such conditions, and thus must be stored at lower temperatures.

Many proteins sold commercially, in particularly high value, low volume products such as vaccines, therapeutic enzymes, hormones, antibodies and diagnostic reagents, are often marketed in freeze-dried from. The technique is also routinely used at a research laboratory scale. The average moisture content of a freeze-dried protein preparation is in the order of 3 per cent.

However, domains often exist within the product which contains much higher moisture content. This can contribute to product inconsistency.

The first step in the freeze-drying process involves freezing the protein solution in suitable containers, generally glass vials or flasks. As the temperature decreases, ice crystals begin to form. Such crystals contain only pure water molecules. As the ice crystals grow, the protein concentration, and the concentration of all other solutes present in the remaining liquid phase, steadily increases. Any solute species, such as salts, buffer components, other chemical additives or proteases present in the product are concentrated many-fold. The greater the solute concentration, the greater the reaction rate between such solutes. Proteins that are damaged by high concentrations of these solutes may be inactivated at this. Such inactivation may be due to chemical or biological modification of the protein, or may be caused by protein aggregation.

Table 2.12 Chromatographic Techniques Most Commonly Used in Protein Purification Protocols with Basis of Separation

Techniques	Basis of separation
Ion exchange chromatography	Differences in protein surface charge at a given pH
Gel filtration chromatography (Size exclusion chromatography)	Differences in mass or shape of different protein
Affinity chromatography	Based upon biospecific interaction between a protein and appropriate ligand
Hydrophobic interaction chromatography	Differences in surface hydrophobicity of protein
Chromatofocusing	Separate proteins on the basis of their isoelectric point
Hydroxyapatite chromatography	Complex interactions between proteins and the phosphate-based media.

From Walsh (2002)

2.9 Bromelain extraction and purification

There are many researches involved in the extraction and purification of bromelain from pineapple and pineapple wastes (Table 2.13). Normally, pineapple fruit, core and stem were cut into small pieces, triturated, filtered to obtain filtered crude juice and then subjected to further purification.

Table 2.13 Bromelain Extraction and Purification

Pineapple source	Techniques	References
Flesh	Precipitation with acetone, affinity chromatography	Rowan, 1990
	Semipermeable membrane, ammonium sulfate precipitation, centrifugation and freeze drying	Doko et al., 1991
	Microfiltration, ultrafiltration	Liang et al., 1999
Flesh	Aqueous two phase system	Rabelo et al., 2004
	Aqueous two phase system	Babu et al., 2008
	Expanded bed adsorption	Silveira et al., 2009
Core	Precipitation with ammonium sulfate, ion-exchange chromatography and spray-dried/freeze-dried	Devakate et al., 2009
Peel, core, stem and crown	Reversed micelles system	Umesh et al., 2008
Stem	Gel-filtration chromatography	Wharton, 1974
Stem	Gel-filtration chromatography	Arroyo-Reyna et al., 1995

2.10 Aqueous two phase system (ATPS)

Biphasic systems formed by mixing of two polymers or a polymer and a salt in water can be used for separation of cells, membranes, viruses, proteins, nucleic acids, and other biomolecules. The partitioning between the two phases is dependent on the surface properties and conformation of the materials, and also on the composition of the two-phase system. The

mechanism of partitioning is, however, complex and not easily predicted. Aqueous two-phase systems (ATPS) have proven to be a useful tool for analysis of biomolecular and cellular surfaces and their interactions, fractionation of cell populations, product recovery in biotechnology, and so forth. Potential for environmental remediation has also been suggested. Because ATPS are easily scalable and are also able to hold high biomass load in comparison with other separation techniques, the application that has attracted most interest so far has been the large-scale recovery of proteins from crude feedstock's. As chemicals constitute the major cost factor for large-scale systems, use of easily recyclable phase components and the phase systems generated by a single-phase chemical in water are being studied (Hatti-Kaul, 2001).

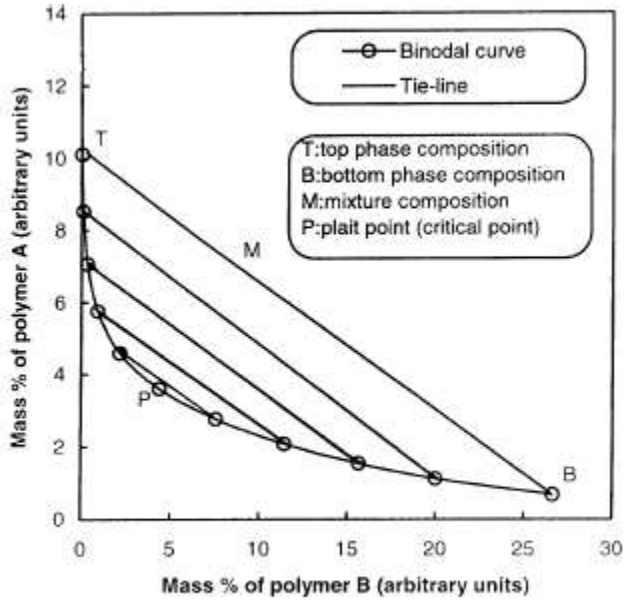
ATPE involves two phases that are basically formed by the addition of two or more water-soluble polymers or a polymer and low molecular weight solutes such as salts above their critical concentration (Figure 2.7). Both phases are basically hydrophilic but of varying degree. Due to this, these systems posses low interfacial tension. This characteristic makes them suitable for separation of labile biomolecules such as proteins, enzymes, nucleic acids etc.

ATPS has been used for the partitioning and recovery of various proteases such as bromelain, papain, trypsin, chymosin and pepsin, protease from fermentation broth and other protease as shown in Table 2.14.

2.10.1 Theory of aqueous two phase system

The simplest procedure of this technique is the one-step extraction. The phase system is prepared and the mixture to be separated is added. After mixing, phase separation is accomplished either by settling under gravity or by centrifugation. The phases are separated and analysed or used to recover the separated components of the initial mixture. The target product (e.g. particulate, biomolecule, cells) should be concentrated in one of the phases and the contaminants in the other. The theoretical yield in the top phase, Y_t, can be calculated in relation to the volume ratio of the phases, VR (volume top/volume bottom), and the partition coefficient K of the target biomaterial ($K = C_{top}/C_{bottom}$) (Equation 1):

$$Y_t = \frac{100}{1+(1+VR)(1/K)}(\%). \qquad \text{……..(1)}$$



From Sinha et al. (2000)

Figure 2.7 Phase diagram for two polymers in water forming an aqueous two-phase system

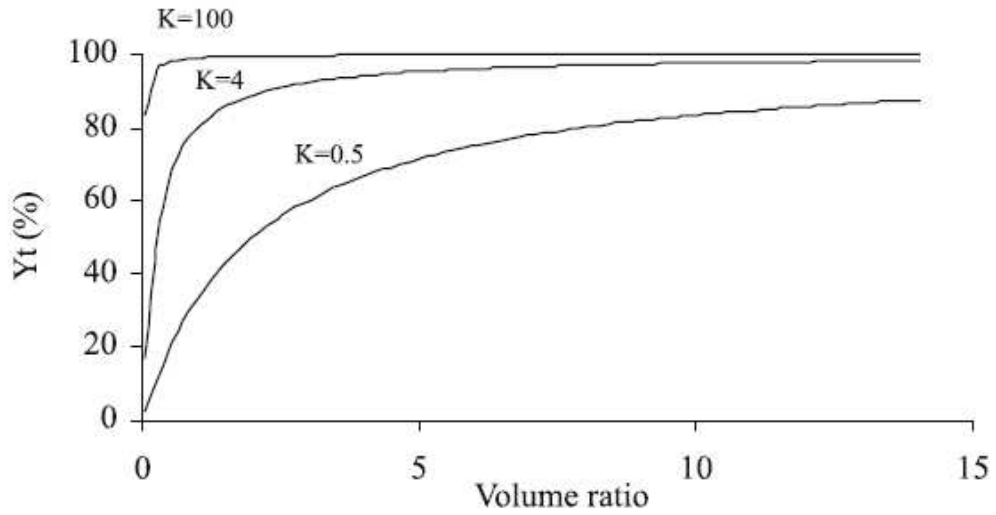
The theoretical concentration factor in the top phase, CF_t, of a biomaterial is defined as the ratio between the target biomaterial concentration in the top phase and the target biomaterial concentration in the input mixture. This can be given as a function of the theoretical yield, volume ratio and the weight percentage of media added to the separation system (Equation 2):

$$CF_t = \frac{Y_t}{100}\frac{\%media}{100}(1+1/VR). \qquad \text{.....(2)}$$

In Figure 2.8 and 2.9 the theoretical yield and concentration factor are depicted against the volume ratio for several *K* values of the target and total biomaterial, and different mixture loads, respectively. For high volume ratios, higher yields can be achieved. However, this is accomplished at a cost of increased dilution of the input mixture to be separated, lower purification factors and less usage of the chemicals per unit weight of mixture, which is economically unfavourable.

Table 2.14 Systems Commonly Used in Aqueous Two Phase Extraction

System	Product	Reference
1. Polymer/salt/water Polyethylene glycol / Potassium sulphate /Water	Bromelain	Babu et al., 2008
2. Polymer/salt/water Polyethylene glycol / Potassium phosphate /Water	Alkaline Protease from *Bacillus subtilis* NS99	Chouyyok et al., 2005
3. Polymer/salt/water Polyethylene glycol / Ammonium sulphate /Water	Papain	Nitsawang et al., 2006
4. Polymer/salt/water Polyethylene glycol / Magnesium sulphate /Water	Proteinase from tuna spleen	Klomklao et al., 2005
	protease from stomach of albacore tuna (*Thunnus alalunga*)	Nalinanon et al., 2009
5. Polymer/salt/water Polyethylene glycol / Sodium citrate /Water	Protease from *Clostridium perfringens* broth	Porto et al. 2005
	Trypsin from bovine pancreas	Tubio et al., 2009
6. Polymer/salt/water Polyethylene glycol/ Potassium phosphate/Water	alkaline protease from *Norcadiopsis sp.*fermentation broth	Porto et al., 2005
7. Polymer/salt/water Polyethylene glycol / Amnonium Cabamate /Water	Trypsin	Dallora et al., 2007
8. Polymer/salt/water Polyethylene glycol / Magnesium sulphate /Water	Protease from the latex of *Calotropis procera*	Rawdkuen et al., 2010 Chaiwut et al., 2010



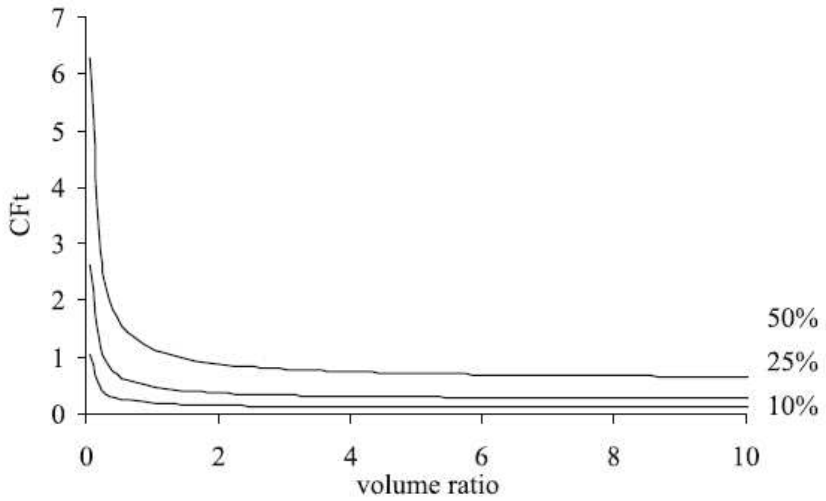
From Cabral (2007)

Figure 2.8 Theoretical Product Yield in the Top Phase for Different Partition Coefficients of the Target Biomaterial

2.10.2 Phase separation and system properties

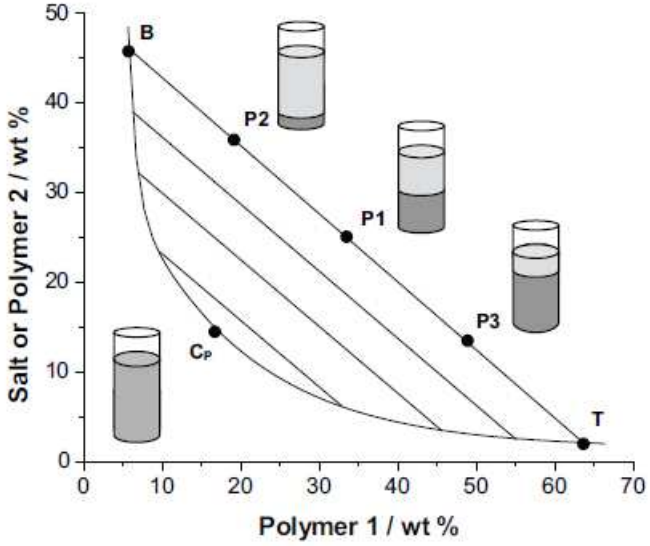
The phase components in aqueous two-phase systems (ATPSs) may be either two different hydrophilic polymers, such as polyethylene glycol (PEG) and dextran, or one polymer and one low molecular weight solute, usually a salt, such as potassium phosphate (Table 2.15). Above certain critical concentrations of these components, phase separation occurs. Separation is dependent on the molecular weight (MW) of the polymers, additives, pH and temperature. Each of the phases is enriched in one of the components. The composition of each phase can be determined for the total system composition from the phase diagram (Figure 2.9).

In Figure 2.10, the three systems A, B and C differ in their initial compositions and in the volume ratios. However, they all have the same top phase equilibrium composition (Xt,Yt) and the same bottom phase equilibrium composition (Xb,Yb). This is because they are lying on the same tie-line, whose end points determine the equilibrium phase compositions and lie in a convex curve, named the binodal, which represents the separation between the two immiscible phases.



From Cabral (2007)

Figure 2.9 Theoretical Product Concentration Factor in the Top Phase for Different Mixture Loads (Weight Percentage) Considering a Partition Coefficient for the Target Biomaterial of 20



From Cabral (2007)

Figure 2.10 Two-Phase Diagrams for Y-X- Water System

It should be noted that commercial polymers are usually polydisperse and their molecular weight distributions may vary from lot to lot, even when obtained from the same manufacturer. The phase diagrams for systems formed by different lots differ accordingly.

The tie-line length (TLL) and the slope of the tie-line (STL) can be related to the equilibrium phase composition as Equation 3 and 4:

$$TLL = \sqrt{(X_b - X_t)^2 + (Y_t - Y_b)^2} \qquad \text{.....(3)}$$

$$STL = \frac{(Y_t - Y_b)}{(X_t - X_b)} = \frac{\Delta Y}{\Delta X} \qquad \text{.....(4)}$$

2.10.3 Polymer-salts Systems

The most important factor for phase separation is the chemical nature of polymers and salts. In polymer-salts ATPS, the phase separation is due to small repulsive interactions between types of monomers and salts in the solution. The total interaction between the polymers and salts is large since each one is composed of several monomers.

2.10.3.1 Molecular weight

The higher the MWs of the polymers, the lower is the polymer concentration required for phase separation, i.e. the binodal is depressed. The effect of polymer MW and temperature in the phase composition of a PEG/dextran ATPS was studied. The TLL, ΔDextran and ΔPEG were found to increase with the MW, and this effect was higher the greater the difference in molecular size between the two polymers, with a consequent increase of the diagrams' asymmetry.

2.10.3.2 Temperature

The concentration of phase polymers required for phase separation usually increases with increasing temperature. For the PEG/dextran ATPS, the effect of the polymer MWs is further increased with increasing temperature. The STL increased with the temperature due to the fact that ΔDextran decreased with increasing temperature, while ΔPEG remained nearly constant.

Table 2.15 Polymer Systems Capable of Phase Separation in the Water Solutions

Polymer	**Polymer**
Polyethylene glycol	Polyvinyl alcohol
	Dextran
	Hydroxypropyl strach
	Ficoll
Polypropylene glycol	Methoxypolyethylene glycol
	Polyethylene glycol
	Dextran
Ethylhydroxyethylcellulose	Dextran
	Hydroxypropyl strach
Ethylene oxide-propylene oxide	Dextran
	Hypoxypropyl strach
Polymer	Low molecular weight solute
Polypropylene glycol	Potassium phosphate
	Glucose
Polyethylene glycol	Inorganic salts, e.g. K^+, Na^+, Li^+, $(NH_4)^+$, PO^3_4, SO^2_4
Stimuli-responsive polymer	
Ethylene oxide-propylene oxide	-
Poly-*N*-isopropylacrlamide	-
Methacrylic-methyl methacrylate	-

From Cabral (2007)

2.10.3.3 Inorganic salts

The hydrophobic (water structure breaking) salts (e.g. $KClO_4$, KI, KSCN) generally elevate the binodal of a two-polymer ATPS, as does the temperature increase, while the hydrophilic (water structure making) salts (e.g. K_2SO_4, KF) depress the binodal of the system.

The PEG/dextran system is much less susceptible to the salt effects when compared to polyvinylpyrrolidone (PVP)/dextran or Ficoll/dextran ATPSs. These effects on the phase separation of PEG/dextran seem to be similar to the ones observed on the lower critical solution temperatures (LCSTs) in the dextran-free aqueous solutions of PEG.

The addition of a given salt affects the polymer composition of the two phases depending on the type and total amount of the salt. The salt composition of the phases is also influenced by the total polymer concentration of the system. PEG rejects phosphate, sulphate and to a lesser extent chloride, while the effect of dextran on the distribution of either salt is much smaller. The magnitude of the PEG effect on the salt distribution behavior was found to be proportional to the polymer concentration. The following empirical showed relationship between the partition coefficient of the salt (*P*salt) and the polymer concentration difference of PEG (PVP or Ficoll) in both phases (Equation 5):

$$\ln P_{salt} = B_{salt} \Delta C\text{PEG} \qquad \text{.....(5)}$$

where B_{salt} is a constant depending on the type of the phase polymers and the type and total concentration of salt additive. Hydrophobic salts were found to favour the PEG (PVP or Ficoll)-rich phase, while hydrophilic salts favored the dextran-rich phase. The STL was related to the total salt concentration in the ATPS.

In an ATPS, anions and cations distribute unequally across the interface. To keep the electroneutrality between the phases, a potential difference results. Water structure making ions (Li^+, Na^+, $NH4^+$, Ca^{2+}, Mg^{2+}, F^-, SO^{2-}_4, CO^{2-}_3 ,PO^{3-}_4, CH_3COO^-) favour the more hydrophilic phase, whereas water structure breaking ions (K^+, Rb^+, Cs^+, Cl^-, Br^-, I^-, SCN^-, NO^-_3, ClO^-_4) favour the more hydrophobic phase.

In summary, salt additives partition between the two phases and lead to a redistribution of the polymers between the phases, i.e. a change in the phase polymer composition. Therefore, when evaluating the partitioning of proteins in two-polymer ATPSs with salt additives, the interrelationship between the polymer and the ionic composition of the coexisting phases should be taken into account (Cabral, 2007)

2.10.4 Benefit of ATPS

The main advantages have been summarized by Albertsson (1986) and are given below:

2.10.4.1 Both phases of the system are of an aqueous nature

2.10.4.2 Rapid mass transfer and mixing until equilibrium requires little energy input

2.10.4.3 Enables the processing of solid-containing streams

2.10.4.4 Polymers stabilize proteins

2.10.4.5 Separation can be made selective

2.10.4.6 Easy and reliable scale-up from small laboratory experiments

2.10.4.7 Possibility of continuous operation

2.10.4.8 It is cost effective

In addition to the other advantages it offers, ATPE is gaining increasing popularity as an environmentally benign technique because (1) it does not employ volatile organic compounds as solvents, (2) it involves phase-forming polymers that rely on the structuring properties of liquid water not only for the formation of phase systems but also for solubilizing the otherwise relatively insoluble hydrophobic solutes, (3) it exploits the remarkable properties of polymers such as polyethylene glycols, polyvinyl pyrolidone (PVP) etc. and (4) it makes use of phase-forming components which could be recovered and recycled to a limited degree. In some modified form, ATPE is finding application in environmental remediation (Cabral, 2007).

2.11 Muscle physiology

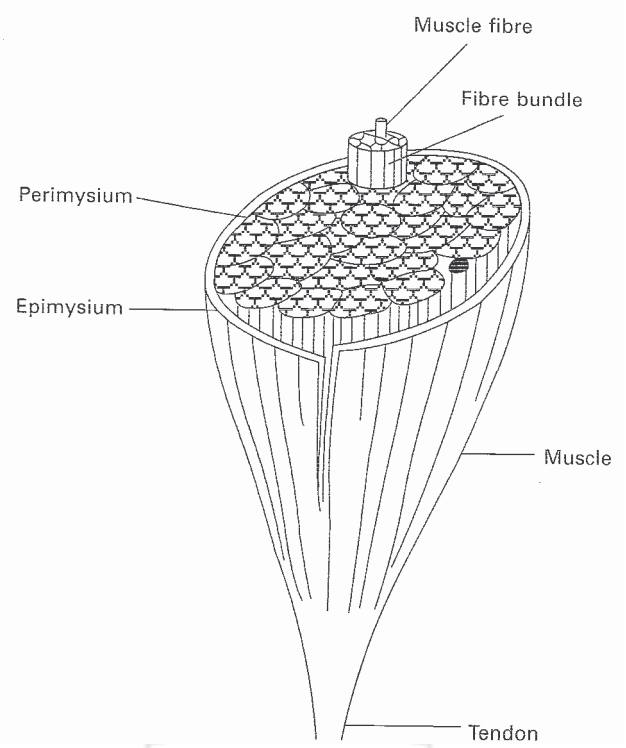
Muscle of slaughtered mammals and poultry and of caught fish become meat. The broad definition of meat comprises all body parts of mammals, birds, fish, and invertebrate's edible by man. For that reason, meat also comprises certain internal organs of animals and skin, e.g., in poultry or swine, and adipose tissues (subcutaneous and internal) (Kijowski, 2001).

A single muscle (Figure 2.10) is covered by a thin layer of connective tissue called the epimysium, which is the extension of the tendon. Muscle is divided into muscle fibre bundles and another thin layer of connective tissue, called the perimysium, covers each fibre bundle, in turn, each fibre bundle is furthermore made out of individual muscle fibres, which are covered by a

membrane of connective tissue known as the endomysium. Underneath the endomysium is another layer known as the sarcolemma, which is of net-like structure and is directly connected to the filaments actin and myosin, the major components of a muscle fibre. A liquid, called sarcoplasm (cytoplasm), is the intracellular substance in a muscle fibre and consists of around 80% water as well as proteins, enzymes, lipids, carbohydrates, inorganic salts as well as metabolic by-products (Feiner, 2006).

Lean muscle tissue contains between 70 and 75% water, 22% protein, around 2-4% intramuscular fat and around 2% of other components such as phosphates and minerals. The 22% protein can be divided into around 13% myofibrillar protein (salt soluble), 7% sarcoplasmic proteins (water soluble or soluble at very low salt concentrations) and around 2% structural proteins such as connective tissue (insoluble in salt and water). Expressed in percentages, the protein in lean muscle tissue consists of 55-60% myofibrillar protein, around 30% sarcoplasmic protein and around 10-15% connective tissue. The main myofibrillar proteins, adding up to 55-60% of the total myofibrillar protein, are myosin (around 42%) and actin (around 16%) as well as tropomyosin, troponin and actinin. Despite that, actin and myosin account only for around 7-8% of the total muscle weight, and only around 70 g per kilogram of meat is soluble protein coming from actin and myosin. Actin and myosin are also known as the myofilaments and are responsible for muscle contraction and relaxation (Feiner, 2006).

Albumins and globulins are the main sarcoplasmic proteins and around 90 different proteins belong to the group of sarcoplasmic proteins. Albumins are fully soluble in water whilst globulins are soluble in weak salt solutions only but insoluble in water. Myoglobin (colour of meat) and haemoglobin (colour of blood) are the most important types of globulin. Sarcoplasmic proteins are responsible for the metabolism in an animal cell. The main representative in the group of connective tissue is collagen (40-60%) and some tropocollagen, and elastin (around 10%) is present as well. Hence, a major part of connective tissue, around 30%, is made from other insoluble proteins. Myofibrillar proteins denature at around 67-72°C whilst sarcoplasmic proteins denature generally at around 62-70°C. Some sarcoplasmic proteins denature at temperatures as low as 50°C. Connective tissue shrinks at 60-65°C and continuous moist heat treatment up to around 90-95°C turns collagen into gelatin. As stated earlier, prolonged heat treatment turns gelatin into individual strands of procollagen (Feiner, 2006).



