CHAPTER 3

RESULTS AND DISCUSSION

1. Characterization of black tiger shrimp and white shrimp enzymes

1.1 Proteases from muscle

1.1.1 Temperature and pH profiles

Temperature profiles of proteases from black tiger shrimp and white shrimp muscle assayed at pH 7.0 are shown in Figure 5. The optimum temperature of muscle proteases from both shrimps was 70°C, which was similar to those of trypsins from shrimp (*Penaeus orientalis*) (Oh *et al*., 2000), Atlantic blue crab (Dendinger and O'Connor, 1990) and sand crab (Dionysius *et al*. 1993), and *P. japonicus* (Kim *et al*., 1996b). A decrease in activity was observed at temperature above 70°C, most likely due to thermal denaturation. From the result, the muscle proteases from both shrimps might require high energy to expose their active sites for activity as evidenced by high optimal temperature. The optimal temperature of proteases has been reported to be varied, depending on species and anatomical locations. Proteases from grass shrimp (*Penaeus monodon*) digestive tract had the maximal activity at 55°C and 65°C (Jiang *et al*., 1991), and that from lobster also had high optimum temperature (60°C) (Galgani *et al*., 1984). Additionally, the optimal temperature of proteases from shrimp (*Penaeus monodon*) hepatopancreas was 60-70°C (Chukiatwattana, 2002). It was likely that proteases from both shrimps, tropical species, exhibited the higher optimal temperature than the temperate counterpart. Trypsin from Greenland cod (Simpson and Haard, 1984), capelin (Hjelmeland and Raa, 1982), sardine (Murakami and Noda, 1981), and Atlantic croaker (Parlisko *et al*., 1997) had the optimal temperatures in the range of 40-45°C, and the optimal temperature range of 45-55°C was observed for proteases from five species of crab (Galgani and Nagayama, 1987). The optimum temperature of the protease has the impact on shrimp processing. If internal temperature is not high enough to inactivate the protease during cooking, the protease will cause the substantial softening of tail meat during storage or distribution (Galgani *et al*., 1984).

The effects of pH on the protease activity from black tiger shrimp and white shrimp muscles are depicted in Figure 6. The optimal pH for proteolytic activity of crude extract from black tiger shrimp muscle was 7.0. Therefore, the major proteases were considered as the neutral proteases. Proteases from the white shrimp muscle showed the maximal activity at pH 8.0 and could be classified as alkaline proteases. These optimal pHs were similar to those reported for proteases from grass shrimp digestive tracts (7.0 and 8.0) (Jiang *et al*., 1991). Proteases from whole digestive tract of brown shrimp (*Penaeus californiensis*) had the optimal pH around 8.0 (Vega-Villasante *et al*., 1995). The similar optimal pH might be associated with similar pK values for prototropic groups around the active sites of those enzymes (Jiang *et al*., 1991). Cao *et al*. (2000) reported that proteases from Atlantic blue crap hepatopancreases had an optimal activity at pH 9.0, while proteases from hepatopancreas of shrimp (*Penaeus orientalis*) had proteolytic activity in the broad pH range of 6.0-9.0 (Oh *et al*., 2000). The apparent pH profiles of the protease from *Penaeus orientalis* were similar to those of trypsin from *Penaeus indicus* (Honjo *et al*., 1990), *Penaeus clarkia* (Kim *et al*., 1996a) and the proteinase from Japanese spiny lobster (Galgani and Nagayama, 1987). Trypsins from anchovy intestine showed the highest caseinolytic activities in the pH range of 9-4-10.0 (Heu *et al*., 1995). From the result, differences in optimal pH between both species might be attributed to the different accessibility of the substrate to the active site at the particular pH environment. From the result, the activity of proteases decreased at very acidic or alkaline pHs, most likely owing to the denaturation of enzyme under the harsh condition. Enzyme structure might undergo the modification or unfolding at those pHs, caused by the repulsion of charged molecules. The sharp decrease in hydrolysis of casein by the protease from hepatopancreas of crayfish and sand crab at low pH may be caused by the irreversible denaturation of enzymes (Kim *et al*., 1992; Dionysius *et al*., 1993).

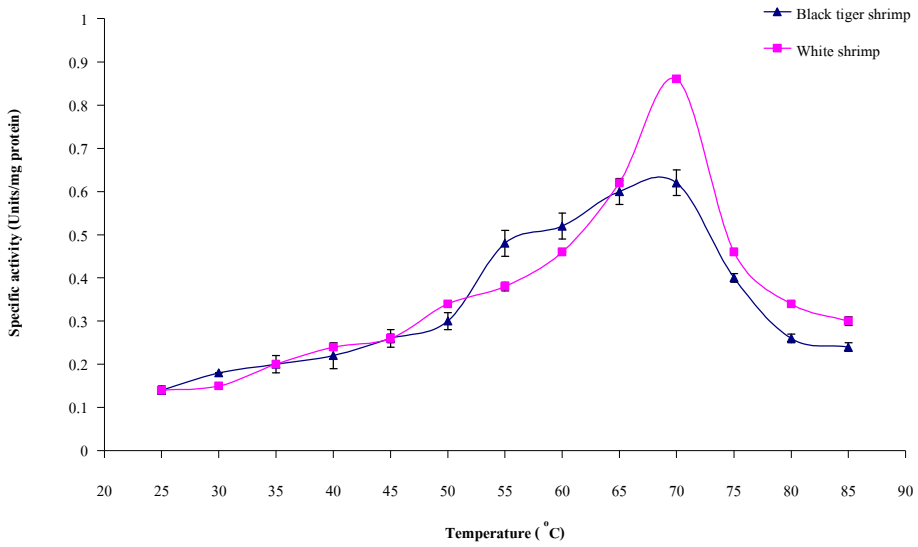

Figure 5. Temperature profiles of proteases from black tiger shrimp and white shrimp muscles. Protease activity was determined at pH 7.0 using casein as a substrate. TCA-soluble peptides released were determined by the Lowry assay and one unit of activity was defined as µmol tyrosine/min.

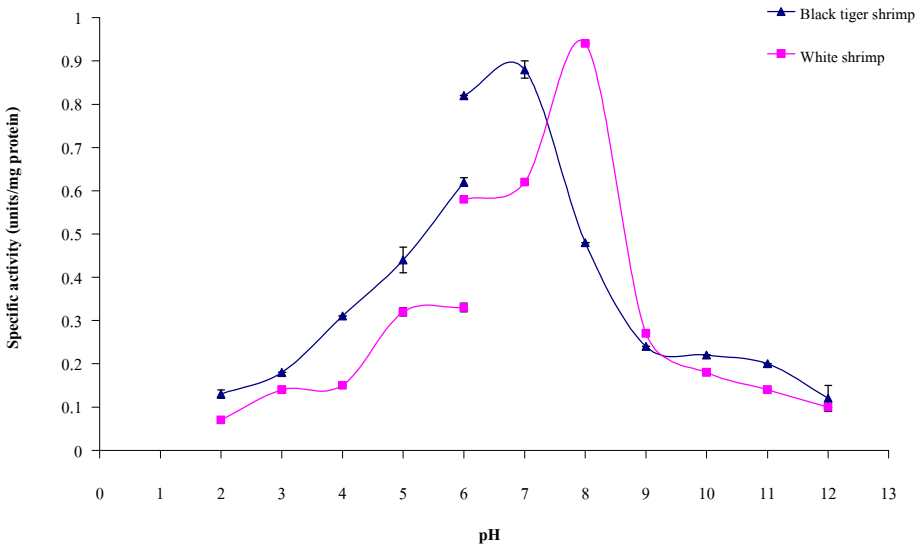

Figure 6. pH profiles of proteases from black tiger shrimp and white shrimp muscles. The activity was determined at 70°C over the pH range of 2.0-12.0 (0.2 M McIlvaine's buffer for pHs 2.0-7.0 and 0.1 M NaH_2PO_4-0.05 M $Na_2B_4O_7$ for pHs 8.0-12.0). Protease activity was determined using haemoglobin and casein as substrates for 60 min. TCA-soluble peptides released were determined by the Lowry assay and one unit of activity was defined as µmol tyrosine/min.

1.1.2 Temperature and pH stability

Proteases from black tiger shrimp muscle were stable when incubated at temperature up to 35°C (Figure 7). Thereafter, the slight decrease in activity was found with increasing temperatures. The sharp decrease in activity was noticeable at temperature above 55°C. At high temperature, enzyme possibly underwent denaturation and lost its activity. The thermal stability of proteases from white shrimp muscle was similar to that of proteases from black tiger shrimp muscle (Figure 3). Thermal stability of proteases from black tiger shrimp and white shrimp muscle was similar to those reported for grass shrimp digestive tracts proteases (Jiang *et al*., 1991) and trypsins from brine shrimp (50°C) (*Artemia salina*) (Olalla *et al*., 1978), which showed the stability at the temperature below 50°C. Trypsin from Atlantic blue crab was stable at temperature ranging from 30 to 50°C for 30 min but activity was rapidly lost at the temperature above 50°C (Dendinger and O'Connor, 1990). From the result, the losses in activity were observed at the optimal temperature (70°C) (Figure 5). After being heated at 70°C, which was the optimal temperature, the activity decreased by 40% and 43%, for proteases from black tiger shrimp and white shrimp muscles, respectively. This might have been attributed to protective effects of the substrate during enzyme assay. This result suggested that the surrounding environment influenced enzyme stability. Dionysius *et al*. (1993) found that trypsin from sand crab was thermostable, with 36% of original activity remaining after heat treatment at 70°C for 30 min in the presence of calcium ions. Hence, the presence of protein substrate or calcium ions enhanced thermal stability of the enzyme (Dionysius *et al*., 1993).

pH stability of proteases from black tiger shrimp and white shrimp muscles is shown in Figure 8. Proteases from black tiger shrimp muscle were stable in the pH range of 4.0-9.0, in which residual activity more than 80% was found. The decrease in activity was observed at very acidic or alkaline pHs. The pH stability of proteases from white shrimp muscle was similar to that of protease from black tiger shrimp muscle. Protease from shrimp (*Penaeus orientalis*) was stable in the pH range of 6.0-9.0 after 30 min incubation (Oh *et al*., 2000). Three trypsins from Atlantic krill were stable in the pH range of pH 7.0-9.5, when incubated for less than 1 h (Osnes and Mohr, 1985). At extremely acidic or alkaline condition, proteolytic activity was decreased due to the conformational change of enzyme. Apparently, inactivation at acid pH is a phenomenon common to anionic trypsins from various species including, crayfish (Kim *et al*.,

1992), *Penaeus setiferus* (Gates and Travis. 1969), and Atlantic blue crab (Dendinger and O'Connor. 1990).

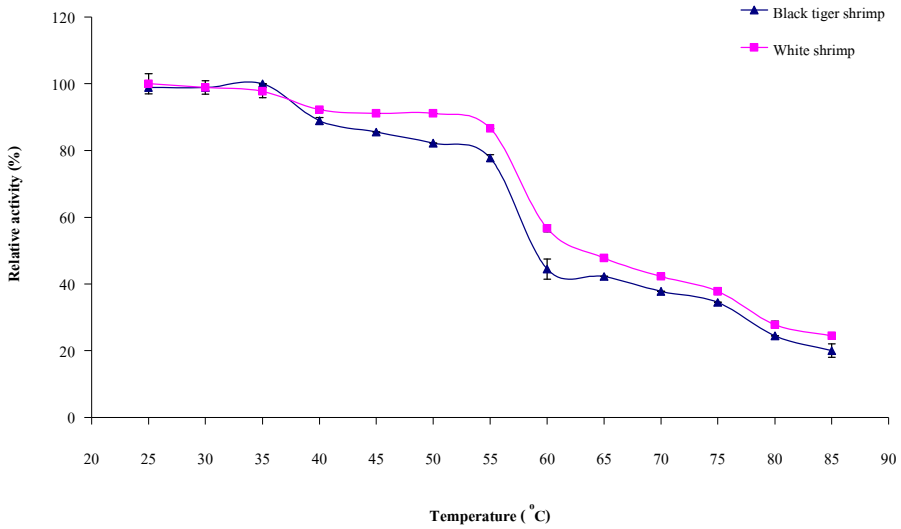

Figure 7. Thermal stability of proteases from black tiger shrimp and white shrimp muscles. Protease activity was determined after incubating crude extract for 15 min at various temperatures. The residual activity was determined at 70°C and pH 7.0 for 60 min using casein as a substrate.

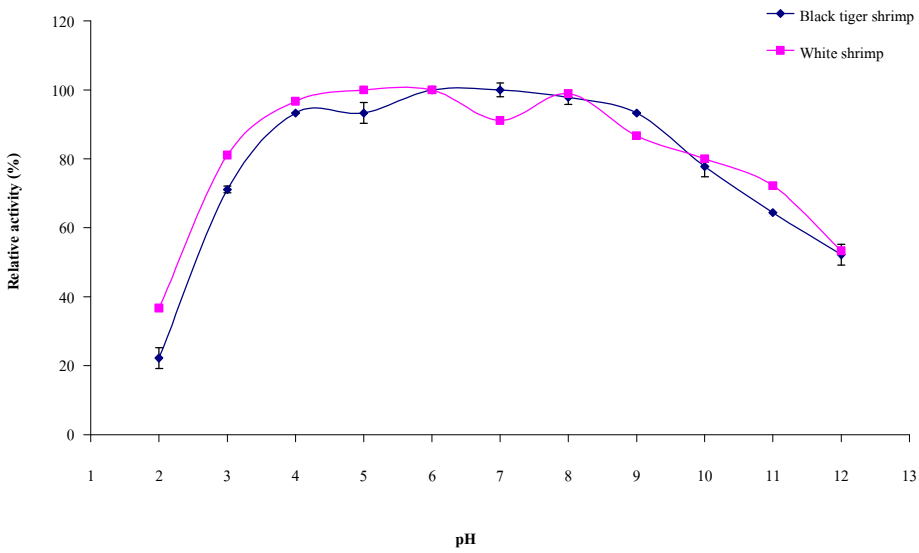

Figure 8. pH stability of proteases from black tiger shrimp and white shrimp muscles. Protease activity was determined after incubating crude extract for 15 min at various pHs. The residual activity was determined at 70°C and pH 7.0 for 60 min using casein as a substrate.

