Chapter 3

Results and Discussion

1. Autolytic degradation of bigeye snapper muscle

Autolytic degradation of mince and washed mince from two species of bigeye snapper is shown in Figure 7 and 8. Higher autolytic activities in both mince and washed mince from two species were observed at 60°C, compared to those obtained at 50°C. Different fish had different modori-inducing proteinase (MIP), which had the different distribution as well as optimum temperature (Kinoshita *et al.*, 1990a). Kinoshita *et al.* (1990a) classified MIP based on extractability and optimum temperature into 4 groups as 1) sarcoplasmic-50°C-MIP, 2) sarcoplasmic-60°C-MIP, 3) myofibriilar-50° C-MIP and 4) myofibriilar-60°C-MIP. Higher degradation products were observed as the incubation time increased ($p<0.05$). The results indicated that thermal activated proteinases were present in both sarcoplasmic fluid as well as were associated with myofibrils, causing the degradation of myofibrils. It was evident that both sarcoplsmic proteinase (Kinoshita *et al.*, 1990b) and myofibrill-associated proteinase (Toyohara *et al.*, 1990c) were responsible for the modori. The myofibril associated proteinase is regarded as much more important since it still retains after washing process (Cao *et al.*, 1999). Consequently, those enzymes directly contributed to the degradation of proteins, resulting in poor gel network. When comparing the autolytic activity between mince and washed mince, it was found that washed mince showed a lower activity. This was presumed to be due to a removal of sarcoplamic proteinase. Nevertheless, the proteinases associated with myofibrils still remained. Approximately one-fifth of the proteinase activity in Pacific whiting was removed by the washing and dewatering steps (Morrissey *et al.*, 1995).

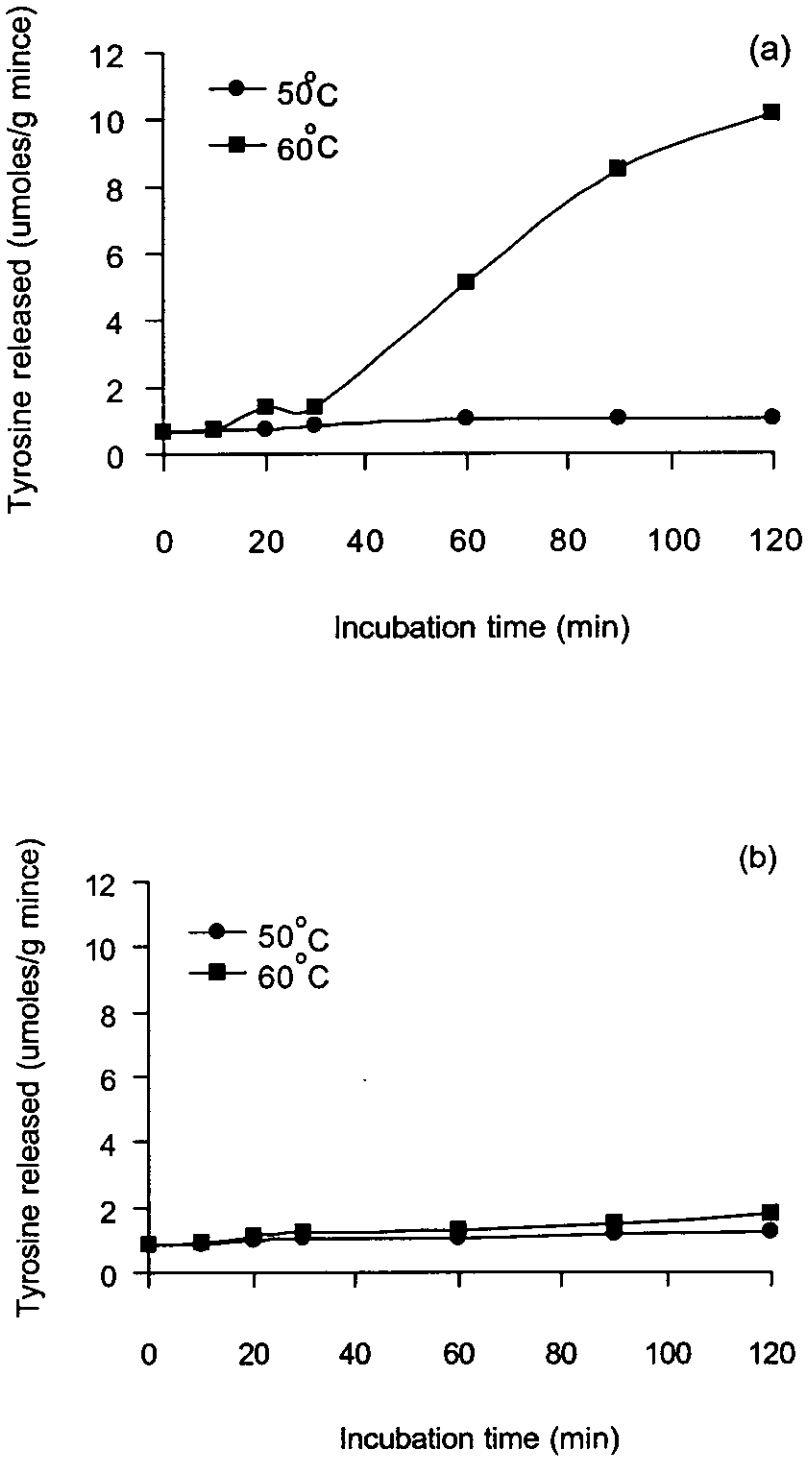

Figure 7 Autolytic degradation products in mince (a) and washed mince (b) of *P. macracanthus* incubated at 50 and 60°C for different times

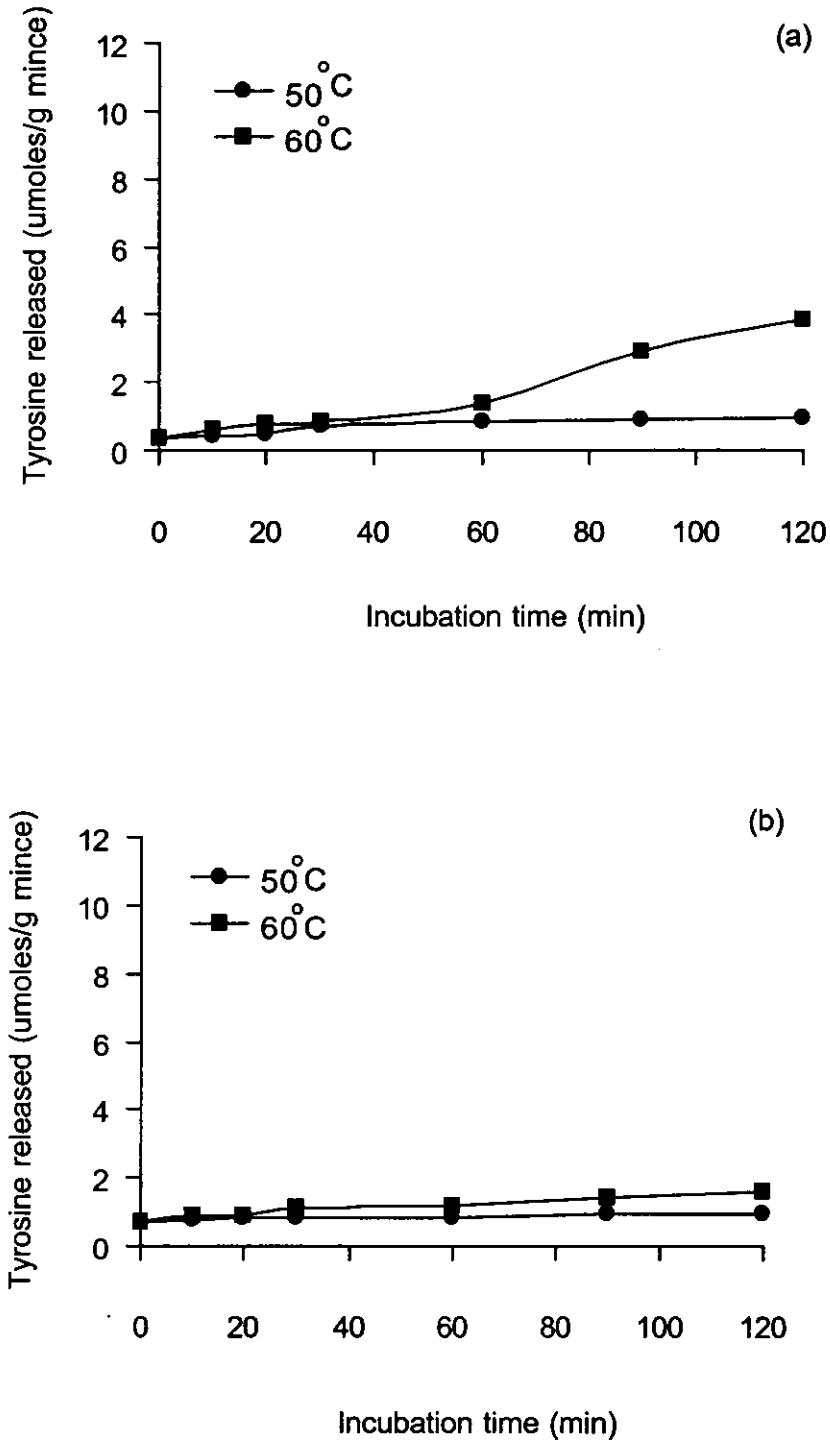

Figure 8 Autolytic degradation products in mince (a) and washed mince (b) of *P. tayenus* incubated at 50 and 60°C for different times

Benjakul *et al.* (1996) reported that cathepsin L was found as a major enzyme in Pacific whiting surimi wash water. Therefore, the proper washing is a means to reduce the proteolytic activity, especially sarcoplasmic proteinases. From the result, autolysis of mince from *P. macracanthus* at 60°C was much higher than from *P. tayenus*, however no marked differences in autolytic activity in washed mince between two species were observed. This result suggested that *P. macracanthus* muscle had a much higher sarcoplasmic proteinase activity than *P. tayenus* muscle. Kinoshita *et al.* (1990a) reported the diversity of modori inducing proteinase in fish muscle. This may lead to the remarkable species-specificity of modori phenomenon. Generally, *P. macracanthus* mince exhibited the higher autolytic activity, compared to *P. tayenus* mince. This was possibly due to the differences in proteinase activity as well as proteinase types between two species.