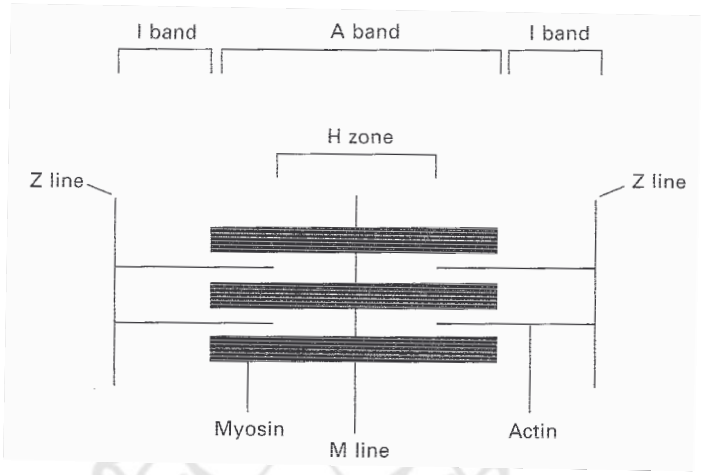
From Feiner (2006)

Figure 2.11 Structure of a Muscle.

Water within muscle tissue is bound more or less firmly and in different ways. Protein-bound water within meat, around 4-6% of the water within the muscle tissue overall, is bound so firmly to protein that, even at a temperature of around -45°C, protein-bound water is still not frozen. Around 55-60% of the water present between the myofibrils is bound in relation to the pH value of meat. Such fibril-bound water is known as immobilized (or not freely available) water but is not bound as firmly as protein-bound water. Water present in the sarcoplasm, around 20-25%, is freely available and is known as 'free water'. Finally, extracellular water accounts for around 8-14% of the total water and is held outside cellular membranes in capillaries (Feiner, 2006).

The contractile unit of a muscle fibre is a sarcomere (Figure 2.11) and, for example, the human biceps exhibits around 10 sarcomeres. A sarcomere, which is around 2 mm long, lies between two Z lines. Actin is connected to the Z line and comes to an end there. Myosin, on the

other hand, is connected to the M line. The I band is the zone where no myosin overlaps with actin, and the H zone is the space where no actin overlaps with myosin. The A band represents myosin. The special arrangement of actin and myosin give the fibre a striated appearance under the microscope, and actin and myosin are arranged in a structured hexagonal pattern (Feiner, 2006).



From Feiner (2006)

Figure 2.12 A Sarcomere

2.12 Muscle protein categories

Muscle proteins can be divided into categories on the basis of the following:

1. location in the structure of muscle and of muscle fiber
2. physicochemical properties, e.g., solubility
3. functionality in regard to further processing of meat.

The main muscle fiber structures that are seen by electronic microscope are thick and thin filaments and M-line, Z-line, and N-line.

Muscles are rich in numerous proteins of different molecular weights, amino acid composition, and spatial structure. Those differences affect the physicochemical properties of proteins.

Muscle proteins form three large groups of proteins of different positions in the muscle and different solubilities, i.e., myofibrillar proteins, connective tissue or stromal proteins, and sarcoplasmic proteins.

Proteins of the muscle fiber, also called myofibrillar proteins, form the muscle fiber structure, and its up-to-date concept is presented in Figure 2.12. Myofibrillar proteins form the largest group, accounting for 55-60% of the total quantity of muscle proteins. On the other hand, myofibrils amount to around 80% of fiber volume. The majority of myofibrillar proteins can be isolated from comminuted muscle tissue using a salt solution of ≤0.6 ionic strength, but they are not soluble at low ionic strength.

Taking into account the physiological and structural roles of proteins in the muscle of a live animal, they can be further divided into the following:

1. contractile proteins, including myosin and actin, that are responsible for muscle contraction and that form myofibrillar structure

2. proteins that control muscle contraction, among them, tropomyosin, troponin complex, and other proteins with the same function but present in lower quantities

3. proteins of the intracellular structure, including titin, nebulin, desmin, and other small proteins that form the inner and outer supporting structure of muscle fiber sarcomere (Kijowski, 2001).

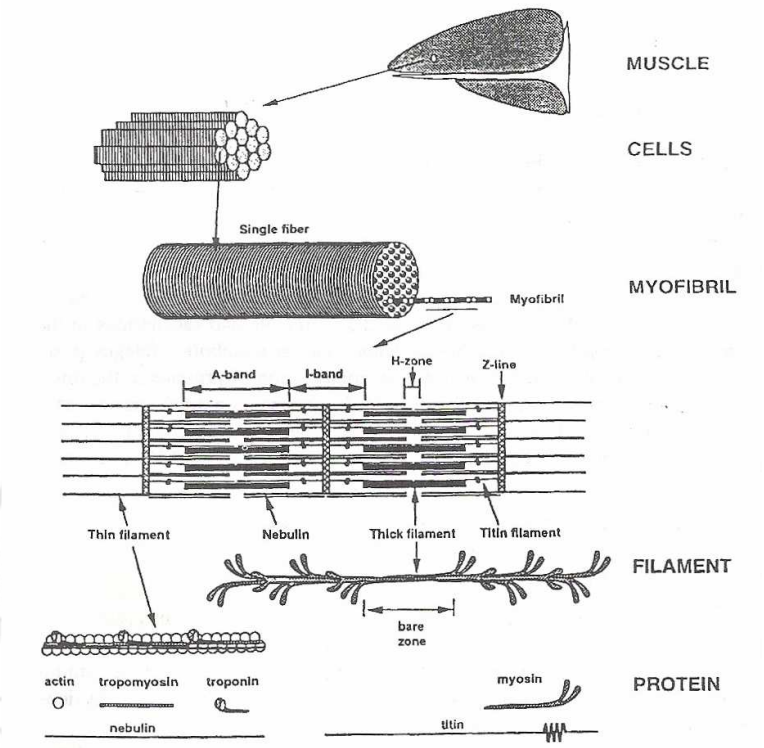
2.12.1 Contractile proteins

2.12.1.1 Myosin

Myosin can be extracted with KCI solution of concentration higher than 0.15 mol/dm^3 from the myofibrillar muscle structure after previous removal of water-soluble proteins. In the course of isolation of myosin, it is to be protected from chemical changes and simultaneous extraction of actin. Myosin of the mammalian muscles has to be isolated by extraction for a couple of minutes, whereas in the fish muscle, actomyosin is extracted under those conditions. Simultaneous extraction of actin can be prevented by $MgCl_2$ and ATP or pyrophosphate addition. Myosin is susceptible to aggregation due to oxidation of thiol groups. The addition of

ethylenethaminetetraacetic acid (EDTA) and mercaptoethanol can prevent its aggregation. Myosin molecule under the effect of sodium dodecylsulfate (SDS) dissociates into subunits of high and low molecular weight that are separated by electrophoretic technique.

Myosin is a large molecule of fiber-like form and high molecular weight that reaches 500 kDa. In the meat of mammals, birds, and fish, it predominates and accounts for around 45% of myofibrillar proteins.



From Kijowski (2001)

Figure 2.13 Proteins of Myofibrillar Structure

Myosin exhibits three important functional properties:

1. It is an enzyme of ATP-ase activity.
2. It forms natural complexes with actin.
3. Myosin molecules react with each other and build filaments.

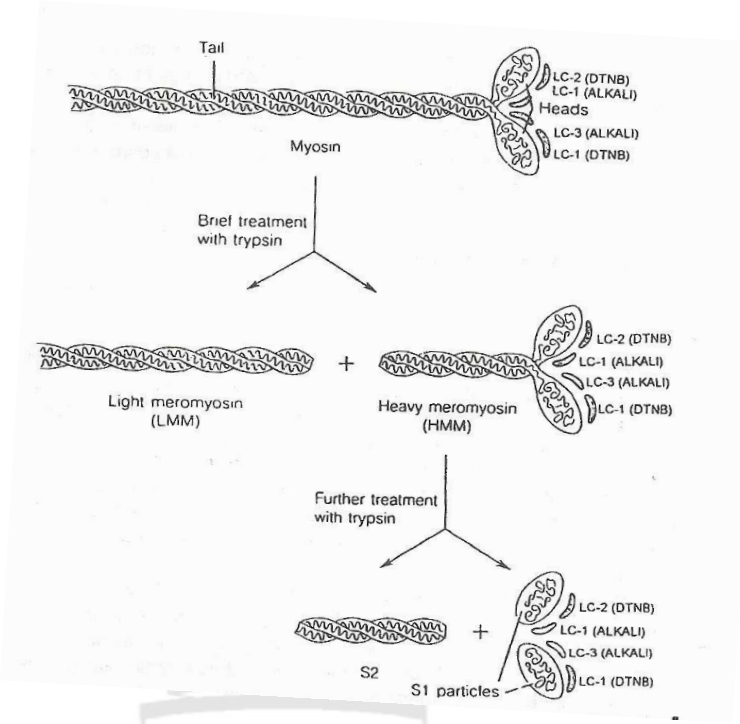
A myosin molecule contains six polypeptide chains, and in that number, two identical heavy chains of 220 kDa each are included. The molecule has a double-headed, globular region attached to a very long rod. The tail is a double-thread helically twisted superhelix formed by heavy chains (Figure 2.13). Rod-like a-helical tails participate in building thick filaments. The heads of the myosin molecules protrude on the filament surface. At physiological ionic strength and PH, 200-400 myosin molecules aggregate to form filaments. The surface hydrophobicity of myosin is attributed to the head region. Associated with globular head are two pairs of light chains (LC) in the size of 19-25 kDa. There are three types of LC: LC-1, LC-2, and LC-3.

Myosin contains a large amount of aspartic acid and glutamic acid residues and a fair amount of the basic residues histidine, lysine, and arginine. It is to be expected that myosin will be a negatively charged protein at physiological pH. The isoelectric point of myosin is 5.3, which means it is a negatively loaded protein that affects meat pH value which typically ranges from 5.4 to 6.2. The head of a myosin molecule demonstrates a large content of hydrophobic amino acids. Most of the 40 sulfhydryl groups in a myosin molecule are located in the head region. Neither the head nor the rod contain -S-S- bond.

Myosin is encoded by genes that apparently vary among species, between red and white muscles and between skeletal and cardiac muscles, producing various isoforms. This results in differences in physicochemical and functional properties between fast and slow myofibrils as well as between skeletal and heart muscles. At least four types of myosin are present in chicken muscles: two embryonic and one fast and one slow adult. The amino acid composition of fast and slow myosin demonstrates differences. Myosin of white, fast-twitch fibrils contains methylhistidine, a unique basic amino acid. The content of methyl amino acids varies and is dependent on muscle type and animal species. (Kijowski, 2001)

2.12.1.2 Actin

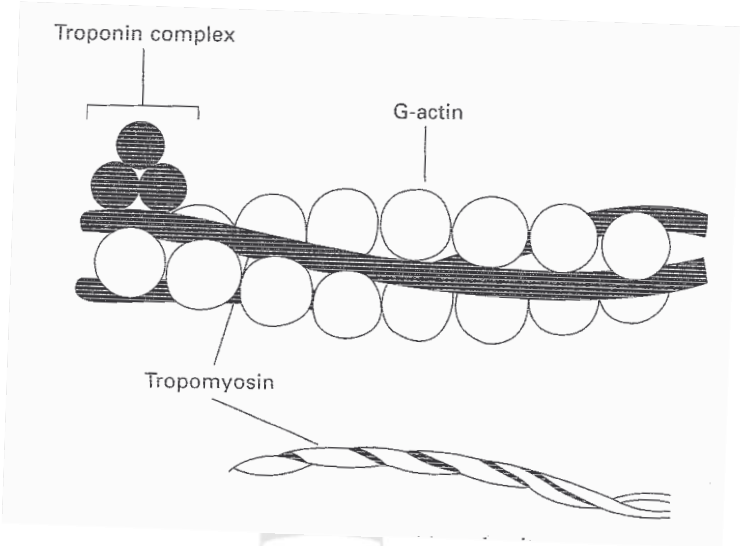
Actin is the second in quantity among myofibril proteins and accounts for above 20% of their weight. It belongs to the contractile proteins of the muscle fiber. Actin is present in all eukaryotes and is a component of thin filaments. It occurs in the muscle in a polymerized F-actin fibrillar form. In solutions of low ionic strength, actin is present as a monomer of 42 kDa weight, in the so-called G-actin globular form. Actin is an ATP-ase too, however, it does not cause muscle contraction but participates in polymerization anddepolymerization of filaments.



From Kijowski (2001)

Figure 2.14 Schemes of the Myosin Molecule and of the Generation of LMM, HMM, S1, S2 Subfragments

G-actin molecule of 42 kDa weight forms a single peptide chain. Polymerized actin (F-actin) has two chains formed into a superhelix having weight of several million Dakons. Actin molecule contains 376 amino acid residues of high contribution of praline and glycine. The high content of those two amino acids is, presumably, responsible for the a-helix structure and globular shape of the molecule. The isoelectic point of that protein is 4.8. The thin filament at in the muscle is composed of 400 actin molecules. In the muscle, actin is natulaurally bound to tropomyosin and troponin complex (Figure 2.14). Actin also has a binding site to myosin, facilitating formation of complexes with that protein during muscle contraction and in the rigor mortis phase. (Kijowski, 2001)



From Feiner (2006)

Figure 2.15 Actin and Its Subunits

2.12.1.3 Actomyosin

Actomyosin is a kind of protein complex formed during muscle contraction. In mammals, birds, and fish, actomyosin formation is the result of ATP drop postmortem. The natural complex can be extracted from muscles post-rigor, and in that case, it contains other proteins such as troponin, tropomyosin, and α-actinin.

A complex showing the weight of several dozen of million daltons and high viscosity is formed in the mixture of actin and myosin. That complex is bound not by covalent, but by electrostatic bonding with the contribution of phosphate groups. The reconstituted actomyosin produced from both component proteins demonstrates many biochemical and physicochemical features of myosin. However, the actomyosin complex does not exhibit physicochemical and functional features of F-actin (Kijowski, 2001).

2.12.2 Regulatory proteins

Tropomyosin and troponin are the main protiens that play an important part in regulation of muscle contraction, and their quantity amounts to 5%, on average. Myofibrils additionally contain other proteins; they occur in lower quantities and regulate muscle filament structure. They are present in the myofibril filament structure (Figure 2.15), e.g., A-band, 1-band,

and Z-disc, namely, α-, β-, γ-actinin, C-, M-, H-, and X-proteins, paratropomyosin, and others. Their function in the tissue *in viva* has not, as yet, been established, and the same pertains to their role in the quality and functionality of meat as food (Kijowski, 2001).

2.12.3 Cytoskeletal proteins

The cytoskeleton of the muscle cell is formed by titin and nebulin filaments and intermediate filaments, while the costamers form the submembrane structure. Due to position in the myofibrils the structures can be divided into internal and external categories. Titin and nebulin proteins form the internal supporting skeleton for myosin and actin. The external skeleton is made up of intermediate filaments composed of desmin, synemin and vimentin. Their role is to integrate and connect the neighboring myofibrils at the disc level. Another group of cytoskele tal proteins is located outside myoflbrils they are submembral proteins that form structures called costamers. (Kijowski, 2001)

2.12.4 Stromal proteins

2.12.4.1 Collagen

Collagen is a substantial part of connective tissue and is found in ligaments, tendons, skin and many other types of tissue serving mechanical and structural functions. Collagen accounts for almost one third of the total protein and is made from several different proteins. One of the major components is the amino acid hydroxyproline and this particular amino acid is present within collagen in a concentration of 12.5%. The amino acids proline and glycine are present at around 45-50% within collagen (Feiner, 2006)

Collagen, elastin, and lipoproteins of the cell membrane, including sarcoplasmic reticulum, are among the most important connective tissue proteins in the muscle. All of them demonstrate a fibrous structure, and in the majority of tissues, collagen quantitatively predominates. In the skeletal muscles, connective tissue occurs as thick layer located at the outer side of the muscle, called the epimysium. The fiber bundles are surrounded by the penmysium, a much thinner layer of connective tissue, while a thin layer of connective tissue surrounds the separate muscle fibers called the endomysium. Each of those tissues differs in the content of proteins specified earlier and in diameter of fibers and their spatial configuration. In fresh muscle tissue, collagen is present in the quantity of 1-2% and amounts to 6% weight in the strongly

tendinous muscles. In chicken meat, collagen, in regard to the crude protein content, varies from approximately 2.5% in breast muscle to 6.5% in thigh muscle (Kijowski, 2001)

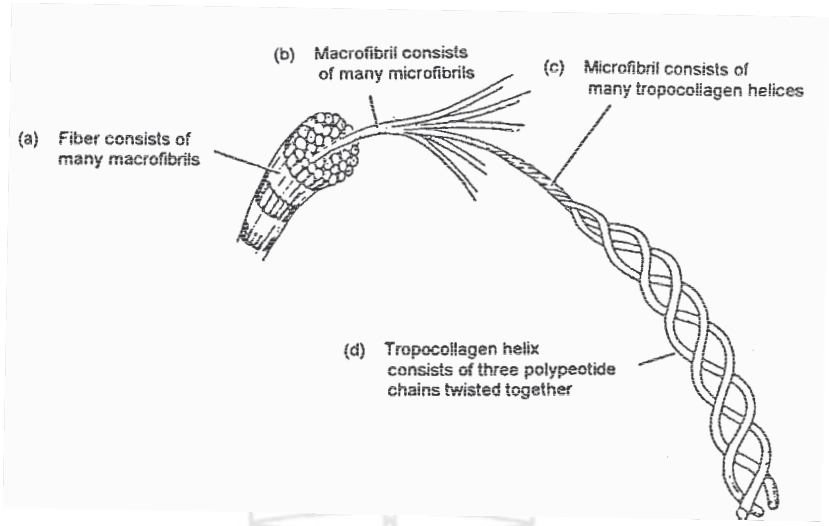
Collagen content in fish meat is lower and varies from 0.2-2.0%, that is from 1-12% crude protein. Collagen is not a homogenous protein. Nineteen genetic types of collagen have been isolated so far and marked I, II, III, etc. Collagen may also be considered in the glycoprotein group because it contains small amounts of galactose and glucose. In amino acid composition, glycine predominates (around 30%). All types of collagen contain hydroxyproline and hydroxylyzine, which are two amino acids not found in other proteins. The amount of hydroxyproline varies and is dependent upon the genetic type of collagen, animal species, and kind of tissue. For those reasons, the amino acid composition of collagen from various sources is different. Tryptophan is not present, and the sulphuric amino acids are at a relatively low level. The common characteristic of collagen molecules of all genetic types is that their structure is made of three polypeptide chains, i.e., the cr constituents that form the structural unit tropocollagen. Its molecular weight is approximately 300 kDa, and it has the shape of three helically twisted polypeptide chains stabilized by hydrogen bonds. The isoelectric point of collagen is at pH 7.0 to 7.8 (Kijowski, 2001)

2.12.4.2 Elastin

Elastin is another component present within connective tissue at around 4% of the amount of collagen and around 0.8% from the total meat protein. In the connective tissue of muscles, it accounts for not more than *5%* of fibrillar constituents. Elastin is the main protein constituent of tendons and muscle ligaments. The amino acid hydroxyproline is found within elastin at 1% and elastin is yellowish in colour, almost insoluble in water and salt and is also resistant towards diluted acids (Feiner, 2006; Kijowski, 2001). The molecule of tropoelastin, i.e., non-cross-linked, is composed of 850 to 870 amino acid residues, and its molecular weight is from 72 to 74 kDa. Glycine, proline, and alanine amount to around 60% of all amino acids (Kijowski, 2001)

Collagen has a regular helical structure, whereas elastin forms a kinetically free random coil network. Elastin contains two untypical amino acids, i.e., desmosine and isodesmosine. Elastin has a highly hydrophobic mature and a tendency to cross-link that make it very stable and slightly soluble. Elastin can be separated from the other meat proteins because it

is insoluble in water at 100 and in hot NaOH solution. *Longissimus dorsi* of beef contain the smallest amount of elastin (0.7% of dry weight), and *Semitendinosus* has the highest (2% of dry weight) (Kijowski, 2001)



From Kijowski (2001)

Figure 2.16 Collagen Fiber Structure

2.13 Meat toughness

Meat tenderness has been resolved into at least 2 different components, “actomyosin toughness” and “background toughness”. Actomyosin toughness is the toughness attributed to the myofibrillar proteins, whereas background toughness is due to the presence of connective tissue and other stromal proteins (Jiang, 1998). 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. 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 (Feiner, 2006).

Muscle toughness segmented into the influence of connective tissue and muscle fiber characteristics. Total amount of connective tissue from within different muscle of the same animal affects tenderness between muscles. For example, the muscles from the round that are used in locomotion of the animal are higher in connective tissue content than muscles from the loin that are mainly used for structural support. Muscles have been generally classified into tender, intermediate, and tough categories. Tender muscles are the *Psoas major*, *Infraspinatus*, *Gluteus medius*, *Longissimus dorsi*, and *Triceps brachii*; intermediate muscles are the *Biceps femoris*, *Rectus femoris*, *Adductor*, *Semitendinosus*, and *Semimembranosus*; and tough muscles are the *Deep pectoral*, *Latissimus dorsi*, *Cutaneous trunci*, *Trapezius*, and *Superficial pectoral*. Between animals, the age-associated increase in collagen cross-linking or decreased collagen solubility significantly affects muscle toughness. As animals increase in age, their meat is tougher. Identification or measurement of animal age, either through ossification of the vertebral column: an animal, identification of dentition, or evaluation of animal chronological has been used as a bench mark for the effect of connective tissue on meat quality between animals (Kinsman, Kotula & Breidenstein, 1994) Besides, several factor affect toughness including pre-slaughter handling treatment, stunning method, carcass chilling rate, carcass suspension system and conditioning time. The production factors likely to affect toughness include breed, carcass weight, fat level and feeding treatment. Breed effects are probably mainly associated with marbling fat and the generic susceptibility to stress (Ledward, Johnston & Knight, 1992).

2.14 Meat tenderization

Of the three factor that determine beef palatability (tenderness, juiciness and flavor), tenderness is considered the most important sensory characteristic of meat. Consumers, retailers and restaurateurs all agree that meat tenderness is one of their top quality concerns.

Tenderness is rated by most consumers as the most important attribute of meat. It is primarily connective tissue collagen that renders meat tough and, hence, necessitates its cooking prior to consumption. Individual collagen molecules in young animals are cross-linked to a low degree and cooking readily promotes their solubilization into gelatin, hence meat from young animals is generally very tender. As animals get older significant additional quantities of collagen

is deposited in their connective tissue (to help support their greater bulk), and individual collagen molecules become progressively more cross-linked. The cooking of meat from older animals thus promotes only partial collagen solubilization and hence the cooked meat remains tough (Walsh, 2002).

Meat tenderness varies considerably among species, among animals within a species, and among different muscles held for different times post mortem. Although the connective tissue content is responsible for some of this variation, the virtual lack of change in this component during postmortem storage while considerable tenderization occurs has led to the conclusion that the proteins in the muscle myofibril primarily control meat texture. The changes in myofibrillar proteins after death are believed to be mainly proteolytic in nature; most efforts to date have focused on identifying the proteases involved and the substrates altered. Different factors have been reported to affect meat tenderness, such as animal age, muscle pH and temperature, sarcomere length, amount and type of collagen, and muscle fiber type and size. With regard to these factors, some authors found that the intensity and rate of these modifications are not only species dependent but also, within a given species, muscle dependent (Walsh, 2002).

It is well known that meat tenderness increases gradually during postmortem storage and it is generally accepted that degradation of myofibrillar proteins and structure disruption by endogenous proteases are responsible for this postmortem tenderness improvement. During postmortem storage of carcasses, numerous changes occur in skeletal muscle, some of which result in the loss of tissue integrity that translates into improved meat tenderness. To further substantiate the argument that proteolysis is the principal reason for meat tenderization during postmortem storage some authors have also found that the major reason for the observed differences in meat tenderness between *Bos taurus* (tender) and *Bos indicus* (tough) breeds of cattle is the reduced rate of myofibrillar protein degradation during postmortem storage. Also, differences in the rate of postmortem tenderization and proteolysis in skeletal muscle from pigs, sheep, and cattle were apparently because of the differences in the rate of myofibrillar protein degradation. The results obtained in these studies clearly indicate that proteolysis of key myofibrillar proteins is the principal reason for the ultra structural changes in skeletal muscle that result in tenderization.