1.1.3 Effects of inhibitors

The effects of various inhibitors on protease activity of shrimp muscles are shown in Table 4. The proteolytic activities of crude extracts from the muscle of both shrimps were strongly inhibited by soybean trypsin inhibitor, followed by E-64 and ethylenediaminetetraacetic acid (EDTA), while pepstatin A showed no inhibition. The inhibition was generally dose-dependent. These results suggested that major proteases from the muscle of both shrimps were both serine protease and cysteine proteases. Pepstatin A is able to inhibit most of aspartic proteases (Umezawa, 1976), while E-64 is an effective irreversible inhibitor of cysteine proteases (Hanada *et al*., 1978). Two kinds of neutral serine proteases from anchovy muscle were inhibited by some trypsin inhibitors, such as soybean trypsin inhibitor, α_1-antitrypsin, aprotinin, leupeptin and antipain (Ishida *et al*., 1994). Therefore, proteases from both shrimps did not belong to aspartic proteases, but most likely to serine protease and cysteine proteases. From the result, EDTA, well-known metal chelator, partially inhibited the protease activity. EDTA might sequester the ion necessary for activity of proteases. This might lower the activity to some extent. Benjakul *et al.* (2003c) reported that lizardfish (*Saurida tumbil*) muscle was markedly inhibited by E-64 and soybean trypsin inhibitor, which was one of the characteristics of cysteine and serine proteinases, respectively. Serine proteinases from bigeye snapper (*Priacanthus macracanthus*) muscle was strongly inhibited by soybean trypsin inhibitor and was partially inhibited by EDTA (Benjakul *et al.*, 2003a).

Table 4. Effect of various inhibitors on the activity of proteases from the muscles of white shrimp and black tiger shrimp**.**

Inhibitors	Concentrations	% inhibition*	
		Black tiger shrimp	White shrimp
Pepstatin A	1 μM	0	0
	10 μM	0	0
E-64	1 μM	5.69 ± 0.49	2.13 ± 4.31
	10 μM	9.09 ± 0.11	8.77 ± 4.96
	50 μM	59.56 ± 0.13	39.11 ± 1.14
	100 μM	65.02 ± 0.65	50.28 ± 0.02
Soybean trypsin inhibitor	1 μM	23.06 ± 1.99	26.80 ± 1.02
	10 μM	34.06 ± 3.07	37.30 ± 0.92
	50 μM	73.90 ± 0.92	51.58 ± 0.43
	100 μM	79.47 ± 0.03	59.30 ± 5.47
EDTA	2 mM	11.70 ± 0.83	4.92 ± 0.29
	10 mM	23.17 ± 0.79	6.35 ± 0.45
	50 mM	30.18 ± 1.51	10.67 ± 1.33
	100 mM	36.26 ± 0.15	22.41 ± 0.41

* Mean ± SD from triplicate determinations.

Residual activity was analyzed using casein as a substrate at 70°C and pHs 7.0 and 8.0 for 60 min for protease from black tiger shrimp and white shrimp muscle, respectively.

1.2 Collagenase from cephalothorax and muscle

1.2.1 Temperature and pH profiles

Temperature profile of collagenase from the white shrimp and black tiger shrimp cephalothorax and muscle are depicted in Figure 9. The maximal activities of collagenase from the cephalothorax and the muscle of black tiger shrimp were noticeable at 55°C when bovine tendon collagen type I was used as a substrate. Moreover, the same optimal temperature was found in collagenase from the cephalothorax of white shrimp. Collagenase from the internal

organs of filefish (*Novoden modestrus*) (Kim and Kim, 1991) and grass shrimp (*Penaeus monodon*) showed the maximal activity at 55-60°C (Jiang *et al*., 1991). The optimal temperature of collagenase from the white shrimp cephalothorax and collagenases from black tiger shrimp cephalothorax and muscle was higher than those of collagenases from hepatopancreas of Northern shrimp (*Pandalus eous*) (Aoki *et al*., 2003) and Antractic krill (*Euphausia superba*) (Turkiewicz *et al*., 1991), which had the optimal temperature ranging from 40 to 45°C. Collagenase from Atlantic cod (*Cadus morhua*) (Kristjansson *et al*., 1995) and greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996) had the optimal temperature at 30°C. However, optimal temperature of collagenase from the tissue of filefish, *Novoden modestrus* was 60°C (Kim and Kim, 1991). From the result, optimal temperature of collagenase from the white shrimp muscle was 40°C. It was presumed that collagenases from white shrimp muscle had less rigid tertiary structures, which would allow them to maintain the conformational flexibility required for efficient catalysis at lower temperatures. The differences in optimal temperature might be associated with the differences in enzymes conformation. The optimal temperature of enzyme is governed by habitat, environment and genetic (Gildberg, 1988).

The effects of pHs on the collagenolytic activity of crude extracts from cephalothorax and muscle of white shrimp and black tiger shrimp are shown in Figure 10. The optimal pHs of collagenase from cephalothorax of white shrimp and black tiger shrimp were 6.0-7.0. Therefore, collagenase from cephalothorax of both shrimps was considered to be a neutral protease. This optimal pH value for collagenase from cephalothorax of both shrimps was similar to those reported for collagenase from various shellfish, e.g. the hepatopancreas of Northern shrimp (*Pandalus eous*) (Aoki *et al*., 2004), fiddler crab (*Uca pugilator*) (Eisen *et al*., 1973), and greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996). Nevertheless, the activity of collagenase from the muscles of white shrimp and black tiger shrimp were found at pH 5, which was similar to the optimal pHs of collagenase from the greenshore crab (*Carcinus maenas*) and the internal organs of filefish (*Novoden modestrus*) (Kim *et al*., 1997), *Paralithodes camtschatica* collagenase (Klimova *et al*., 1990), and *Parahaliporus sibogae* collagenase (Muramatsu and Kariuchi, 1978). At pH 4.0 and 9.0, activities of collagenase from both cephalothorax and muscle markedly decreased, suggesting the denaturation of collagenase at very acidic and alkaline pHs.

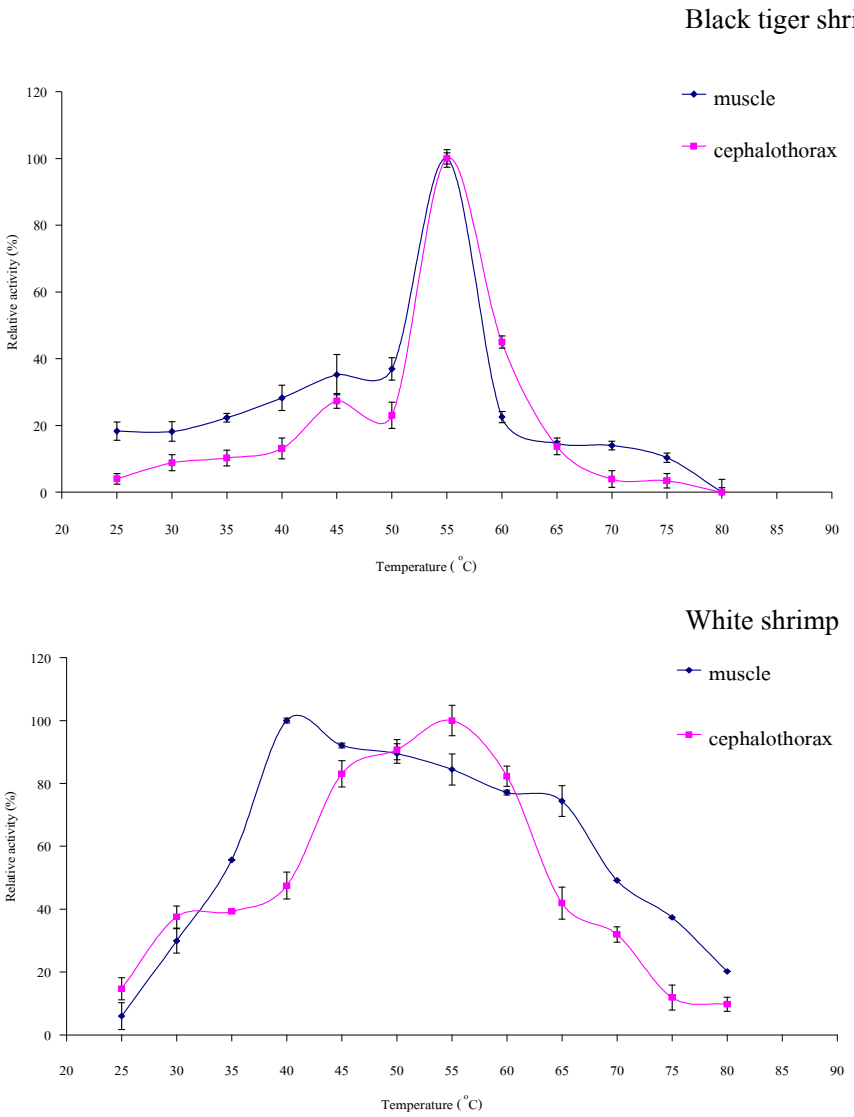

Figure 9. Temperature profiles of collagenase from the cephalothorax and muscle of black tiger shrimp and white shrimp. Collagenase activity was determined at pH 7.0. Collagenase activity was determined using bovine tendon collagen type I as a substrate. One unit of activity was defined as μmol leucine released/5h.

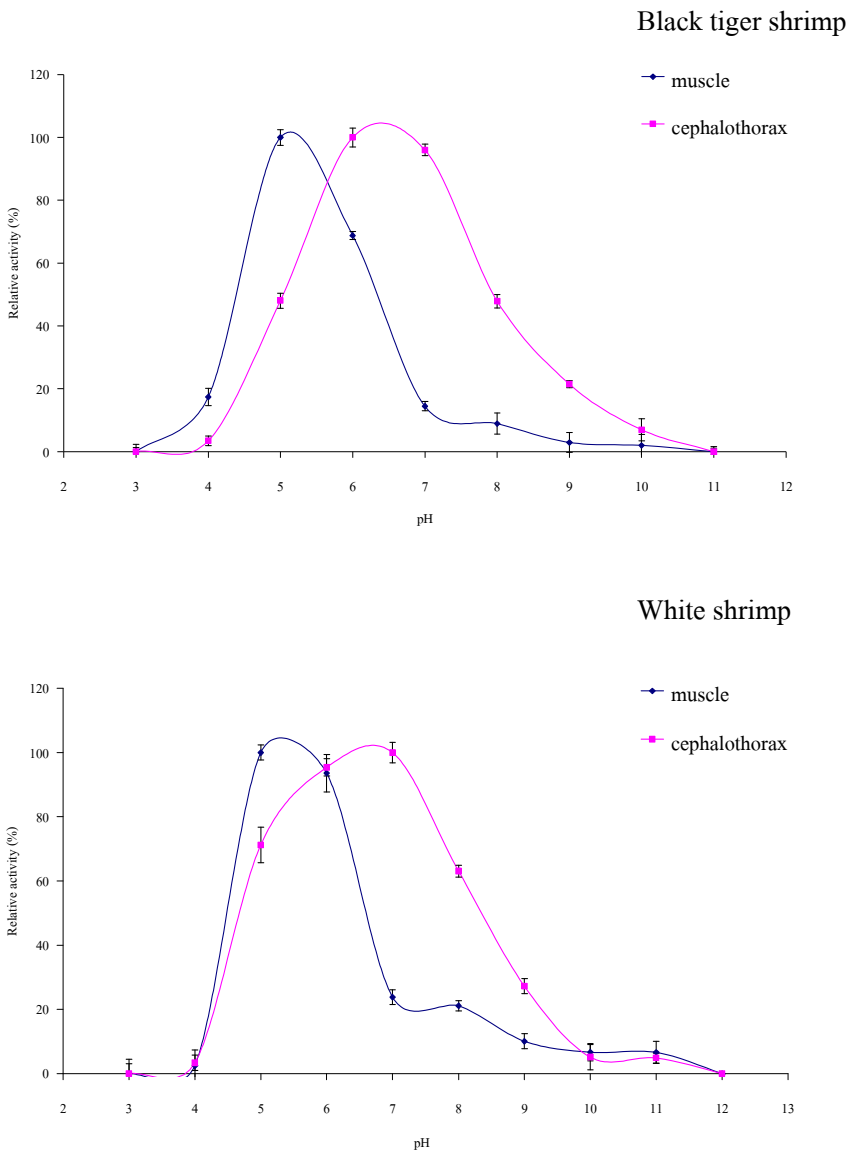

Figure 10. pH profiles of collagenase from the cephalothorax and muscle of black tiger shrimp and white shrimp. The activity was determined at 55°C for collagenase from black tiger shrimp cephalothorax and muscle and at 55°C and 40°C for collagenase from white shrimp cephalothorax and muscle, respectively, over the pH ranges of 2.0-12.0 (0.2 M McIlvaine's buffer for pHs 2.0-7.0 and 0.1 M NaH_2PO_4-0.05 M $Na_2B_4O_7$ for pHs 8.0-12.0). Collagenase activity was determined using bovine tendon collagen type I as a substrate. One unit of activity was defined as µmol leucine released/5h.