When protein pattern of mince and washed mince subjected to autolysis at different temperatures and times were determined by SDS-PAGE, it was noted that myosin heavy chain was more susceptible to degradation, compared to other proteins (Figure 9 and 10). Higher degradation was observed in mince than washed mince, which was associated with the higher degradation products (Figure 7 and 8). From the result, myosin heavy chain in *P. macracanthus* decreased to a higher extent, compared to that in *P. tayenus* in both mince and washed mince. Additionally, myosin heavy chain was more degraded at 60°C than at 50°C. Actin of *P. macracanthus* mince underwent degradation as the incubation time increased, while no changes in actin were obtained in *P. tayenus* mince. Actin was regarded as a proteolytically resistant protein, compared to myosin heavy chain and β-tropomyosin and troponin-T (An *et al.*, 1994b). Myofibril-bound serine proteinase from lizardfish muscle was reported to hydrolyze myosin heavy chain at 55-60°C, whereas α-actinin and actin were not degraded (Cao *et al.*, 2000a). The results suggested that

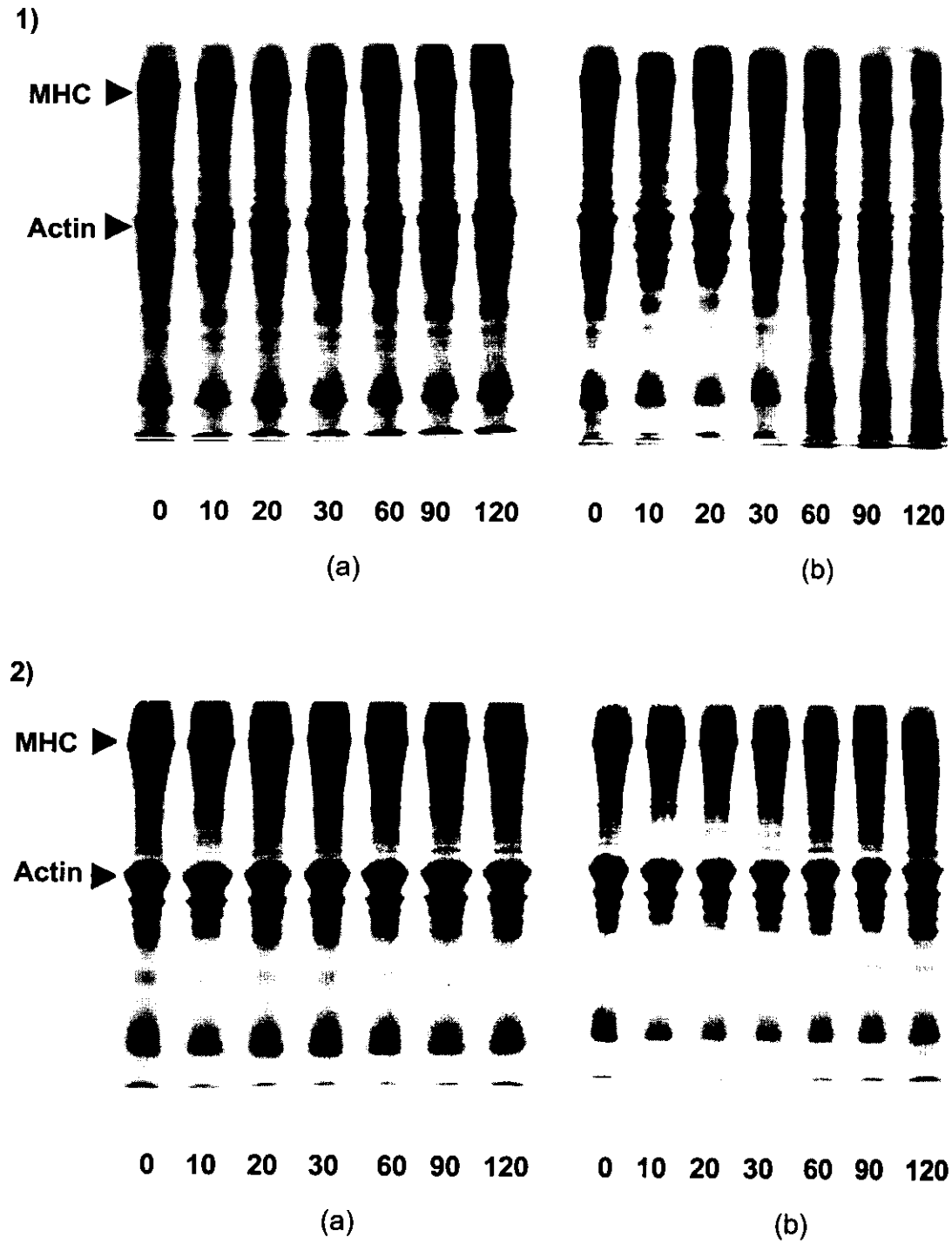

Figure 9 Autolytic pattern of mince (1) and washed mince (2) of *P. macracanthus* at 50 °C (a) and 60 °C (b). Numbers designate incubation time (min)

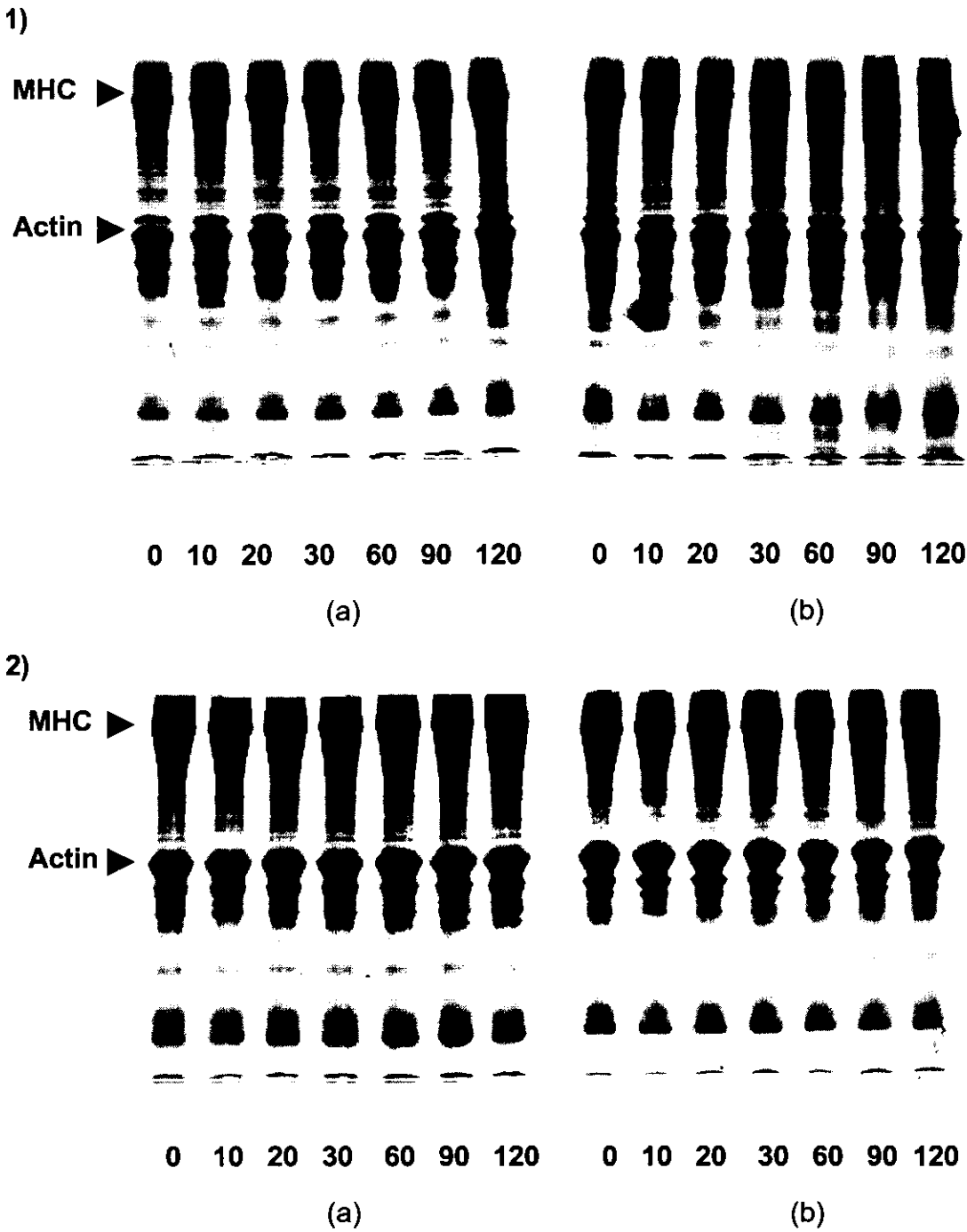

Figure 10 Autolytic pattern of mince (1) and washed mince (2) of *P. tayenus* at 50 °C (a) and 60 °C (b). Numbers designate incubation time (min)

P. macracanthus proteinase was able to hydrolyze actin, especially when no myosin heavy chain was available.

2. Temperature and pH profile of sarcoplasmic proteinase from bigeye snapper

Optimum temperature of sarcoplasmic proteinase in both species was 60°C when assayed at 5.5 and 8.5 (Figure 11 and 12). However, the maximum activity was observed at 45°C, when tested at pH 3.0. Therefore the different groups of proteinase were presumed to be present in sarcoplasmic fluid of both species. At the same temperature tested, the activity assayed at pH 8.5 was much higher than that observed at 5.5. Due to the high activity at pH 8.5 and 60°C, it was postulated to be the heat stable alkaline proteinase. Heat-stable alkaline proteinase has often reported to be responsible to textural degradation of surimi gels. Makinodan *et al.* (1985) reported that only alkaline proteinase could act at the pH of meat paste and 60°C. Alkaline proteinase from white croaker had an optimum activity at 60°C and pH 8.0 (Makinodan *et al.*, 1987). Generally, higher sarcoplasmic proteolytic activity was observed in *P. macracanthus*, compared to *P. tayenus*. The result was in agreement with the higher rate of autolysis in mince from *P. macracanthus*.

Optimum pHs of *P. macracanthus* sarcoplasmic proteinase were found at 6.5 and 8.5. For *P. tayenus*, optimum peaks appeared at 5.0 and 8.5. For both species, the activity was higher at pH 8.5, compared to other pHs (Figure 13). *P. macracanthus* showed a higher activity than *P. tayenus* under all conditions tested. The results suggested that sarcoplasmic proteinases in bigeye snapper possibly were heat stable alkaline proteinase. Choi *et al.* (1999b) reported two alkaline proteinase with optimum pH and temperature of 8.0 and 55°C in Atlantic menhaden muscle, respectively.

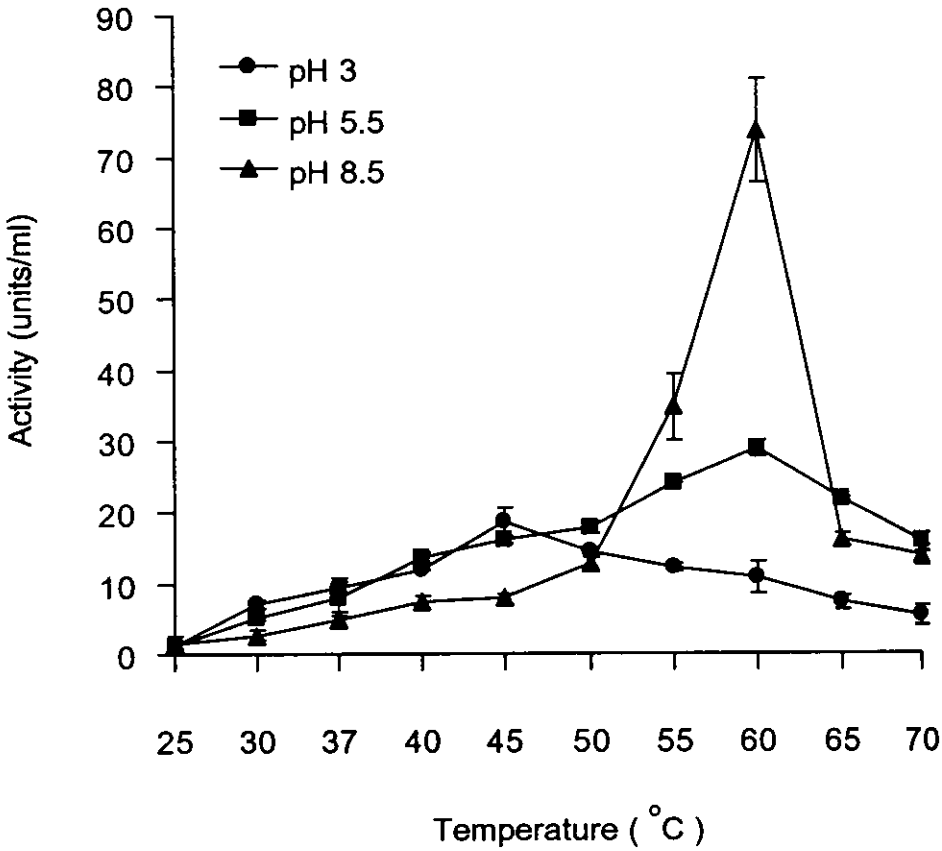

Figure 11 Temperature profiles of sarcoplasmic proteinase from *P. macracanthus*. Proteinase activity was determined by incubating reaction mixture at pH 3.0, 5.5 and 8.5 at various temperatures. TCA-soluble peptides released were determined by Lowry assay and activity was expressed as units/ml