The deliberate action of protease on muscle tissues to make it softer has been practiced for centuries in the form of cooking meat wrapped with papaya leaves. Papain is still the most important enzyme used for meat tenderizing although a considerable number of enzymes have been promoted in this area. The simplest way to apply the enzymes is to sprinkle it over slices of raw meat (Naz, 2002).

Fish meat is much less tough than beef. When proteases are applied to fish muscle, the desired result is generally a thorough degradation of the fish muscle into soluble peptides. The traditional autolysis process leading to the various fish sauces of Southeast Asia exemplify this application.

A number of attempts have been made to tenderize and improve the qualities of such meat, which mainly reduce the amounts of detectable connective tissues either chemically including the use of physical methods, pressure treatments (Palka, 1999), electrical stimulation (Claus, Schilling, Marriott, Duncan, Solomon, & Wang, 2001), blade tenderization (Pietrasik, Aalhus, Gibson & Shand, 2010); chemical addition, salts, phosphates (Baublits, Pohlman, Brown & Johnson, 2005 Pietrasik, Aalhus, Gibson, & Shand, 2010), calcium chloride (Gerelt, Ikeuchi, & Suzuki, 2000) and the use of enzymes (Melendo, Beltrin, Jaime, Sancho & Roncales, 1996; Ashie et al., 2002, Wada et al., 2002; Naveena et al., 2004; Pawar et al., 2007) or the combination among physical, chemical and enzyme treatment.

Table 2.16 Methods in Meat Tenderization

Method	Muscle type	Description	Reference
Physical	Rabbit *semimembranosus* muscle	Wrapped with *Pteridium aquilinum* fern	Sotelo, Perez-Munuera, Quiles, Hernando, Larrea & Lluch, 2004
	Thai indigenous chicken *Pectoralis* muscle	Heat treatment	Wattanachant, Benjakul & Ledward, 2005
	Beef semitendinosus and *longissimus* muscle	Ultrasonic processing	Jayasooriya, Torley, Arcy & Bhandari, 2007

Table 2.16 (continued)

Method	Muscle type	Description	Reference
Chemical	*Pectoralis profundus* muscle from cull cows	Lactic acid injected	Berge, Ertbjerg, Larsen, Astruc, Vignon & Moller, 2001
	Shoulder part of a culled-cow	Calcium chloride after osmotic dehydration	Gerelt, Ikeuchi, Nishiumi & Suzuki, 2002
	Pork *sternohyoid* muscle	Salt-fermented sauce from shrimp processing by-products	Kim, Shahidi & Heu, 2005
	Beef *biceps femoris*	Sodium chloride, phosphates	Baublits, Pohlman, Brown & Johnson 2005
Enzyme	Shoulder part of a culled-cow	Elasetase from Bacillus strain	Qihe, Guoqing, Yingchun, & Hui, 2006
	Beef top round and briskets muscle	Papain and *Aspergillus* species protease	Ashie, Sorensen & Nielsen, 2002
	Bovine *longissimus dorsi* muscle	Protease from *Sarcodon aspratus*	Shin, Choi, Kim, Ryu, Lee & Kim, 2008
	Lean thigh strips of beef	Pineapple Juice	Lizuka & Aishima, 1999
	Spent adult Murrah buffaloes *Biceps femoris* muscles	Proteases from *Cucumis trigonus Roxb* and *Zingiber officinale roscoe*	Naveena, Mendiratta & Anjaneyulu, 2004
	Coarse dry sausage	Bromealin	Melendo et al., 1996
	Squid *(Loligo vulgaris* and *Illex coindetii*	Bromelain and bovine spleen lysosomal extract	Melendo, Beltrin & Roncales, 1997

Table 2.16 (continued)

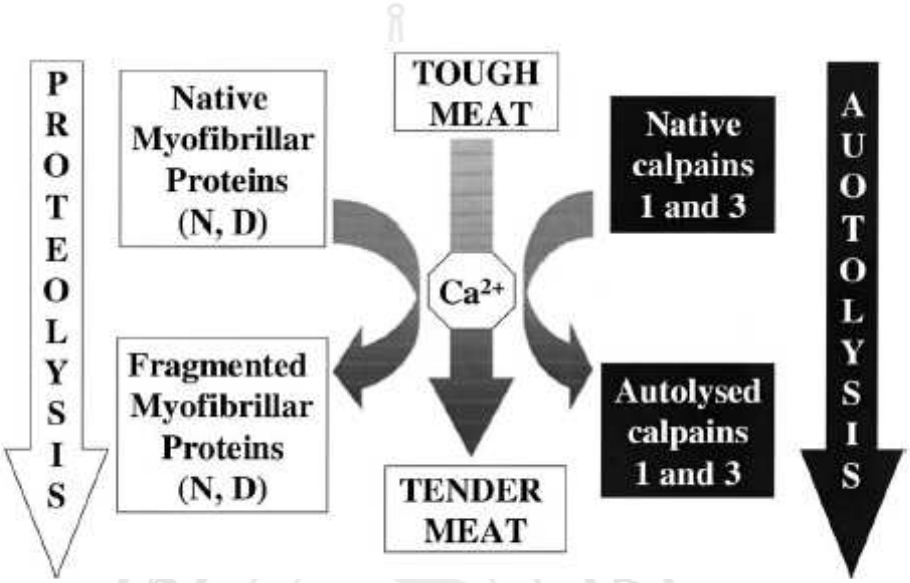
Method	Muscle type	Description	Reference
	Biceps femoris muscle of Osmanabadi goat (Chevon meat)	Protease Ginger rhizome extract	Pawar, Mule & Machewad, 2007
	Breast and leg muscles of spent hen	Protease Ginger rhizome extract	Bhaskar, Sachindra, Modi, Sakhare & Mahendrakar, 2006
Combination	Beef *semitendinosus* muscle	pressure treatments and kiwi fruit protease	Wada, Suzuki, Yaguti & Hasegawa, 2002
	Beef *semitendinosus* muscle	salt/phosphate moisture enhancement, pancreatin enzyme and blade tenderization	Pietrasik et al., 2010
	Lean meat from the shoulder part of a culled-cow	Osmotic dehydration, *Aspergillus sojae* and *Aspergillus oryzae* protease	Gerelt, Ikeuchi & Suzuki, 2000

Enzymatic mechanisms of meat tenderization

In living muscles, intracellular protein degradation is mediated by a number of different endogenous proteolytic enzymes. Because most changes occurring in the course of meat tenderization are currently believed to be the result of proteolysis, every proteinase located inside muscle cells could be a potent contributor to meat aging.

In the proteolytic systems detected in skeletal muscle, proteinases have been noted that are able to degrade the myofibrillar proteins (Figure 2.16). These proteinases are calcium-dependent cysteine proteinases located in the cellular cytosol, calpain I and calpain II. They are active at micromolar (50 to 70 μmol) and millimolar (1 to 5 mmol) concentrations of Ca^{2+} respectively. The calcium-dependent proteases, calpain I and calpain II, also referred to as μ-

calpain and m-calpain,have an optimum pH of and have been shown to degrade Z-disk, troponin-T, and desmin. The calpain proteolytic system also includes a tissue-specific calpain, calpain 3. Although calpain has fold more mRNA in muscle cells than calpain I or calpain II, the calpain 3 enzyme has never been identified. Other proteinases have been isolated, such as cathepsins B, H, and L but their importance to meat tenderization is not completely clear.



From Ilian, Bekhit and Bickerstaffe (2004)

Figure 2.17 Schematic Illustrations of the Changes in Intramuscular Connective Tissue during Postmortem Aging

The tenderness of meat may be maximized by storing fresh carcasses in a cold room for several days (ideally up to 10) post-slaughter. This process is termed conditioning (also ageing or ripening). During storage, proteolytic and other hydrolytic enzymes are released as the physiological integrity of some muscle cells (i.e. the meat) breaks down.

Artificial tenderization (particularly of meat from older animals) may be achieved using papain, a plant derived cysteine protease. Papain tenderizes the meat by degrading myofibrillar (contractile elements of muscle fibre) and connective tissue proteins. Its tenderization action

occurs during the cooking process. Papain displays little activity against native, intact collagen. However, the enzyme has an unusually high optimum temperature (50°C) and is quite thermostable. At temperatures in excess of 50°C, the native collagen structure is loosened, facilitating attack by near maximally active papain. Collagen breakdown occurs during cooking when the meat temperature is between 55°C and 65°C. The papain is likely fully inactivated when meat temperature reaches 80-90°C.

Commercially available powdered papain preparations may be rubbed or dusted onto the meat before cooking, although this will mainly promote a surface action. For larger meat cuts, the enzyme must be injected into the joint by commercially available equipment. An alternative approach entails injecting a papain solution into the animal approximately 30 min before slaughter. This facilitates even body distribution of the enzyme. Injection of an active protease into an animal's bloodstream will promote activation of the serum complement system, leading to shock and death. Papain injected into live animals is first chemically oxidized. Oxidation of the essential catalytic site cysteine residue inactivates the enzyme. As long as the animal is alive the papain remains oxidized and hence inactive. After slaughter, cellular glycolysis continues for several hours. This quickly consumes muscle oxygen, resulting in the generation of a reducing environment. This in turn reduces the papain, restoring its catalytic activity. Ficin and bromelain, addition plant proteases, have also been used to tenderize meat. Papain, however, remains the enzyme of choice, mainly on economic grounds.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Raw Materials

Pineapples (*Ananus. comosus* L.) *Nang Lae* and *Phu Lae* cultivars were collected from a plantation in the Nang Lae sub district of Chiang Rai province, Thailand during June to July, 2009. These pineapples were used for comparative study for bromelain extracted from pineapple wastes.

Pineapple peels of *Nang Lae* and *Phu Lae* cultivars were obtained from the vendor in Nang Lae subdistrict, Muang, Chiang Rai, Thailand during July 2009 to April 2010. These peels were used as raw material for studying the effect of extractants on bromalain extraction, partitioning of bromelain by aqueous two-phase system, and application in muscle foods.

3.1.2 Chemicals

Table 3.1 Chemical Lists

Step	Step
Extraction	Extraction (continued)
- Distilled water	-L- cysteine (Fluka)
- Sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$), Di-sodium hydrogen phosphate (Na_2HPO_4) (Univar)	- Ethylenediaminetetraacetic acid (EDTA) (Merck)

Table 3.1 (continued)

Step	Step
Characterization	Aqueous two phase partition
Enzyme activity determination	- Polyethylene glycols (PEG-2000, 4000, and 6000 Da) (Sigma)
- Sodium phosphate buffer	- Ammonium sulfate ($(NH_4)_2SO_4$) (Univar)
- Casein from bovine milk (Fluka)	- Magnesium sulphate ($MgSO_4$) (Univar)
- L-Tyrosine (Fluka)	- Potassium hydrogen phosphate (K_2HPO_4) (Univar)
- Trichloroacetic acid (TCA) (Merck)	- Urea (CH_4N_2O) (Unilab)
Protein content determination	*Electrophoresis*
- Bovine serum albumin (BSA) (Fluka)	- Sodium Dodecyl Sulfate (SDS) (Fluka)
- Sodium potassium tartate ($KNaC_4H_4O_6$) (Univar)	- Polyacrylamide (Sigma)
- Copper sulphate pentahydrate ($CuSO_4$. $5H_2O$) (Uniivar)	- Bis-acrylamide (Sigma)
- Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) (Univar)	- N,N,N',N'-tetramethyl ethylene diamine (TEMED) (Bio-Rad)
- Sodium carbonate (Na_2CO_3) (Univar)	- Coomasie Brilliant Blue R-250 (Bio-Rad)
- Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) (Univar)	- Methanol (CH_4O) (J.T. Beaker)
- Folin-Ciocalteu reagent (BDH AnalaR)	- Bromophenol Blue (Sigma)
	- Beta-mercaptoethanol (βME) (sigma)
	- Glycerol ($C_3H_5(OH)_3$) (Merck)
	- Protein standard marker (Thermo Scientific)
	- (hydroxymethyl)aminomethane (Tris) ($(HOCH_2)_3CNH_2$) (Merck)
	- Glacial acetic acid (CH_3COOH) (J.T. Baker)
pH profile and stability	Meat application
-Sodium phosphate (NaH_3PO_4) (Univar)	-Sodium chloride (NaCl) (Univar)
-Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) (Univar)	-Glutaraldehyde ($CH_2(CH_2CHO)_2$) (Sigma)

Table 3.1 (continued)

Step	Step
pH profile and stability (continued)	Meat application (continued)
-Sodium bicarbonate ($NaHCO_3$) (Univar)	-Ethanol (C_2H_6O) (J.T. Baker)
-Sodium acetate ($C_2H_3NaO_2$) (Univar)	
-Glycine (NH_2CH_2COOH) (Univar)	
-Hydrochloric acid (HCl) (J.T. Baker)	
-Sodium hydroxide (NaOH) (Merck)	

3.1.3 Instruments

Table 3.2 Instrument Lists

Instrument	Model	Company, Country
Water bath	WNE-22	Memmert, Schwabach, Germany
Spectrophotometer	Libra S22	Biochrom, Cambridge, USA
Micro centrifuge	VS 15000N	Vision, Korea
Table top centrifuge	PLC-05	Industrial Corp., Taipei, Taiwan
Electrophoresis equipment	Miniprotean Tertra cell	Bio Rad Laboratories, Inc, Richmond, CA, USA
Hot Plate with magnetic stirrer	Unimag, AREX2	Scott Instrument, GMBH Germany
Ultrasonic centrifuge	Avanti J-30I	Beckman Coulter, USA
pH meter	pH 510	Eutech Instrument, Ayer Rajah Crescent, Singapore
Shaker	KS 4001IC	IKA® Werke GmbH & Co. KG, Germany
Blender	HR 2011	Philips Electronics,China

Table 3.2 (continued)

Instrument	Model	Company, Country
Food Processor	MK5086M	Panasonic, Selangor, Malaysia
Homogenizer	IKA Ultra Turrax, T25 D	IKA® Werke GmbH & Co. KG, Germany
Vortex	G560E	Scientific Industries Inc, New York, USA
Digital balance 4 digits	Sartorius, ED224S	Sartorius AG, Germany
Digital balance 2 digits	Adventer, ARC120	Ohaus,New Jercy,USA.
Hot air oven		Memmert, Schwabach, Germany
Sonicator	Sonorex digitex, DT255H	Bandeline electronic, Berlin, Germany
Texture analyzer	TA-XT2	Stable Micro system, Surrey,UK
Refrigerator	P2003	Sanyo, Bangkok, Thailand
Refrigerator	Tiara	Misubhishi, Thailand
Freeze dryer	FD-8 drywinner	Heto, Cambridge, UK.
Scanning electron microscope	LEO01450VP	Cambridge, UK

3.2 Methods

3.2.1 Comparative study of bromelain extraction from *Nang Lae* and *Phu Lae* pineapple wastes

3.2.1.1 Pineapple wastes preparation

The pineapple fruits were washed, air dried and then manually peeled. The different wastes portions including peel, core, crown, and stem were separated and then stored at 4ºC for the experiment. Each waste portion was weighed, recorded and reported as a percentage of the proportion of a pineapple.

3.2.1.2 Crude enzyme preparation

Each pineapple waste was blended with cold distilled water at a 1:1 ratio for 3 min. The resulting blend was filtered through a cheese cloth and then centrifuged at 10,000×g at 4 °C for 20 min. The obtained supernatant (crude enzyme extract; CE) was collected, recorded and used for pH measurement by using a pH meter. The total soluble solids (TSS) were measured by using a hand refractometer (Atago N1-E, Tokyo, Japan) and it was reported as degrees Brix. Biochemical characteristic of the CE was also studied.

3.2.1.3 Characterization of crude enzyme

1. Proteolytic activity determination

The proteolytic activity of the crude enzyme extracts was determined by the Murachi method (Murachi, 1976), using casein and *L*-tyrosine as a substrate and a standard, respectively. The extract (1.0 ml) was mixed with 1.0 ml of a reaction cocktail (contained 1% (w/v) of casein, 0.03 M cysteine, 0.006 M EDTA in 0.05 M phosphate buffer pH 7.0). The reaction was carried out at 37ºC and was stopped by the addition of 3 ml of 5% (w/v) TCA. The reaction mixture was then centrifuged at 8,000×g for 10 min. The obtained supernatant was measured and showed an absorbance of 275 nm indicated by the soluble peptides. One unit of protease activity was defined as the amount of enzyme, releasing a product equivalent to 1 µg of tyrosine min^{-1} mL^{-1} under the standard assay conditions.

2. Protein content determination

Proteins present in the crude enzyme extract was measured according to the Bradford method (Bradford, 1976) using BSA as a standard.

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

1) Protein pattern

SDS-PAGE was carried out by the method in Laemmli (1970) using 15% separating and 4% stacking gels. The samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, 0.5% bromophenol blue, 10% glycerol, and 2% SDS) with and without βME at a ratio of 1:1 for reducing and non-reducing condition, respectively. The mixture was then boiled for 3 min. Four micrograms of protein were loaded in each well and then subjected to separate at 15 mA/gel by using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc, Richmond, CA,

USA). After separation was achieved, the protein was stained with Coomassie Brilliant Blue R-250 and destained with a methanol-acetic acid solution.

2) Activity staining

The bromelain activity in the protein band separated on the SDS-PAGE was verified by using activity staining according to the method of Garcia-Carreno, Dimes and Haard (1993) with slight modification. Each well was loaded with two micrograms of the protein. After electrophoresis, the gel was immersed in 50 ml of 2% (w/v) casein in 0.05 M sodium phosphate buffer (pH 7.0), containing 0.03 M cysteine and 0.006 M EDTA, with a constant agitation at 4ºC for 45 min. The gel was then incubated at 37ºC for 30 min and then rinsed with distilled water, fixed, stained and destained as mentioned above. The bromelain activity was observed by developing of clear zones against a dark background. The apparent molecular weight (MW) of the bromelain was estimated by comparing the reference distance (Rf) with those of a broad-range molecular weight standard marker containing myosin (215 kDa), phosphorylase B (120 kDa), bovine serum albumin (84 kDa), ovalbumin (60 kDa), carbonic anhydrase (39.2 kDa), trypsininhibitor (28 kDa), and lysozyme (18.3 kDa).

3.2.2 Effect of extractants on bromalian extraction from *Nang Lae* and *Phu Lae* peels

3.2.2.1 Crude bromelain extract preparation

According to the peel portions of both cultivars provided the largest quantity of wastes, they were considered for using as raw materials in this study and further experiments. The pineapple peels of *Nang Lae* and *Phu Lae* cultivars were washed, air dried and chopped into small pieces. Four extractants in the study were distilled water (DI), distilled water with 15mM cysteine and 2mM EDTA (DI-CE), 100mM sodium phosphate buffer pH 7.0 (PB) and 100mM sodium phosphate buffer pH 7.0 with 15mM cysteine and 2mM EDTA (PB-CE). The pineapple peel was blended in a cold extractants at 1:1 ratio (w/v) for 3 min and then filtered through a cheese cloth. The filtrate was centrifuged at 10,000xg for 20 min at 4°C. The obtained supernatant was referred to as "**crude bromelain extract: BE**'' and used for further experiments.

3.2.2.2 Characterization of bromelain extract

1. Determination of protease activity

The protease activity of the bromelain extract was determined according to Murachi (1976) by using tyrosine as a standard as previously described.

2. Protein determination

Protein concentration of the samples was determined by using the Bradford method (Bradford 1976) and BSA was used as a standard as mentioned above.

3. Protein patterns

The molecular weight distribution was determined by using SDS-PAGE according to the method of Laemmli (1970) as previously described.

4. Activity staining

The protein was separated by electrophoresis as mentioned in 2.3.3 and was verified for bromelain by using activity staining (casein-substrate gel electrophoresis) as done in Garcia-Carreno et al. (1993) as mentioned before.

3.2.2.3 Effect of bromelain extract in meat samples

1. Marination of meat samples

Two grams of meat samples (beef, chicken, and squid) were marinated with BE (1000, 2000 µl, the activity of 6.36, 12.72 units, respectively) at room temperature for one hour. The control samples were marinated by using DI-water instead of BE. The marinated samples were used to determine the effect of BE on protein degradation by using TCA-soluble peptides content and hydrolysis patterns by SDS-PAGE.

2. Determination of TCA-soluble peptides content

TCA-soluble peptides content of treated samples incubated at room temperature for an hour were measured by the method used in Rawdkuen and Benjakul (2008). Two grams of treated samples were homogenized in 18 ml of 5% (w/v) TCA for 1 min. The homogenate was kept at 4°C for 1 h and centrifuged at 8000×g for 5 min. The soluble peptide in the supernatant was measured according to the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). The TCA- soluble peptide content was calculated as mM Tyrosine/g of sample.

3. Determination of muscle protein degradation

The marinated samples (2 grams) were solubilized with 18 mL of 5% (w/v) SDS. The mixture was then homogenized using a homogenizer (IKA Ultra Turrax, T25D, KG, Germany). The homogenate was incubated at 85^{o}C in a water bath for 1 h to dissolve the protein

and then centrifuged at 8,000×*g* for 5 min at room temperature using a centrifuge (PLC-05, Industrial Corp., Taipei, Taiwan) to remove undissolved debris. The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3min. The samples (20 µg protein) were loaded into the polyacrylamide gel (12% running and 4% stacking gels) then they were subjected to an electrophoresis set at a constant current of 15mA 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. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

3.2.3 Partitioning of bromelain by using aqueous two-phase system (ATPS)

3.2.3.1 Crude bromelain extract preparation

Crude bromelain extracts were prepared as previously described in 3.2.2.1 by using extractant (PB-CE) containing phosphate buffer solution, cysteine and EDTA that stabilize enzyme activity, then crude bromelain extract was supposed to partition by aqueous two phase system.

3.2.3.2 Aqueous two phase partitioning

ATPS was run for both *Nang Lae* and *Phu Lae*. ATPS was performed in 50 ml centrifuge tubes according to the method of Nitsawang et al. (2006) with slight modification. To study the effect of PEG on the partitioning of bromelain, the concentrations (12, 15 and 18%, w/w) of PEG 2000, 4000, and 6000 Da were added into the system with a constant 17% $MgSO_4$. Salt affecting the bromelain partitioning was also investigated. Each salt ($(NH_4)_2SO_4$, $MgSO_4$, and K_2HPO_4) at each concentration (14, 17, 20%, w/w) was mixed with the PEG that gave the highest yield in the above studied. Fifty percentages of crude bromelain extract were used in the ATPS. The mixture was mixed thoroughly for 3 min using a Vortex mixer. Phase separation was achieved by centrifugation at 7000x*g* at 4ºC for 20 min. The protein content and protease activity in the obtained fractions were determined. The partitioning parameters were calculated as; partitioning coefficient of protein (K_P), specific activity (SA), purification factor (PF), and bromelain recovery (%Yield). The phase that gave the highest bromelain recovery was chosen for characterization.

3.2.3.3 Characterization of bromelain extract

1. Determination of protease activity

The bromelain activity was determined by the Murachi method (Murachi 1976) by using tyrosine as a standard as previously described.

2. Protein determination

Protein concentration of the sample was determined by using the Bradford method (Bradford 1976), and BSA was used as a standard as previously described.

3. Protein patterns

The molecular weight distribution was determined by using SDS-PAGE according to the Laemmli method (Laemmli 1970) as previously described.

4. Activity staining

The protein separated by electrophoresis was verified for bromelain by using activity staining as the method employed in Garcia Carreno et al. (1993) as mentioned before.

5. Determination of pH profile and stability

The pH profile was tested by assaying the protease activity in the different pHs (3-12). The buffers of 0.05M Glycine-HCl (3.0), 0.05M Na-acetate (4.0-5.0), 0.05M Na-phosphate (6.0-7.0), 0.05M Tris-HCl (8.0-10.0) and 0.05M Na-carbonate (11.0-12.0) were all used. The residual activity was measured and expressed as the relative bromelain activity.

The pH stability was determined by incubating the bromelain extract at different pH buffers as mentioned above at room temperature for 20 min. The casenolytic activity was expressed as the relative activity when compared with that of the control.

6. Determination of thermal profile and stability

Bromelain extract (200μL) was subjected to assay the casenolytic activity at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C). The residual activity was measured and expressed as the relative bromelain activity.

The effect of temperature on the activity of the isolated bromelain was investigated by incubation the enzyme sample (200μL) at a temperature of 90ºC for 0-60 min. The casenolytic activity was expressed as the relative activity when compared with that of the control.

7. Effect of salt on bromelain activity

The bromelain extract (200μL) was incubated at room temperature for 20 min in the presence of NaCl at different concentrations(0, 0.25, 0.50, 0.75, 1.00 and 1.50% (w/v)) with the ratio of 1:1 (v/v). The residual activity of the bromelain was measured as previously described and expressed as the relative activity when compared with that of the control.