1.2.2 Temperature and pH stability.

Thermal stability of collagenase from the cephalothorax and the muscle of white shrimp and black tiger shrimp is depicted in Figure 11. Collagenase from the cephalothorax of both species was stable up to 40°C, while those from muscle were stable at temperature lower than 50°C. Nevertheless, the sharp decrease in activity was noticeable when incubated at temperature above those mentioned. The relative activity lower than 20% was observed when incubated at temperature greater than 75°C. This was mainly due to thermal denaturation of enzyme. Collagenases from viscera and liver from cod, saithe, haddock, tusk and ling were stable after incubation for 10 min at 35°C, and lost their activity completely after heating for 10 min at 70°C (Sovik and Rustad, 2006). Additionally, Kristjansson *et al*. (1995) found that collagenolytic serine protease purified from the intestines of Atlantic cod was stabilized against thermal inactivation by calcium ions. However, the enzyme was unstable when held for 30 min at temperature above 30°C even in the presence of calcium. For collagenase from the muscle of white shrimp, no changes in activity was observed when heated up to 50°C. Thereafter, the sharp decrease in activity was noticeable. When heated at 60°C, activity lower than 20% was retained. For collagenase from cephalothorax of white shrimp, it was noted that activity remained constant when heated up to 40%, followed by the drastic decrease with higher temperature of heating. No activity was found for collagenase from both portions after heating at 70°C

Collagenases from white shrimp cephalothorax and muscle were stable in the pH range of 7.0-9.0 and 6.0-9.0, respectively (Figure 12). Collagenases from white shrimp cephalothorax and muscle were unstable at pH 4.0 and no activities remained after subjected to pH 3.0. However, most activities of collagenase from black tiger shrimp was retained at pH 4.0. From the result, the collagenase might undergo denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly. Fish collagenolytic enzyme was stable within the pH range of 6.0-8.0, whereas bacterial collagenase was most stable within the pH range of 7.0-9.0 (Teruel and Simpson, 1995). At alkali pHs, a slight decrease in activity of collagenases from white shrimp cephalothorax and muscle was found at pH 10.0, suggesting some losses in activity at very alkaline pH. On the other hand, the losses in activity were noticeable in collagenases from black tiger shrimp at pH lower than 8.0-9.0. No activity was found after being subjected to pHs 10-11. The result indicated that

collagenases from black tiger shrimp were vulnerable to activity loss at alkali pH. The differences might be associated with the differences in amino acid composition and bonding stabilizing the enzyme structure.

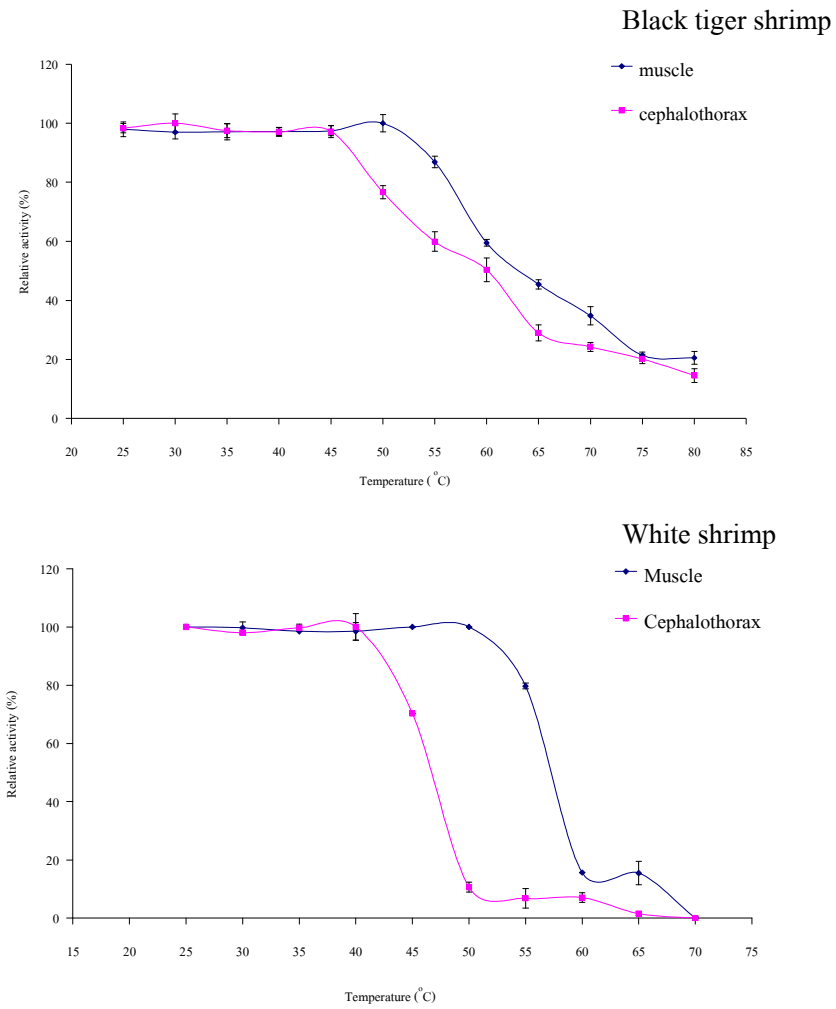

Figure 11. Thermal stability of collagenase from the cephalothorax and muscle of black tiger shrimp and white shrimp. Collagenase activity was determined after incubating crude extract for 15 min at various temperatures. The residual activity was determined at 55°C, pH 5.0 and 55°C, pH 6.0 for 5 h, for collagenase from black tiger shrimp muscle and cephalothorax, respectively and at 40°C, pH 5.0 and 55°C, pH 7.0 for 5 h, for collagenase from white shrimp muscle and cephalothorax, respectively using bovine tendon collagen type I as a substrate.

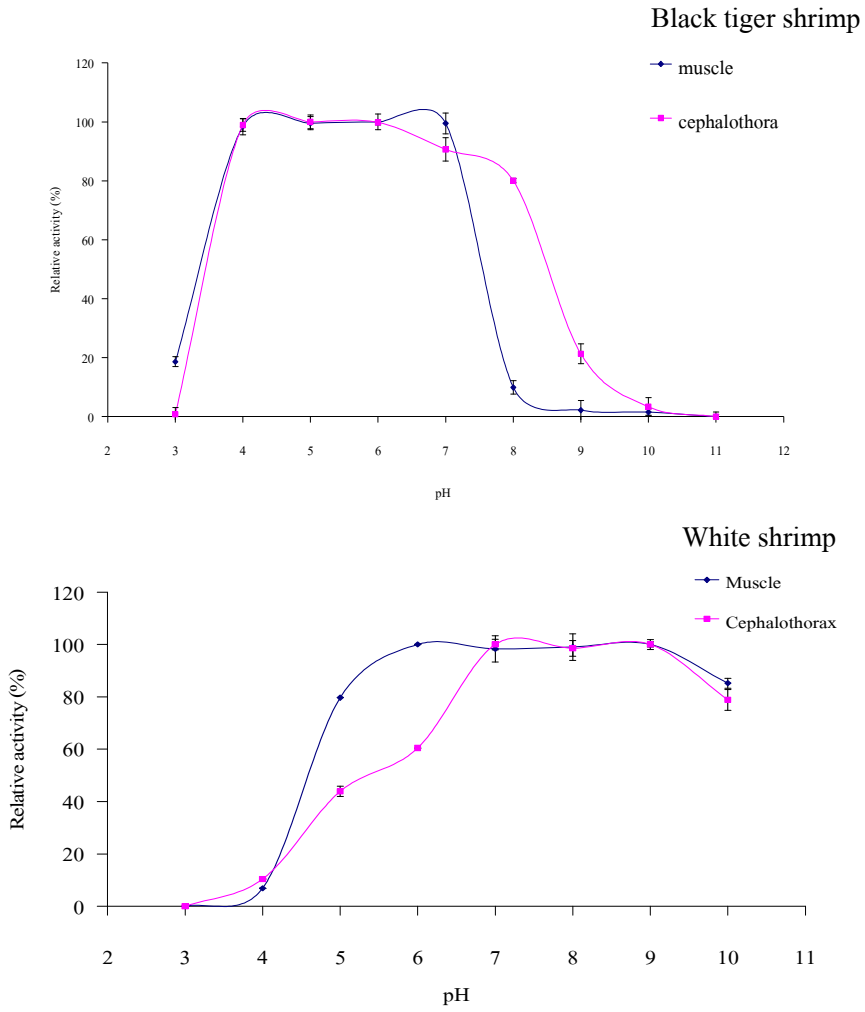

Figure 12. pH stability of collagenase from the cephalothorax and muscle of black tiger shrimp and white shrimp. Collagenase activity was determined after incubating crude extract for 15 min at various pHs. The residual activity was determined at 55°C, pH 5.0 and 55°C, pH 6.0 for 5 h, for collagenase from black tiger shrimp muscle and cephalothorax, respectively and at 40°C, pH 5.0 and 55°C, pH 7.0 for 5 h, for collagenase from white shrimp muscle and cephalothorax, respectively using bovine tendon collagen type I as a substrate**.**

1.2.3 Effects of inhibitors

The effects of the various inhibitors on activity of collagenase from the cephalothorax and muscle of black tiger shrimp and white shrimp are shown in Table 5. The activities of collagenase from both portions of black tiger shrimp and white shrimp were completely inhibited by 10 μM soybean trypsin inhibitor, a specific inhibitor toward serine protease. EDTA in the range of 1-10 mM partially inhibited the collagenase from white shrimp but showed no inhibitory effect towards collagenase from black tiger shrimp. E-64, specific inhibitors of cysteine proteinases, and pepstatin A, specific inhibitors of aspartic proteinases, had no inhibitory effect on collagenase from white shrimp. However, E-64 at the same range of concentration slightly inhibited the collagenase activity from black tiger shrimp. The result confirmed that collagenase from white shrimp cephalothorax and muscle was mainly serine collagenase. Serine collagenases have been found in fiddler crab (Eisen *et al*., 1973), greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996), Atlantic krill (*Euphausia superba*) (Turkiewicz *et al*., 1991) and Northern shrimp (*Pandalus eous*) (Aoki *et al*., 2004). EDTA showed the partial inhibition towards collagenase from both portions of black tiger shrimp. A result indicates that these enzymes possibly required metal ions for their activity.

Table 5. Effects of various inhibitors on the activity of collagense from cephalothorax and muscle of white shrimp and black tiger shrimp.

Inhibitors	Concentrations	% Inhibition*			
		White shrimp		Black tiger shrimp	
		Muscle	Cephalothorax	Muscle	Cephalothorax
Soybean trypsin	1 µM	95.25 ± 0.24	97.87 ± 0.59	97.52 ± 2.60	96.30 ± 0.72
inhibitor	10 µM	100.00	100.00	100.00	100.00
Pepstatin A	1 µM	0.00	0.00	0.00	15.40 ± 0.63
	10 µM	0.00	0.00	0.00	17.90 ± 1.55
EDTA	1 mM	12.02 ± 0.93	15.53 ± 3.23	0.00	0.00
	10 Mm	18.20 ± 0.35	21.46 ± 2.04	0.00	0.00
E-64	1 µM	0.00	0.00	12.60 ± 3.35	16.80 ± 2.32
	10 µM	0.00	0.00	17.42 ± 2.28	18.30 ± 1.09

* Mean ± SD from triplicate determinations.

Residual activity was analyzed using bovine tendon collagen type I as a substrate for 5 h at pH 5.0 and 55°C, pH 6.0 and 55°C for collagenase from muscle and cephalothorax of black tiger shrimp, respectively and at 40°C, pH 5.0 and 55°C, pH 7.0 for 5 h, for collagenase from white shrimp muscle and cephalothorax, respectively.

1.3 PPO from cephalothorax

1.3.1 Temperature and pH profiles

The optimal temperature of PPO from the cephalothorax of black tiger shrimp was 45°C (Figure 13). The activity increased with increasing temperature up to 45°C. This was suggested that the reaction was accelerated by the enhanced kinetic energy (Montero *et al*., 2001a). However, the activity decreased when the temperature increased. The decrease in activity at high temperature was most likely associated with the thermal denaturation of PPO. The optimal temperature of PPO has been reported to be varied, depending on species and habitat temperature. PPO from carapace of shrimp (*Penaeus setiferus*) had the maximal activity at 45°C (Simpson *et al*., 1987), whereas the optimal temperature of PPO from carapace of shrimp (*Penaeus japonicus*)

cultured in Spain was 55°C (Simpson *et al*., 1988). From the result, PPO from the white shrimp cephalothorax had the maximal activity at 50°C. PPO from the cephalothorax of black tiger shrimp showed slightly lower optimal temperature than did that from white shrimp. It was suggested that lower activation energy was required for the activity of PPO from the black tiger shrimp cephalothorax, compared with that needed for PPO from white shrimp.

The maximal activity of PPO from the cephalothorax of black tiger shrimp was observed at pH 6.5 (Figure 14). The activity markedly decreased in very acidic and alkaline pH ranges. The unfolding of PPO molecules might occur at the extreme pHs owing to the increase in electrostatic repulsion, leading to the losses in their activity. The result was in accordance with Benjakul *et al*. (2005) who reported that the optimal pH of PPO from the cephalothorax of kuruma prawn was 6.5. The optimal pH of PPO from different crustaceans was different. The maximal PPO activity from the cephalothorax of white shrimp was observed at pH 5.5 (Figure 10). PPO from the carapace of shrimp (*Penaeus setiferus*) showed the maximal activity at pH 7.5 (Simpson *et al*., 1987). PPO from the prawn (*Penaeus japonicus*) had two pronounced peaks at pHs 5.0 and 8.0 (Montero *et al*., 2001a), whereas the optimal pH of black tiger prawn PPO was 6.0 (Rolle *et al*., 1991). Moreover, the optimal pH depends to a large extent on the physiological pH, in which the enzyme activity occurs in nature. Optimal pH of PPO from the carapace of cephalothorax was 7.16±0.07, whereas that of PPO from the abdominal cuticle was 8.76±0.04. Enzyme probably possessed different optimal pH characteristics, depending on the locus of extraction (Montero *et al*., 2001a). The differences in optimal pH might be due to the different nature of prototropic groups in the active site of enzymes (Whitaker, 1972).