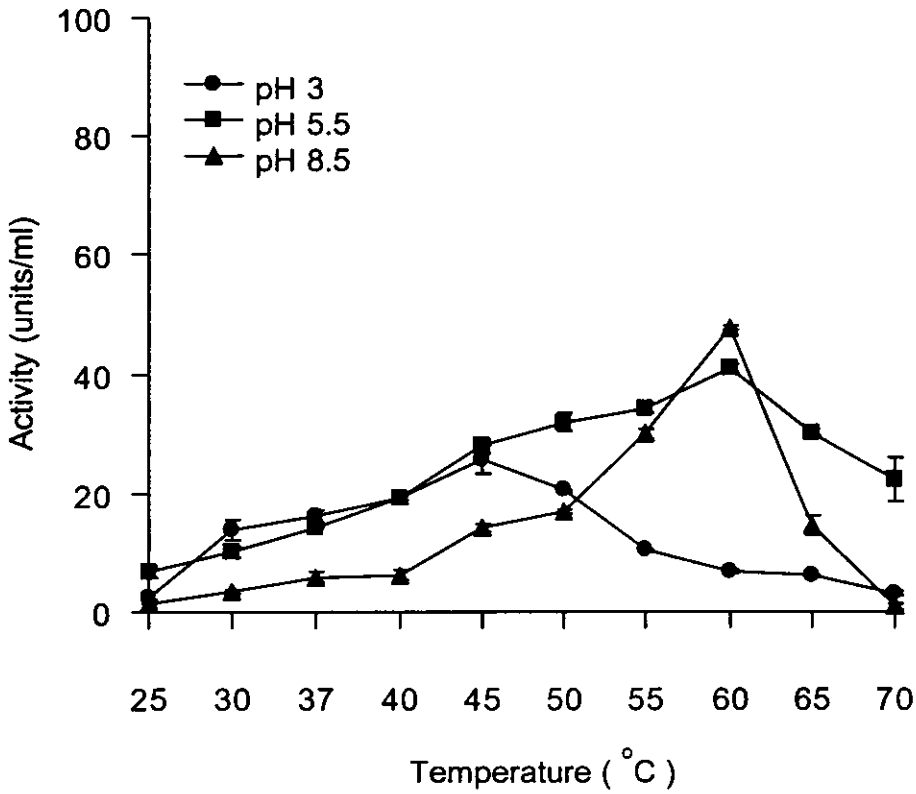

Figure 12 Temperature profiles of sarcoplasmic proteinase from *P. tayenus*. Proteinase activity was determined by incubating reaction mixture at pH 3.0, 5.5 and 8.5 at various temperatures. TCA-soluble peptides released were determined by Lowry assay and activity was expressed as units/ml

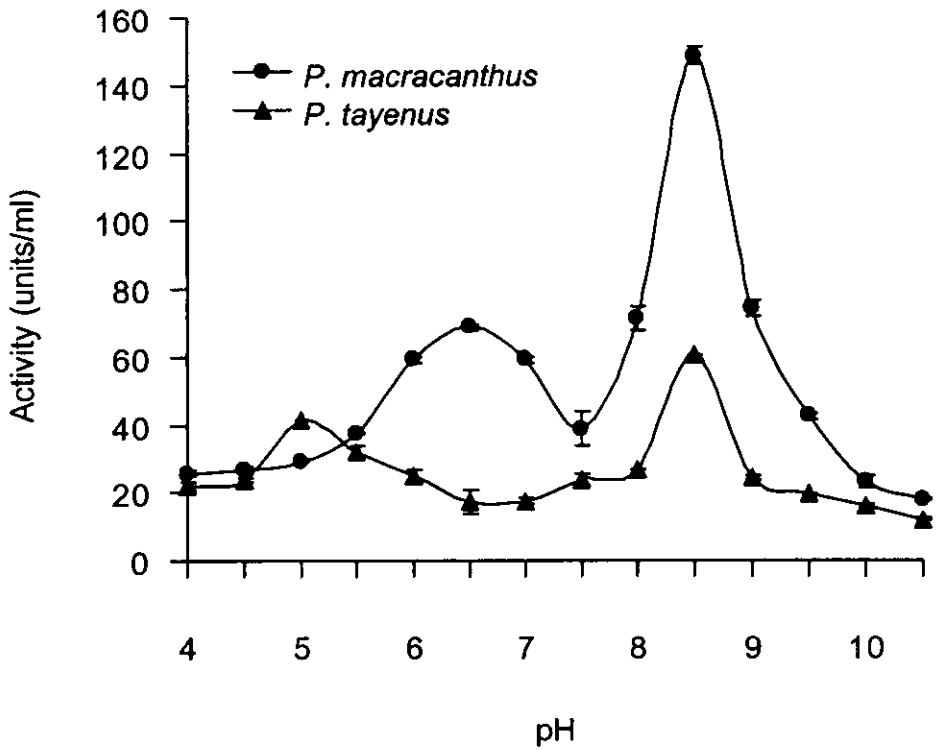

Figure 13 pH profiles of sarcoplasmic proteinase from *P. macracanthus* and *P. tayenus*. Proteinase activity was determined by incubating reaction mixture at 60°C at various pHs. TCA-soluble peptides released were determined by Lowry assay and activity was expressed as units/ml

3. Effect of inhibitors on activity of sarcoplasmic proteinase from bigeye snapper

The effect of various inhibitors on sarcoplasmic proteinase of two species of bigeye snapper is shown in Table 5. *P. macracanthus* proteinase was mainly inhibited by soybean trypsin inhibitor. Activity of 67-81% was inhibited in the presence of 0.01 mM soybean trypsin inhibitor, when tested at pH 6.5 and 8.5. For *P. tayenus*, pepstatin A showed the highest inhibition (75%) when tested at pH 5.0, while various inhibitors including soybean trypsin inhibitor, E-64 and iodoacetic acid rendered the high inhibition when assayed at pH 8.5. E-64 and iodoacetic acid are considered to be cysteine proteinase inhibitors (Hanada *et al.*, 1978; North, 1989). Soybean trypsin inhibitor and PMSF are serine proteinase inhibitors (Birk, 1976; North, 1989). Pepstatin A can inhibit most of the aspartic proteinases (Umezawa, 1976), EDTA, EGTA and 1,10-phenanthroline are metallo-proteinases inhibitors (Tsuchiya *et al.*, 1994), and lactacystin is specific for proteasome (Dick *et al.*, 1996). Thus, the results indicated the presence of various types of proteinases in the muscle of two species. Proteinase from threadfin bream was classified as a serine proteinase, based on inhibitory study (Kinoshita *et al.*, 1990c). However, proteinase from Pacific whiting was classified as cathepsin L, cysteine proteinase (Seymour *et al.*, 1994). From the inhibitory study, it can be inferred that serine proteinase was found to be a major sarcoplasmic proteinase in *P. macracanthus* muscle. Nevertheless, different sarcoplasmic proteinases were present in *P. tayenus* muscle.

Effect of different inhibitors on autolysis of washed mince from two species was compared (Figure 14). Myosin heavy chain in washed mince from *P. macracanthus* underwent much more degradation, compared to that from *P. tayenus*. For *P. macracanthus*, soybean trypsin inhibitor showed an effective inhibition, resulting in the retained myosin heavy chain. No myosin heavy chain was remained in sample without inhibitors and those added with

E-64 or iodoacetic acid. For *P. tayenus*, soybean trypsin inhibitor also exhibited the highest inhibition, compared to E-64 and iodoacetic acid. Myofibril associated serine proteinases were found in carp and lizardfish (Osatomi *et al.*, 1997; Cao *et al.*, 2000a). The degradation of oval-filefish myofibrillar gel at 50 and 65°C was inhibited by soybean trypsin inhibitor and by leupetin, respectively (Toyohara *et al.*, 1990c). From the result, it can be concluded that major myofibril-associated proteinases in both *P. macracanthus* and *P. tayenus* belonged to serine proteinase. However, *P. macracanthus* had a greater activity than *P. tayenus.* The differences in proteolytic activity may lead to the differences in gel quality of surimi or mince between two species.

From the result, sarcoplasmic proteinases from *P. macracanthus* muscle showed a higher activity than those from *P. tayenus* muscle. Therefore, proteinase from *P. macracanthus*, which had highest activity at pH 8.5 and 60°C was further purified and characterized.

4. Purification of sarcoplasmic proteinase of *P. macracanthus*

To identify the major proteinase responsible for autolysis of bigeye snapper mince, particularly *P. macracanthus*, at elevated temperatures, purification of sarcoplasmic proteinase was carried out.

Purification of proteinase from *P. macracanthus* muscle is summarized in Table 6. Approximately 60% of activity remained after heat treatment. Purification fold of 3.1 was obtained by heat treatment at 60°C for 6 min, which was used to denature and remove heat-labile proteins as well as sarcoplasmic proteins. From the result, activity loss of 38% was noted after heat treatment. This indicated that some proteinases, especially heat labile proteinases, were inactivated caused by thermal denaturation. Heat stable

Table 5 Effect of inhibitors on the sarcoplasmic proteinases activity

Inhibitors	Concentration	% Inhibition			
		Priacanthus macracanthus		*Priacanthus tayenus*	
		pH 6.5	pH 8.5	pH 5.0	pH 8.5
Control		0a	0a	0a	0a
Pepstatin A	1 μM	6.79 ± 1.43 [a b] a	1.62 ± 1.68a	75.18 ± 4.63e	9.76 ± 12.40a
Soybean trypsin Inhibitor	10 μM	80.89 ± 4.82c	67.25 ± 5.79e	18.62 ± 1.95bc	74.28 ± 7.27f
PMSF	1 mM	18.04 ± 5.90b	4.64 ± 3.50ab	40.00 ± 1.95d	20.17 ± 1.17bc
E-64	10 μM	0a	9.59 ± 5.33bc	45.52 ± 1.95d	67.08 ± 1.16ef
Iodoacetic acid	1 mM	16.61 ± 1.97b	4.64 ± 3.20ab	38.62 ± 5.85d	61.32 ± 1.75e
EDTA	10 mM	21.61 ± 0.90b	35.89 ± 0.15d	0a	30.87 ± 2.33cd
EGTA	10 mM	7.50 ± 1.07a	38.47 ± 0.76d	31.73 ± 3.90cd	38.89 ± 3.78d
Phenanthroline	1 mM	0a	14.87 ± 0.61c	19.31 ± 4.87bc	22.64 ± 5.82c
Lactacystin	10 μM	6.25 ± 1.25a	10.99 ± 4.57bc	14.49 ± 7.80b	36.42 ± 3.78d

[a] Average ± SD from duplicate determinations.

[b] The different letters in the same column denote the significant differences ($p<0.05$).