3.2.3.4 Effect of bromelain extract on collagen hydrolysis

The hydrolysis of collagen from bovine achilles tendon (C9879-5G; Sigma Chemical Co., St Louis, MO, USA) and giant catfish skin collagens obtained by the procedure described in Thitipramote & Rawdkuen (2010) by using bromelain extract was performed according to the method of Saito, Kunisaki, Urano and Kimura (2002) with a slight modification. The collagen was dissolved with a buffer solution (0.02M sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS and 3.5M urea). The bromelain extract (0-0.3 units) was added to the collagen solutions. The mixture was incubated at 37ºC for 10 min and then terminated by submerging the mixture in boiling water for 3 min. Generated peptides were determined by SDS-PAGE using 7.5% separating gel and 4% stacking gel as previously mentioned.

3.2.4 Application of bromelain extract in muscle foods

3.2.4.1 Bromelain extract preparation

According to the activity recovery, TCA soluble peptides content and hydrolysis of muscle proteins, *Phu Lae* peel was selected for application study of muscle food tenderization. The crude extract derived by blending the pineapple peel with a cold extraction buffer (PB-CE) was subjected to partition with an 18% PEG 6000–17%$MgSO_4$ system as mentioned above. A powder from the bromelain extract (BE) was obtained by freeze drying the top phase of the aqueous two-phase system fraction.

3.2.4.2 Marination of meat samples by bromelain extract

The uniform-sized meat chunks (10 pieces for each treatment) of beef and chicken (3×3×3 cm) and squid (3×3×0.5 cm) were weighed and then sprayed with 0, 3, 7, 10 and 20 % (w/w) of BE. After thoroughly mixing, the chunks of meat were placed in a plastic box and then left at room temperature for 60 min before determining the physico-chemical and quality characteristics as described below.

3.2.4.3 Physical properties determinations

1. Textural properties

The texture of meat sample was analyzed using a TAxT2 texture analyzer (Stable Micro Systems, Surrey, UK), equipped with a Warner–Bratzler blade (square shape) in accordance with the method used in Espe, Ruohonen, Bjornevik, Froyland, Nortvedt and Kiessling (2004). Seven rectangular shaped samples of beef, chicken and squid were prepared. Each sample was cut perpendicular to the longitudinal orientation of the muscle fibers. The blade was pressed down at a constant speed of 2 mm s^{-1} through the sample. Maximum shear force (N) and total work (N.Sec) were recorded. They are the maximum resistance (toughness or breaking point) and total forces needed to cut the sample, respectively.

2. Water-holding capacity (WHC)

The WHC of meat sample was determined according to 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 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 in percentage as the following equation:

$$\%\text{WHC} = \frac{\text{Volume of NaCl before centrifuge} - \text{Volume of NaCl after centrifuge}}{\text{Volume of NaCl before centrifuge}} \times 100$$

3. 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 reweighed using an analytical balance. The cooking yield was calculated by the difference in raw and cooked weights as following:

$$\text{Cooking Yield (\%)} = \frac{\text{Weight of cooked chunks}}{\text{Weight of raw chunks}} \times 100$$

4. Scanning electron microscopy (SEM)

Microstructures of the samples were determined by using a scanning electron microscope (LE01450VP, Cambridge, UK). Muscle samples with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in an 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) and 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 SEM with a magnification of 250× or 2000× at an acceleration voltage of 10 kV.

3.2.4.4 Chemical properties determinations

1. pH

To determine pH, 10 g of the samples were homogenized with 50 ml of chilled distilled water. The pH values were measured with a digital pH meter.

2. Moisture content

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

3. TCA-soluble peptides content

The TCA-soluble peptide content of the samples was measured by the method used in Rawdkuen and Benjakul (2008) as mention before.

4. Electrophoresis

Samples (2 g) from different treatment conditions were mixed with 18 ml of 5% (w/v) SDS solution (85^{o}C). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (20 µg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gels) according to the method in Laemmli (1970) as previously described. After electrophoresis, the gel was stained overnight with staining solution (0.02% (w/v) Coomassie Brilliant Blue G-250 in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Gels

were destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 40 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 20 min.

3.2.5 Statistical analysis

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. $P<0.05$ was the accepted level of significance for all comparisons.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Comparative study of bromelain extraction from *Nang Lae* and *Phu Lae* pineapple wastes

4.1.1 Proportion of pineapple wastes

The *Nang Lae* and *Phu Lae* fruits were divided into different parts as presented in Table 4.1 and Figure 4.1. Of the pineapple wastes, the peels were the largest portion (30-42%, w/w), especially those of the *Phu Lae* cultivar (42%, w/w). Other proportions including the cores, stems and crowns were 9-10, 2-5 and 2-4% w/w, respectively. In addition, the *Nang Lae* fruits had around 50% flesh while that of *Phu Lae* was about 42%. As a result, the pineapple wastes (peel, core, stem and crown) accounted for 50% of total pineapple weight. Due to increasing production, approximately 2.8 million tons of the peels and 370,000 tons of the crowns are generated annually (FAO, 2009). *Nang Lae* pineapples have more edible portions than *Phu Lae* because they are bigger in size.

4.1.2 Characteristics of crude extracts

4.1.2.1 pH, total soluble solid, protein content and enzyme activity

Crude extracts were prepared by extracting the pineapple wastes with distilled water at a ratio of 1:1 (w/v) and then the pH, total soluble solid, protein content and enzyme activity were all measured. The results are presented in Table 4.2. The pH of the crude extracts from *Nang Lae* was similar to that of the same as *Phu Lae* at around 4.0-5.0. The crown portion showed the highest pH (4.8-5.19) while the peels of both cultivars gave the lowest pH (4.01-4.02).

The main organic acids of ripe pineapple fruit are citric and malic acid (Bartolome, Rupbrez & Carmen, 1995). The low pH value indicated the high acidity from the presence of citric and malic acid in the extracts. In both cultivars, the pH value was close to the pH of a *Smooth Cayenne* cultivar, which was 3.54 (Bartolome et al., 1995). The total soluble solid of *Nang Lae* and *Phu lae* crude extracts were 3.00-4.73 and 2.63-6.27 °Brix, respectively. Bartolome et al. (1995) reported a total soluble solid of *Smooth Cayenne* of 12.48 °Brix, and the total soluble sugars detected in the cultivar were sucrose, fructose and glucose in the amounts of 4.50g/L, 2.21g/L, 1.45g/L, respectively. Distinction in total soluble solids might be due to the differences of cultivars and plantation area. Bartholomew et al. (2003) reported that the cultivar and cultivation affect the pH and total soluble solids.

Table 4.1 Proportion of Pineapple Fruits *Nang Lae* and *Phu Lae* Cultivars

Cultivar	***Nang Lae***		***Phu Lae***	
Proportion	**Weight (g)***	**%**	**Weight (g)**	**%**
Peel	143.40 ± 8.53^{b**}	30.09 ± 3.96^{b}	159.86 ± 2.64^{a}	42.20 ± 3.51^{a}
Core	44.61 ± 0.69^{c}	9.36 ± 0.76^{c}	40.60 ± 3.38^{b}	10.72 ± 1.46^{b}
Stem	26.55 ± 4.05^{d}	5.57 ± 1.20^{c}	9.26 ± 3.67^{c}	2.44 ± 0.45^{c}
Crown	22.48 ± 1.62^{d}	4.72 ± 0.38^{c}	10.20 ± 0.98^{c}	2.69 ± 0.15^{c}
Flesh	239.86 ± 0.10^{a}	0.33 ± 4.2^{a}	158.94 ± 1.04^{a}	41.95 ± 1.02^{a}
Total	**476.54 ± 8.26**	**100.00 ± 8.22**	**378.86 ± 11.32**	**100.00 ± 5.53**

Note. *Means ± SD from triplicate determinations.

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

The extract from the crown of both cultivars exhibited the highest total protein contents (141 and 220 mg for *Phu Lae* and *Nang Lae*, respectively) and total proteolytic activity (1,782 and 955 units for *Phu Lae* and *Nang Lae*, respectively). The lowest protein content was found in the core extract of the *Phu Lae* fruit (28 mg) and the stem extract of the *Nang Lae* fruit (30 mg). Protein content was much lower in the stem and the core portion compared with the other wastes

($P<0.05$). Same with the protein content, the highest total protease activity was found in the crown portion of both cultivars, and the lowest activity was found in the stem. The pineapple peel provided the second major source of bromelain activity after the crown. The differences in enzyme activity and protein content in each portion are probably due to the different types of enzymes consisted in the pineapple, such as enzymes from the stem: stem bromelain, and from the fruit: fruit bromelain, ananain and comosain (Maurer, 2001; Hale, Greer, Trinh & James, 2005).

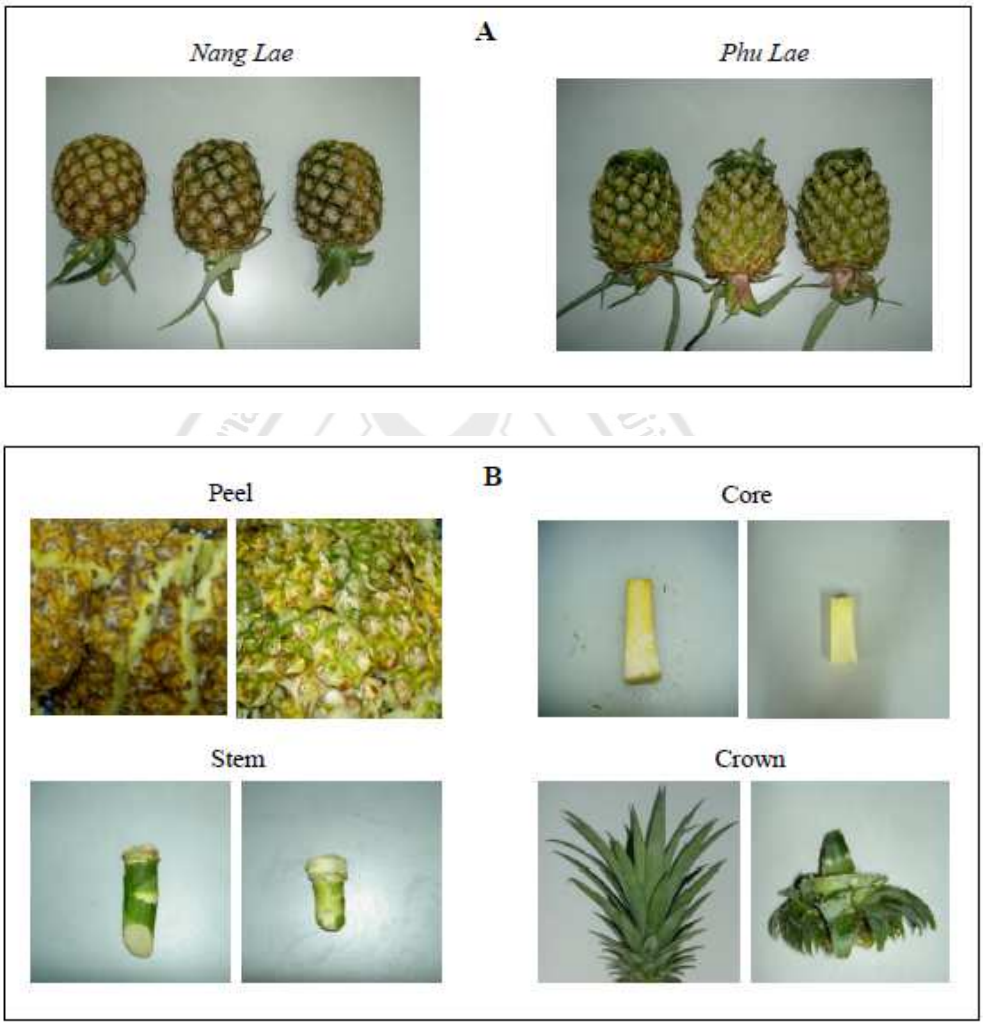


Figure 4.1 Morphology of *Nang Lae* and *Phu Lae* Pineapple Fruits (A) and Their Wastes (B)

Table 4.2 Chemical Characteristics of Crude Extract from 100 g Pineapple Wastes

Proportions	Extract (ml)	pH	TSS (°Brix)	Total protein (mg)	Total activity (unit)
Nang Lae					
Peel	163.50 ± 0.71[b**]	4.02 ± 0.30[b]	4.33 ± 0.58[ab]	132.40 ± 1.40[b]	500.43 ± 1.08[b]
Core	175.50 ± 3.54[a]	4.27 ± 0.24[b]	4.73 ± 0.87[a]	45.40 ± 0.87[c]	199.35 ± 1.62[c]
Stem	140.50 ± 10.61[bc]	4.76 ± 0.16[b]	3.00 ± 1.00[b]	29.80 ± 0.76[d]	76.69 ± 2.26[d]
Crown	133.50 ± 13.44[c]	5.19 ± 0.26[a]	3.00 ± 0.00[b]	220.50 ± 3.65[a]	954.86 ± 1.29[a]
Phu Lae					
Peel	154.50 ± 3.53[a]	4.01 ± 0.13[b]	4.43 ± 0.81[b]	70.70 ± 0.46[b]	656.51 ± 2.95[b]
Core	164.00 ± 5.65[a]	4.09 ± 0.22[b]	6.27 ± 0.64[a]	27.10 ± 1.83[c]	234.53 ± 2.22[c]
Stem	149.90 ± 12.25[a]	4.64 ± 0.22[a]	2.63 ± 0.15[c]	28.10 ± 1.85[c]	94.23 ± 0.44[d]
Crown	113.20 ± 15.47[b]	4.80 ± 0.13[a]	3.43 ± 0.40[bc]	141.00 ± 3.30[a]	1,781.96 ± 1.67[a]

Note. [*]Means ± SD from triplicate determinations.

[**]Different superscripts in the same column indicate the significant differences ($P<0.05$).

4.1.2.2 Electrophoresis analysis

1. Protein patterns

Protein patterns of the crude extracts from *Nang Lae* and *Phu Lae* pineapple wastes under non-reducing and reducing conditions are shown in Figure 4.2 A. The results show that the protein components in the crude extract were almost the same for both cultivars. From the protein patterns under non-reducing condition, the main protein components in the waste extracts showed molecular weights (MW) of 39.2, 28.0, and 18.3 kDa. Stem bromelain (lane 9) was used as a reference to show the MW of ~28 kDa. High band intensity of protein with an MW of 28 kDa was found in the crown portion. Interestingly, this protein band (MW of 28 kDa) was the major component in the crown extract of both cultivars (lane 5 and 6). Umesh et al. (2008) reported that the bromelain extracted from pineapple cores was found to be around 26 kDa by

using SDS-PAGE analysis. Maurer (2001) reported that the bromelain extracted from stems and fruits were 23.8 kDa and 23 kDa, respectively.

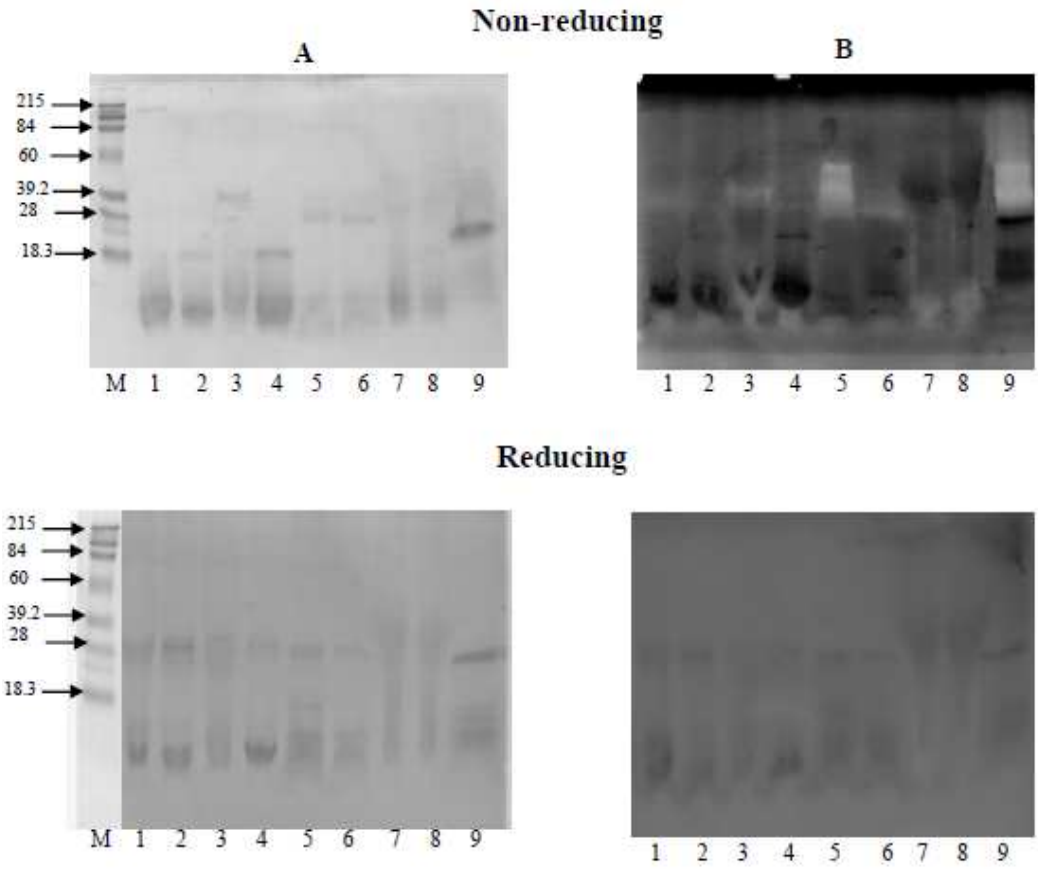


Figure 4.2 Protein Patterns (A) and Activity Staining (B) of Enzyme Extracts from *Nang Lae* and *Phu Lae* Pineapple under Non-Reducing and Reducing Conditions. *Nang Lae* (lane 1,3,5,7) and *Phu Lae* Cultivars (lane 2,4,6,8) Pineapple. Protein (6 μg and 4 μg protein) were loaded in to the gel for protein patterns and activity staining, respectively. M: molecular weight marker (kDa), lane 1, 2: peel, lane 3, 4: core, lane 5, 6: crown, lane 7,8: stem, lane 9: commercial stem bromelain

2. Activity staining

To verify the band of bromelain, activity staining was performed by substrate (casein) gel electrophoresis. Figure 4.2B shows the activity staining of the crude extracts from *Nang Lae* and *Phu Lae* pineapple wastes. No clear zone was observed in the crude extract from the peels or the stems of both cultivars as well as the *Phu Lae* pineapple core. This might be due

to 2% SDS containing in sample buffer appears to be induced structural deformity and inactivation of the enzyme (Bhattacharya & Bhattacharyya, 2009). In contrast, the crude extract from the core of *Nang Lae* pineapple and the crowns of both cultivars showed the clear zone at MW 39.2 kDa. The presence of this clear band in activity staining of non-reducing condition suggested that proteases content in those extracts, especially in the crown, were higher than those of other portions. There were some protein bands that did not show caseinolytic activity staining (Figure 4.2B). This can be explained by the presence of other non-proteases in the pineapple extracts. There are consistent reports of the presence of peroxidase, acid phosphatase, and several protease inhibitors resulting in no protease activity (Bitange, Zhang, Shi & Wenbin, 2008; Umesh et al., 2008).

There was no clear zone observation of all crude extracts in activity staining under the reducing condition (Figure 4.2B). This result indicated that the bromelain is stabilized by disulfide bond. The presence of reducing agents disrupted this type of bonds and the enzyme became denatured.

4.2 Effect of extractants on bromelain extraction from *Nang La*e and *Phu Lae* pineapple peels

4.2.1 Bromelain extraction from pineapple peels

The peels, the largest wastes portion, of both NL and PL were used in this study. Bromelain extract (BE) from pineapple peels with pH, protein content and protease activity are all presented in Table 4.3. The volume of BE ranged between 143.7-149.7 mL for NL. The highest volume amount was observed when it was extracted by DI-CE (148.7 mL). The volume of CE of PL ranged from 152.0 to 161.6 mL from 100g of the peel. Both NL and PL showed greater extraction volume when using the extractant containing Cys and EDTA when compared to the extractant without these compounds. For the pH values of BE in both cultivars, PB and PB-CE seemed to retain the most pH of the crude extract at around 5.1 in NL and 5.7 in PL, while DI and DI-CE exhibited pH of 3.6 and 3.7 for NL and PL, respectively. The PB has a buffering capacity to retain the system pH solution by slightly changing the pH value (Bonner, 2007). Therefore, it is beneficial to extracts enzyme by promoting the maximum activity. The use of buffer solution is

often very efficient and is acceptable for some proteins/enzymes without causing excessive denaturation (Bonner, 2007). The best extractant used in the extraction process of any enzymes should maintain the system pH close to the optimum pH of the target enzyme. It should not alter the enzyme activity. As already known, bromelain has an optimum pH of around 4.0-4.5 and 6.8 for stem and fruit bromelain, respectively (Harrach, Eckert, Schulze-Forster, Nuck, Grunow & Maurer, 1995; Rowan et al., 1990), so the extractant buffer that can keep the system pH close to that value should be selected.

Extracting bromelain by using PB allowed for higher protein content and protease activity compared to the extractions using DI. High protein content and protease activity could be attributed to the effect of salts present in the buffer that could solubilize the protein out of the compact structure in the sample. BE from NL showed the highest protein content (100.79 mg) when PB was used, while PB-CE provided the highest protease activity (886.88 units). The highest specific activity of bromelain was found in the extract using PB-CE (10.54) and DI (13.26) for NL and PL, respectively. However, the protease activity obtained from the DI of PL was still 2.3 times lower than that of PB-CE. The different protein and enzyme recovery when using different extractants was probably due to the ability and quantity of active compounds contained in the samples. As already known, most proteases in pineapple belong to cysteine proteases and contain cysteine residue in their active sites, so when the extractant is contained with their activator, a higher enzyme recovery was obtained. This might be because proteolytic activities are elevated by additions of small amounts of reducing agents such as cysteine and chelating agents like EDTA (Chaiwut, Nitsawang, Shank & Kanasawud, 2007). In the presence of cysteine, it prevents the disulphide bonds of the cysteine residues in the protein/enzymes from oxidizing (Sluyterman, 1967). EDTA can trap heavy metals that enhance the oxidation of thiols with molecular oxygen, and they can form complexes with specific groups, which may cause problems like lowering enzyme activity (Jansan & Ryden, 1998). Between NL and PL peels extract, the latter provided higher total activity than that of the former. This is because PL peels gave a high volume of the extract when using the same amount of the starting material. According to the results, the highest total activity of the extract was found when PB-CE was used for both NL and PL peels.

Table 4.3 Characteristics of Crude Extracts from 100 g of Pineapple Peels Using Different Extractants

Extractant	Volume* (ml)	pH	Protein Content (mg/ml)	Total Protein (mg)	Activity (Unit/ml)	Total Activity (unit)	Specific Activity (Unit/mg protein)
Nang Lae Peel							
DI	143.67 ± 1.00^{a**}	3.64 ± 0.18^{b}	0.337 ± 0.02^{a}	48.48 ± 2.85^{a}	2.28 ± 0.02^{b}	327.71 ± 3.51^{b}	6.77 ± 0.35^{b}
DI-CE	148.67 ± 0.50^{c}	3.58 ± 0.12^{a}	0.335 ± 0.01^{a}	49.81 ± 2.19^{a}	2.16 ± 0.04^{a}	321.01 ± 6.07^{a}	6.45 ± 0.34^{b}
PB	143.67 ± 2.78^{a}	5.15 ± 0.01^{c}	0.702 ± 0.01^{c}	100.79 ± 1.18^{c}	2.76 ± 0.04^{c}	396.44 ± 7.53^{c}	3.93 ± 0.05^{a}
PB-CE	146.00 ± 0.87^{b}	5.14 ± 0.02^{d}	0.564 ± 0.02^{b}	82.41 ± 3.55^{b}	5.74 ± 0.06^{d}	866.88 ± 3.73^{d}	10.54 ± 0.47^{c}
Phu Lae Peel							
DI	155.67 ± 1.73^{c}	3.75 ± 0.02^{a}	0.215 ± 0.01^{a}	33.47 ± 1.46^{a}	2.85 ± 0.08^{a}	443.66 ± 16.47^{a}	13.26 ± 0.34^{d}
DI-CE	152.00 ± 1.73^{a}	3.75 ± 0.03^{a}	0.257 ± 0.01^{b}	40.89 ± 1.86^{b}	3.15 ± 0.12^{b}	478.90 ± 13.68^{b}	11.79 ± 0.89^{c}
PB	153.00 ± 2.29^{b}	5.83 ± 0.03^{c}	0.574 ± 0.02^{c}	87.79 ± 2.89^{c}	5.97 ± 0.19^{c}	913.77 ± 20.84^{c}	10.41 ± 0.27^{a}
PB-CE	161.60 ± 2.50^{d}	5.74 ± 0.03^{b}	0.625 ± 0.02^{d}	101.00 ± 4.56^{d}	6.98 ± 0.11^{d}	1,031.94 ± 21.51^{d}	10.23 ± 0.26^{b}

Note. *Means ± S.D. from triplicate determinations.