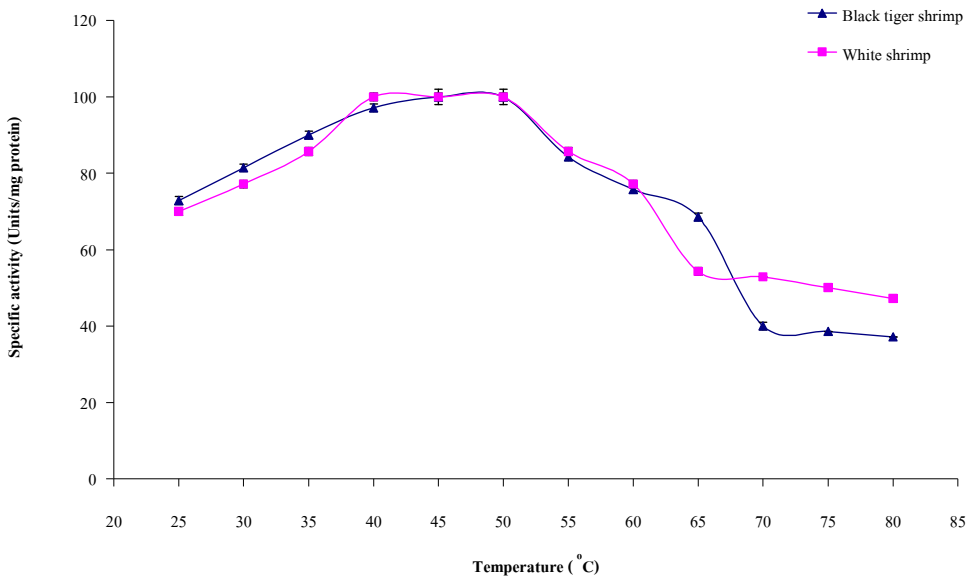

Figure 13. Temperature profiles of PPO from the cephalothorax of black tiger shrimp and white shrimp. PPO activity was determined at pH 7.0 using *L*-DOPA as a substrate. One unit of PPO was defined as the change in 0.001 unit of A_{475}/min.

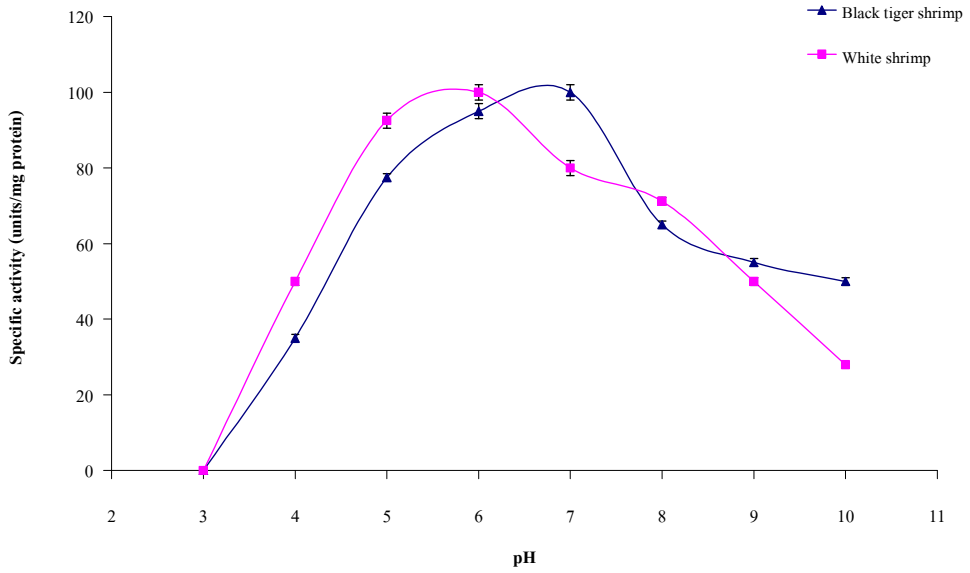

Figure 14. pH profiles of PPO from the cephalothorax of black tiger shrimp and white shrimp. PPO activity was determined at 45°C and 50°C for PPO from the cephalothorax of black tiger shrimp and white shrimp respectively, over the pH ranges of 2.0-12.0 (0.2 M McIlvaine's buffer for pHs 2.0-7.0 and 0.1 M NaH_2PO_4-0.05 M $Na_2B_4O_7$ for pHs 8.0-12.0) using *L*-DOPA as a substrate. One unit of PPO was defined as the change in 0.001 unit of A_{475}/min.

1.3.2 Temperature and pH stability

Thermal stability of PPO from the cephalothorax of black tiger shrimp is depicted in Figure 15. The activity was stable up to 45°C and the loss in activity was observed at higher temperature. The result indicated that PPO from the cephalothorax of black tiger shrimp was not stable at high temperature. PPO most likely underwent denaturation and lost its activity at high temperature. PPO from the cephalothorax of white shrimp was stable up to 40°C but the greater loss in activity was found at temperature above 40°C (Figure 13). The result was in agreement with Simpson *et al*. (1987) who reported that PPO from the carapace of shrimp (*Penaeus setiferus*) was stable up to 50°C but unstable at temperatures higher than 50°C. Pink shrimp PPO was not stable at temperatures higher than 40°C (Simpson *et al*., 1988). Even within the same species, there were differences in enzyme stability, depending on the state of activation (Ferrer *et al*., 1989).

PPO from cephalothorax of the black tiger shrimp was stable in the pH range of 5.5-6.5 (Figure 16). The activity decreased in the alkaline pH ranges. Also, activity tended to decrease at the acidic pHs, in which activity of approximately 70% was retained. The result was in agreement with Rolle *et al*. (1991) who found that the PPO from black tiger shrimp was not stable at acidic pHs. PPO from the cephalothorax of white shrimp was stable in the pH range of 5.5-6.0. The instability at acidic pH of PPO suggested that treatment of shrimps with acid solutions would inhibit melanosis to a large extent (Montero *et al*., 2001). The differences in pH stability indicated the different molecular properties including the bondings stabilizing the structure as well as the enzyme conformation among the various species and anatomical locations. In addition, the stability of PPO varied, depending on a number of factors such as temperature, pH, substrate used, ionic strength, buffer system and time of incubation (Montero *et al*., 2001).

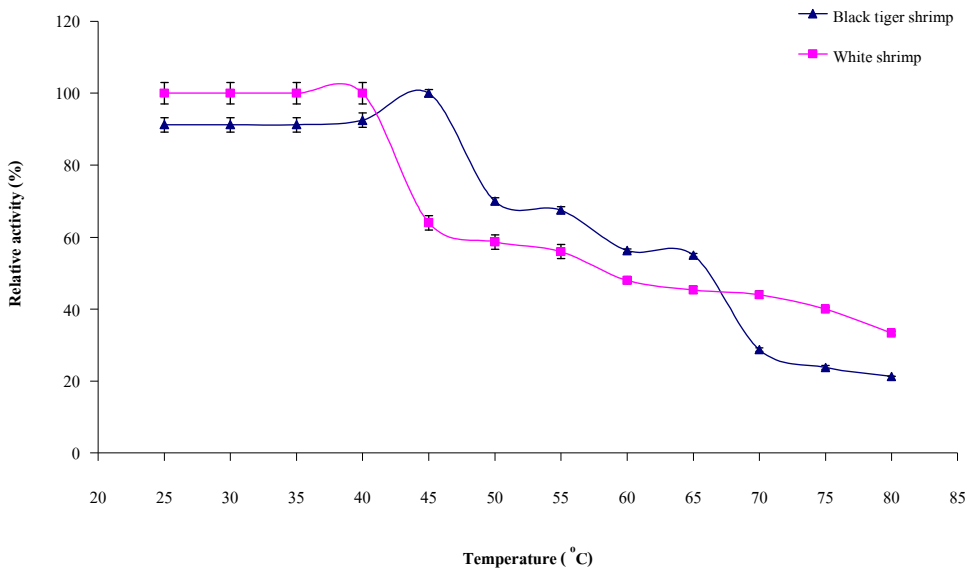

Figure 15. Thermal stability of PPO from the cephalothorax of black tiger shrimp and white shrimp. PPO activity was determined after incubating crude extract for 15 min at various temperatures. The residual activity was determined at 45°C, pH 6.5 and 50°C, pH 5.5 for 3 min for PPO from the cephalothorax of black tiger shrimp and white shrimp cephalothorax, respectively, using *L*-DOPA as a substrate. One unit of PPO was defined as the change in 0.001 unit of A_{475}/min.

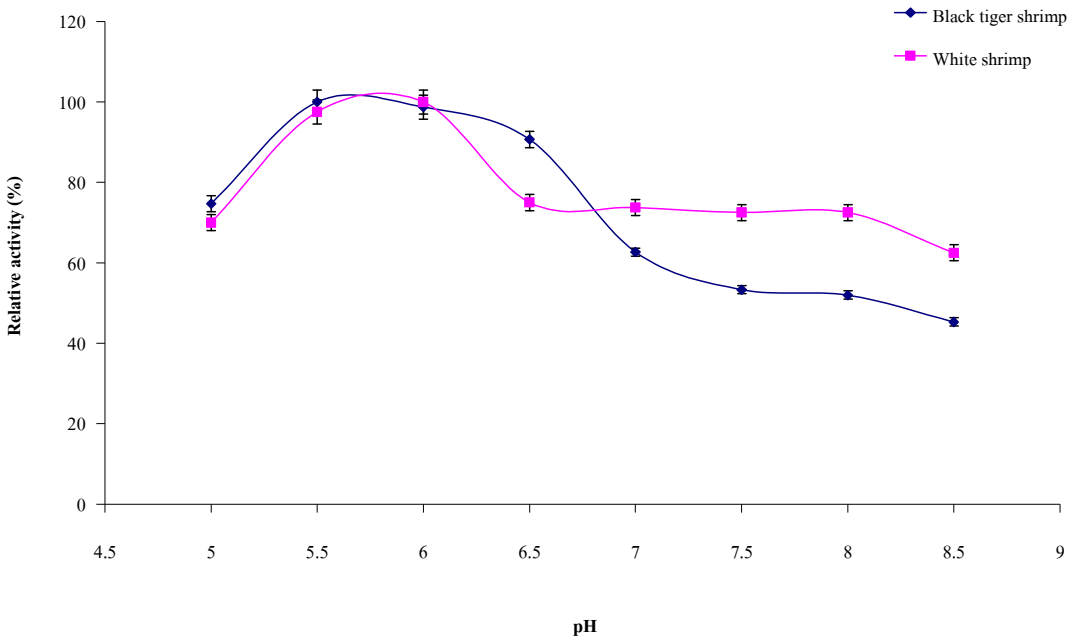

Figure 16. pH stability of PPO from the cephalothorax of black tiger shrimp and white shrimp. PPO activity was determined after incubating crude extract for 15 min at various pHs. The residual activity was determined at 45°C, pH 6.5 and 50°C, pH 5.5 for 3 min for PPO from black tiger shrimp and white shrimp cephalothorax, respectively, using *L*-DOPA as a substrate. One unit of PPO was defined as the change in 0.001 unit of A_{475}/min.

1.3.3 Effects of inhibitors

Various chemicals exhibited the inhibitory activity towards PPO from the cephalothorax of black tiger shrimp and white shrimp differently (Table 6). From the result, PPO activities from both shrimps were strongly inhibited by ascorbic acid, 4-hexylresorcinol and citric acid. Conversely, EDTA and sodium benzoate showed the low inhibitory effect towards PPO. The greater inhibition was generally noticeable as the higher concentrations were used. These results suggested that ascorbic acid and citric acid might cause the destabilizing effect on PPO by lowering the pH and ability to reduce quinones back to phenolic compounds before they can undergo further reaction to form pigments. At high concentrations, ascorbic acid also can directly inhibit PPO (Markis and Embs, 1966). EDTA, widely used chelating agent, also inhibits the oxidative degradation of ascorbic acid, and browning of grapefruit juice (Sapers and Hicks,

1989). 4-hexylresorcinol has been used as a specific PPO inhibitor to prevent melanosis in postmortem shellfish (Montero *et al*., 2004). Much lower concentrations of 4-hexylresorcinol was required to exhibit melanosis inhibition in shrimp, compared with sulfite (McEvily *et al*., 1991). Montero *et al*. (2001b) reported that kojic acid, sodium benzoate and 4-hexylresorcinol could potentially be used as sulfite substitutes to prevent melanosis in shrimp.

Table 6. Effect of various inhibitors on the activity of PPO from the cephalothorax of black tiger shrimp and white shrimp.