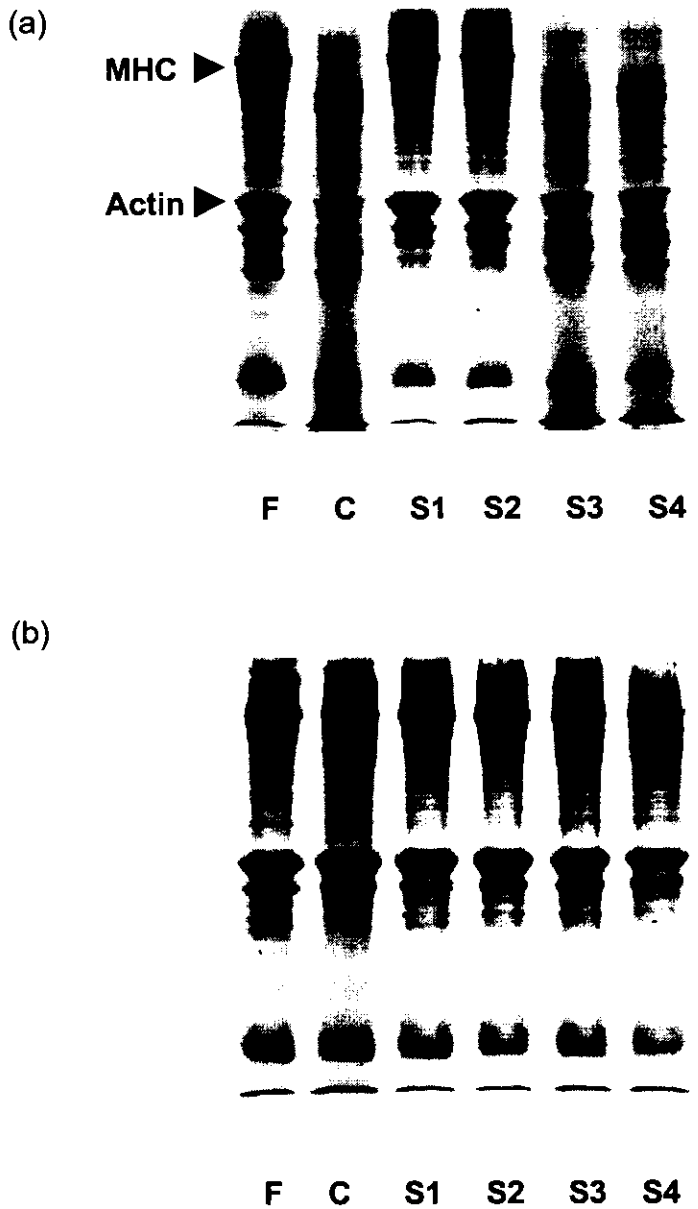

Figure 14 SDS-PAGE pattern of washed mince of *P. macracanthus* (a) and *P. tayenus* (b) in absence or presence of various proteinase inhibitors. F, washed mince; C, control (without inhibitor); S1, 0.1 mM soybean trypsin inhibitor; S2, 0.01 mM soybean trypsin inhibitor; S3, 0.01 mM E-64; S4, 1 mM iodoacetic acid

proteinases are detrimental to gel formation, particularly when the temperature was raised to 50-60°C, which modori phenomenon commonly occurs. Seymour *et al.* (1994) found that heat-treatment of Pacific whiting flesh juice at 60°C for 3 min resulted in the increased proteinase activity by 4.1 folds.

Heat-treated sarcoplasmic fluid was further purified by hydrophobic chromatography on phenyl-Sepharose 6 fast flow column. Among hydrophobic interaction chromatography (HIC) media, phenyl-Sepharose 6 fast flow was shown to be most suitable for proteinase recovery (data not shown). The heat-treated sarcoplasmic fluid was added with ammonium sulfate to obtain a concentration of 1 M to increase the hydrophobic interaction between enzymes and the phenyl group of resin. Proteins adsorbed on HIC column were eluted by decreasing linear gradient from 1 M to 0 M ammonium sulfate (Figure 15). Fractions having proteolytic activity (fraction 26-35) were pooled for further purificarion. Specific activity increased about 4 times, compared to that obtained in heat-treated sarcoplasmic fluid (Table 6). Seymour *et al.* (1994) purified cathepsin L from the sarcoplasmic fluid of Pacific whiting (*Merluccius productus*) using heat treatment followed by hydrophobic chromatography on phenyl-Sepharose, leading to an increase in specific activity by 13.5 folds.

Pooled phenyl-Sepharose 6 fast flow fractions were dialyzed against 20 mM Tris-HCl, pH 7.5, prior to loading to anion exchanger, Source 15Q column. After loading and washing, column was eluted by using a 0-0.5 M NaCl linear gradient. Two proteinase activity peaks were found. Major peak of proteolytic activity (fraction 49-61) was eluted at about 0.2 M NaCl, while the minor peak (fraction 67-77) was eluted at about 0.3 M NaCl on Source 15Q chromatography (Figure 16). The result suggested that two major groups of proteinase were present in the phenyl-Sepharose 6 fast flow fraction. However, only a major peak was used for further purification using second Source 15Q column chromatography. Even though approximately half of the

activity remained, a large amount of contaminated proteins were removed, resulting in a substantial increase in purification fold. Purification fold of 3,411 with a yield of 10.3% was obtained from this step. Ishida *et al.* (1995) found that purification of two kinds of neutral serine proteinases from muscle of anchovy (*Engraulis japonica*) by using anion exchanger, TSK gel SuperQ-Toyopearl 650M column was achieved. Purification fold of 293.5 and 304 were obtained for proteinases type I and II, respectively.

To refine the pooled fraction obtained from previous step. Pooled activity fractions were subjected to the second Source 15Q column chromatography. A slight increase in purification fold was obtained. Only one proteolytic peak was found at the same NaCl concentration appeared in the first Source 15Q column chromatography (Figure 17). Therefore, fractions 49-61 were pooled. Purification fold of 4,260 with a yield of 7.3% was obtained from this step. Choi *et al.* (1999b) was also rechromatographed the proteinase on anion exchanger, DEAE-Sephacel column for purification of two alkaline proteinase (A and B) from Atlantic menhaden muscle. Specific activity of proteinase type A and B were increased by 1.1 and 10.4, respectively, compared to that obtained from the 1st DEAE-Sephacel.

When second Source 15Q fraction with proteolytic activity was subjected to gel filtration on Superose 12 HR 10/30, a single peak was obtained (Figure 18). Purification fold of 5,180 with a yield of 0.8% was observed. Choi *et al.* (1999a) found that the use of gel filtration on Sephacryl S-200 in the final step of purification process of two tryptic serine type proteinases (A and B) from Atlantic menhaden muscle led to an increase in proteinase activity by 26.0 folds (type A) and 21.7 folds (type B).

Table 6 Purification of sarcoplasmic proteinase from *P. macracanthus* muscle

Purification steps	Total activity* (units)	Total protein (mg)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude extract	8,521	32,674	0.3	1	100
Heat treatment	5,295	6,652	0.8	3.1	62
Phenyl-Sepharose	1,741	495	3.5	13.5	20
1st Source 15Q	879	0.99**	887	3,411	10
2nd Source 15Q	619	0.56	1,108	4,260	7.3
Superose 12 HR 10/30	65	0.05	1,347	5,180	0.8

* The unit of enzyme activity is expressed as nmoles of tyrosine liberated per min.

** Protein concentration was measured by Bradford method

5. Protein pattern and activity staining of proteinase

Protein pattern of sarcoplasmic fluid during purification process is shown in Figure 19. Crude extract contained a variety of proteins with different molecular weight (lane 1). After heat treatment, proteins with MW of 93,500, 42,000, 36,000 and proteins with MW lower than 30,000 were removed (lane 2). Those proteins disappeared with heating process were presumed to be heat labile proteins. After phenyl-Sepharose chromatography, proteins with MW of 51,000 and 30,000-33,000 were removed with a concomitant increase in protein with a MW of 55,000-63,000 (lane 3). This indicated that phenyl-Sepharose column effectively remove hydrophilic protein and concentrate the hydrophobic proteins. After subjecting to both Source 15Q (lane 4,5), it was noted that proteins with MW of 55,000-63,000 disappeared and the smaller bands with a wide range of MW were observed. When SEC fraction was analyzed, two protein bands with MW of 66,000 and 13,700 were obtained (lane 6). From SDS-PAGE, it indicated that a large amount of proteins was

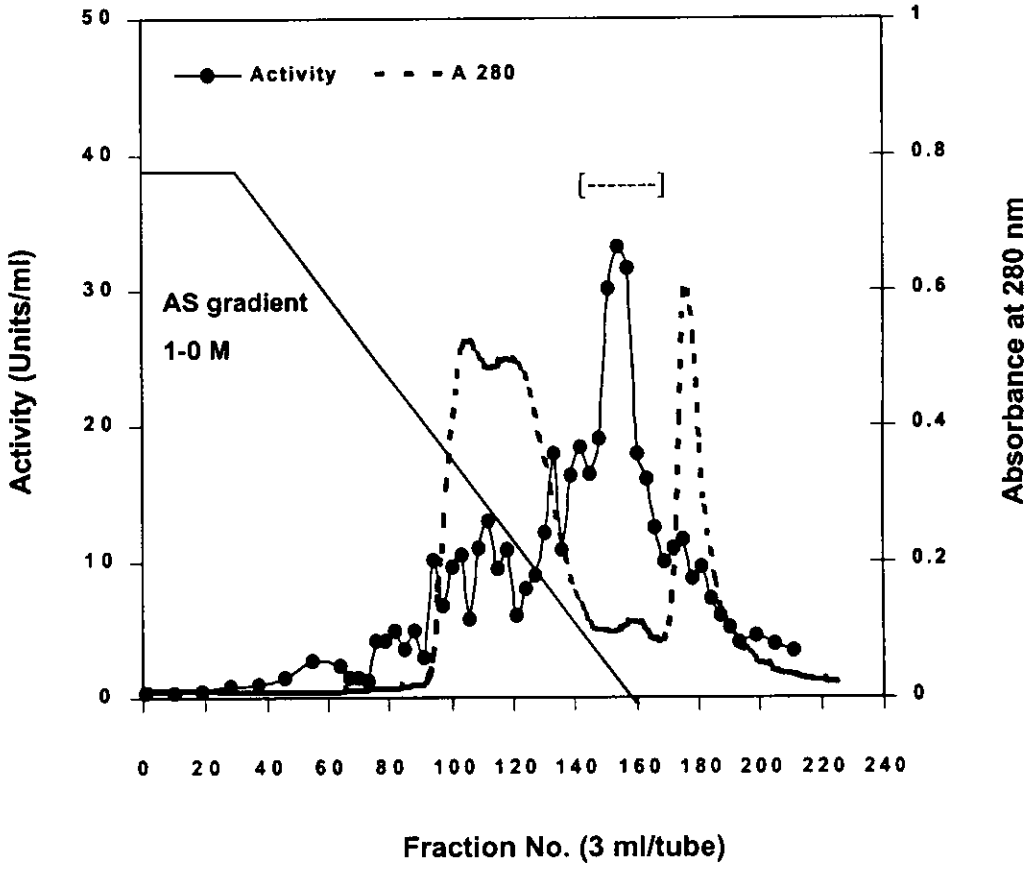

Figure 15 Elution profile of heat-treated *P. macracanthus* sarcoplasmic proteinase on phenyl-Sepharose column. Enzyme solution was adjusted to 1 M AS in SB and applied to a phenyl-Sepharose column at room temperature. After the column was washed with 1 M AS in SB, elution was carried out with a linear gradient of 1 to 0 M AS in SB

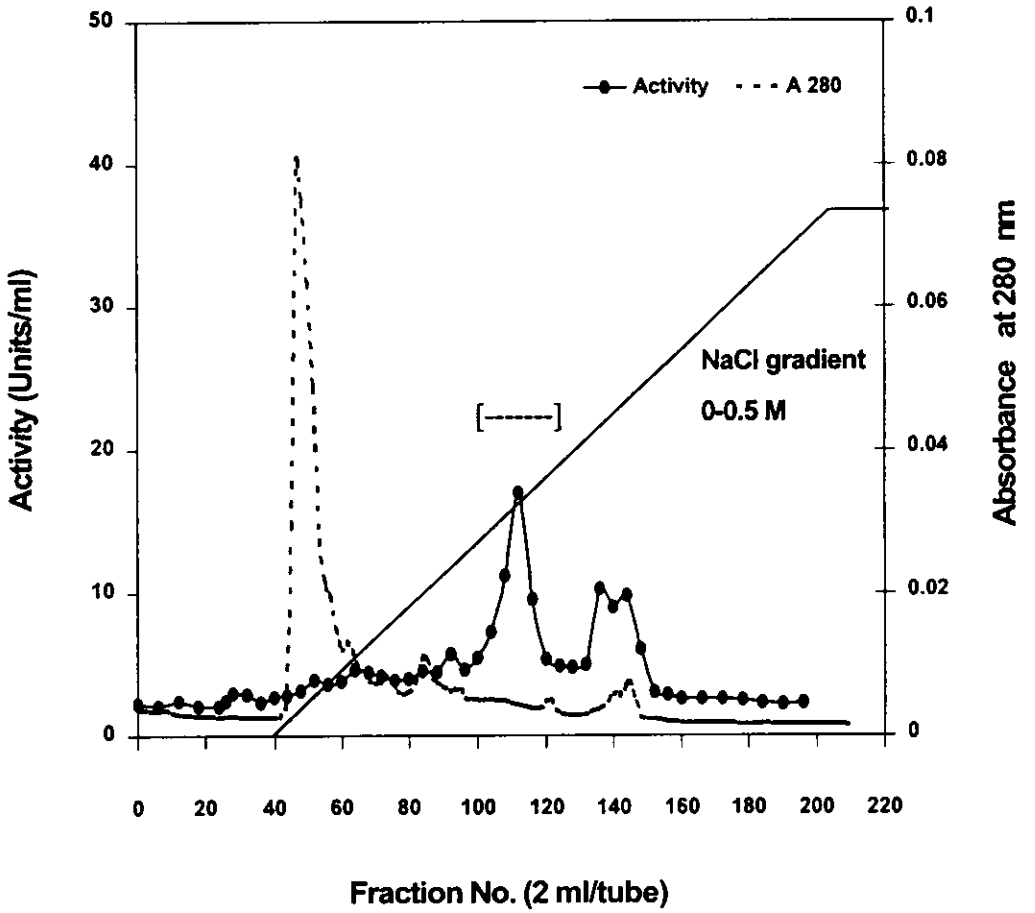

Figure 16 Elution profile of proteinase on the 1st Source 15Q column. Pooled fractions from phenyl-Sepharose chromatography were dialyzed against SB and applied onto a Source 15Q column. Fractions were eluted with a linear gradient of 0 to 0.5 M NaCl in SB