**Different superscripts in the same column indicate the significant differences ($P< 0.05$). DI: Distilled water, DI-CE: Distilled water with 15mM Cystein and 2mM EDTA, PB: 100mM phosphate buffer pH 7.0, PB-CE: 100mMphosphate buffer pH 7.0 with 15mM

4.2.2 Protein patterns

The protein patterns of BE from NL and PL with different extractants are shown in Figure 4.3. In the reducing condition (Figure 4.3A), the migrations of proteins in BE were quite similar in all of the extractants used. BE from both cultivars showed the proteins composition molecular weight (MW) to be in the range of 24-28 kDa. The main protein component found in BE of NL and PL has a MW of around 28 kDa. However, the smear protein bands with the MW lower than 18.3 kDa were also observed in all samples. The reducing agent (βME) could split the protein molecule, which stabilized by disulfide bond into different low MW proteins (Walsh, 2002). The commercial stem bromelain (lane ST) exhibited the protein band at a MW of 28 kDa. For the non-reducing condition (Figure 4.3B), the migrations of proteins in BE were quite similar to that of the reducing condition. Different protein patterns using DI (number 1-2) and PB (number 3-4) were observed in the non-reducing condition. The two major protein bands in non-reducing condition of the samples extracted with DI (number 1-2) were found at around 28 kDa and below 18.3 kDa, while only a single protein band (MW~28 kDa) was observed when using PB as an extractant. Nonetheless, the BE of NL extracted by PB and PB-CE showed other major protein bands at around 30 and also 18.3 kDa. Differences in protein components among those BE were probably due to the differences in the amount and characteristics of interfering proteins obtained from using different extractants.

4.2.3 Activity staining

To verify the band of bromelain, activity staining was performed and the results (blue background) are also shown in Figure 4.3. No clear zones were observed for the protein band in the reducing condition. The presence of a reducing agent, such as βME or dithiothreitol, breaks the disulfide bonds in the protein structure lead to protein with less conformationally stable and loss of functional and/or structurally important elements of the domain tertiary structure in place (Walsh, 2002; Ahmed, 2005). This reason can be used to explain why there was no apparent of clear zone in the substrate-gel of the reducing condition. The clear zone generated by the proteolytic activity on the blue background was presented in the protein band at around 28 kDa. Clear zones were observed in all BE and in the commercial stem bromelain (lane ST), but not in the BE of NL using PB as an extractant (N3 in Figure 4.3B). This is probably due to a rather low bromelain content and purity. Besides, it might be due to 2% SDS containing in sample buffer

appears to be induced structural deformity and inactivation of the enzyme (Bhattacharya & Bhattacharyya, 2009). Comparing the bromelain extracted from pineapple peels with the stem bromelain, the MW of bromelain extracted from the peels gave a slightly lower MW than from the stem bromelain as indicated in lane N2, P1-P4 and lane ST. Pineapple extract also contains a peroxidase, acid phosphatase organically bound calcium and several protease inhibitors which may play a role in bromelain inhibition or in the cleavage protein structure, resulting in no activity (Umesh et al., 2008; Bitange et al., 2008). It is noticed that the major protein band with a MW of around 28 kDa exhibited caseinolytic activity (N3 and N4). This implies that the pineapple peel possibly contains other protease apart from the bromelain, which is showing activity at a MW of around 28 kDa.

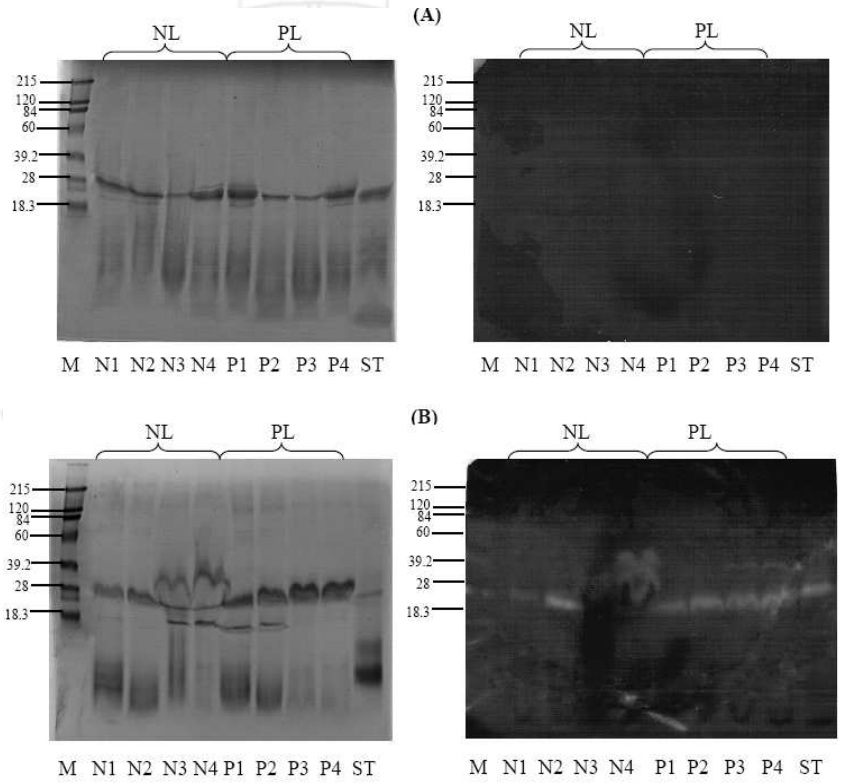


Figure 4.3 SDS–PAGE Patterns and Activity Staining of Protease from *Nang Lae* (NL) and *Phu Lae* (PL) Pineapple Peel Extracted by 1: DI, 2: DI-CE, 3: PB, 4 PB-CE in Reducing Condition (A) and Non-Reducing condition (B). Protein 6 μg and 4 μg were loaded into the gel for protein and activity staining, respectively. ST; stem bromelain, M; molecular weight makers.

4.2.4 Effect of bromelain extract on muscle proteins

4.2.4.1 TCA-soluble peptides content

Effect of the addition of bromelain extracts to proteins degradation was investigated in beef, chicken and squid muscles. The TCA-soluble peptides content of treated samples are shown in Table 4.4.

Table 4.4 TCA-Soluble Peptides Contents in Muscle Samples Treated with Crude Bromelain Extract from Pineapple Peel

Pineapple	Muscle	BE (µl)	TCA-soluble peptides (mmole Tyrosine/g sample)*			
			DI**	DI-CE	PB	PB-CE
Nang Lae	Beef	1000	1.11 ± 0.01^{bA}	1.37 ± 0.01^{cA}	1.09 ± 0.01^{aA}	1.86 ± 0.01^{dB}
		2000	1.50 ± 0.02^{bA}	1.79 ± 0.01^{cB}	1.32 ± 0.01^{aA}	2.02 ± 0.01^{dB}
	Chicken	1000	1.27 ± 0.02^{bB}	1.38 ± 0.01^{cA}	1.10 ± 0.01^{aB}	1.37 ± 0.01^{cA}
		2000	1.52 ± 0.01^{cA}	1.60 ± 0.01^{dA}	1.32 ± 0.01^{aA}	1.61 ± 0.01^{dA}
	Squid	1000	1.64 ± 0.01^{aC}	2.04 ± 0.00^{bB}	2.55 ± 0.01^{cC}	2.68 ± 0.01^{dC}
		2000	1.97 ± 0.01^{aB}	2.21 ± 0.01^{bC}	2.86 ± 0.01^{cB}	2.77 ± 0.07^{dC}
Phu Lae	Beef	1000	0.97 ± 0.00^{aA}	1.21 ± 0.01^{bA}	1.76 ± 0.01^{dB}	1.55 ± 0.01^{cB}
		2000	1.30 ± 0.00^{aA}	1.58 ± 0.02^{bA}	2.07 ± 0.01^{dB}	1.78 ± 0.01^{cB}
	Chicken	1000	1.12 ± 0.01^{aB}	1.32 ± 0.01^{cB}	1.55 ± 0.01^{dA}	1.17 ± 0.01^{bA}
		2000	1.32 ± 0.01^{aB}	1.65 ± 0.15^{cB}	1.77 ± 0.02^{dA}	1.59 ± 0.01^{bA}
	Squid	1000	1.88 ± 0.00^{aC}	2.03 ± 0.02^{bC}	2.42 ± 0.11^{cC}	2.45 ± 0.01^{dC}
		2000	1.92 ± 0.01^{aC}	2.39 ± 0.01^{bC}	2.50 ± 0.01^{cC}	2.87 ± 0.01^{dC}

Note. *Means ± S.D. from triplicate determinations.

**Different superscripts (a, b,) in the same row and (A, B,) in the same row column significant differences ($P<0.05$)

DI: distilled water, DI-CE: distilled water with 15mM Cystein and 2mM EDTA100mM, PB: phosphate buffer pH 7.0 100mM, PB-CE: phosphate buffer pH 7.0 with 15mM Cystein and 2mM EDTA

The highest TCA-soluble peptides content in all of the muscle samples was found in the sample treated with BE extracted using PB-CE followed by DI-CE, PB and DI, respectively. When the amount of crude bromelain extract (BE) was increased, the TCA-soluble peptides content increased in every muscle sample. TCA-soluble peptides content in the squid muscle was the highest when compared with other muscle foods. High values of TCA-soluble peptides in squid may be caused by the synergistic effects of endogenous proteases presented in this muscle (Cortés-Ruiz, Pacheco-Aguilar, Lugo-Sánchez & García-Sánchez, 2008). Ramirez-Olivas, Rouzaud-Sánchez, Haard, Pacheco-Aguilar & Ezquerra-Brauer (2004) have suggested that jumbo squid mantle muscle possesses high proteolytic activity. Degradation of the myofibrillar proteins resulted in an increase in peptides and free amino acids (Naveena et al. 2004). The tyrosine level indicated that the endogenous oligopeptides and/or free amino acids, as well as degradation products accumulated after being marinated with BE. From the result, high TCA-soluble peptides content indicated greater hydrolysis of the muscle proteins generated by proteolytic enzymes in BE. In addition, high hydrolytic activity was found in the samples treated with the BE using extractants containing an enzyme activator.

4.2.4.2 Protein patterns of muscle samples

The hydrolytic patterns of muscle proteins treated with BE from NL and PL are shown in Figure 4.4 and 4.5 for BE from NL and PL, respectively. The two main protein components in the beef and chicken muscle were myosin heavy chain (MHC) and actin (AC), while only AC was found as the major one in the squid muscle. The decrease in MHC and AC was observed in all of the meat samples when the amount of BE added increased (number 1-2). Small peptide or protein bands with a MW lower than the myosin light chain (MLC) were clearly observed when BE was applied. Compared to the BE of NL (Figure 4.4) and PL (Figure 4.5), higher hydrolytic activity was found in the samples treated with BE from PL (Figure 4.5) as indicated by the low band intensity of the major protein components. Muscle protein proteolysis was increased in all enzyme treated samples as evidenced by the reduction in the number and intensity of protein bands when the BE concentration was increased. Squid muscle was more hydrolyzed when compared with others. This result agreed with to the TCA-soluble peptides content as previously mentioned. In addition, the beef muscle was more susceptible to BE than that of the chicken muscle as indicated by the low number and intensity of the protein bands

especially in the degradation bands of proteins. From the results, it is also evident that cleavage of high MW proteins into low MW proteins was generated. In addition, large amount of high protein breakdowns was observed when the sample was treated with BE containing an enzyme activator. Jorgova, Danchev & Kostov (1989) reported that the reduction of high MW fractions in muscle proteins treated with proteolytic enzymes was related to increasing meat tenderness. Proteolytic degradation of myofibrillar proteins, especially myosin, resulted in a reduction in MW and the loss of their functional properties (Visessanguan, Benjakul & Tanaka, 2003).

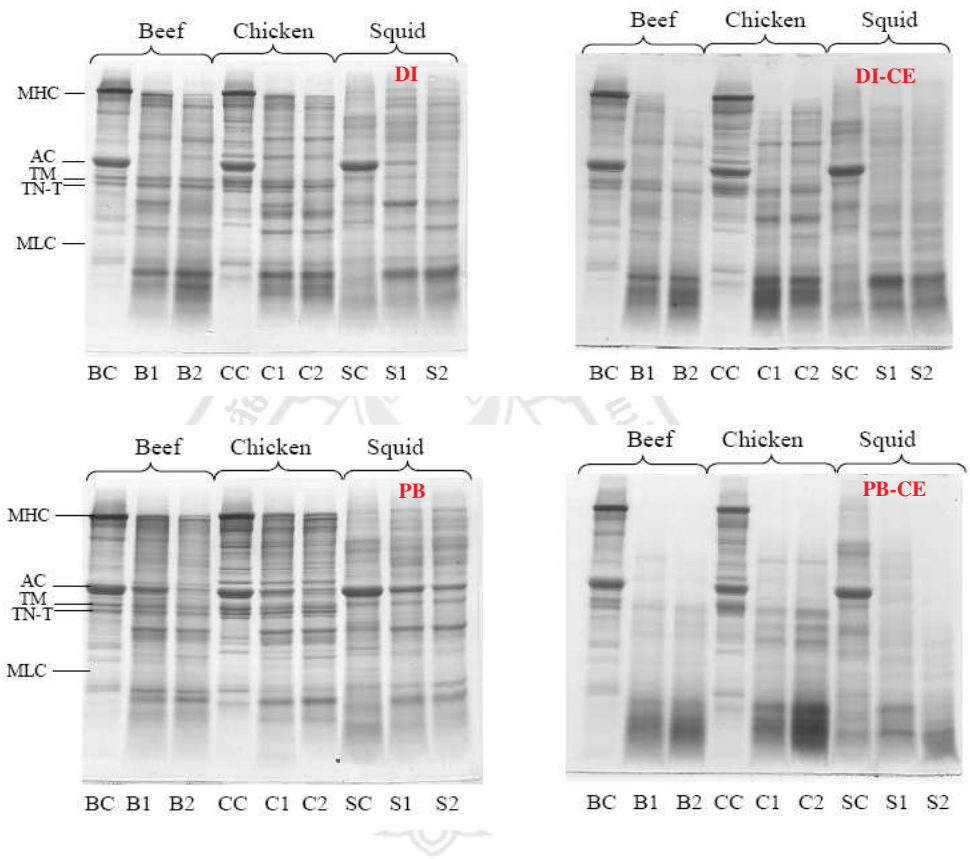


Figure 4.4 SDS–PAGE Patterns of Muscles Treated with Crude Bromelain Extract from *Nang Lae* Pineapple Peel Extracted by Different Extractants. C; control without extract, B; beef, C; chicken, S; Squid. The number of 1 and 2 indicate the amount of extract 1 and 2 ml, respectively. (6 μg and 4 μg) was loaded in to the gel for protein and activity staining, respectively. Protein (20 μg) under reducing condition was loaded into the gel. MHC: myosin heavy chain, AC: actin, TM: tropomyosin, TN: 19 troponin-T, MLC: myosin light chain

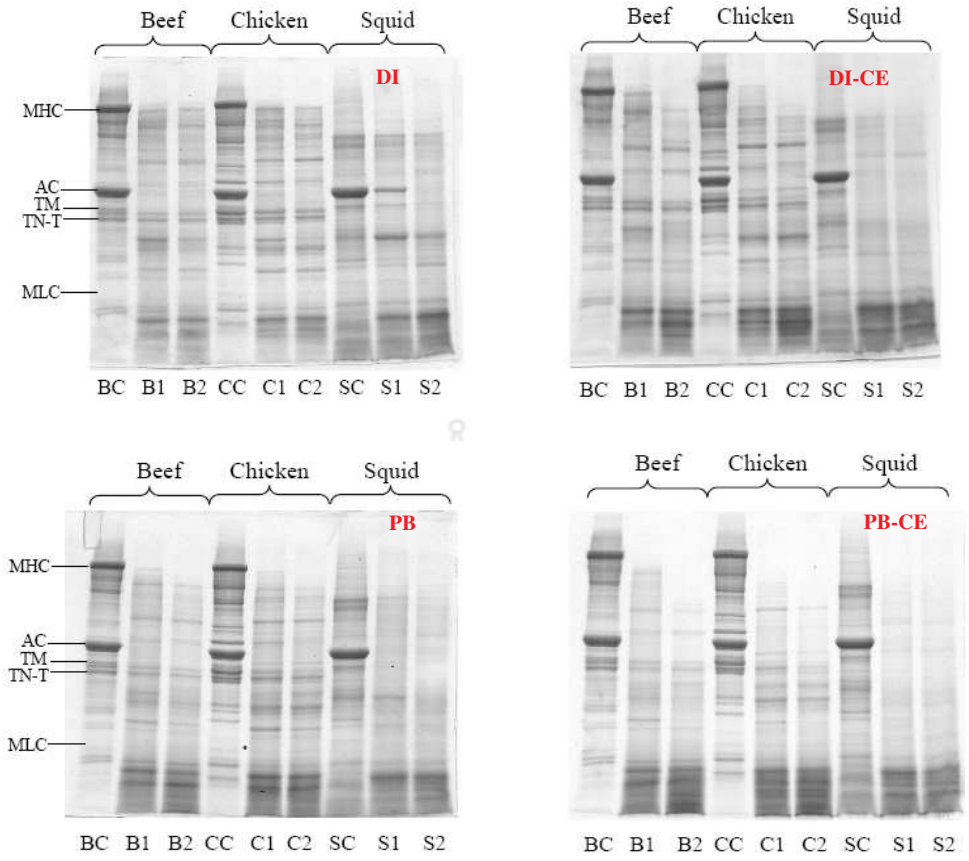


Figure 4.5 SDS –PAGE Patterns of Muscles Treated with Crude Bromelain Extract from *Phu Lae* Pineapple Peel Extracted by Different Extractants. C; control without extract, B; beef, C; chicken, S; Squid. The number of 1 and 2 indicate the amount of extract 1 and 2 ml, respectively. Protein (20 µg) under reducing condition was loaded into the gel. MHC: myosin heavy chain, AC: actin, TM: tropomyosin , TN: 19 troponin-T, MLC: myosin light chain

4.3 Partitioning of bromelain by using aqueous two-phase system (ATPS)

4.3.1 Isolation of bromelain by ATPS from *Nang Lae* pineapple peel

4.3.1.1 Effect of PEG on bromelain partitioning

The bromelain partitioning in the ATPS containing different molecular weight (MW, 2000, 4000 and 6000 Da) and concentrations of PEG (12, 15 and 18%, w/w) were studied with a constant 17% $MgSO_4$. As shown in Table 4.5, partitioned bromelain was strongly dependent on

the MW and the concentration of PEG. The result showed that the V_R of the system ranged from 0.84 to 1.19 (Table 4.5). Distribution of the protein and protease in ATPS are reported by partition coefficient of protein (K_P) and partition coefficient of enzyme (K_E), respectively. High in K_P values indicating most of proteins were partitioned more to the top phase, while high in K_E implying only that the target enzyme was partitioned to the top phase (Chaiwut, Rawdkuen & Benjakul, 2010). The K_E tended to be increased with an increase in PEG concentration and MW (Table 1). This was a result from the reduction of protease partitioning to the salt-rich bottom phase. It has been stated that an increase in polymer concentration or MW resulted in the reduction of biomolecule partition to the bottom phase (Chaiwut et al., 2010). The K_E obtained from all systems were higher than 1, indicating that bromelain preferentially partitioned the top phase. The increase in concentration of bromelain is due to more dominant effect of volume exclusion over salting out. Among all ATPS tested, the system comprising 15%PEG-2000 with 17%$MgSO_4$ effectively partitioned the bromelain to the top PEG-rich phase with 106.29% yield providing approximately PF of 2.14 folds. The highest bromelain recovery was found when the medium concentration (15%, w/w) of PEG 2000, 4000 and 6000 was used. It has been reported that the system comprising of polymer with high concentration or high MW polymer and high salt concentration resulted in partitioning of biomolecules at the interphase due to the influence of both volume exclusion and salting out effect (Babu et al., 2008). From the result, PEG 2000 provided the higher bromelain recovery than those of PEG 4000 and 6000. This implied that lower MW of PEG was preferable for bromelain partitioning to the top phase. It has been found that the system consisted of PEG 1000 using for penicillin acylase isolation gave the high yield compared with that of PEG 3350 and 8000 (Marcos, Fonseea, Ramalho, & Cabrae, 1999). Similarly, the lower MW of PEG 4000 produced the higher yield of *Calotropis procera* protease than that of PEG 6000 and 8000 (Chaiwut et al., 2010). Based on the highest activity recovery of 15% PEG-2000-17% $MgSO_4$, the 15% PEG2000 was chosen for investigation the effect of salts on bromelain partitioning

Table 4.5 Effect of Phase Composition in PEG-17% $MgSO_4$ ATPS on Bromelain Partitioning.

Phase composition (%,w/w)	V_R	K_P *	K_E	SA	PF	Yield (%)
12% PEG2000–17% $MgSO_4$	0.84	2.43 ± 0.27^{b}	5.48 ± 0.08^{b}	18.00 ± 0.26^{c}	1.93 ± 0.02^{c}	93.72 ± 0.28^{g}
15% PEG2000–17% $MgSO_4$	1.10	2.51 ± 0.61^{b}	10.65 ± 0.29^{g}	19.92 ± 0.20^{d}	2.14 ± 0.03^{d}	106.29 ± 0.33^{i}
18% PEG2000–17% $MgSO_4$	1.10	2.45 ± 0.25^{b}	8.28 ± 0.17^{f}	20.37 ± 0.14^{e}	2.19 ± 0.03^{e}	96.53 ± 0.12^{h}
12% PEG4000–17% $MgSO_4$	0.72	2.04 ± 0.13^{a}	5.09 ± 0.10^{a}	18.22 ± 0.24^{c}	1.96 ± 0.02^{c}	69.33 ± 0.33^{a}
15% PEG4000–17% $MgSO_4$	1.00	2.33 ± 0.03^{b}	7.10 ± 0.12^{d}	20.93 ± 0.18^{f}	2.25 ± 0.04^{f}	88.88 ± 0.32^{f}
18% PEG4000–17% $MgSO_4$	1.19	2.60 ± 0.08^{b}	7.33 ± 0.88^{e}	17.29 ± 0.23^{b}	1.86 ± 0.03^{b}	85.90 ± 0.16^{d}
12% PEG6000–17% $MgSO_4$	0.70	2.02 ± 0.10^{a}	5.46 ± 0.07^{b}	21.75 ± 0.19^{g}	2.34 ± 0.04^{g}	81.59 ± 0.23^{c}
15% PEG6000–17% $MgSO_4$	0.87	2.53 ± 0.14^{b}	6.03 ± 0.05^{c}	17.14 ± 0.09^{b}	1.84 ± 0.01^{b}	87.43 ± 0.29^{e}
18% PEG6000–17% $MgSO_4$	1.19	3.39 ± 0.03^{c}	7.01 ± 0.26^{d}	11.75 ± 0.05^{a}	1.27 ± 0.15^{a}	70.70 ± 0.29^{b}

Note. * Means ± S.D. from triplicate determinations.

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

V_R: volume ratio; K_P: partition coefficient of protein; K_E: partition coefficient of bromelain;

SA: specific activity (unit/mg protein); PF: purification factor (fold); Yield: activity recovery (%).