Inhibitors	Concentrations	% inhibition*	
		White shrimp	Black tiger shrimp
Sodium benzoate	0.1 g/l	2.14 ± 1.25	1.22 ± 0.31
	1.0 g/l	4.58 ± 0.52	9.18 ± 0.21
4-hexylresorcinol	1.0 g/l	22.77 ± 2.32	24.85 ± 0.64
	10.0 g/l	59.64 ± 0.64	63.99 ± 0.99
Citric acid	0.5 g/l	8.50 ± 0.72	19.37 ± 1.45
	5.0 g/l	36.10 ± 0.17	46.15 ± 0.61
Ascorbic acid	0.5 g/l	22.82 ± 0.22	27.85 ± 0.37
	5.0 g/l	67.99 ± 0.88	68.46 ± 0.10
EDTA	0.25 g/l	1.94 ± 0.13	1.51 ± 0.56
	2.5 g/l	2.35 ± 0.12	5.12 ± 1.25

* Mean ± SD from triplicate determinations.

Residual activity was analyzed using *L*-DOPA as a substrate for 3 min at 45°C, pH 6.5 and 50°C, pH 5.5 for 3 min for PPO from the cephalothorax of black tiger shrimp and white shrimp, respectively.

2. Effect of different MAP conditions on melanosis and quality of black tiger shrimp and white shrimp during refrigerated storage.

Changes in total viable count

Changes in TVC of black tiger shrimp and white shrimp kept under MAP with and without ascorbic acid pretreatment during storage at 4°C are depicted in Figure 17 A and 17 B, respectively. TVC of fresh black tiger shrimp and white shrimp were 2.48 log CFU/g and 2.95 log CFU/g, respectively. TVC increased rapidly in both species stored in air and the lowest counts were found in sample stored under MAP3 (80% CO_2, 10% O_2, 10% N_2). However, white shrimp kept under MAP3 had the similar TVC to the sample stored in air at day 12 of storage. Major mechanism of MAP techniques is to lower the level of oxygen in a food environment, in which growth of aerobic bacteria can be retarded (Patsias *et al*., 2006). Aerobic microorganisms are generally sensitive to CO_2; therefore, MAP delayed the spoilage of black tiger shrimp and white shrimp. Hoz (2000) indicated that CO_2 has an important effect on microbial growth, exerting a selective inhibitory action. To achieve inhibitory effect toward microorganism, the storage temperature of MAP products should be as low as possible, since solubility of CO_2 decreases with an increase in temperature (Daniels *et al*, 1985). Ozogul *et al*. (2004) reported that significant differences in TVC were found between sardines kept in air and MAP.

At the end of storage (12 days), TVC of black tiger shrimp stored in MAP containing higher concentration of CO_2 was lower than that of shrimp kept under MAP containing lower concentration of CO_2 ($P<0.05$). CO_2, which is water and lipid soluble, can penetrate through bacteria membranes to cause changes in intracellular pH and results in disruption of internal enzymatic equilibrium (Aickin and Thomas, 1975; Farber, 1991). Marshall *et al* (1991) found that high-CO_2 modified atmospheres (76% CO_2, 13.3% N_2 and 10.7% O_2 or 80% CO_2, and 20% N_2) inhibited the growth of *L. monocytogenes* in chicken nuggets stored at 3°C. Carbon dioxide extends the lag phase of Gram-negative aerobic bacteria (Reddy *et al*., 1992a). These bacteria play a major role in fish spoilage by generating of off-colors, off-odors and a bad visual appearance of fish. Additionally, CO_2 can flavor the growth of some Gram-positive bacteria, i.e. lactobacillus that would compete with spoilage bacteria (Stenstrom, 1985). The effectiveness of the MAP system in the preservation lies in the combined inhibitory effect of low storage temperature and the carbon dioxide atmosphere in the package on microbial activity (Gray *et al*.,

1983). CO_2, the inhibitory gas, has a strong antimicrobial action, which varies between different microorganisms (Gould, 2000). It is responsible for extending the lag phase of bacterial growth and for decreasing the growth rate during the log phase (Farber, 1991). The bacteriostatic effect of CO_2 depends on the gas concentration, the initial bacterial count and the type of fish product (Finne, 1982; Gray *et al*., 1983). CO_2 is highly soluble in water, forming carbonic acid that may lower the pH (Smith *et al*., 1990). The inhibitory effect of CO_2 on gram negative bacteria is directly related to the percentage of CO_2 in the gas mixes used (Lannelongue *et al*., 1982). Reddy *et al*. (1996) found that initial aerobic bacteria counts and anaerobe bacteria count in tilapia packed under MAP (75% CO_2, 25% N_2) were 4.3 log and 3.2 log CFU/g, respectively and reached 4.6 log CFU/g after 10 days of storage. Psychotropic bacteria count of under MAP (25% CO_2/ 75% N_2) packed gutted cod are lower than that of air packed samples during the storage at 0°C (Villemure *et al.*, 1986). Ozogul *et al.* (2002) reported that the amount of mesophilic bacteria count in herring flesh at the time of rejection in MAP (60% CO_2/ 40% N_2) (10 days) and in vacuum (8 days) is < 6 log CFU/g. TVC of iced sardines reached the limit counts of 6 log to 7 log CFU/g at day 3, in air; at day 8, for vacuum packing and at day 10, for MAP (60% CO_2/ 40% N_2) (Ozogul *et al*., 2004). When considering, the effect of ascorbic acid pretreatment on TVC, samples kept in air and treated with 0.5% ascorbic acid had lower TVC when compared to those without ascorbic acid, most likely due to a direct lethal effect of ascorbic acid. This was evidenced after 3 days of storage. Weak acids like ascorbic acid penetrate the cell membrane and act by releasing a proton and acidifying the cytoplasm of the cell, which may result in detrimental effects (Ita and Hutkins, 1991). For samples kept under MAP, most of samples had low TVC when the samples were treated with ascorbic acid. From the results, it was suggested that combined effects of MAP and ascorbic acid during in both species storage could retard the microbial growth. From the result, TVC exceeded log 6 CFU/g 12 days of storage for the control without ascorbic acid pretreatment, which is the limit for acceptability (ICMSF, 1986). When comparing TVC of both species, white shrimps had the higher TVC at day 12 of storage than black tiger shrimps. Thus, white shrimps might be more prone to microbial spoilage than black tiger shrimps, which could be due to the different compositions. It was noted that TVC determination was conducted at 37°C and the value represented mostly mesophilic bacteria. This

might not indicate the microbial load in the shrimp stored at low temperature. Those psychrophilic microorganisms most likely played a role in spoilage of those shrimps.

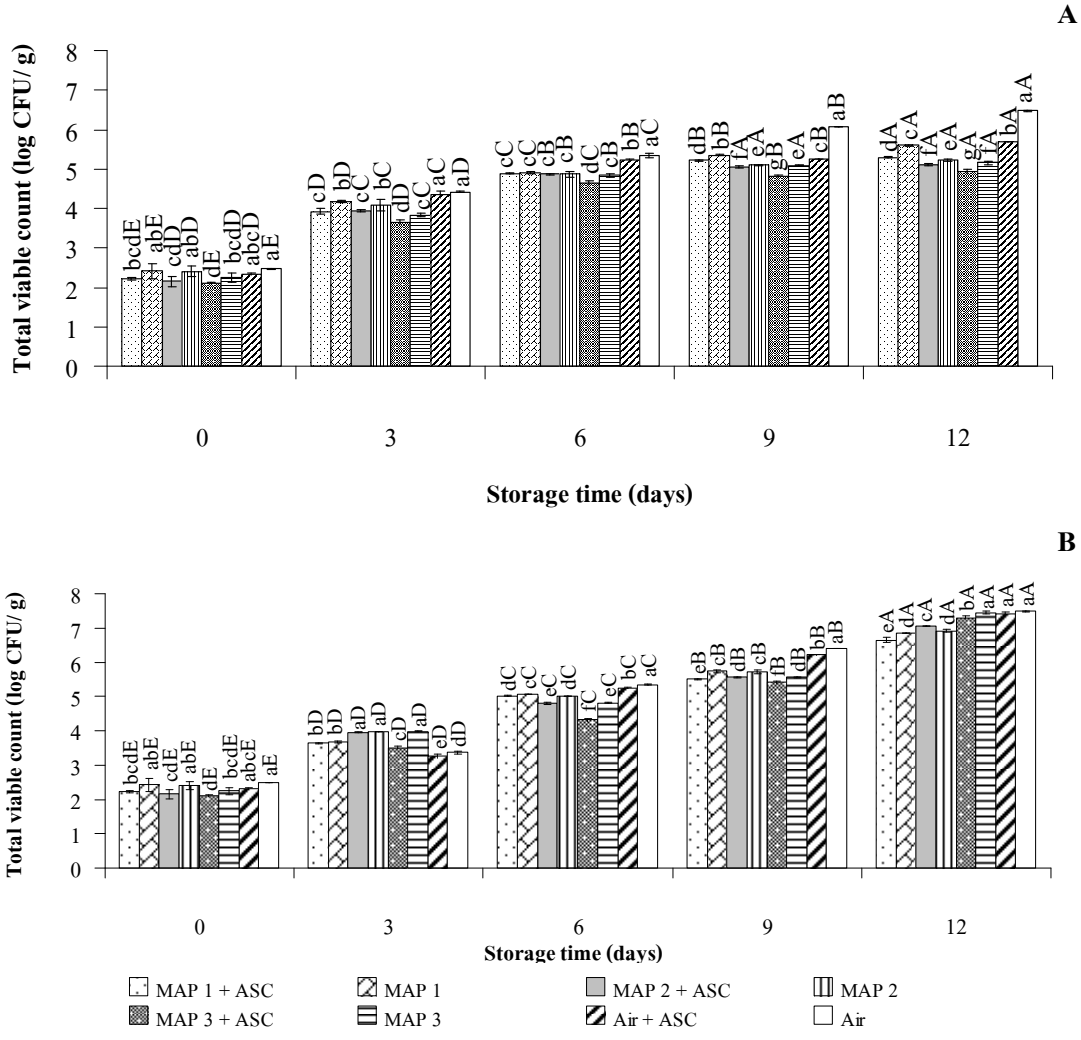

Figure 17. Changes in TVC (CFU/g) of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in lactic acid bacteria count

Changes in lactic acid bacteria (LAB) count of black tiger shrimp and white shrimp kept in air or under MAP at 4°C with and without ascorbic acid pretreatment are depicted in Figure 18 A and 18 B, respectively. Both shrimps stored in MAP had higher LAB counts than those stored in air throughout the storage. Samples kept under MAP with higher CO_2 content had the greater LAB count than the sample stored under MAP having lower CO_2 content. At day 12 of storage, LAB count of black tiger and white shrimps kept in air ranged from 2.56 log to 2.59 log CFU/g. For both species stored under MAP, LAB counts were 4.45 log to 6 log CFU/g. LAB do not dominate as the microflora of the raw material, and only certain species have been found in freshwater fish and their surrounding environment (Stiles and Holzapfel, 1997; González *et al*., 1999; Ringo *et al*., 2000). LAB have usually been found predominating or occurring in spoiled vacuum-packaged cold-smoked fish (Joffraud *et al*., 2001). Aerobic spoilage bacteria such as Gram-negative bacteria like *Pseudomonas* and *Flavobacterium,* Gram-positive bacteria like *Micrococcus* as well as moulds and yeasts are very CO_2 sensitive, whereas facultatively anaerobes, such as; *Enterobacteriaceae*, are less sensitive. Gram positive bacteria, including LAB and *Brochothrix thermospacta* and obligatory anaerobic bacteria are usually resistant to CO_2. Under anaerobic conditions at temperatures below 20°C, psychrotrophic LAB, which can grow at 5°C or below, could compete successfully with other psychrotrophic spoilage microoganisms (Adams and Moss, 2000). For all MAP treatment, O_2 at 20% was used. The main effect of O_2 is to maintain the organoleptic properties, such as the color and taste of the fish product and to reduce the drip (Stammen *et al*., 1990). During modified atmosphere storage, a shift from an initial Gram-negative aerobic microflora to a predominantly Gram-positive facultatively anaerobic spoilage microflora takes place and usually psychrotrophic LAB become the dominating spoilage flora (Stenstrom, 1985 and Farber, 1991). The LAB selection is generally recognized as beneficial for shelf-life because their growth can inhibit potent spoilers due to antagonistic activities such as the production of organic acids, hydrogen peroxide and CO_2, and the synthesis of antibiotics and bacteriocins (Daeschel, 1989; Lindgren and Dobrogosz, 1990). For ascorbic acid treatment, it was noted that ascorbic acid treatment resulted in the lower LAB count of both shrimps stored under MAP1. Ascorbic acid did not show inhibitory effect on LAB of black tiger shrimp stored in

MAP2 or MAP3. Nevertheless, ascorbic acid treatment yielded the sample kept in air with the lower LAB than those with no ascorbic acid pretreatment.