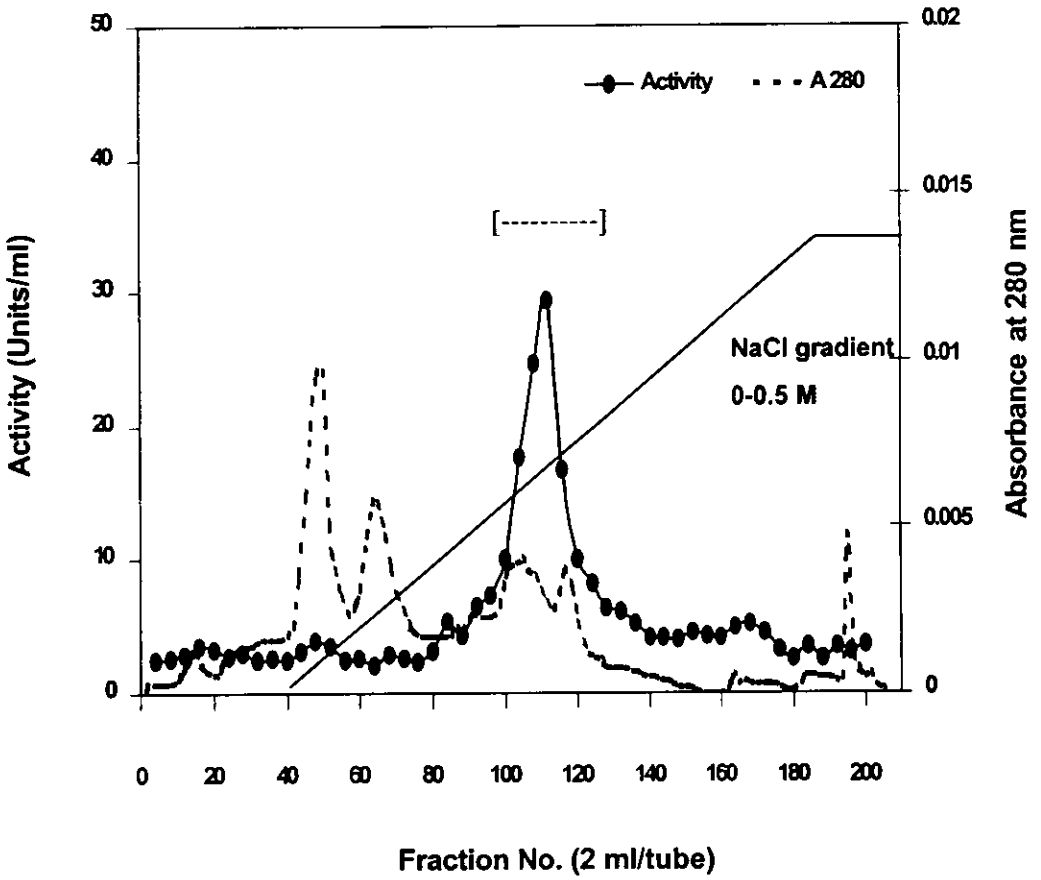

Figure 17 Elution profile of proteinase on the 2nd Source 15Q column. Pooled fractions from 1st Source 15Q chromatography were dialyzed against SB and applied onto a 2nd Source 15Q column. Fractions were eluted with a linear gradient of 0 to 0.5 M NaCl in SB

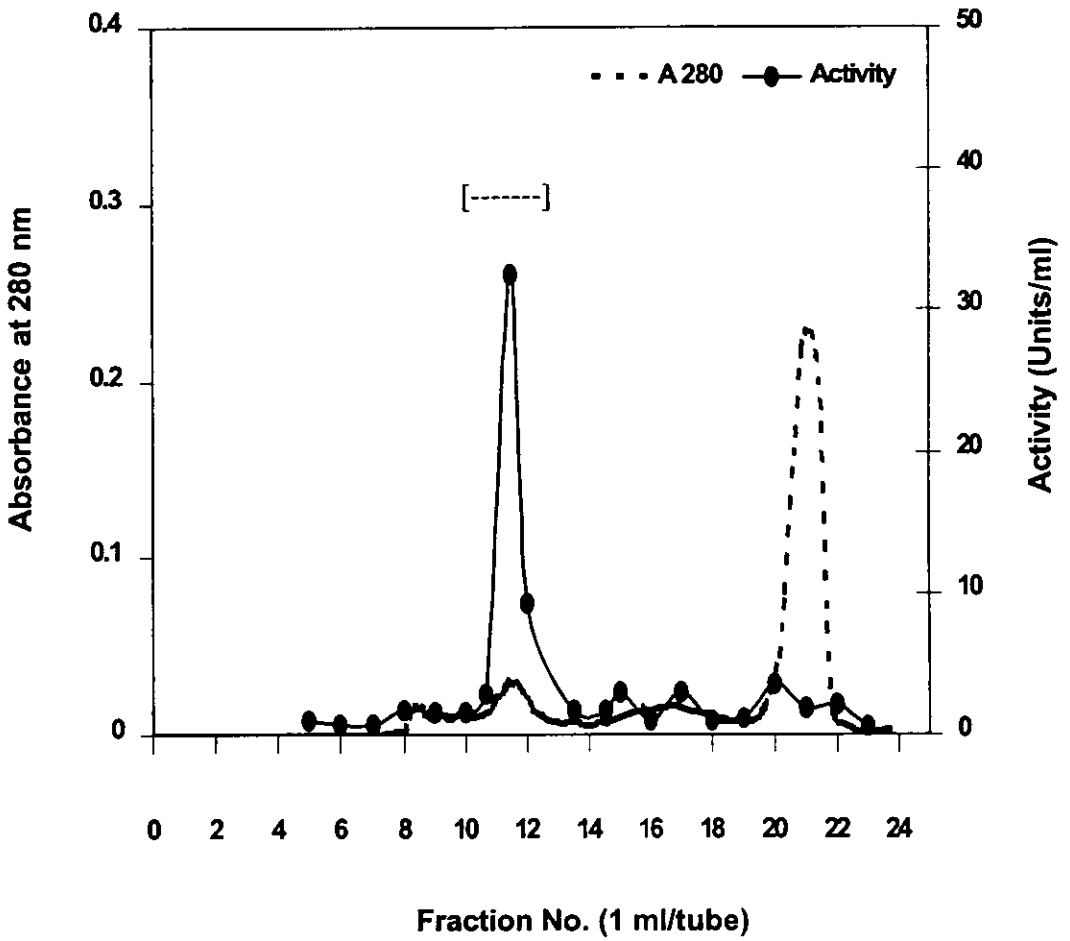

Figure 18 Elution profile of proteinase on Superose 12HR 10/30 column. Pooled fractions from 2nd Source 15Q chromatography were dialyzed against SB and applied onto a Superose 12 HR 10/30 column. Fractions were eluted with 20 mM Tris-HCl, pH 7.5 at a flow rate of 0.2 ml/min

removed during purification. Subsequently, higher purity of interested proteinase was observed as shown in Table 6.

Proteolytic activities of sarcoplasmic fluid and various fractions obtained from purification process were analyzed on SDS-substrate gel (Figure 20). Two clear zones were observed for 2nd Source 15Q fractions at MW of 66,000 and 13,700. The same pattern of clear zone was observed with SEC fractions. However, no ciear zone were found in sarcoplasmic fluid and heat-treated fraction. This was possibly due to the lower activity of proteinase loaded into the gel. As a consequence, no marked clear zone was observed. From SDS-substrate gel, it indicated that two proteolytic activities were present in heat-treated sarcoplamic fluid. This possibly suggested the presence of two different proteinases or different subunits of the proteinase. In other words, the enzyme possibly consisted of two nonidentical subunits.

To verify hypothesis, activity staining was performed on native-substrate gel (Figure 21). The clearing of the gel by the proteolytic activity was reconfirmed by treatment with various inhibitors. It was noted that only one clearing zone was found on the native gel. Soybean trypsin inhibitor, a specific inhibitor for serine proteinase, at a concentration of 0.1 mM completely eliminated clearing on the gel, while the decrease in clearing was found with the presence of 0.01 mM soybean trypsin inhibitor. No changes in clearing were observed with the addition of E-64, cysteine proteinase inhibitor (Hanada *et al.,* 1978) or pepstatin A, aspartic acid proteinase inhibitor (Umezawa, 1976). EDTA, a metalloproteinase inhibitor, showed a slight effect on the clearing. It was noted that two clearing zones with similar MW were observed with addition of EDTA. This was postulated that EDTA might dissociate the subunits, which were stabilized by ionic interaction, leading to the additional clearing zone.