4.3.1.2 Effect of salts on bromelain partitioning

The effect of types and concentrations of salts on the partitioning parameters (V_R, K_p, K_E, SA, PF and yield) are shown in Table 4.6. Salts are frequently used in ATPS to improve the partitioning of the target molecules between the phases (Klomklao, Benjakul, Visessanguan & Kishimura, 2005; Nalinanon, Benjakul, Visessanguan & Kishimura, 2009). The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecule because of the water structure breaking effects (Nalinanon et al., 2009). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecule (Nalinanon et al., 2009). From the result, the V_R was decreased when increased in salt concentration. Enhancement of salt quantity provided the higher proportion of salt-rich bottom phase leading to practically reduced of V_R. Johansson (1998) reported that the partition of a protein is influenced by the presence of salts and this effect is enhanced with the net charge of the protein increase. The K_P and K_E increased with higher salt addition. This phenomenon can be explained by salting out effect (Babu et al., 2008) which both bromelain and impurity proteins were more partitioned to the PEG-rich top phase and resulted in decreases of SA and PF (Table 4.6). The shift of protease as well as the non-desired proteins in *C. procera* latex to the top phase when raising the salt concentration is probably due to similar hydrophobic properties on their surface (Chaiwut et al., 2010). It is noticed that the phase comprising of K_2HPO_4 was more selection for bromelain partitioning. Increase of K_2HPO_4 concentration selectively caused more the most bromelain to move to the top phase, while mainly non-target proteins preferred the bottom phase. Consequently, increased in the K_E and PF could be detected. Babu et al. (2008) reported that the system of 18%PEG1500- 20% K_2HPO_4 provided the highest activity recovery of bromelain from pineapple fruit. Molecular weight, shape, surface hydrophobicity and specific binding sites of biological materials have also been reported to affect the partitioning profiles (Raghavarao et al., 2003).

The highest K_P (9.43) and the highest K_E (20.94) were found in the system of 15% PEG2000-20% $(NH_4)_2SO_4$. In contrast, the phase composition of 15% PEG2000–17% $MgSO_4$ showed the lowest K_P (2.51), indicating that both desired protease and contaminant proteins was preferable to the lower phase. The phase containing 15% PEG2000–14%$MgSO_4$ gave the highest PF (2.23) and maximal yield of 113.54%. Magnesium sulfate has been documented to provide

substantial results of ATPS. Rawdkuen, Pintathong, Chaiwut & Benjakul (2010) reported that the system of 12% PEG3000 and 14% $MgSO_4$ showed the highest activity recovery of protease *C. procera*. The system consisting of 20% PEG1000-20% $MgSO_4$ gave the highest SA and PF in partitioning of protease from tuna spleen (Klomklao et al., 2005) and stomach (Nalinanon et al., 2009). In view of industrial application, the highest recovery was attractive for further study. Therefore, the extracted bromelain obtained from the ATPS composing 15% PEG2000-14% $MgSO_4$ was chosen for investigation the biochemical properties.

4.3.1.3 Biochemical properties of extracted bromelain

1. Protein patterns and activity staining

Protein patterns of crude extract and bromelain fraction from the system of 15% PEG2000–14% $MgSO_4$ are shown in Figure 4.6. In non-reducing condition, migration of proteins in the crude extract, top phase and bottom phase fractions were quite different. Crude extract showed the MW of protein compositions ranging of 24-28 kDa (Figure 4.6A). Proteins with MW around 28-30 kDa were found in the top phase, while those with lower than 18 kDa were mainly presented in the bottom phase. The commercial stem bromelain exhibited the protein band at MW of around 28-30 kDa and below 18 kDa. For reducing condition (without βME), the migration of protein in the crude extract, the top phase and the bottom phase fractions were quite similar which possessed the major protein band around 28 kDa and below 18 kDa.

Activity staining was performed to verify the band of bromelain. The clear band by proteolytic activity on the blue background was present only at the protein band of 28 kDa. This hydrolysis zone was observed in all samples with non-reducing condition (Figure 4.6B). It is presumable that this band possibly belonged to bromelain. Umesh et al. (2008) reported that the bromelain extracted from pineapple core showed the MW ~26 kDa. High intensity of protein band from the top phase of 15% PEG2000-14% $MgSO_4$ fraction was agreement with the highest PF and the yield as shown in Table 4.6

Table 4.6 Effect of Phase Composition in 15% PEG 2000–Salt ATPS on Bromelain Partitioning.

Phase composition (%, w/w)	V_R	K_P *	K_E	SA	PF	Yield (%)
15% PEG2000–14%$MgSO_4$	1.38	$1.93 \pm 0.24^{b**}$	10.18 ± 0.04^{a}	21.26 ± 0.79^{e}	2.23 ± 0.06^{g}	113.54 ± 0.84^{g}
15% PEG2000–17%$MgSO_4$	1.10	2.51 ± 0.61^{a}	10.65 ± 0.29^{b}	19.92 ± 0.20^{d}	2.14 ± 0.03^{de}	106.29 ± 0.33^{f}
15% PEG2000–20%$MgSO_4$	0.83	2.76 ± 0.67^{c}	11.20 ± 0.13^{c}	18.31 ± 0.15^{c}	2.10 ± 0.02^{d}	93.32 ± 0.54^{b}
15% PEG2000–14%$(NH_4)_2SO_4$	0.65	3.68 ± 0.41^{e}	14.40 ± 0.15^{e}	18.82 ± 0.07^{c}	2.21 ± 0.02^{fg}	94.06 ± 0.31^{c}
15% PEG2000–17%$(NH_4)_2SO_4$	0.54	6.22 ± 1.03^{f}	20.54 ± 0.13^{h}	14.26 ± 0.14^{a}	1.68 ± 0.01^{a}	81.52 ± 0.63^{a}
15% PEG2000–20%$(NH_4)_2SO_4$	0.47	9.43 ± 0.61^{g}	20.94 ± 0.07^{i}	16.61 ± 0.08^{b}	1.79 ± 0.02^{b}	82.53 ± 0.44^{a}
15% PEG2000–14%K_2HPO_4	0.64	3.45 ± 0.35^{d}	13.02 ± 0.07^{d}	13.44 ± 0.05^{a}	1.81 ± 0.02^{b}	101.68 ± 0.50^{e}
15% PEG2000–17%K_2HPO_4	0.58	3.23 ± 0.36^{d}	17.55 ± 0.17^{f}	24.36 ± 0.04^{f}	1.99 ± 0.01^{c}	95.61 ± 0.51^{d}
15% PEG2000–20%K_2HPO_4	0.50	2.75 ± 0.12^{c}	18.65 ± 0.31^{g}	16.32 ± 0.18^{b}	2.05 ± 0.01^{cd}	82.45 ± 0.41^{a}

Note. * Means ± S.D. from triplicate determinations.

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

V_R: volume ratio; K_P: partition coefficient of protein; K_E: partition coefficient of bromelain;

SA: specific activity (unit/mg protein); PF: purification factor (fold); Yield: activity recovery (%).

There was an absent of clear band in all fractions under reducing condition. This might be due to disulfide linkages in the enzyme molecule were broken resulting in loss of proteolytic activity. The present of a reducing agent, such as 2-mercaptoethanol or dithiothreitol (DTT), breaks the disulfide bonds in the protein structure lead to protein less conformationally stable and loss of functional and/or structurally important elements of domain tertiary structure in place (Ahmed, 2005; Roe, 2000). Electrophoretic result clearly showed that this ATPS condition substantially primarily purified the bromelain from *Nang Lae* pineapple peels.

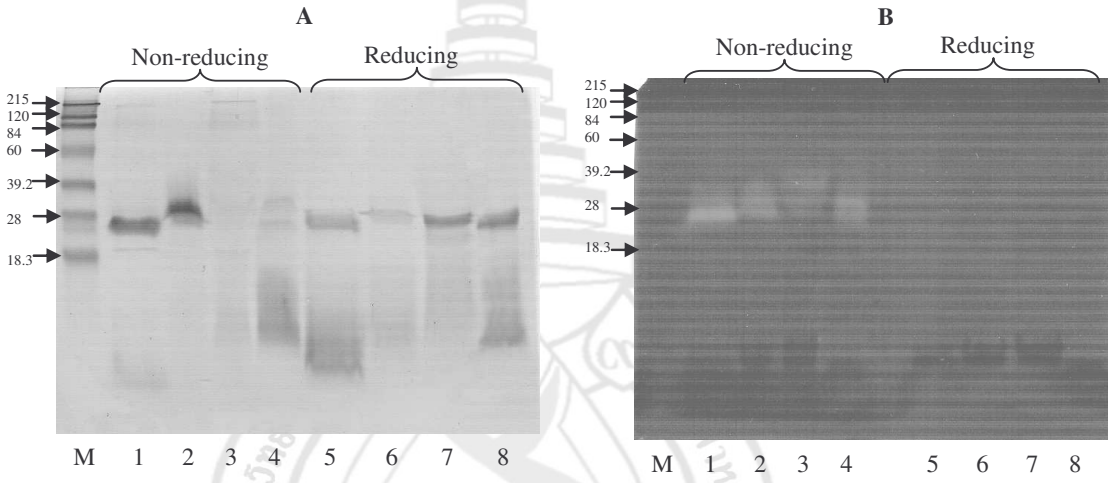


Figure 4.6 SDS–PAGE Patterns (A) and Activity Staining (B) of Protease from *Nang Lae* Pineapple Peel Partition with 15%PEG-2000–14%$MgSO_4$ ATPS. 1-4 non-reducing condition, 5-8 reducing condition [6 μg and 4 μg protein were loaded in to the gel for protein patterns and activity staining, respectively]. Lane 1, 5: crude extract, 2, 6: top phase, 3, 7: bottom phase, 4, 8: standard stem bromelain, M: molecular weight protein makers

2. pH profile and stability

The effect of pH (3-10) on the activity of bromelain was measured and reported as a relative protease activity (Figure 4.7A). The bromelain exhibited a broad range of pH on its activity and its maximum was found at pH 7.0. The activity of bromelain gradually decreased at acidic and also alkaline condition. This is supported by the result of Liang et al. (1999) that the bromelain has a wider pH range for optimum activity 6.8-9.0. The results revealed that the

isolated bromelain is more stable at a wide range of pH. The stability of bromelain incubated with various pH buffers is shown in Figure 4.7B. Interestingly, the isolated bromelain still showed its high activity (more than 50%) at very extreme pH (7.0-11.0). It would be pointed out that the bromelain molecule was stabilized by the presence of PEG2000. In this regards, the isolated bromelain-PEG is unique, and therefore might be useful for application in the food, cosmetic and pharmaceutical industries.

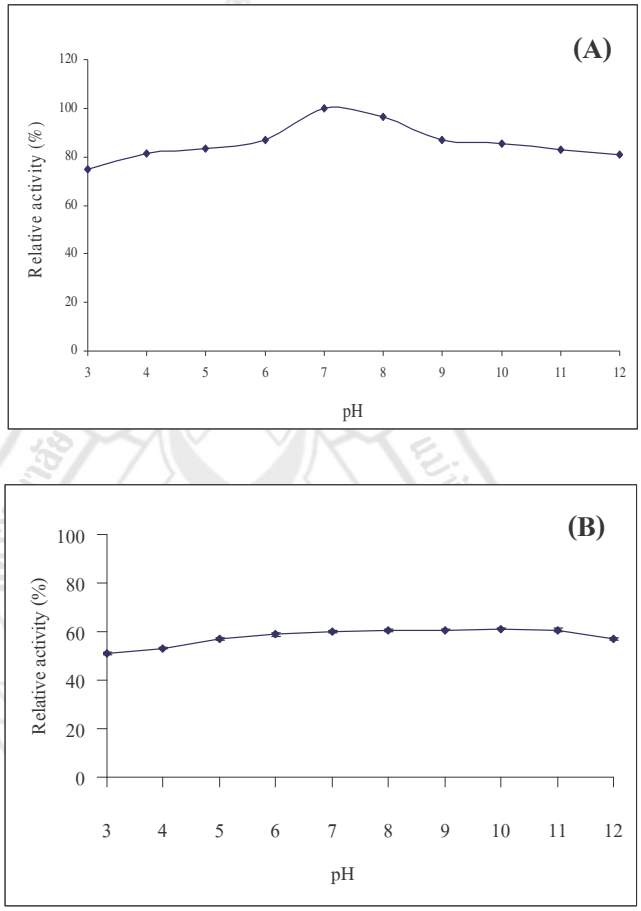


Figure 4.7 Effect of pH on the Activity (A) and pH Stability (B) of Bromelain from *Nang Lae* Pineapple Peel Partition with 15% PEG2000–14%$MgSO_4$

3. Thermal profile and stability

Thermal profile of the isolated bromelain from *Nang Lae* was assayed at a temperature ranging from 30 to 100°C (Figure 4.8A). The highest activity of bromelain was

found at 50-60°C. Temperature higher than 60°C, drastically decreased the bromelain activity until reached a plateau at around 70°C. This result is similar to the report of Liang et al. (1999) that the optimum temperature for the activity of bromelain from pineapple juice was found to be at about 55°C. As the temperature increases, more molecules have enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to be disrupted (Naz, 2002).

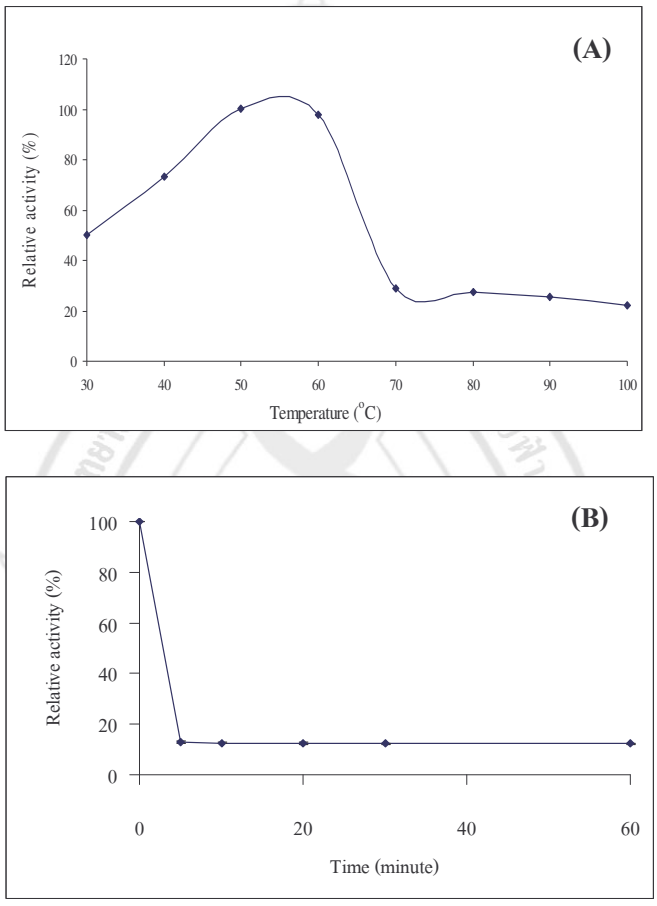


Figure 4.8 Effect of Temperature on the Activity (A) and Thermal Stability (B) of Bromelain from *Nang Lae* Pineapple Peel Partition with 15% PEG2000–14%$MgSO_4$

Therefore, a decrease in activity was detected. The result showed bromelain attached to PEG2000 was remarkably heat stable which expressed its activity even at 100°C where most of

enzymes are destroyed, or denatured. Isolated bromelain was also measured its thermal stability at 90°C at different times. The results are presented in Figure 4.8B. The relative activity of bromelain decreased about 80% after 5 min of incubation and seemed to be constant until 60 min. High temperature is found to be an irreversible-denaturant of proteins and enzymes. Enzymes are inactivated at high temperature due to the partial unfolding of their molecules (Naz, 2002).

4. Effect of salt on bromelain activity

The ability of isolated bromelain to retain its activity under growing ionic strength was tested by exposing it at different concentration of NaCl (0-1.5%, v/v). The activity of bromelain decreased with increase of salt concentrations (Figure 4.9). At 0.75% NaCl, the activity of enzyme decreased about 50% when compared with the control (without NaCl). Loss of bromelain activity might be due to changes in enzyme structure induced by the 'salting out effect' (Naz, 2002).

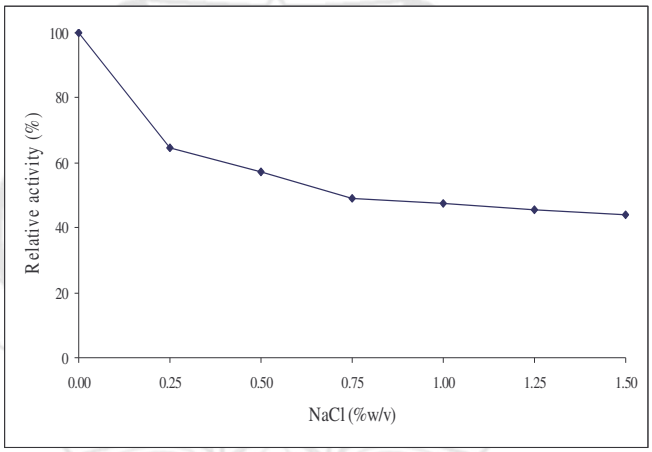


Figure 4.9 Effect of Salt Content on the Activity of Protease from *Nang Lae* Pineapple Peel Partitioned with 15% PEG2000–14% $MgSO_4$

This effect results from the competition between the protein and the salt ions for the water molecules necessary for their respective salvations. At high concentrations of salt, there are not enough water molecules are available for protein salvation, since the majority of the water molecules are strongly bound to the salts. Thus protein-protein interactions become more powerful than protein-water interactions and this may lead to aggregation, followed by

precipitation of protein molecules (Walsh, 2002). In addition, NaCl at higher concentrations possibly competed the enzyme in water binding, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation (Switzer & Garrity, 1999). The result can be useful for further application in food and cosmetic in which the formulation might contain high amount of salt.

4.3.1.4 Effect of bromelain on collagen hydrolysis

The peptide mapping of bovine achilles tendon and giant catfish skin collagens digested by bromelain are shown in Figure 4.10.

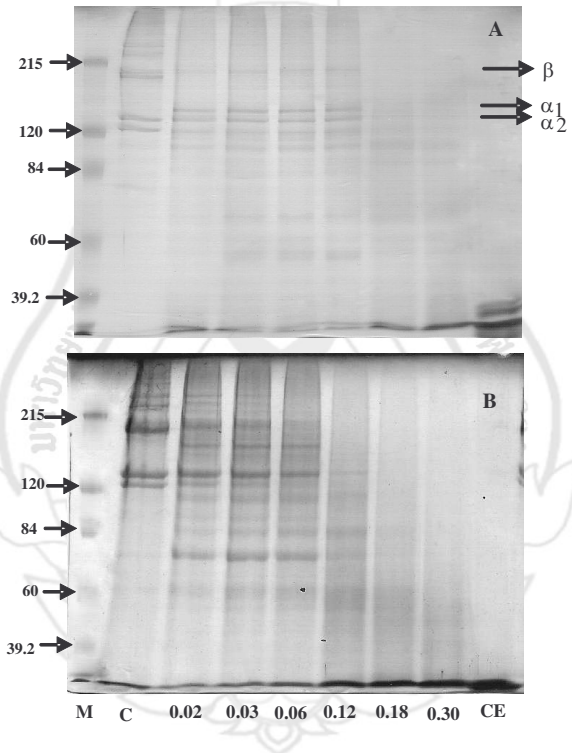


Figure 4.10 Peptide Mapping of Bovine Achilles Tendon Collagen (A) and Giant Catfish Skin Collagen (86.28% protein) (B) after Degraded by Bromelain from *Nang Lae* Pineapple Peel Partition with 15%PEG2000–14%$MgSO_4$. C: control, 0.02-0.30: the activity units of treated bromelain, M: molecular weight protein makers and CE: crude extract

Similar hydrolyzed pattern between bovine and giant catfish skin collagens were observed, when using bromelain 0.18 units. Compare to giant catfish skin collagen, bovine achilles tendon collagen was more resistant to bromelain hydrolysis. Collagen hydrolysis depended on bromelain amount. When the bromelain activity increased, high degradation of collagen constituent was clearly observed. Disappearance of all collagen components was noticeable when the bromelain was added up to 0.30 and 0.12 units for bovine and giant catfish skin collagens, respectively. The bromelain exhibited a high degree of both collagen hydrolyses, though bovine skin collagen was more resistant to hydrolysis than these of giant catfish. The results contribute to the use of this enzyme as meat tenderizer and collagen hydrolysate preparation.

4.3.2 Isolation of bromelain by ATPS from *Phu Lae* pineapple peel

4.3.2.1 Effect of PEG on bromelain partitioning

The bromelain partitioned by in the ATPS containing different molecular weights (MW; 2000, 4000 and 6000) and concentrations of PEG (12%, 15% and 18% w/w) with 17%$MgSO_4$ was studied. As shown in Table 4.7, partitioning of bromelain was strongly dependent on the MW and the concentration of PEG. The highest bromelain recovery at each MW of PEG was found when the highest concentration (18%, w/w) of PEG was applied. In addition, the increase of PEG concentration resulted in the higher recovery of bromelain ($P<0.05$). The K_p obtained from most systems were higher than 1, indicating that the proteins preferentially partitioned to the top phase. High protein content in the top phase is probably due to the effect of volume exclusion over salting out (Nalinanon et al., 2009). Among all of the ATPS tested, the system comprising of 18% PEG6000-17% $MgSO_4$ was effective for partitioning the bromelain to the top PEG-rich phase with a recovery of 205.78% and providing an approximate PF of 3.44 folds. The bromelain activity recovery was very high (>>100%) for the system studied. It was probably due to the structural alteration of the enzyme active sites in the presence of PEG (Babu et al., 2008).The increase in PF was due to relatively more partitioning of bromelain to the top phase when compared to that of other proteins partitioned in the bottom phase of the system. It has been reported that the system comprising of high concentration of polymer or high MW polymer, and high salt concentrations resulted in the partitioning of biomolecules at the interphase. This was due to the influence of both volume exclusion and the salting out effect (Nalinanon et al. 2009). Babu et al. (2008) reported that the MW of PEG1500

produced a higher yield of bromelain than that of PEG4000. In addition, Rawdkuen et al. (2010) found that the system comprising of a MW of PEG1000 produced a higher yield of protease from *Calotropis procera* latex than those of PEG2000 and 3000. Klomklao et al. (2005) also reported that a system consisting of PEG1000 achieved a high yield of proteinase yellow fin tuna spleen when compared with that of PEG4000. Based on the highest activity recovery (206%) of ATPS, the 18% PEG6000 was chosen for investigating the effect of salts on bromelain partitioning.

Table 4.7 Effect of PEG Molecular Weights and Concentrations on Partitioning of Bromelainfrom *Phu Lae* Pineapple Peel

Phase composition (%,w/w)	K_p*	SA	PF	Yield (%)
12% PEG2000–17% $MgSO_4$	$1.23 \pm 0.01^{cd**}$	19.02 ± 0.13^{e}	2.08 ± 0.03^{e}	118.54 ± 1.27^{cd}
15% PEG2000–17% $MgSO_4$	1.13 ± 0.03^{b}	16.62 ± 0.29^{cd}	1.82 ± 0.03^{cd}	115.11 ± 1.69^{c}
18% PEG2000–17% $MgSO_4$	1.15 ± 0.01^{bc}	17.32 ± 0.33^{d}	1.90 ± 0.04^{d}	128.11 ± 2.26^{f}
12% PEG4000–17% $MgSO_4$	1.16 ± 0.03^{bc}	16.49 ± 0.34^{cd}	1.81 ± 0.05^{cd}	101.62 ± 2.51^{b}
15% PEG4000–17% $MgSO_4$	1.20 ± 0.03^{bc}	15.74 ± 0.36^{c}	1.72 ± 0.03^{c}	122.97 ± 2.85^{e}
18% PEG4000–17% $MgSO_4$	1.41 ± 0.05^{e}	14.60 ± 0.78^{b}	1.60 ± 0.02^{b}	134.12 ± 1.30^{g}
12% PEG6000–17% $MgSO_4$	1.27 ± 0.02^{d}	16.11 ± 0.37^{c}	1.76 ± 0.05^{c}	120.53 ± 2.37^{de}
15% PEG6000–17% $MgSO_4$	0.90 ± 0.00^{a}	12.60 ± 0.19^{a}	1.38 ± 0.03^{a}	86.29 ± 1.71^{a}
18% PEG6000–17% $MgSO_4$	0.87 ± 0.06^{a}	31.39 ± 1.52^{f}	3.44 ± 0.19^{f}	205.78 ± 2.97^{h}

Note. *Means ± S.D. from triplicate determinations.

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

K_p: partition coefficient of protein; SA: specific activity (unit/mg protein); PF: purification factor (fold); Yield: activity recovery (%).