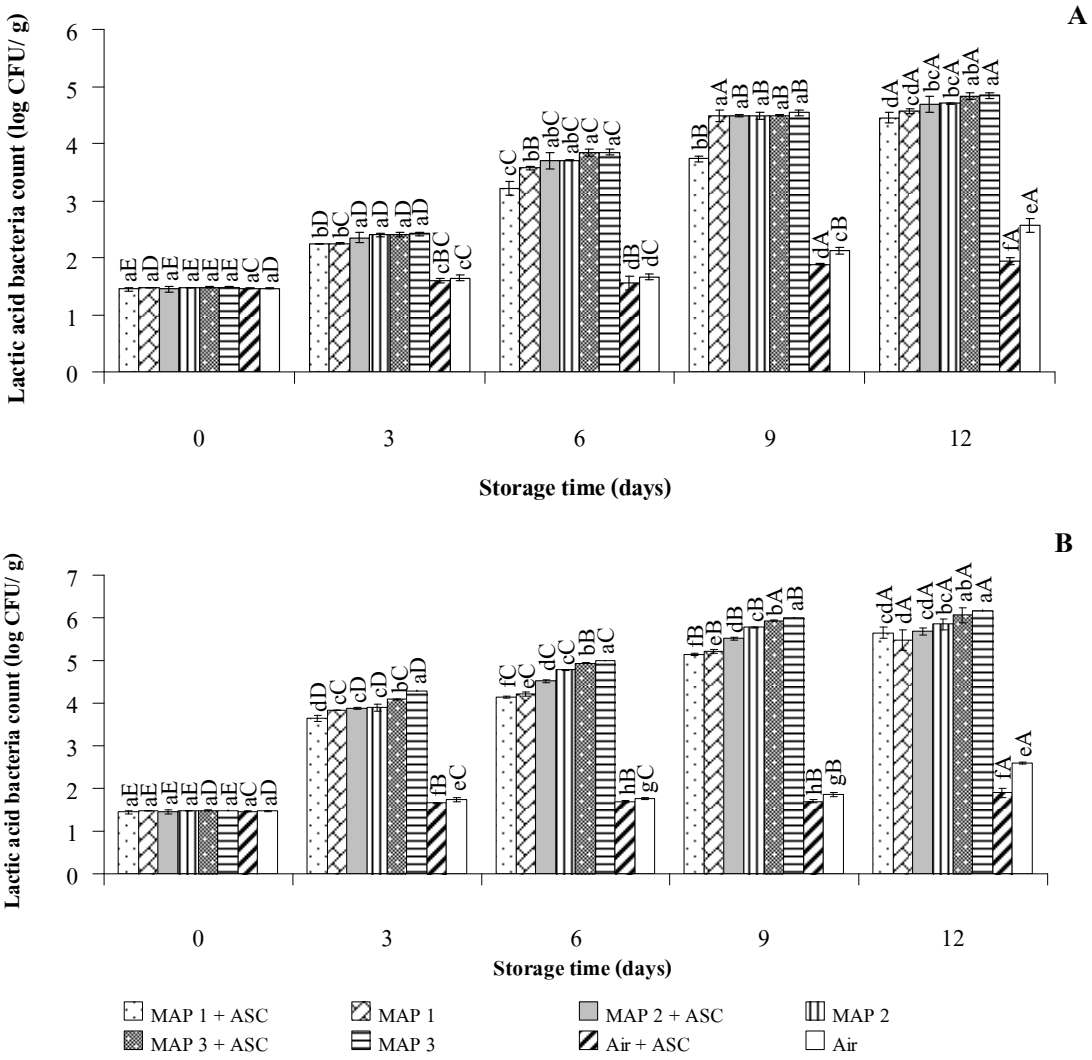

Figure 18. Changes in Lactic acid bacteria (CFU/g) of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in pH

Changes in pH of black tiger shrimp and white shrimp with and without ascorbic acid pretreatment during storage in air or under different MAP conditions are presented in Figure 19 A and 19 B, respectively. The initial pH of black tiger shrimp and white shrimp were 7.13 and 6.69, respectively. For the samples pretreated with ascorbic acid, pH values were 6.71 and 6.48, respectively (Figure 19 A and 19 B). The pH value was in the range of 7-8 reported in shrimp muscle (Layrisse and Matches, 1984; Mendes *et al*., 2002). Generally, the pH value of crustacean is higher than that of fish and mammal species because of the higher content of nonprotein nitrogenous compounds (Shahidi *et al*., 1994). From the result, both shrimps kept in air had the continuous increase in pH throughout the storage up to 12 days, reaching pHs approximately of 8.49 and 8.11, respectively after 12 days of storage. The increase in pH is associated with bacteria growth and is probably caused by the formation of basic amines (Wang and Brawn, 1983; Debevere and Boskou, 1996; Pastoriza *et al*., 1996b). Among all samples, those kept in air had the highest pHs, followed by those stored under MAP 1. Samples stored under MAP3 showed the lowest increase in pH throughout the storage of 12 days ($P<0.05$). The changes in pH depend on the amount of CO_2 dissolved and the buffering capacity of the shrimp muscle. Lannelongue *et al*. (1982) reported a decrease in surface pH of brown shrimp packaged under several enriched CO_2 atmospheres (35% to 100%). However, Layrissw and Matches (1984) found a constancy of the pH value of 6.8 in spotted shrimp packed in 100% CO_2. Changes in pH also depended on a variety of factors such as species, fishing ground, feeding, storage temperature and buffering capacity of meat (Pacheco-Aguilar *et al*., 2000). When comparing the changes in pH between both species, black tiger shrimp muscle had a higher increase in pH than did white shrimp muscle during storage times. This might be due to the different buffering capacity and the different activity of enzymes converting glycogen into lactic acid between species. From the result, ascorbic acid pretreatment did not show the pronounced effect on lowering the increase in pH of both shrimps, especially as the storage time increased.

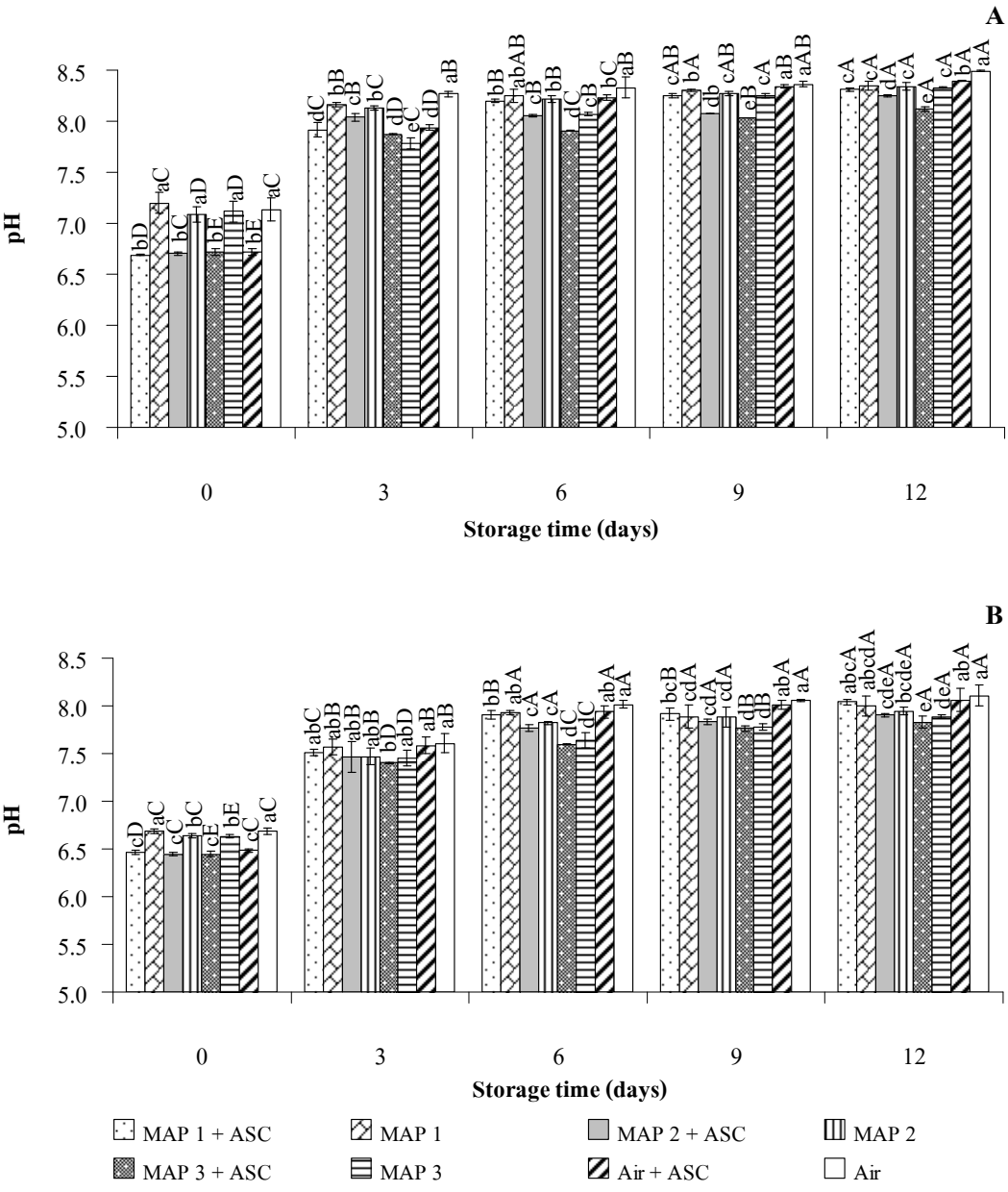

Figure 19. Changes in pH of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30%N_2; MAP3 = 80% CO_2, 10% O_2, 15% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in total volatile bases and trimethylamine contents

Total volatile bases (TVB) contents of black tiger shrimp and white shrimp during storage at 4°C up to 12 days are depicted in Figures 20 A and 20 B, respectively. The initial values of TVB of fresh black tiger shrimp and white shrimp were 3.52 mg N/ 100 g sample and 2.53 mg N/ 100 g sample, respectively. TVB content in all samples increased with increasing storage times ($P<0.05$). Generally, samples stored in air had the higher TVB, compared with samples kept under MAP, regardless of ascorbic acid pretreatment, throughout the storage ($P<0.05$). Among all MAP conditions used, MAP3 exhibited the lowest increases in TVB for both shrimps ($P<0.05$). The increases in TVB of sample kept in air were more pronounced after 3 days of storages, compared with those stored under MAP. At day 12 of storage, TVB content of sample kept in air with ascorbic acid pretreatment of black tiger shrimp and white shrimp were 11.19 mg N/ 100 g sample and 8.85 mg N/ 100 g sample, respectively. TVB of 11.41 mg N/100 g sample and 9.76 mg N/100 g were found in the sample kept in air without ascorbic acid treatment. From the result, ascorbic acid might show the antimicrobial effect in most samples since a much lower TVB content was obtained with ascorbic acid treatment, possibly caused by acidulant effect and oxygen scavenging property of ascorbic acid. As a consequence the growth of aerobic spoilage bacteria might be retarded. TVB limit of acceptability (35 mg/100 g) for consumption of hake steaks was reported by Ordonez *et al*. (2000). Hake steaks could be extended to 7 days when stored in air, 11 days when packaged and stored in 20% CO_2 and 14 days when stored in 40% CO_2. TVB usually include trimethylamine and ammonia. Ammonia is supposed to be the first compound found in the TVB fraction (Gram and Huss, 1996; Huss, 1995). TMA and TVB are products of bacterial spoilage and the content is often used as an index to assess the keeping quality and shelf-life of seafood products (Lannelongue *et al*., 1982). TVB values of fresh and good quality fish are generally less than 12 mg/100g. Higher TVB-N values in the range of 25-35 mg/100 g indicate that the fishes ranged from slightly decomposed/edible to decomposed/inedible (Ababouch *et al*., 1996). However, it is known that the concentration of TVB never reaches 20 mg/100 g in many fatty fishes (Sikorski *et al*., 1990). Considering the TVB content, it can be concluded that black tiger shrimp and white shrimp still had a good quality for consumption up to 12 days. Boskou and Debevere (2000) reported that cod fillets treated with 10% acetic acid and packed under 50% CO_2/ 45% O_2/ 5% N_2 atmosphere had the retarded formation of TMA and

TVB during storage at 7°C for 12 days. From the result, a 0.5% (v/w) ascorbic acid in combination with MAP was effective to inhibit the microbial activity as indicated by low TVB values. The increases in TVB content in all samples especially the sample stored in air correlated well with the increases in pH. However, Lannelongue *et al.* (1982) found that TVB content in shrimp stored under MAP (100% CO_2) was similar to that found in shrimp stored under MAP (34% CO_2, 60% O_2). Based on TVB, both shrimp could be justified as acceptable for consumer since TVB content was lower than 10 mg N / 100 g in all samples stored in different conditions up to 12 day. However, TVB values of black tiger shrimp stored in air was higher than 10 mg N /100 g sample at day 12 of storage. Limits of TVB of 30 mg N / 100 g in shrimp have been reported (Cobb *et al*., 1973).

Trimethylamine (TMA) contents of black tiger shrimp and white shrimp samples stored in air were higher than those of sample kept under MAP, regardless of ascorbic acid pretreatment (Figure 21 A and 21 B). For the same packaging atmosphere used, the samples treated with ascorbic acid possessed lower than TMA content for most samples. It was suggested that ascorbic acid might function as antimicrobial toward TMAO reducing bacteria. Trimethylamine oxide (TMAO), found in a large number of marine fish and shellfish, is broken down to trimethylamine (TMA) either by the endogenous enzymes or by the bacterial enzyme (Debevere *et al.*, 2001). Enzymes from spoilage microorganisms primarily metabolize the extractive fraction of the fish muscle, producing a wide variety of volatile compounds, resulting in off-flavors and odors (Ryder *et al*., 1984). TMA content is also used as a quality indicator for fish. The quantitative level of TMA in fish is considered a major index of the quality of marine fish (Mendes *et al*., 1999). The rejection limit is usually 5-10 mg TMA/100 g muscle; however, in numerous fatty fishes, the concentration of TMA never reaches the limit of 5 mg TMA-N/ 100 g (Sikorski *et al*., 1990). Lannelongue *et al*. (1982) reported that a TMA value of 5 mg TMA-N/ 100 g was the limit for acceptability of swordfish. Ordonez *et al*. (2000) reported that TMA formation was inhibited when hake steaks were stored in CO_2-enriched atmospheres. The concentration of TMA was found to be 13.5 mg in herring kept in MAP (60% CO_2/ 40% N_2) for 10 days at 2°C (Ozogul *et al*., 2002) and Ozogul *et al.* (2004) found that the highest concentration of TMA was found in sardines kept in air, followed by sardines stored in vacuum atmosphere. The lowest TMA was observed in sardines stored under MAP. Debevere and Boskou (1996) indicated that

MAP (60% CO_2/ 30% O_2/ 10% N_2) prevented TMA production in cod kept under MAP. Similar results have been reported by de la Hoz *et al*. (2000) and Ordonez *et al.* (2000) for salmon steak and hake slices stored under carbon dioxide enriched atmospheric conditions, in which TMA content was lower than that kept in air. Reddy *et al.* (1995) found that TMA value of tilapia increased from, 0.07 mg/100 g to 2.59 mg/100 g after 9 days (rejection time) when stored in air and to 1.83 mg/100 g after 13 days (rejection time) for samples stored under MAP (25% CO_2/ 75% N_2). From the results, TMA levels associated with spoilage of both shrimps were very low and did not exceed the limit reported. The slow rate of TMA production in samples stored under MAP was most likely due to a reduction in growth of aerobic, Gram-negative bacteria, including TMA producing microorganisms, by CO_2 and ascorbic acids. However, Photobacterium phosphoreum, Gram-negative and CO_2 resistant bacterium is able to reduce TMAO to TMA and causes the spoilage of modified atmosphere packed marine fish from temperate waters (Dalgaard, 1995).