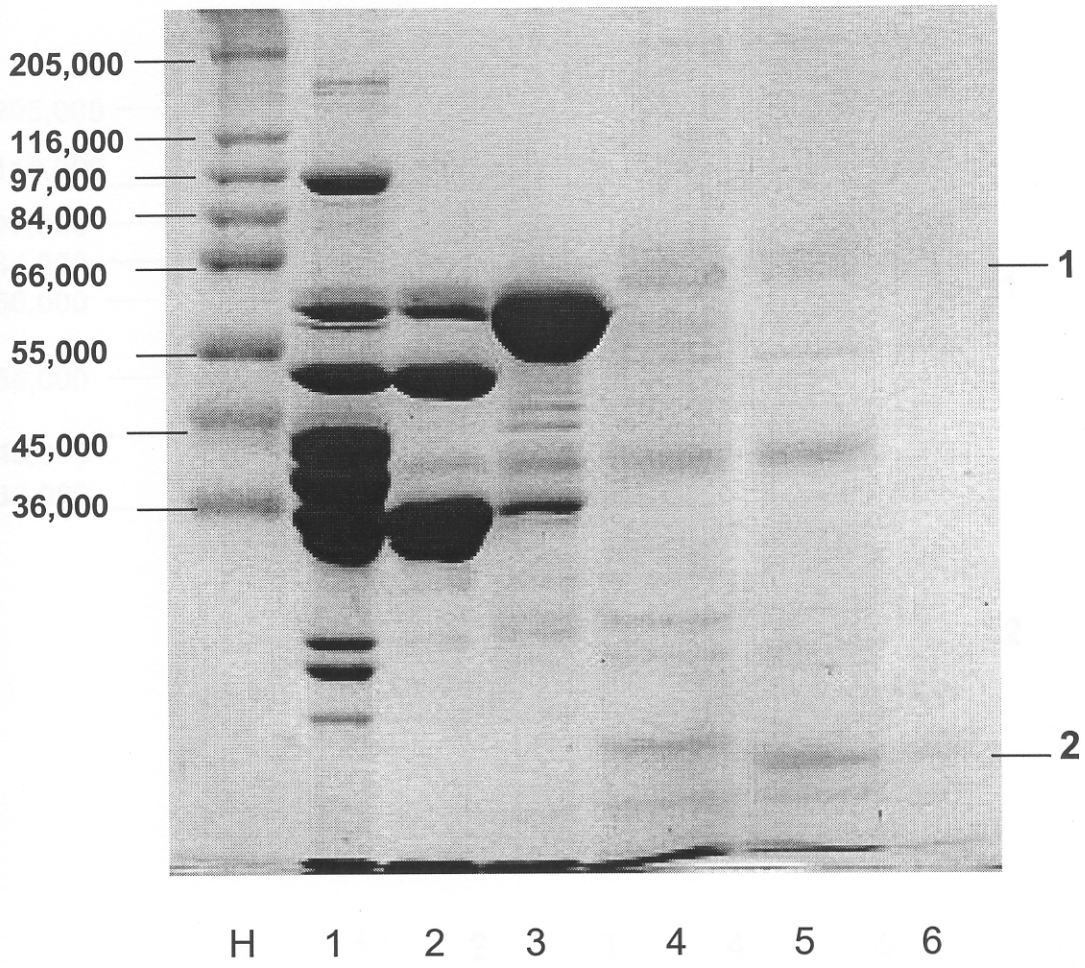

Figure 19 SDS-PAGE pattern (with reducing agent) of purified sarcoplasmic proteinase from *P. macracanthus* muscle. H, high molecular weight standard; lane 1, crude extract, 20 μg; lane 2, heat treated fraction, 20 μg; lane 3, phenyl-Sepharose fraction, 20 μg; lane 4, 1st Source 15Q fraction, 1.98 μg; lane 5, 2nd Source 15Q fraction, 1.83 μg; lane 6, SEC fraction, 0.187 μg

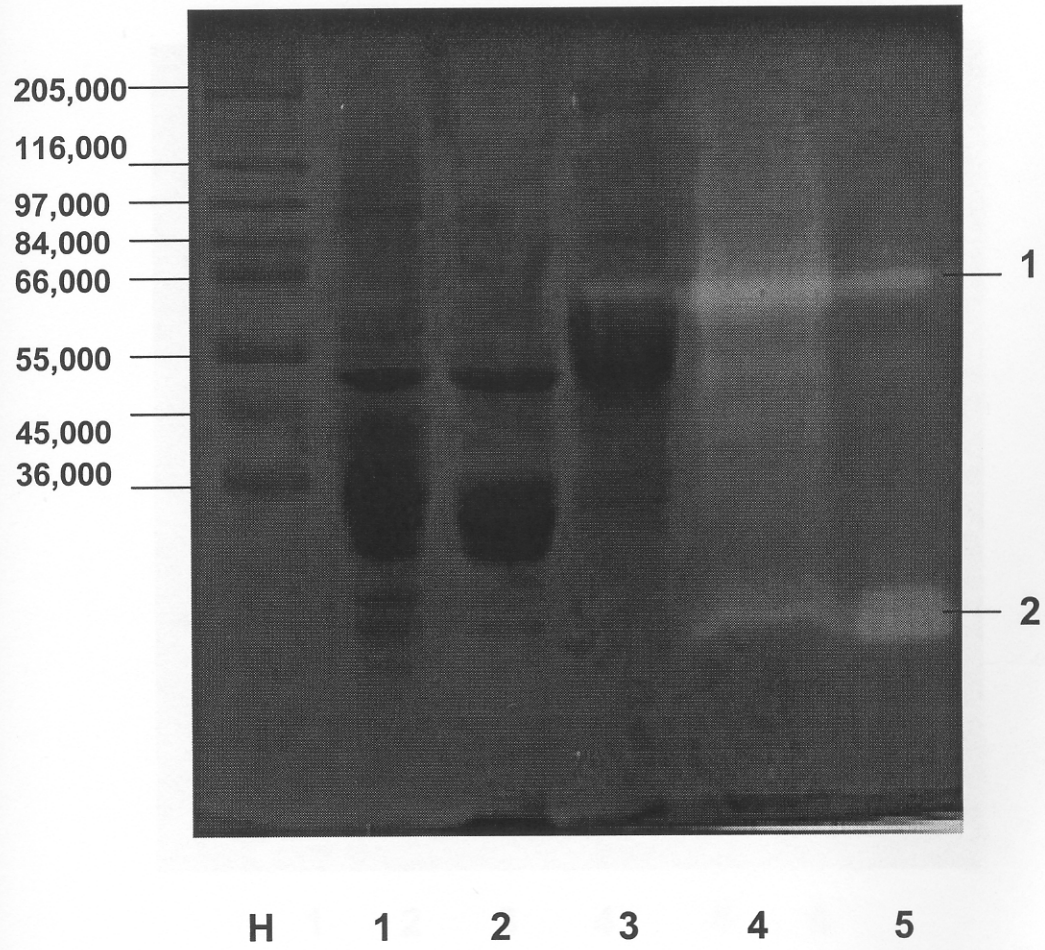

Figure 20 Activity staining (without reducing agent) of purified sarcoplasmic proteinase from *P. macracanthus* muscle. H, high molecular weight standard; lane 1, crude extract, 20 μg; lane 2, heat treated fraction, 20 μg; lane 3, phenyl-Sepharose fraction, 20 μg; lane 4, 2nd Source 15Q fraction, 1.83 μg; lane 5, SEC fraction, 0.187 μg

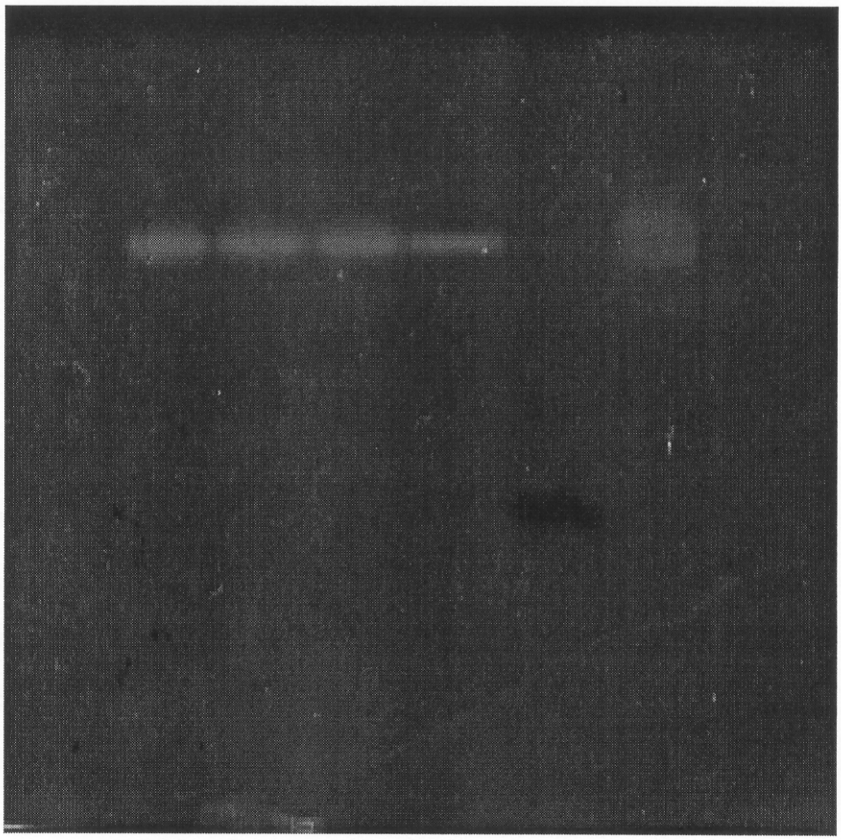

Figure 21 Effect of proteinase inhibitors on purified sarcoplasmic proteinase from *P. macracanthus* muscle on native discontinuous gel electrophoresis followed by staining for proteolytic activity at pH 8.5, 60°C. Lane 1, control (without inhibitors); lane 2, 1 μM pepstatin A; lane 3, 10 μM E-64; lane 4, 0.01 mM soybean trypsin inhibitor, lane 5, 0.1 mM soybean trypsin inhibitor; lane 6, 10 mM EDTA

From the gel, it can be inferred that the sarcoplasmic proteinase from *P. macracanthus* was composed of two subunits with different MW. In the absence of SDS or reducing agent, only one clearing was found. Conversely, the dissociation of enzyme occurred in presence of SDS, resulting in two different clearing zones.

Molecular weight of purified proteinase was estimated by gel filtration on Superose 12 HR 10/30 (Figure 22). From the logarithmic plots of the molecular mass versus available partition coefficient (K_{av}) of the proteins, the molecular mass of the enzyme was estimated to be 72 kDa (Figure 22). According to the non-reducing SDS-substrate gel electrophoresis, purified proteinase had two activity bands with the MW of 66 and 13.7 kDa shown as clear zones on the dark background (Figure 20). Binding of SDS leads to interference with native hydrophobic and ionic interaction, causing the dissociation of most oligomeric protein into their monomer subunit (Robyt and White, 1987). Consequently, it was concluded that this purified enzyme was heterodimer, which are linked by hydrophobic or ionic interaction. Different molecular weights have been reported for purified serine proteinases from fish muscle, depending on fish species. The molecular weight of a serine proteinase from white croaker muscle was estimated to be about 68,000 by gel filtration chromatography and about 32,000 and 71,000 by SDS-PAGE with or without a reducing reagent, respectively (Yanagihara *et al.,* 1991). The molecular weight of a novel serine proteinase from threadfin bream muscle was estimated to be 77,000 daltons on SDS-PAGE analysis (Kinoshita *et al.*, 1990c). The molecular weight of two alkaline proteinases from menhaden muscle were estimated to be about 707,000 and 450,000 daltons, respectively, by gel filtration chromatography (Choi *et al.*, 1999b). Molecular weight of two tryptic serine proteinases (A and B) from Atlantic menhaden were estimated to be about 112,000 and 90,500 daltons, respectively by gel filtration and about 55,000 and 45,000 daltons, respectively by SDS-PAGE

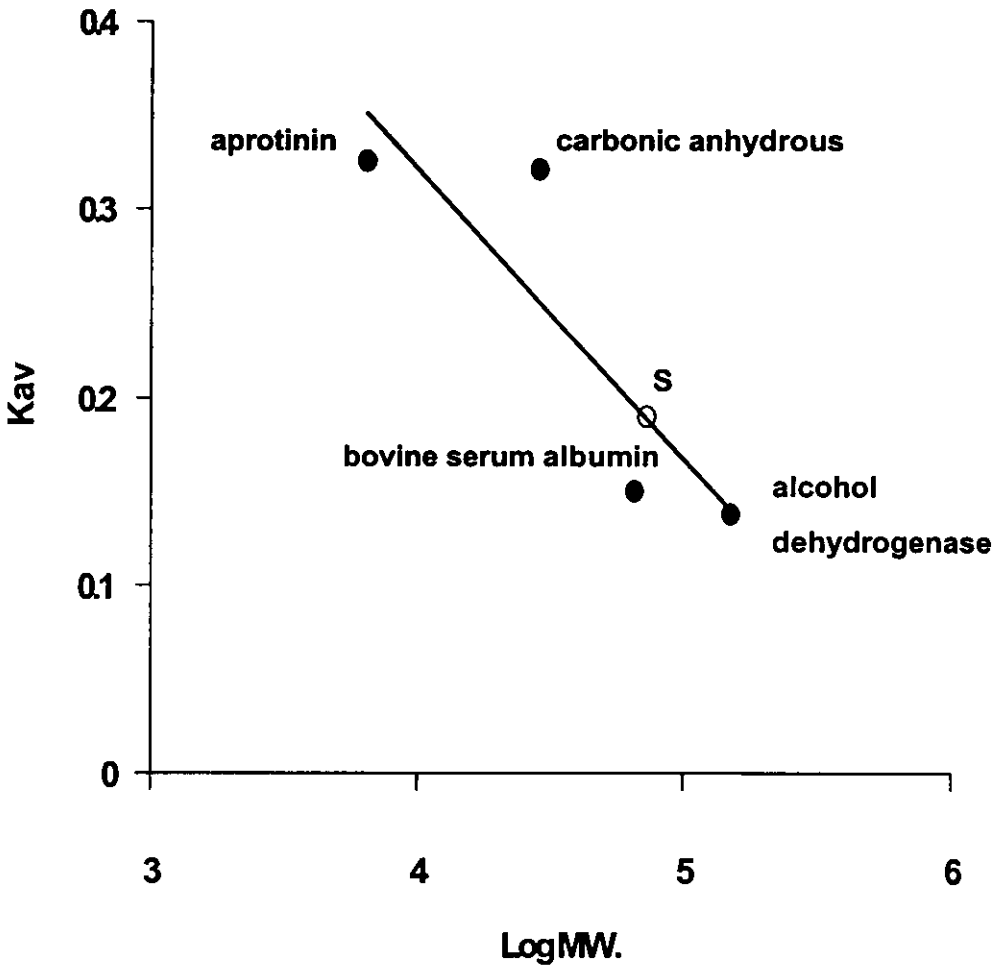

Figure 22 Calibration curve for the molecular weight determination of the purified proteinase on Superose 12 HR 10/30 chromatography. S, the purified proteinase, 72,000