4.3.2.2 Effect of salts on bromelain partitioning

The effect of salt types and concentrations on the partitioning factors (K_p, SA, PF and Yield) are shown in Table 4.8. Salts are frequently used in ATPS to improve the partitioning

of the target molecules between the phases (Nalinanon et al., 2009; Klomklao et al., 2005). The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecule because of the water structure breaking effects (Nalinanon et al., 2009). From the results, the K_p of the whole ATPS system was in the range of 0.60 to 6.46. The increase in salt concentration from 14 to 20% contributed to an increase in bromelain partition coefficients. This phenomenon could be due to the "salting out effect", which resulted in increased bromelain partitioning to the top phase (Babu et al., 2008). The highest K_p (6.46) was found in the system of 18% PEG6000-20% K_2HPO_4. It is implied that not only that most bromelain partitioned to the top phase, but also other proteins as indicated by low of SA. A phase containing 18%PEG6000 and 17%$MgSO_4$ gave the highest SA (31.39) and PF (3.44). The activity yield of bromelain by using ATPS was in the ranges of 101 to 206%. The maximal yield of 206% was obtained from the system containing 18%PEG6000-17%$MgSO_4$. $MgSO_4$ provided the highest bromelain recovery when compared to the other 2 salts. The effectiveness of the salt is mainly determined by the nature of the anion. Multicharged anions are the most effective their order: $SO4^{2-}$ > $HPO4^{2-}$ > acetate > Cl^{-1}. The order of cations is usually given as $NH4^{+}$ > K^{+} > Na^{+} > Li^{+} > Mg^{2+} > Ca^{2+} (Roe, 2000). Increasing the salt concentration in the range of 14 to 20% could not provide a good trend of bromelain purity. It may have needed a wider range of salt concentration to achieve a high PF.

However, Magnesium salt has been reported to be successful in partitioning of protease from several raw materials. Klomklao et al. (2005) reported that using 20% PEG1000 20% $MgSO_4$ gave the highest SA and PF in partitioning protease from tuna spleen. Besides, Nalinanon et al. (2008) reported the same result in partitioning of protease from the stomach of albacore tuna. For the present results, the system comprising 18% PEG6000-17%$MgSO_4$ was chosen and the top phase of this system was used as the "isolated bromelain" for characterization.

Table 4.8 Effect of Salts on Partitioning of Bromelain from *Phu Lae* Pineapple Peel

Phase composition (%,w/w)	K_p *	SA	PF	Yield (%)
18% PEG6000–14%$MgSO_4$	$0.60 \pm 0.01^{a**}$	25.93 ± 0.24^{g}	2.85 ± 0.03^{f}	162.88 ± 0.12^{h}
18% PEG6000–17%$MgSO_4$	0.87 ± 0.06^{b}	31.39 ± 1.52^{h}	3.44 ± 0.19^{g}	205.78 ± 2.97^{i}
18% PEG6000–20%$MgSO_4$	0.92 ± 0.03^{b}	21.41 ± 0.26^{e}	2.34 ± 0.03^{e}	117.42 ± 0.64^{c}
18% PEG6000–14%$(NH_4)_2SO_4$	1.67 ± 0.06^{c}	18.00 ± 0.52^{d}	1.97 ± 0.11^{d}	101.69 ± 0.90^{a}
18% PEG6000–17%$(NH_4)_2SO_4$	2.82 ± 0.15^{e}	22.08 ± 0.20^{f}	2.43 ± 0.15^{e}	112.76 ± 0.70^{b}
18% PEG6000–20%$(NH_4)_2SO_4$	7.23 ± 0.46^{g}	18.61 ± 0.12^{d}	2.04 ± 0.15^{d}	158.06 ± 1.54^{g}
18% PEG6000–14%K_2HPO_4	2.48 ± 0.15^{d}	12.49 ± 0.02^{a}	1.38 ± 0.01^{a}	133.88 ± 0.48^{e}
18% PEG6000–17%K_2HPO_4	2.80 ± 0.06^{e}	16.59 ± 0.06^{c}	1.82 ± 0.02^{c}	141.83 ± 1.14^{f}
18% PEG6000–20%K_2HPO_4	6.46 ± 0.28^{f}	15.59 ± 0.01^{b}	1.73 ± 0.01^{b}	125.39 ± 13.45^{d}

Note. *Means ± S.D. from triplicate determinations.

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

K_p: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery.

4.3.2 Characteristics of isolated bromelain

4.3.2.1 Protein patterns and activity staining

The protein patterns of the crude extract and the isolated bromelain from the 18%PEG6000–17%$MgSO_4$ system are shown in Figure 4.11. The migration of the proteins (Figure 4.11A) in the crude extract, top phase and bottom phase fractions were quite different. In the non-reducing condition, the crude extract (lane 1) showed a major protein composition with the MW of 28 kDa. In addition, the proteins with the MW range of 28-30 kDa clearly partitioned to the top phase (lane 2), while others did not clearly present in the bottom phase (lane 3). The commercial stem bromelain exhibited a protein band at a MW of 28 and 30 kDa (lane 4). The differences in the protein component fractions are probably due to the differences in the amount and characteristics of the interfering proteins in each phase. In reducing condition (with βME),

the protein migration in the crude extract for the top and the bottom phase fractions were quite different. The crude extract (lane 5) and the bottom phase (lane 7) showed two small protein bands with a MW in the range of 18.3-28 kDa, while this protein disappeared in top phase. Absence of the major components in the top phase (lane 6) may be because the alteration of its molecular weight or surface residues by reducing agent. The present of a reducing agent, such as β-mercaptoethanol or dithiothreitol, breaks the disulfide bonds in the protein structure leading to protein with less conformationally stable and losing of functional and/or structurally important elements of the domain tertiary structure in place (Roe, 2000; Walsh, 2002). Umesh et al. (2008) reported that the bromelain extracted from pineapple core showed the MW ~26 kDa.

To verify the band of bromelain, activity staining was performed and is shown in Figure 4.11B. The clear zone caused by hydrolytic activity was clearly observed on the blue background in the crude extract (lane 1) and the top phase (lane 2). The proteolytic activity was not only found in the crude extract and the top phase, but also in the bottom phase (lane 3). Shifting of protease band (clear zone) in each fraction, resulted in different positions of clear zone was observed. It means that each fraction contained different types of protease that could hydrolyze the casein in the SDS-PADE gel. To prove this obtained bromelain, commercial stem bromelain was also used as reference. A clear zone of this sample appeared at a MW of 28 kDa (lane 4). The pineapple plant was shown to contain at least four distinct cysteine proteinases. The major proteinase present in plant stem extracts was stem bromelain (23.8 kDa), whilst fruit bromelain (23 kDa) was the major proteinase in the fruit. Two additional cysteine proteinases were detected only in the stem: ananain (23.46 kDa) and comosain (24.50 kDa) (Maurer, 2001). There was no observation of clear zone in the crude extract, top phase and bottom phase fractions under reducing condition. This is probably because the stabilizing disulfide bonds in the bromelain structure were broken and resulting in a loss of enzyme structure. Consequently, hydrolytic activity was not observed.

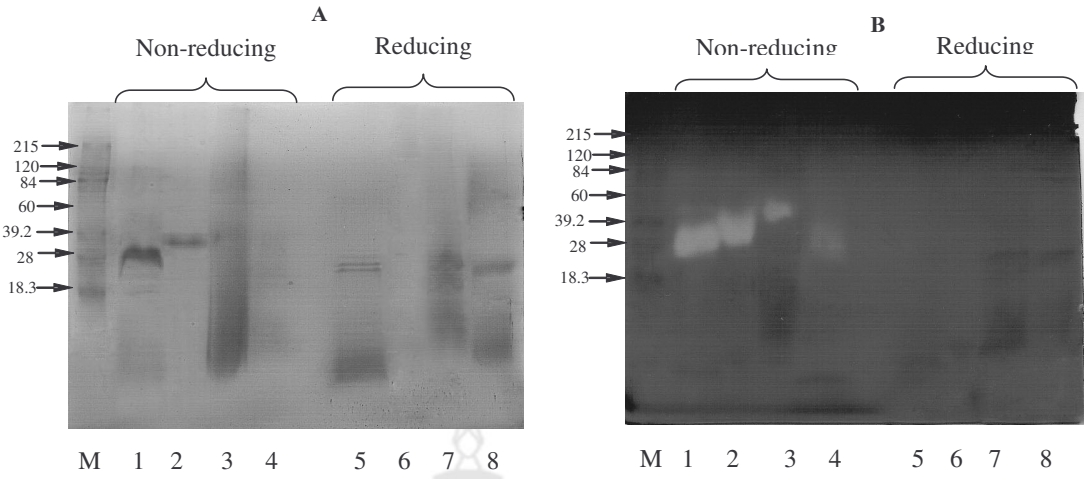


Figure 4.11 Protein Patterns (A) and Activity Staining (B) of Bromelain from *Phu Lae* Pineapple Peel Partitioned with 18% PEG6000–17% $MgSO_4$ ATPS. (6 μg and 4 μg protein were loaded in to the gel for protein patterns and activity staining, respectively); lane 1, 5: crude extract, lane 2, 6: top phase, lane 3,7: bottom phase, lane 4,8: stem bromelain and M: molecular weight protein makers

4.3.2.2 pH profile and stability

The effect of pH (3-10) on the activity of bromelain was measured and reported as a relative protease activity against the control (Figure 4.12A). The bromelain exhibited a broad pH activity profile, as indicated by still high relative activity (>60%) at all tested pHs. The maximum activity was found at pH 8.0. The bromelain activity gradually decreased at acidic pH of 3-4 and also at an alkaline pH of 10-12. This is supported by the results in Liang, Huang & Kwok (1999): the bromelain has a wider pH range for optimum activity at 6.8-9.0. In the case of the other plant proteases, Vallés et al. (2007) reported that proteolytic enzymes from ripe fruits of *Bromelia antiacantha* Bertol exhibited high caseinolytic activity (higher than 80%) in a broad pH range (5–9) as well. In addition, enzymes from unripe fruits of *Bromelia hieronymi* Mez and Asclepain cI from the Latex of *Asclepias curassavica* L. showed the optimum pH activity on casein between pH 7.5 and 9.0 (Bruno, Sebastian, Trejo, Nestor, Caffini, Laura & Lope, 2006; Liggieri, Cecilia, Sebastia, Canals, Avile & Priolo, 2004). For the result in this experiment, high relative activity (>80%) was found in the pH range of 5-11. In this regard, the isolated enzyme is unique, and therefore might be useful for application in the food and pharmaceutical industries.

The stability of bromelain incubated with various pH buffers is shown in Figure 4.12B. Bromelain was able to retain most of its activity (>70%) at pH 9.0. The enzyme stability slightly declined (around 10%) in an acidic and alkaline areas. In addition, the pH stability of bromelain was similar to that of the protease from Asclepain cI (pH 8.5) (Liggieri et al., 2004) and *Funastrum clausum* latex (pH 8.0-9.0) (Morcelle, Caffini & Priolo, 2004). The activity of this enzyme sharply deceased (<70%) in an acidic pH range, possibly due to the denaturation of the enzyme. Under a very acidic and alkaline pH, there is a charge repulsion, which decreases electrostatic bonds (Naz, 2002). For the present result, it should be noticed that the enzyme seems to be more stable in an alkaline rather than in an acidic condition.

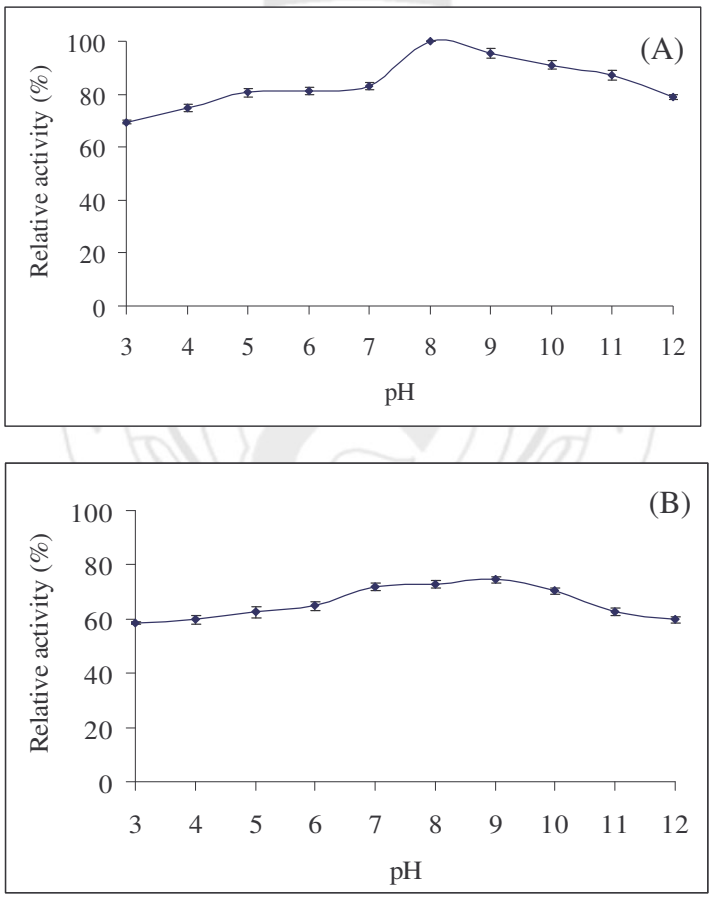


Figure 4.12 Effect of pH on the Activity of and pH Stability Bromelain from *Phu Lae* Pineapple Peel Partitioned with 18% PEG6000–17% $MgSO_4$ ATPS.

4.3.2.3 Thermal profile and stability

For the thermal profile, the isolated bromelain was assayed at a temperature ranging from 30 to 100°C. The relative proteolytic activity against the casein was calculated and is presented in Figure 4.13A. The highest relative bromelain activity (>80%) was found at 50-60°C. As the incubation temperature increased higher than 60°C, the bromelain activity dramatically decreased and then reached a plateau at 60°C. This result is similar to previous reports (Laing et al., 1999; Koh et al., 2006; Valles et al., 2007). As the temperature increases, more molecules gain enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to be disrupted (Naz, 2002). Therefore, a decrease in activity was detectable when the temperature was increased. The knowledge of optimum temperature is of use to explore the usefulness of the enzyme for possible applications. As reported, bromelain is remarkably heat stable, retaining proteolytic activity (>80%) between 50 and 60°C where most enzymes are destroyed or denatured.

The thermal stability of isolated bromelain was also measured at 90°C at different times. The results are presented in Figure 4.13B. The relative activity of bromelain decreased about 70% after 5 min of incubation and seemed to be constant until 60 min. However, some plant proteases showed different loss of activity. Vallés et al., (2007) reported that proteolytic enzymes from ripe fruits of *Bromelia antiacantha* Bertol retained 80% of its initial activity after incubation at 60 °C for 30 min. In addition, the caseinolytic activity of Asclepain cI became almost completely lost after heating for 40 min at 70°C (Liggieri et al., 2004). High temperature is found to be an irreversible-denaturant of proteins and enzymes. Enzymes are deactivated at high temperature due to the partial unfolding of their molecules (Naz, 2002).

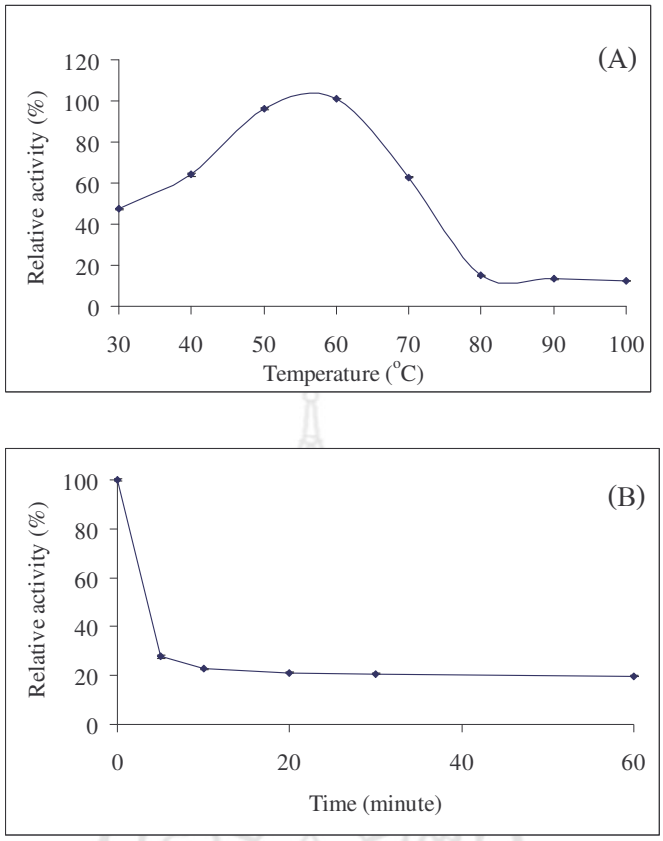


Figure 4.13 Effect of Temperature on the Activity and Thermal Stability of Bromelain from *Phu Lae* Pineapple Peel Partitioned with 18% PEG6000-17% $MgSO_4$ ATPS. The enzyme was incubated at 90°C for 0-120 minute before determined its activity at 37°C, pH 7.

4.3.3.4 Effect of salt on bromelain activity

The ability of extracted bromelain to retain its activity under growing ionic strength was tested by exposing it at different concentrations of NaCl (0-1.5%, v/v). The activity of the bromelain decreased when NaCl concentrations increased (Figure 4.14). At 0.25% NaCl, the enzyme activity decreased about 20% when compared with the control (without NaCl). However, when the salt reached 1.5% approximately 60% of relative activity was still obtained. The enzyme was stable enough at moderate ionic strength values. The extracted bromelain showed less relative activity than Asclepain cI which declined only by 30% when NaCl rose to 5.84% concentration (Liggieri et al., 2004). However, bromelain extract from *Phu Lae* was more resist in NaCl than that from *Nang Lae* which reduced its activity around 50 at the presence of 1.5% NaCl

(Figure 4.8). A loss of bromelain activity might be due to the changes in the enzyme structure. NaCl at higher concentrations possibly competed with the enzyme in water binding, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation (Switzer, 1999). In this regard, the isolated enzyme can be used with salt marination in the meat industry due to its stability in salt concentration.

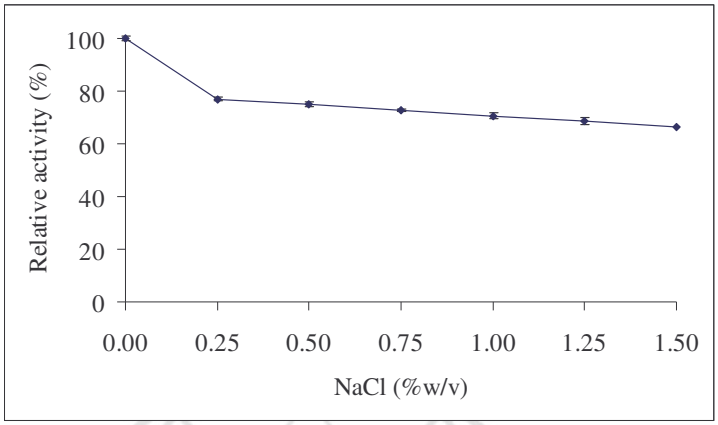


Figure 4.14 Effect of Salt Content on the Activity of Bromelain from *Phu Lae* Pineapple Peel Partitioned with 18% PEG6000-17% $MgSO_4$ ATPS.

4.3.3 Effect of bromelain on collagen hydrolysis

The protein patterns of bovine (A) and giant catfish skin (B) collagens hydrolyzed by isolated bromelain are shown in Figure 4.15. Similar hydrolyzed patterns between bovine and giant catfish skin collagens were observed. Hydrolytic patterns of collagen were clearly observed when the unit of isolated bromelain increased. Compared to giant catfish skin collagen, bovine collagen was more resistant to bromelain hydrolysis. A disappearance of major components (α and β) of collagen were observed when the addition level was 0.3 and 0.12 units for bovine achilles tendon collagen (A) and giant catfish skin collagen (B), respectively. Collagen hydrolysis was bromelain concentration dependent. The bromelain exhibits a high degree of efficiency at hydrolyzing both types of collagens, while giant catfish skin collagen was more susceptible to hydrolysis than the other. From the result, it was found that the extracted bromelain exhibited a high degree of hydrolysis for collagen from both sources. This contributes to the use of this enzyme as a meat tenderizer. As reported in previous works, bromelain has been used to decrease

the toughness of beef (Lizuka et al., 1999; Wada et al., 2002), coarse dry sausage (Melendo et al., 1996) and tenderized squid (Melendo et al., 1997).

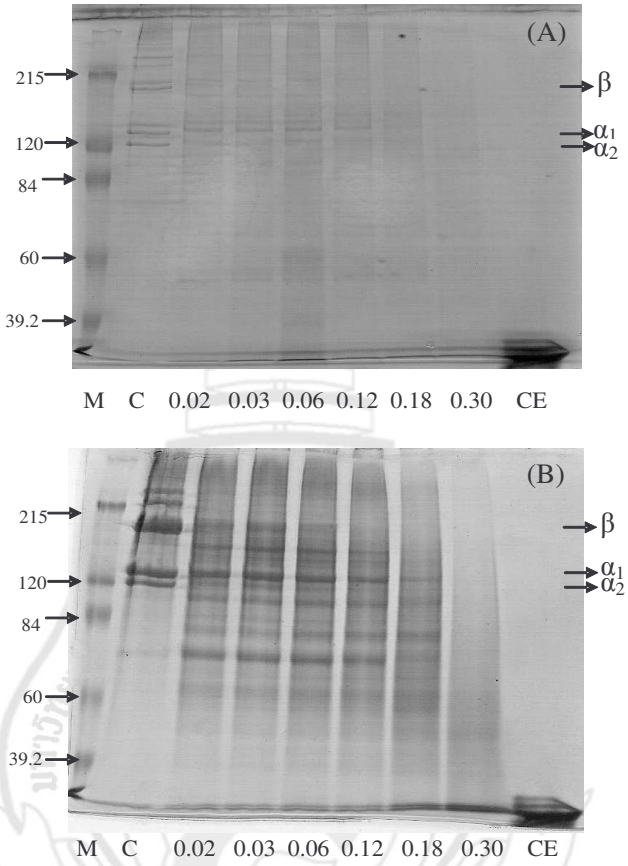


Figure 4.15 Peptide Mapping of Bovine Achilles Tendon Collagen (A) and Giant Catfish Skin Collagen (86.28% protein) (B) Digested with Bromelain from *Phu Lae* Pineapple Peel Partition with 18% PEG6000-17% $MgSO_4$ ATPS; M: molecular weight protein makers; C: control; Numbers represent the activity units of bromelain; CE: crude extract.

4.4 Application of bromelain extract in muscle foods

In this experiment, crude extract derived from blending pineapple peels (*Phu Lae*) with a cold extraction buffer (0.1 M sodium phosphate buffer with 15 mM cysteine and 2 mM EDTA,

pH 7.0) was used as a raw material for bromelain partitioning with 18% PEG6000-17% $MgSO_4$ ATPS. The bromelain extract (BE) powder obtained from freeze drying the top phase fraction of the system was applied in different muscle food sample.

4.4.1 Effects of bromelain extract on the physical properties of muscle foods

4.4.1.1 Textural properties

Textural properties of meat samples (beef, chicken and squid) after treating with various amount of BE were studied. The shear force values significantly decreased in all of the BE-treated samples when compared to control (without BE addition) ($P<0.05$). It was observed that the shear force values continuously decreased in the entire treated samples when the level of BE increased (Figure 4.16A). The firmness for the staring beef, chicken and squid muscles were 570, 111, and 314 N, respectively. Adding 3% (w/w) BE to the samples reduced the firmness of the samples by about 20, 28, and 31% when compared to the control for beef, chicken and squid, respectively. When 20% (w/w) of BE was added to the sample, firmness reduction of more than 61% was found when compared to the control. Reduction of meat firmness resulting from the action of the enzyme presented in the BE on myofibrillar proteins. When the breakdown of myofibrillar protein occurred, small peptides, or low MW of proteins were generated. This resulted in the decrease in the firmness of meat samples.

Data on the toughness of the treated meat samples is presented in Figure 4.16B. Similar results were obtained compared to the firmness values as mentioned above. The toughness indicated by the amount of intramuscular connective tissue, intramuscular fat, and the length of the sarcomere (Kemp, Sensky, Bardsley, Buttery & Par, 2010). This value is related to the acceptable meat quality by the consumer. The eating quality of muscle foods is directly related to meat toughness. Older animals normally produce the tougher meat than that of young ones. The tougher meat, the more connective tissues are present. Larger size animals also normally give a larger amount of connective tissues rendering the meat to be tougher. Tenderization associated with postmortem aging is due to the action of enzymes that are endogenous to the muscle. Proteolytic enzymes such as plant proteases can cause the meat tenderization (Kemp et al., 2010). According to Naveena et al. (2004a, 2004b), Warner–Bratzler shear force values were diminished by plant proteases, including cucumis and ginger powders. They also observed a decrease in shear force values when ginger rhizome extract was added to

buffalo meat with extensive degradation of muscle fibers and connective tissue layers surrounding the muscle fibers. Reduction in shear force values with ginger extract treatment was also reported in chevon muscles (Pawar et al., 2007), buffalo (Syed Ziauddin, Rao & Amla, 1995) and spent hen meat (Naveena & Mendiratta, 2001).