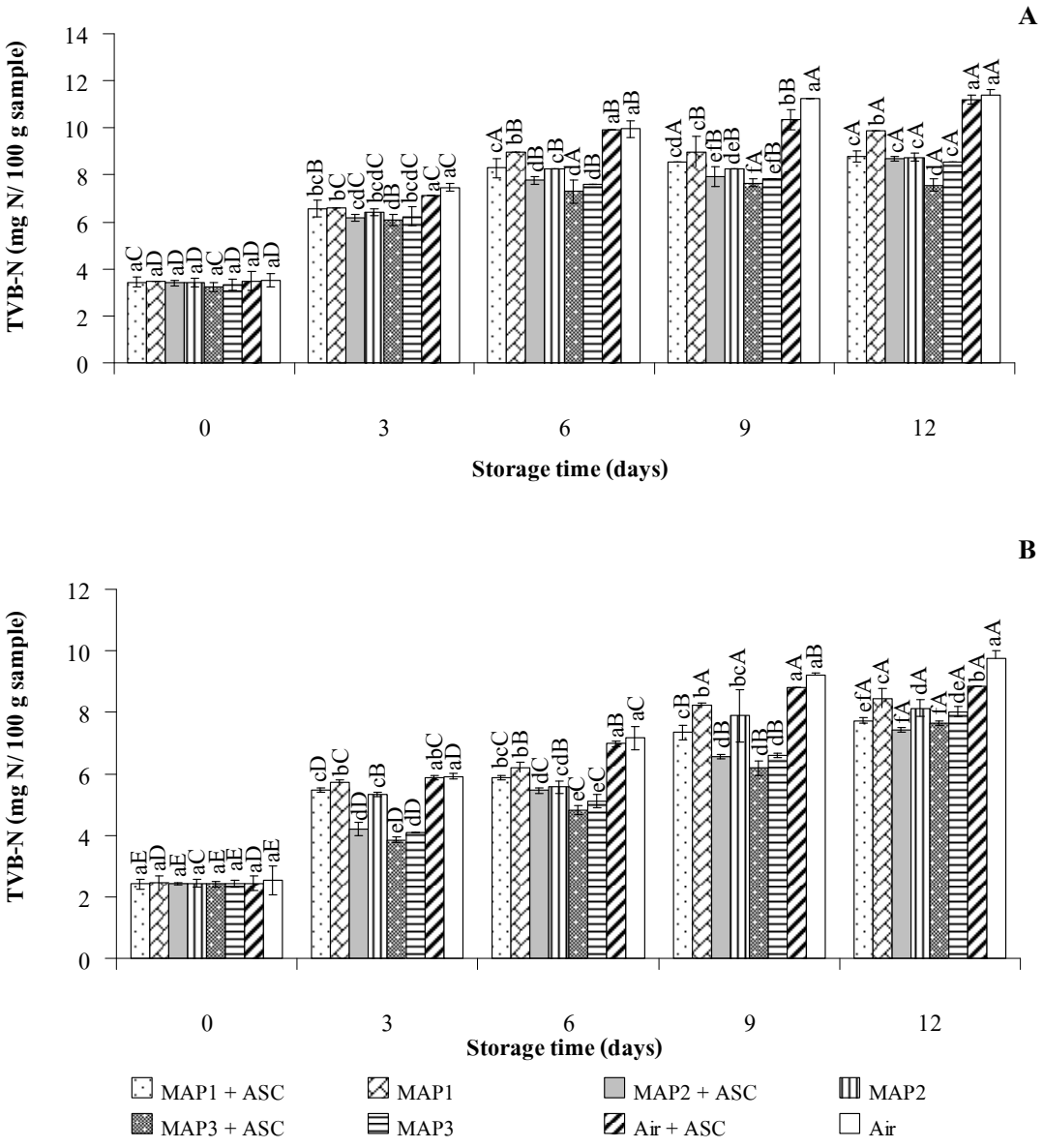

Figure 20. Changes in TVB contents of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

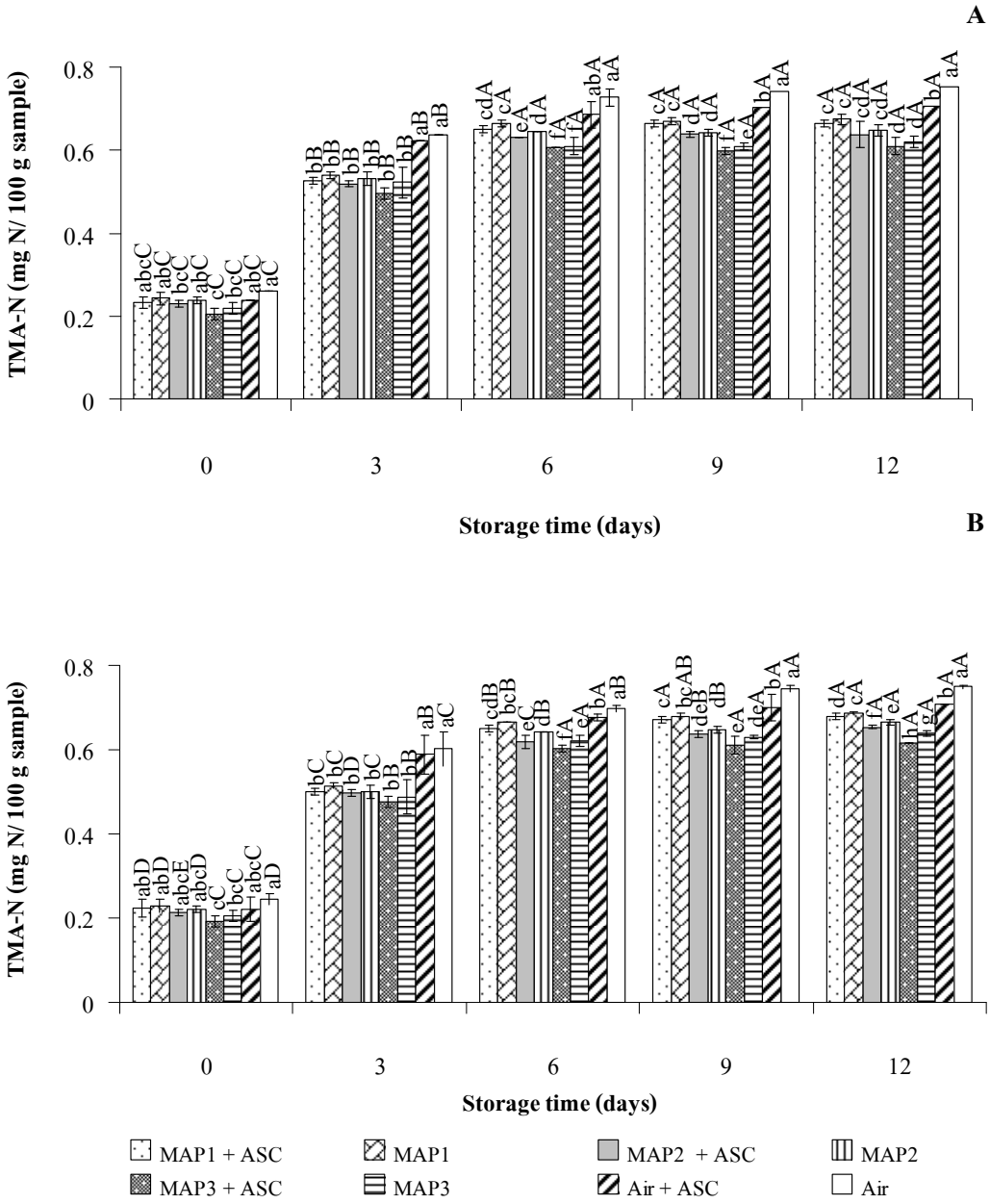

Figure 21. Changes in TMA contents of black tiger (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TBARS

Changes in TBARS values of black tiger shrimp and white shrimp stored in air or under MAP with and without ascorbic acid pretreatment are shown in figure 22 A and 22 B, respectively. The initial values of TBARS of fresh black tiger shrimp and white shrimp were 1.13 mg MDA/ kg sample and 1.02 mg MDA/ kg sample, respectively. The increase in TBARS was observed in all samples when the storage time increased ($P<0.05$) (Figure 22 A and 22 B). Among all samples, those stored under MAP with higher CO_2 (MAP3) showed the higher TBARS than those with MAP having lower CO_2 after 3 days of storage. Moreover, the sample stored in air had the lowest TBARS than others throughout the storage of 12 days ($P<0.05$). From the results, the higher of TBARS values of both shrimps stored under MAP might be associated with the low pH values of the samples, which accelerated the rate of lipid oxidation. Lowering the pH values of the samples accelerated the increase in TBARS values (Chen and Waimaleongora-Ek, 1981). Under acidic condition caused by dissolved CO_2, the pro-oxidant might be more released. This might expedite lipid oxidation of shrimps. The result was in accordance with Masniyom *et al*. (2006) who found that seabass slices stored under MAP had the higher TBARS values than those kept in air. Thus, some antioxidants should be applied to retard the oxidation of sample kept under MAP. At high content of CO_2 (80%), carbonic acid formed in muscle probably caused the inactivation of antioxidative enzymes, e.g. gluthathione peroxidase, resulting in the higher oxidation in the muscle (Renerre *et al*., 1996). Therefore, CO_2-enriched packaging effectively inhibited the spoilage caused by microorganisms, but it somehow accelerated lipid oxidation. Furthermore, the lower TBARS values of samples kept in air might result from the direct microbial utilization of malonaldehyde and other TBARS (Wang and Brown, 1983). Treatment of shrimps with ascorbic acid tended to yield the samples with the lower TBARS, regardless of MAP condition and storage time. Ascorbic acid has been known to act as antioxidant (Majchrzak *et al*., 2004). Ascorbic acid could scavenger O_2 remaining in the package. This might result in the lowered oxidation. Oxidative rancidity has been recognized as a major cause of seafood and food spoilage. The process involves oxidation of unsaturated fatty acids or triglycerides in seafood (Hamre *et al*., 2003). Factors that may influence lipid oxidation, and thus seafood spoilage, include free radical mechanism, various biochemical substances, temperature, water activity, pH, and chemical environment (Chytiri *et al*., 2004). TBARS consists of

secondary lipid oxidation products, mainly malondialdehyde as representative of aldehydes (Hamre *et al*., 2003). Gokodlu *et al*. (1998) correlated sensory evaluation with oxidation in muscle of sardine and reported values of TBARS > 5 mgmalonaldehyde/ kg muscle for moderate oxidation. It was suggested that a maximum level of TBARS value indicating the good quality of the fish frozen, chilled or stored with ice is 5 mg Malonaldehyde/kg (Nunes *et al*., 1992). Sinnhuber and Yu (1958) reported that TBARS value of 4-7 mg malondialdehyde/ kg indicated the poor quality fish. However, the both shrimps stored under MAP with and without ascorbic acid pretreatment had TBARS below 4 mg malondialdehyde/ kg. From the results, white shrimp had the higher TBARS values than black tiger shrimp, compared with same treatments, particularly after 6 days of storage. The differences in TBARS between both species might be associated with the differences in lipid compositions, especially unsaturated fatty acids (Sriket *et al*., 2007).