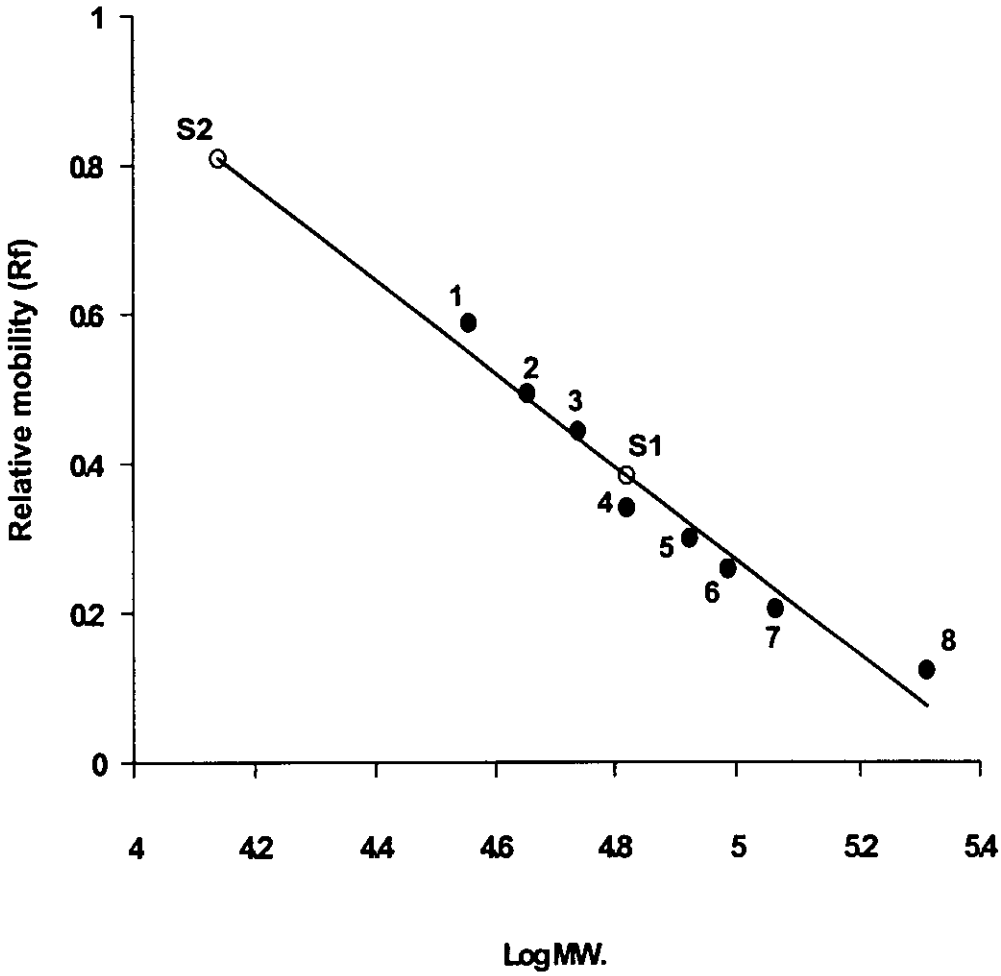

Figure 23 Calibration curve for determination of molecular weight (using non-reducing SDS-PAGE). S1, first activity band, 66,000; S2, second activity band, 13,700; 1, glyceraldehyde-3-phosphate dehydrogenase, 36,000; 2, ovalbumin, 45,000; 3, glutamic dehydrogenase, 55,000; 4, albumin, 66,000; 5, fructose-6-phasphate kinase, 84,000; 6, phosphorylase b, 97,000; 7, galactosidase, 116,000; 8, myosin, 205,000

with 2- mercaptoethanol (Choi *et al.*, 1999a). A novel myofibril-bound serine proteinase (MBP) from ordinary muscle of the carp (*Cyprinus carprio*) muscle had a molecular weight of 30 kDa as estimated by SDS-PAGE and gel filtration (Osatomi *et al.*, 1997). The molecular weight of myofibril-bound serine proteinase (MBSP) from the skeletal muscle of lizard fish was estimated to be about 29 kDa under reducing conditions, while 60 kDa under non-reducing conditions (Cao *et al.*, 2000a). Molecular weight of type-I and type-II serine proteinase were estimated to 25,000 and 37,000 daltons, respectively, on electrophoretic analysis (Ishida *et al.*, 1995). According to the above data, the molecular weight of purified proteinase was similar to that purified enzyme from threadfin-bream and white croaker muscle. This may be due to the similar habitat or climate where fish live, leading to similar physiological or biochemical system among those species.

6. Characterization of purified proteinase

6.1 Optimum pH

The effects of pH on the enzyme activity are shown in Figure 24. The optimum pH for caseinolytic activity was found at pH 8.0-8.5. Therefore, this proteinase is considered to be a alkaline proteinase. The activity of purified proteinase sharply decreased above optimum pH due to the denaturation of enzyme, especially the unfolding of α-helix (Dufour *et al.*, 1988). The decrease in activity was also found in the acidic pH ranges. The optimum pH value of purified proteinase was similar to that of myofibrillar-bound serine proteinase obtained from carp (*Cyprio carprio*) muscle, which had an optimum pH of 8.0 on Boc-Phe-Ser-Arg-MCA (Osatomi *et al.*, 1997). Two alkaline proteinase serine proteinases (A and B) from Atlantic menhaden had an optimum of pH 8.0 when casein was used as substrate (Choi *et al.*, 1999b). Myofibril-bound serine proteinase from lizardfish (*Saurida wanieso*) muscle had an optimum pH of 7 to 8 on Boc-Phe-Ser-Arg-MCA (Cao *et al.*, 2000a).

Optimum pH can be varied, depending upon protein substrates (Seymour *et al.*, 1994). Kinoshita *et al.* (1990c) reported that serine proteinase from threadfin bream had optimum of pH 7.0 on Boc-Leu-Thr-Arg-MCA as well as myosin heavy chain in the presence of 2-4% NaCl. Choi *et al.* (1999a) also reported that two tryptic serine proteinases (A and B) from Atlantic menhadenmuscle had optimum pH at 7.4 when Z-Phe-Arg-NMec was used as substrate. Differences in optimum pH have been attributed to the accessibility of the substrate to the active site at the particular pH environment (Mason *et al.*, 1984). Therefore, optimum pH can be changed when other substrate is used instead of casein.

6.2 Optimum temperature

The optimum temperature of caseinolytic activity at pH 8.5 was 60°C (Figure 25). A very sharp decrease in activity was observed above 60°C, due to thermal denaturation. The optimum temperature of purified proteinase was similar to that of a novel serine proteinase from threadfin bream muscle, which had optimum temperature of 60°C (Kinoshita *et al.*, 1990c). A novel myofibril-bound serine proteinase from carp (*Cyprinus carpio*) muscle had optimum temperature of 55°C (Osatomi *et al.*, 1997). Choi *et al.* (1999b) reported that two alkaline proteinases (A and B) from Atlantic menhaden muscle had optimum temperature of 55°C. Heat-stable alkaline proteinase required some kinds of activation for the *in vitro* assay, such as heating, gamma-irradiation or treatment with several reagents (urea, SDS, some fatty acids, poly-L-Lys or alkylating reagents) in order to expose their active sites (Makinodan *et al.*, 1963; 1987; Wilk and Orlowski, 1983; Tanaka *et al.*, 1986). From the result, purified proteinase may require higher temperatures to expose their active sites for activity.

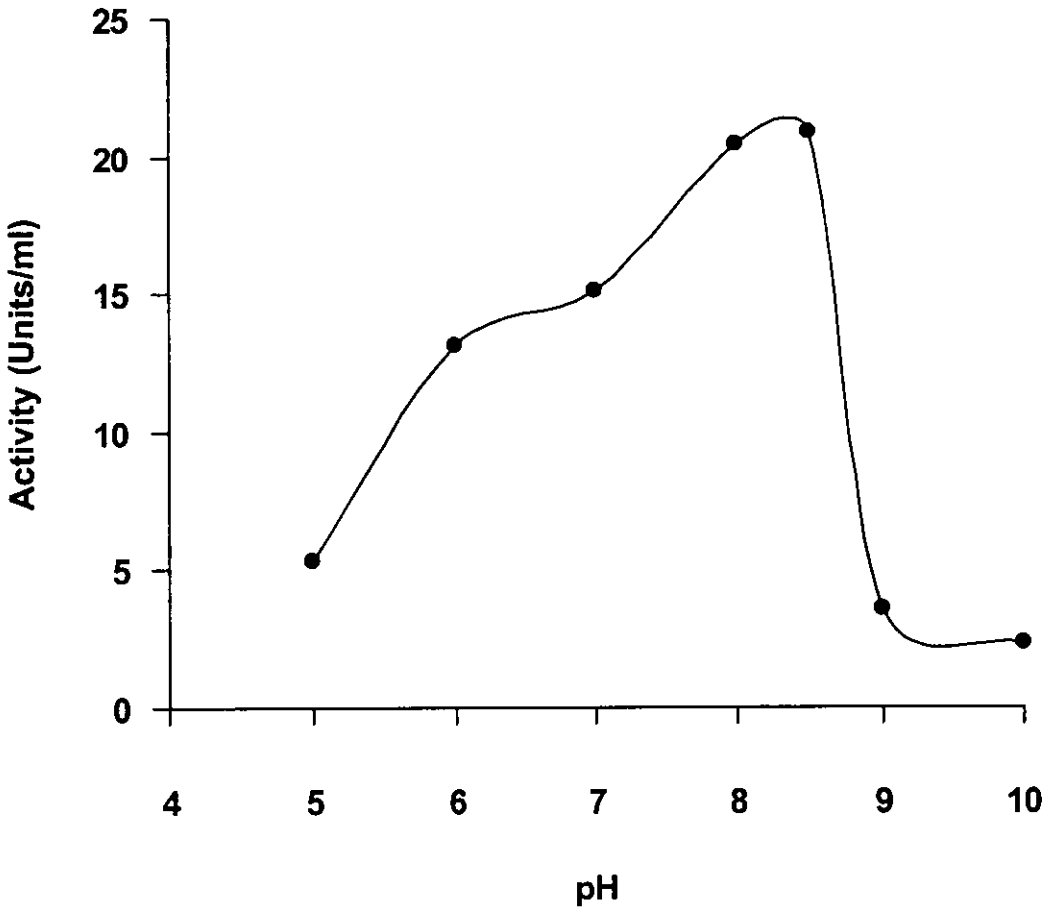

Figure 24 pH profiles of purified sarcoplasmic proteinase from *P. macracanthus* muscle. Proteinase activity was determined by incubating sarcoplasmic fluid at 60°C at various pHs. TCA-soluble peptides released were determined by Lowry assay and expressed as units/ml