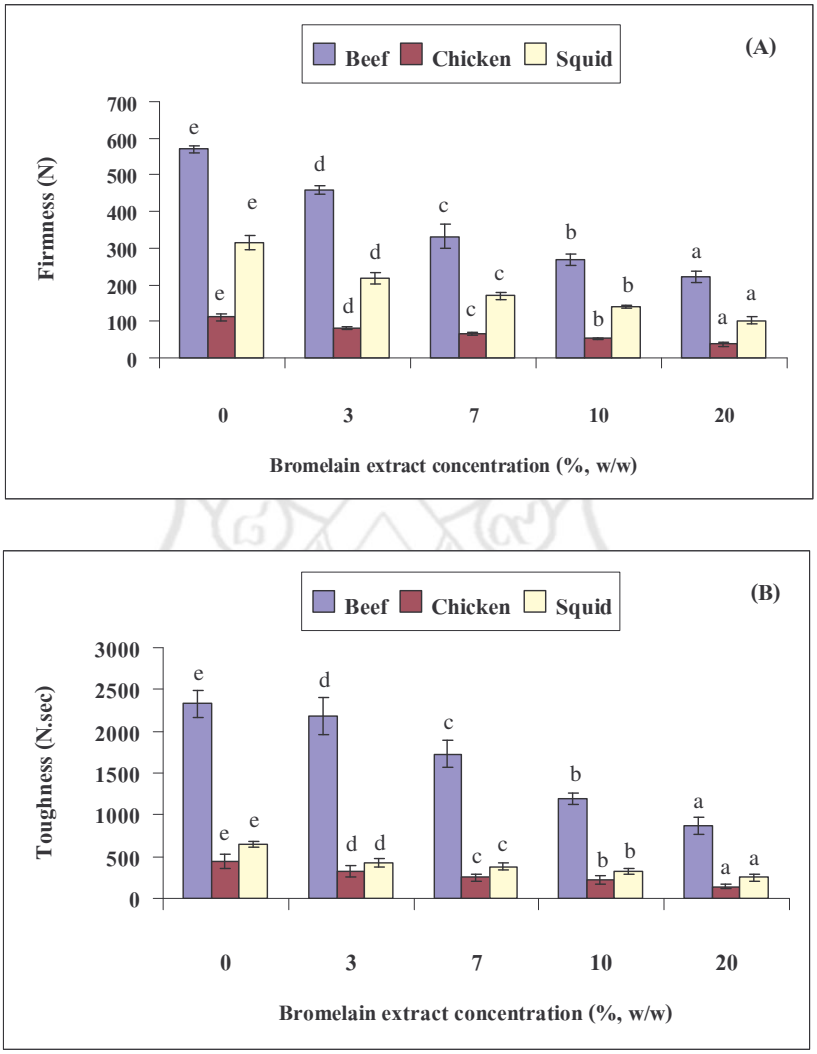


Figure 4.16 Firmness (A) and Toughness (B) of Muscle Samples Treated with Different Bromelain Extract Concentrations. Different letters each bar in the samples indicated significant differences ($P< 0.05$).

4.4.1.2 Water holding capacity and cooking yield

There was a significant reduction of WHC in all of the treated samples, especially when the concentration of BE increased ($P<0.05$). The lowest WHC values were observed in the treated beef muscle when compared with others (Table 4.9). The squid showed the highest WHC, while the highest value for each meat type was found in the untreated samples. The higher WHC in the control sample may be due to the overall reduction in the protein reactive group that is available for water binding (Forrest, Aberle, Hedrick, Judge, & Merkel, 1994). The reduction in WHC in all of the treated samples might be due to lower pH. This drop in pH may be responsible for the overall reduction in protein reactive groups available for water holding. Slight denaturation of sarcoplasmic proteins, which play an important role in determining WHC, could be the reason for lowering of WHC (Joo, Kauffman, Kim, & Park, 1999). The WHC of meat is very important since many physical properties such as color, texture, and firmness of raw meat are partially dependent on the WHC. However, in this experiment, the lowered WHC in the BE treated samples are probably due to the denaturation of myofibrillar proteins, which play a role in water retention. BE could hydrolyze these proteins into small peptides or amino acids, which then could not function their responsibility. Reduced WHC results from myofibrillar shrinkage, as well as the movement of water from the myofilament space the extra-cellular space. There are three main factors involved in the shrinkage and/ or swelling of the myofibrils: the onset of rigor mortis, the extent of decline in pH, and protein fragmentation (Huff-Lonergan & Lonergan, 2005).

In addition, all three BE-treated samples were significantly different from the control samples in the cooking yield ($P<0.05$). Marinated meat samples with BE showed a reduction in cooking yield (Table 4.9). The cooking yield, inversely proportional to cooking loss, was found to decrease with the increase in the level of BE addition in all samples. The highest and lowest cooking yield of staring meat was found in squid and beef muscles, respectively. When treated with 20% (w/w) BE, the highest and lowest value was also found in squid and beef, respectively. This result indicated that thermal treatment could remove more water from treated beef sample than that of squid muscle. It can be implied that the enzyme in BE could hydrolyze protein in beef more than in chicken and squid. Moreover, the addition of BE at different levels could possibly affect tenderization by proteolytic enzymes from the pineapple peel. Pawar et al. (2007) reported

that the reduction of cooking yield in the treated sample when compared to the control may be caused by the degradation of sarcoplasmic and myofibrillar proteins.

4.4.1.3 Muscle microstructure

Scanning electron micrographs of the muscles treated with (20% BE, w/w) and without BE (control) are shown in Figure 4.17. The structures of all the control muscle fibers had well organized structure and were closely bound to one another. Whereas in the BE treated samples, these were broken muscle fibers in different bundles, they were less attached and there was a loss of muscle interaction. Furthermore, there was a disintegration of myofibrillar structure with a lot of exudates. This produced an increase of inter-fibrillar space, or voids between muscle fibers, which grow and revealed big gaps between them. These gaps might be due to the degradation of endomysial collagen and sarcolemma surrounding the muscle fibers. The degradation of the muscle structure becomes evident when that the cell membranes are more severely degraded. The junctions of the myofibrillar package and the sarcolemma have disappeared from most cells. Disruption of the structure intramuscular connective tissue is another cause for meat tenderization by the proteolytic enzymes. The microstructure of the 20 BE samples showed a strong correlation with the previous results in terms of textural properties, TCA-soluble peptides content and SDS-PAGE patterns. Naveena (2004b) studied the effect of ginger extract on the microstructure of buffalo muscle by using SEM and they 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 honeycomb structure observed in the beef treated with elastase from the *Bacillus* strain (Qihe et al., 2006).

Table 4.9 Physico-Chemical Properties of Meat Samples Treated with Bromelain Extract at Difference Concentrations

Sample	Properties	Concentration (%, w/w)**				
		0	3	7	10	20
Beef	pH*	5.28 ± 0.01^{c}	5.27 ± 0.01^{ab}	5.26 ± 0.02^{ab}	5.26 ± 0.02^{ab}	5.24 ± 0.01^{a}
	Moisture (%)*	74.64 ± 0.29^{c}	70.03 ± 0.52^{b}	69.71 ± 0.53^{b}	69.27 ± 1.21^{b}	66.56 ± 1.10^{a}
	WHC (%)***	40.01 ± 1.48^{e}	27.97 ± 0.80^{d}	22.36 ± 0.68^{c}	16.25 ± 0.49^{b}	12.01 ± 0.12^{a}
	Cooking loss (%)***	77.89 ± 2.31^{c}	63.67 ± 1.80^{b}	62.38 ± 1.67^{b}	62.93 ± 1.83^{b}	57.83 ± 2.60^{a}
Chicken	pH	5.93 ± 0.03^{d}	5.81 ± 0.01^{bc}	5.81 ± 0.01^{bc}	5.78 ± 0.02^{ab}	5.76 ± 0.01^{a}
	Moisture (%)	75.78 ± 1.27^{d}	71.17 ± 0.39^{c}	66.58 ± 0.81^{b}	66.26 ± 0.59^{b}	59.63 ± 1.87^{a}
	WHC (%)	89.62 ± 0.97^{e}	84.70 ± 0.27^{d}	61.10 ± 1.42^{c}	43.69 ± 0.18^{b}	34.04 ± 0.25^{a}
	Cooking loss (%)	84.12 ± 1.68^{c}	83.13 ± 3.19^{c}	76.05 ± 1.24^{b}	75.09 ± 2.96^{b}	72.74 ± 3.51^{a}
Squid	pH	7.04 ± 0.01^{e}	6.79 ± 0.01^{d}	6.70 ± 0.06^{c}	6.44 ± 0.01^{b}	6.06 ± 0.01^{a}
	Moisture (%)	84.07 ± 0.10^{e}	80.74 ± 0.22^{d}	78.91 ± 0.44^{c}	77.11 ± 0.31^{b}	75.53 ± 0.60^{a}
	WHC (%)	100.00 ± 0.00^{c}	99.36 ± 0.03^{c}	98.84 ± 0.16^{c}	95.66 ± 0.09^{b}	87.80 ± 1.40^{a}
	Cooking loss (%)	77.09 ± 0.95^{d}	75.04 ± 2.17^{c}	74.31 ± 0.49^{bc}	73.07 ± 1.79^{ab}	72.01 ± 1.28^{a}

Note. *Means ± S.D. from triplicate determinations. **Different superscripts in the same row indicate the significant differences ($P<0.05$). Number of observations = ***10. Bromelain extract (BE) was obtained from the top phase of aqueous two phase system comprising of 18% PEG 6000–17%$MgSO_4$. The muscle samples were treated with difference concentrations of BE and then subjected to incubate at room temperature for 60 min. before properties measurement.

4.4.2 Effect of bromelain extract on chemical properties of muscle foods

4.4.2.1 pH and moisture content

A significant decrease in pH was found in all of the treated samples when compared to the control. The low pH of BE (4.07) was probably caused by the lower pH of the treated samples (Table 4.9). Moreover, hydrolysis of the muscle by BE may result in releasing amino acids that can reduce the pH of the system. The decrease of pH of the treated sample (20%BE, w/w) ranges are 5.28-5.24, 5.93-5.76 and 7.04-6.06, when compared to the control for beef, chicken and squid, respectively. The highest pH of starting material, the highest pH was found in the squid muscle. In addition, the most decreased value of pH was also found in this kind of muscle. The pH value in meat product is highly important because it has a major influence on other physic-chemical and quality properties such as WHC, tenderness, and juiciness (Goli, Abi Nakhoul, Zakhia-Rozis, Trystram, & Bohuon, 2007). Changes in pH are caused by postmortem metabolism and also by the application of added substances to the meat during technological processes (Gault, 1985).

The powder of bromelain extract could absorb more water in the fresh meat samples, which resulted in lower moisture content when compared with the control ($P<0.05$). The moisture content in the BE-treated samples markedly decreased when compared to the untreated samples (Table 4.9). Increased in BE concentration resulted in decreased in moisture content, justifying the improvement of hydrophilic properties by the enzyme treatment in the meat samples. Also, it could probably be due to absorption of moisture in the treated meat samples. It is also probably due to the hygroscopic nature of BE powder when attached to the wet surface of the sample. The low moisture content of the treated samples was related to product acceptability for the consumer. It also affected to the yield of the final product ($p<0.05$). Naveena et al. (2001) reported moisture retention in spent-hen meats treated with ginger rhizome extract as compared with untreated sample. Similar observations were found: 77.18% moisture in ginger rhizome extract was found in a treated buffalo meat sample as compared to 76.51% in an untreated one.

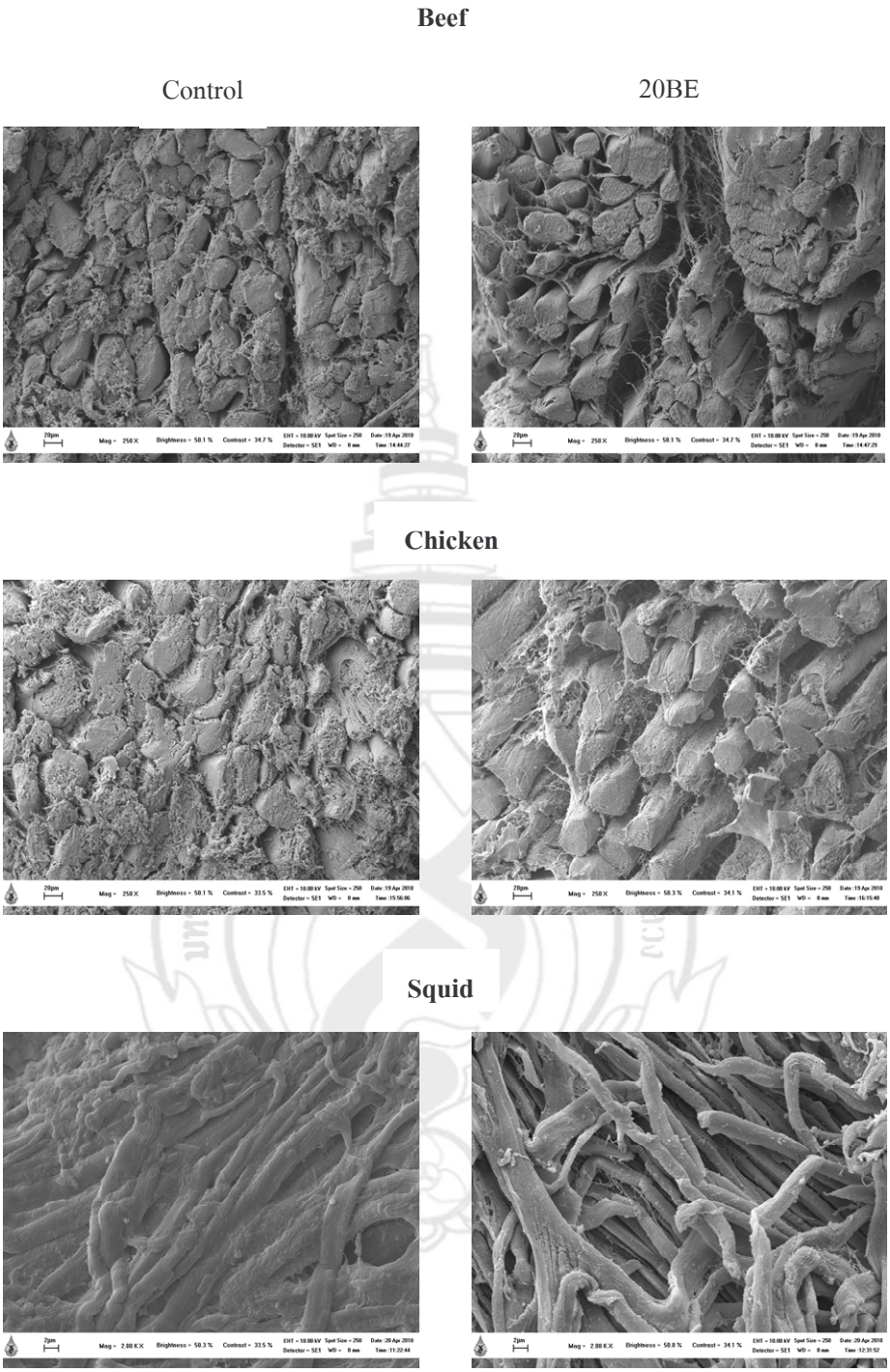


Figure 4.17 Microstructure of the Samples Treated Without (Control) and With 20% (w/w) Powder of Bromelain Extract (20BE). Magnification of 250x for Beef and Chicken Samples and 2,000x for Squid, at Acceleration Voltage of 10 kV

4.4.1.2 TCA-soluble peptides content

The highest content of TCA-soluble peptides was found in the chicken, followed by beef and squid muscle treated with 20% BE (w/w), respectively (Figure 4.18). When the concentration of BE was increased, the TCA-soluble peptide content in all of the treated sample increased ($P<0.05$). The TCA-soluble peptides were the lowest in the squid muscle when compared with others. This result coordinated with the protein patterns show higher intensity for major protein bands (Figure 4.19, lane 1). More than a 27% increase of that TCA-soluble peptides as found in the sample treated with 20% BE as compared to the control. The highest increase of TCA-soluble peptides content was found in the chicken and the lowest was belonged to the squid muscle. Amount of detectable tyrosine indicated that the endogenous oligopeptides and/or free amino acids, as well as degradation products, all accumulated after being marinated with BE. From these results, high TCA-soluble peptide content indicated a greater muscle proteins hydrolysis generated by proteolytic enzymes present in BE. The degradation of the myofibrillar proteins resulted in an increase in peptides and free amino acids.

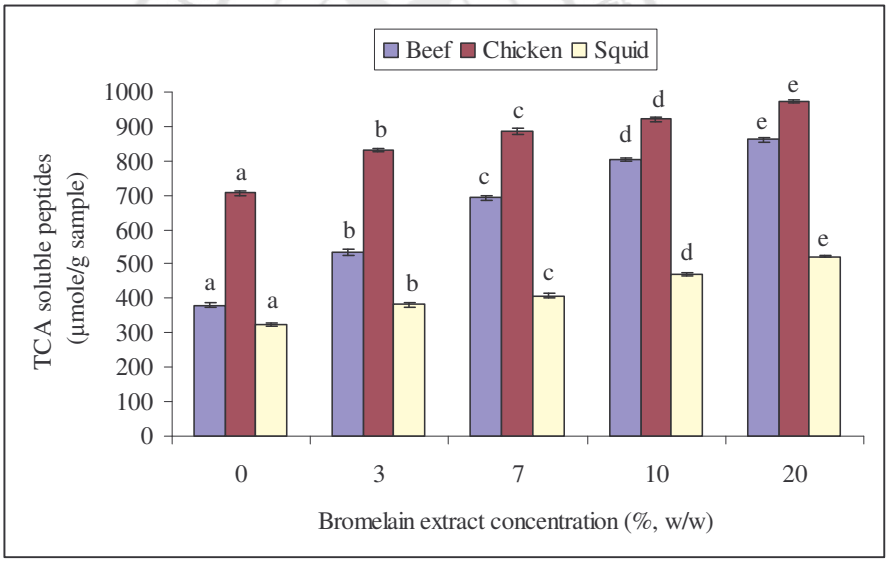


Figure 4.18 TCA-soluble peptides content of muscle samples treated with different concentrations of bromelain extract.

Increased TCA-soluble peptides content and free α-amino acids were coincidental with the continuous decreased in band intensity of the major proteins after being marinated with BE, especially in the chicken muscle (Figure 4.18, lane 5). Rawdkuen et al. (2010) reported that when using protease from *Calotropis procera* in beef, giant catfish and squid muscles, hydrolytic properties were shown to gradually decrease in numbers of protein bands and band intensity as compared with the control sample.

4.4.1.3 Hydrolytic patterns of muscles samples

To elucidate the effect of the BE on myofibrillar proteins degradation, SDS–PAGE patterns are shown in Figure 4.19. Similar protein patterns in beef and chicken were observed (lane 1), while slightly different compositions in squid muscle were observed. Myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle foods. Apart from MHC and AC, a new protein band with high intensity, paramyosin (PM), was observed in the protein patterns of the squid muscle (lane 1). There was an increase in proteolysis of the muscle proteins in all of the BE treated samples as evidenced by the reduction in the number and intensity of the protein bands when the BE concentration was added. When comparing the treatment (BE-treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. Moreover, degradation of the AC from the BE addition were also observed on the SDS–PAGE. 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 (Miller, Strange, & Whiting, 1989). From the result, it is also evident that effective cleavage of high MW proteins into low MW proteins was generated.

The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during the postmortem storage. Myosin constitutes approximately 45% of the total myofibrillar proteins, and it is the major structural protein in muscle (Schiaffino & Reggiani, 1996). Myofibrillar proteins such as troponin-T, titin, and nebulin are considered to be indicators of the overall extent of postmortem proteolysis and predictors of meat tenderness (Huff-Lonergan, Parrish, & Robson, 1995). The results from the current study appear to confirm this.

Also, MHC and AC are very stable and do not degrade under normal postmortem conditions (Bandman & Zdanis, 1988). The BE affected the MHC by fragmenting them into small molecular weight protein bands. The BE-treated meat samples were tender, and showed greater induced fragmentation of MHC. Based on these results, it could be determined that BE potentially has high proteolytic activity, making meat more tender. Sawdy, Kaiser, St-Pierre, & Wick (2004) reported a significant correlation between MHC fragments and meat tenderness in bovine muscle. Similar observations of lower numbers of protein bands in buffalo meat added with ginger and *Cucumis trigonus* extract have been reported. In addition, the increase in proteolysis can be correlated with significantly higher protein solubility. Bhaskar Sachindra, Modi, Sakhare, & Mahendrakar (2006) found cleavage of high MW proteins into lower MW proteins produced when using ginger powder in spent hen meat. Bacterial proteolytic 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, Danchev, & Kostov, 1989).

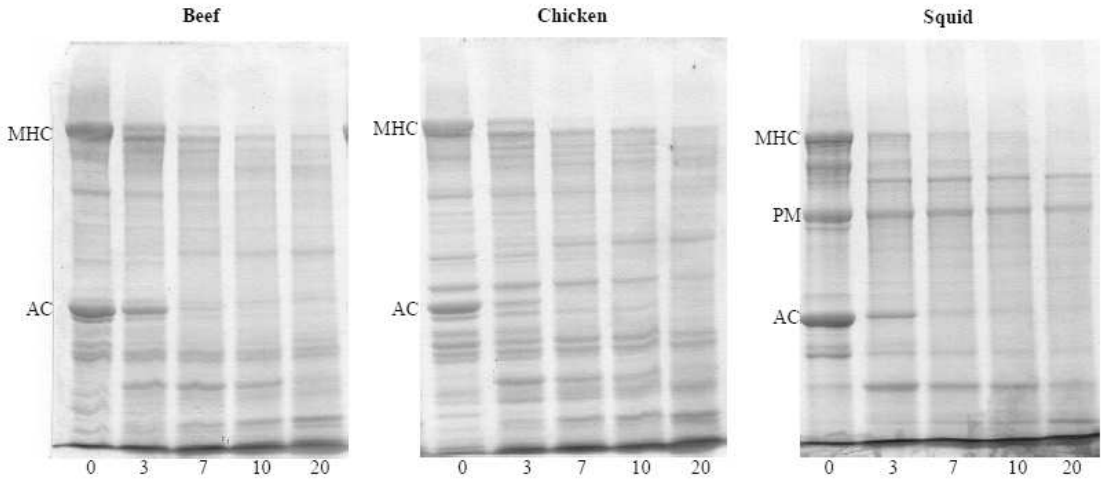


Figure 4.19 SDS-PAGE Patterns of Meat Samples Treated with Different Concentration of Bromelain Extract at Room Temperature for 60 Min. Protein (20 μg) in reducing condition was loaded into the gel. Numbers indicated the concentration of bromelain extract addition (%, w/w), MHC: myosin heavy chains; AC: actin, PM: paramyosin

CHAPTER 5

CONCLUSION

Wastes from *Nang Lae* and *Phu Lae* pineapples were used as the raw material for bromelain extraction. The peel is the largest waste portion followed by the core, stem, and crown for both cultivars. Crown provided the highest protease activity and protein content. However, the peel was selected as a potential staring material for bromelain extraction because it accounts for the largest waste proportion.

For the extraction process, the extractants played an important role in maintaining bromelain activity from the pineapple peel. Phosphate buffer with cysteine and EDTA solution showed to be the most efficient in bromelain extraction from both cultivars by providing the highest activity in the test tube and also muscle model system.

An aqueous two-phase system could be applied for the partial purification of bromelain from both *Nang Lae* and *Phu lae* pineapple peels. The top phase of 15% PEG2000-14% $MgSO_4$ and 18% PEG6000-17% $MgSO_4$ provided the highest bromelain recovery for *Nang Lae* and *Phu Lae*, respectively. According to the biochemical characteristic (pH profile and stability, thermal profile and stability and salt stability) of isolated bromelain, the extract bromelain could be applied in any food processing system, especially the muscle foods.

Physico-chemical properties and some consuming quality of beef, chicken and squid muscles treated with bromelain powder supported that this enzymes could be used as a better alternative tenderizing agent for tough meat not only in the household but also in the industrial level.

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