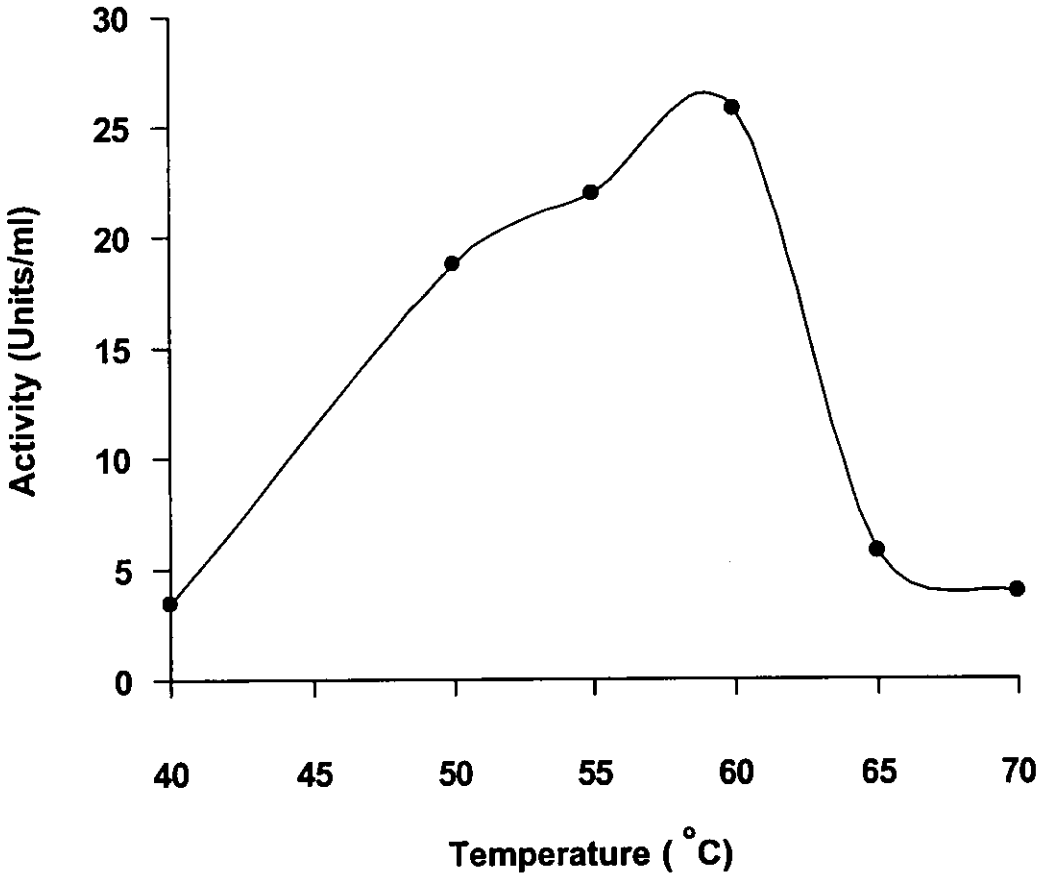

Figure 25 Temperature profiles of purified sarcoplasmic proteinase from *P. macracanthus* muscle. Proteinase activity was determined by incubating sarcoplasmic fluid at pH 8.5 at various temperatures. TCA-soluble peptides were released were determined by Lowry assay and expressed as units/ml

Since the optimum temperature of the purified proteinase was 60°C, this proteinase played an essential role in the modori phenomenon, which specially occurs around 50-70°C in the manufacturing process of fish jelly products.

6.3 Effect of inhibitors

The effect of the various inhibitors on proteinase activity was determined (Table 7). The enzyme activity was strongly inhibited by soybean trypsin inhibitor (82.70%) and partially inhibited by EDTA. Pepstatin A and E-64 had no inhibitory activity on purified proteinase. The result indicated that proteinase from *P. macracanthus* muscle was serine proteinase. Serine proteinase from threadfin bream muscle was inhibited by soybean trypsin inhibitor and leupeptin, while E-64 showed no effect (Kinoshita *et al.*, 1990c). Two kinds of neutral serine proteinases from anchovy muscle was inhibited with some trypsin inhibitors, such as soybean trypsin inhibitor, α_1-antitrypsin, aprotinin, leupeptin and antipain (Ishida *et al.*, 1995). Pepstatin A is able to inhibit most of aspartic proteinase (Umezawa, 1976), while E-64 is an effective irreversible inhibitor of cysteine proteinase (Hanada *et al.*, 1978). Therefore, purified enzyme did not belong to both cysteine and aspartic proteinase family. Lactacystin, specific for proteasome (Dick *et al.*, 1996), had a slight inhibitory effect on proteolytic activity. When EDTA, a metalloproteinase inhibitor (Tsushiya *et al.*, 1994), was tested, it was found that it showed approximately 40% inhibition. This result indicated that EDTA possibly affect the structure of enzyme or active site conformation, leading to the reduced activity. Additionally, Francesco *et al.* (1996) reported that the NS3 protein of hepatitis C virus contains a chymotrypsin-like serine proteinase domain, which indeed contain a Zn^{2+} ion with S_3N ligation. The metal is required for structural integrity and activity of the enzyme. As a result, a decreased activity was observed in the presence of EDTA.

The results were in accordance with those observed in native substrate gel (Figure 21). Activity zone could be completely inhibited by 0.1 mM soybean trypsin inhibitor and partially inhibited by EDTA, but not by pepstatin A and E-64.

From above data, the purified proteinase was considered to be serine proteinase, which possibly required metal ion for activity.

Table 7 Effect of inhibitors on the purified sarcoplasmic proteinase activity from *P. macracanthus* muscle

Inhibitors	Concentration	% Inhibition
Control		0a
Pepstatin A	1 μM	0a
E-64	10 μM	0a
Soybean trypsin inhibitor	10 μM	82.70 ± 1.73 [a] [b] d
Lactacystin	10 μM	20.82 ± 0.99b
EDTA	10 mM	44.69 ± 2.17c

[a] Averages ± SD from duplicate determination. Residual activity was analyzed using casein as a substrate for 30 min at pH 8.5 and 60°C.

[b] The different letters in the same column denote the significant differences ($p<0.05$).

6.4 Substrate specificity

Apart from inhibitor study, proteinase was further identify using different synthetic substrates including Boc-Phe-Ser-Arg-MCA, Z-Phe-Arg-MCA and Z-Arg-Arg-MCA (Table 8). Purified proteinase was highly active on Boc-Phe-Ser-Arg-MCA, but hydrolyzed Z-Phe-Arg-MCA and Z-Arg-Arg-MCA to small extent. Boc-Phe-Ser-Arg-MCA is considered to be the most specific synthetic

substrate for trypsin (Ishida *et al.*, 1995), while Z-Phe-Arg-MCA is a highly specific substrate for cathepsin L and Z-Arg-Arg-MCA can be hydrolyzed by cathepsin L and B (Barrett and Kirschke, 1981). The result indicated that enzyme was a trypsin-type serine proteinase based on substrate specificity and inhibitor study. Similar substrate specificities of serine proteinase from other species have been reported. Myofibril-bound serine proteinase from carp muscle hydrolyzed Boc-Phe-Ser-Arg-MCA and Boc-Gln-Arg-Arg-MCA effectively (Osatomi *et al.*, 1997).

Table 8 Specifity for hydrolysis of peptide methylcoumarylamide substrates

Substrate	Fluorescence intensity*
Boc-Phe-Ser-Arg-MCA	926.35 ± 3.91 [a] [b] a
Z-Phe-Arg-MCA	32.75 ± 1.36b
Z-Arg-Arg-MCA	23.09 ± 1.64c

[a] Averages ± SD from duplicate determination.

[b] The different letters in the same column denote the significant differences ($p<0.05$).

*Assay was conducted for 10 min using 100 μl of purified enzyme at 60°C.

7. Hydrolysis of natural actomyosin by purified proteinase

The degradation of natural actomyosin (NAM) by the purified enzyme was investigated by SDS-PAGE as shown in Figure 26. Myosin heavy chain, the main constituent contributing to gel strength of kamaboko, was most hydrolyzed, particularly as the incubation time increased. Its original band markedly decreased after incubation at 60°C for 60 min. However, no changes in actin were observed, even with an extended incubation time. The result was in agreement with Cao *et al.* (2000a) who reported that degradation

of myosin heavy chain was occurred by myofibril-bound serine proteinase from lizardfish, while α-actinin and actin were not hydrolyzed. For the control (without purified proteinase), a slight degradation of myosin heavy chain was observed (lane 2), suggesting the existence of a myofibril-bound proteinase in NAM. Myofibril-bound serine proteinase was associated with the autolysis of washed mince as described previously.

The similar result has been reported on proteolysis of a myofibril-bound serine proteinase (MBP) from carp (*Cyprinus carpio*) muscle on myofibrillar proteins and their gel formation ability. The optimum degradation temperature of MBP to myosin heavy chain in myofibril and kamaboko gel were 55°C and 60°C, respectively (Cao *et al.*, 1999). Osatomi *et al.* (1997) reported that myofibril-bound serine proteinase hydrolyzed myosin in the presence of 0.5 M NaCl at 55°C.

The result reconfirmed that sarcoplasmic proteinase, which remained to some extent after washing, played a paramount role in hydrolysis of myosin heavy chain, leading to the weakening of gel matrix. Therefore, the uses of food-graded inhibitors can be a promising means to hamper the proteolysis of both sarcoplasmic and myofibril-associated proteinases in surimi, resulting in increased gel strength of surimi gel products.

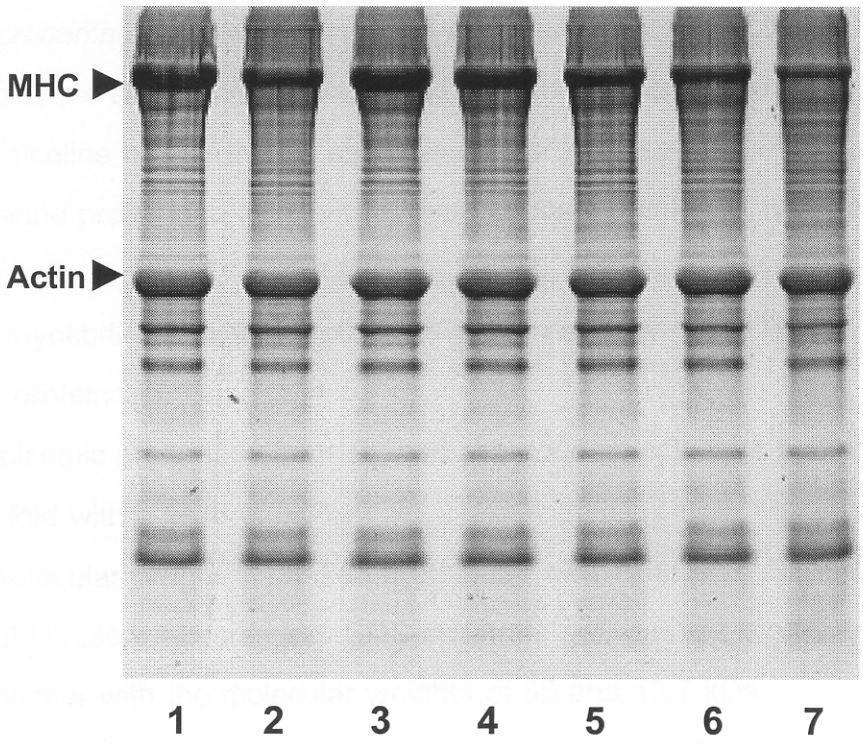

Figure 26 Hydrolysis of natural actomyosin (NAM) by purified proteinase from from *P. macracanthus* muscle at 60°C for various times. Lane 1, NAM incubated at 0°C; lane 2, NAM incubated at 60°C, 60 min; lane 3, 4, 5, 6, 7 represent NAM added with 1 unit purified proteinase and incubated at 60°C for 5, 10, 20, 30 and 60 min, respectively