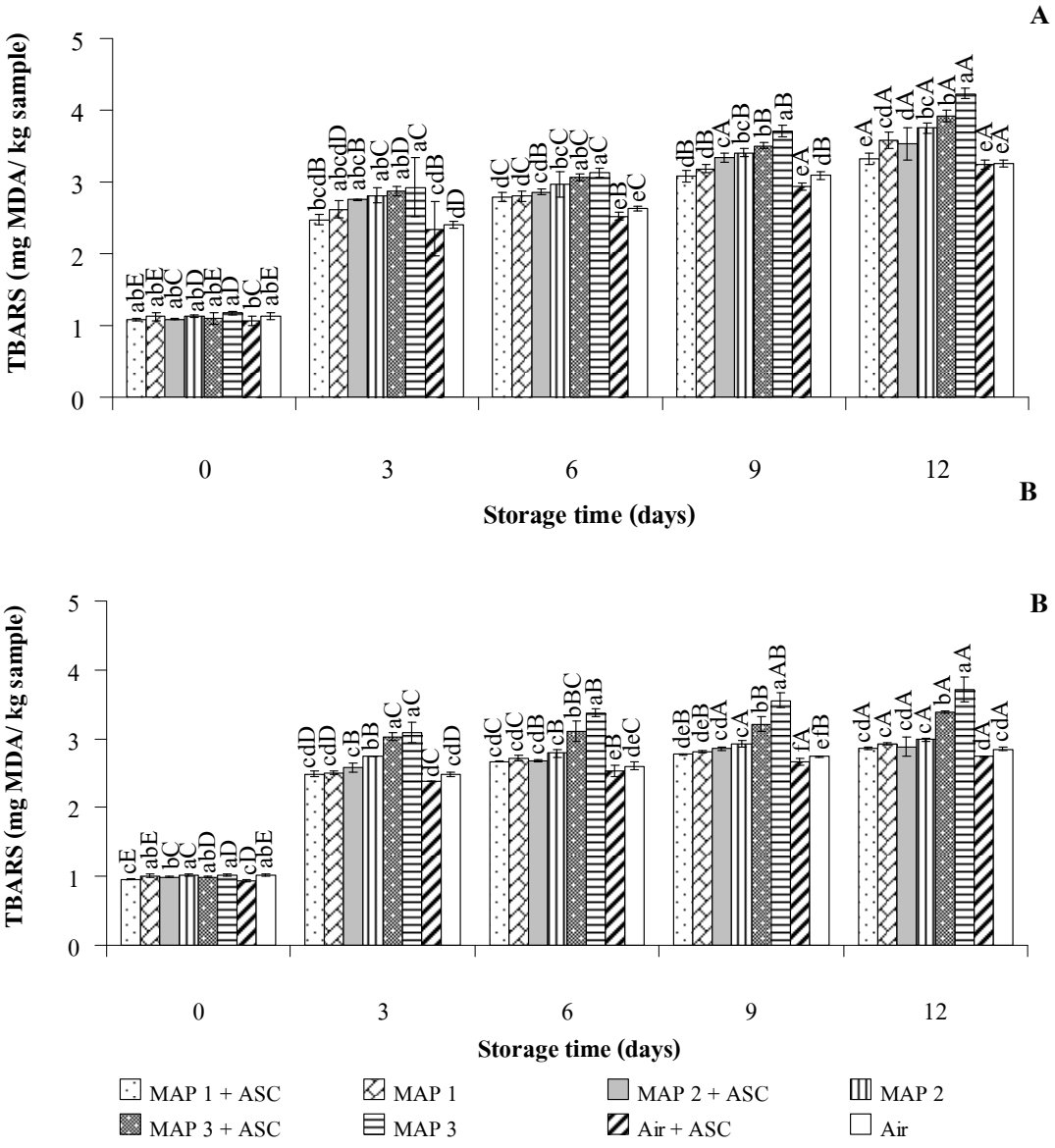

Figure 22. Changes in TBARS of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TCA-soluble peptide content

The continuous increases in TCA-soluble peptide content were observed for both shrimps throughout the storage of 12 days, especially during the first 3 days of storage (Figure 23 A and 23 B). TCA-soluble peptide has been used as the index for the protein degradation of fish muscle (Benjakul *et al*., 1997). At 0 day, TCA-soluble peptide contents in black tiger shrimp and white shrimp were 153.33 and 113.43 μmol tyrosine/ g sample, respectively. For both shrimps, higher TCA-soluble peptides were obtained in sample kept in air, compared with those stored under MAP throughout the storage. In general, TCA-soluble peptide content under CO_2-enriched atmosphere was lower than those found in sample kept under MAP with lower CO_2. In the presence of high content of CO_2, the growth of spoilage microorganism could be inhibited as shown by lowered TVC (Figure 17), resulting in the lower hydrolysis of muscle proteins. Inhibition of microbial growth is attributed to CO_2 because this gas extends the lag phase and reduces the growth rate during the logarithmic phase (Church, 1994; Genigeorgis, 1985). Therefore, MAP was shown to be a promising means to prevent the degradation of muscle proteins during prolonged storage. From the result, samples pretreated with ascorbic acids tended to have slightly lower TCA-soluble peptides, compared with those without ascorbic acid pretreatment throughout the storage. Ascorbic acid might lower the pH, in which the proteinase produced by microorganisms could be lowered. The lower TCA-soluble peptides found in samples stored in CO_2 enriched atmosphere were coincidental with the lower TVB and TMA formation (Figure 20 and 21). From the results, white shrimp generally showed similar increase in TCA-soluble peptides to black tiger shrimp. Therefore, MAP could be used to retard the protein degradation associated with the microbial growth.

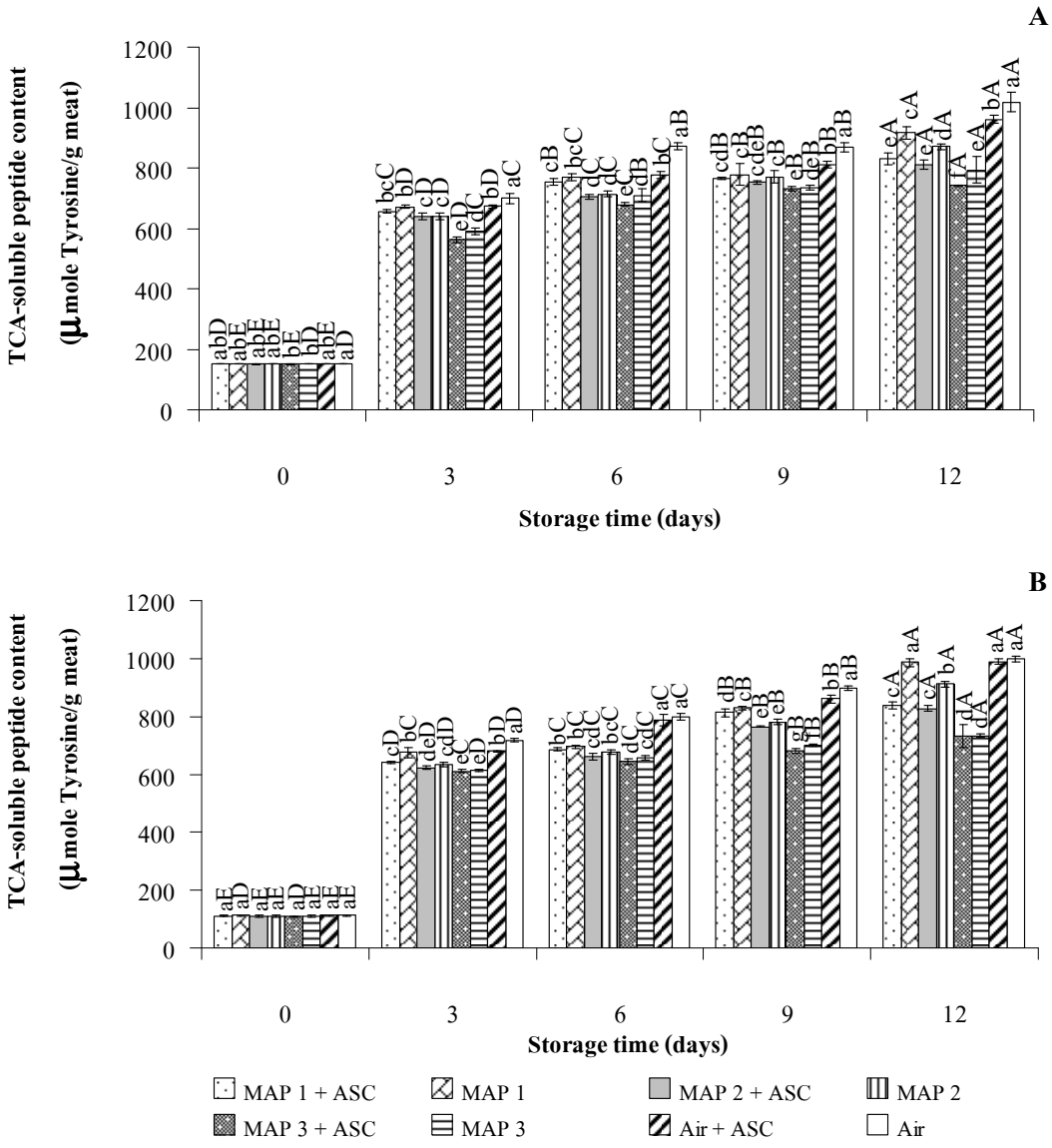

Figure 23. Changes in TCA-soluble peptides of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in formaldehyde

Changes in formaldehyde content of black tiger shrimp and white shrimp kept in air or under MAP with and without ascorbic acid pretreatment are depicted in Figure 24 A and 24 B, respectively. Samples stored in CO_2-enriched atmosphere showed a lower formaldehyde content than the sample kept in air throughout the storage, particularly at day 12 of storage. It was found that the sharp increases in formaldehyde were noticeable in black tiger shrimp and white shrimp after day 0 and 6, respectively. However, the marked increase was found in white shrimp kept in air after day 0. The formaldehyde content of samples kept under MAP with 80% CO_2 was lower than that of samples kept under atmosphere with lower CO_2, indicating that a high CO_2 concentration potentially inhibited the formation of formaldehyde. The inhibitory effect of CO_2 on formaldehyde formation was more pronounced in black tiger shrimps. Formaldehyde is an enzymatic product from trimethylamine oxide, a compound present in most marine species caused by TMAO demethylase. This phenomenon is common for gadoid species (Hebard *et al*., 1982) and causes an alteration in the characteristics of fish muscle protein, particularly changes in functional properties (Careche *et al*., 1998). Formaldehyde of lizard fish stored in ice increased continuously up to 15 days (Benjakul *et al*., 2003b). From the result, the lower pH caused by dissolved CO_2 might result in lowering the activity of TMAO demethylase found in shrimp. From the result, pretreatment with ascorbic acid generally showed no marked effect on formaldehyde formation. The formation of formaldehyde in both shrimps of black tiger shrimp and white shrimp, particularly in the sample kept in air may have led to the changes in texture, especially the toughening of muscle.

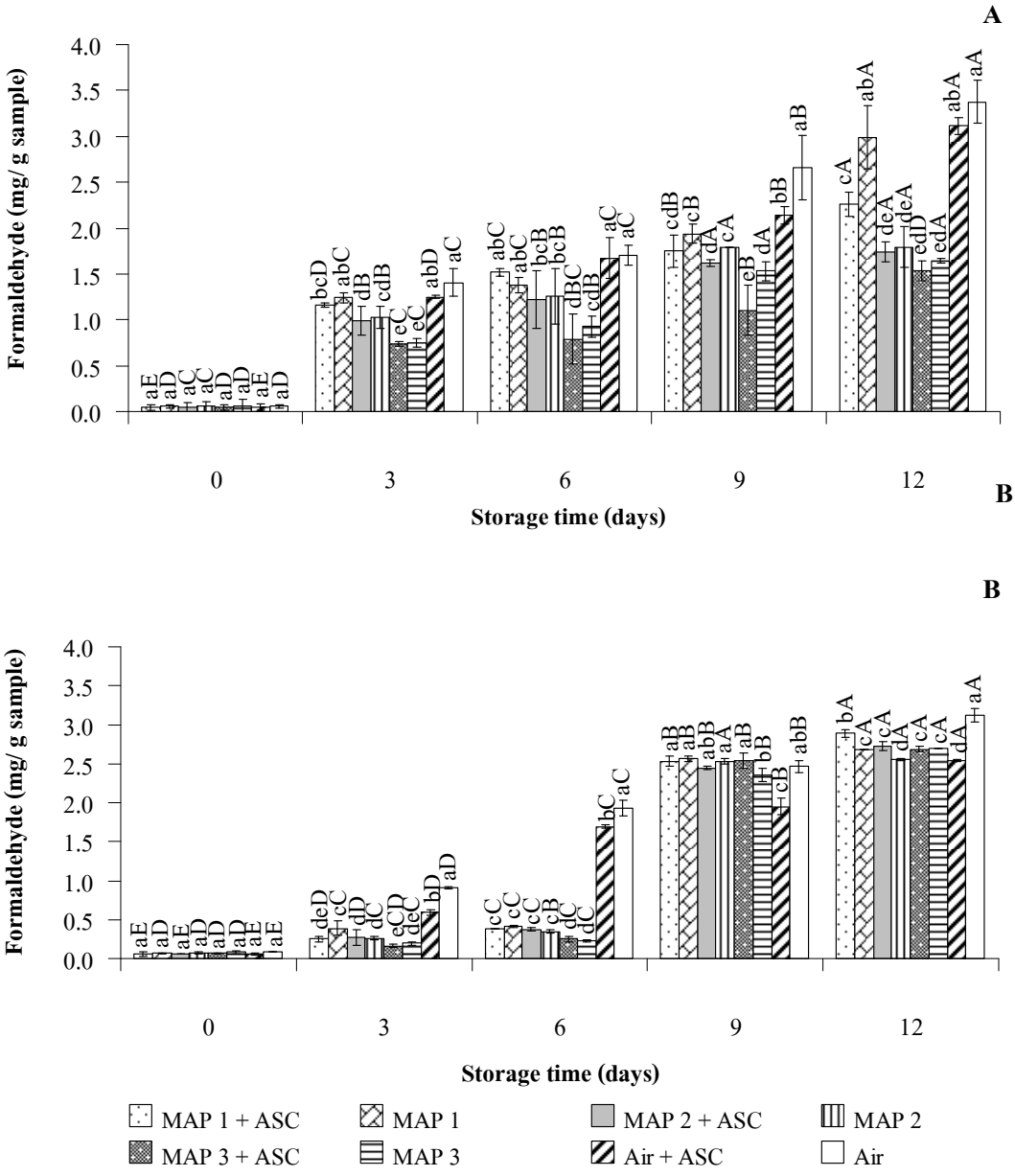

Figure 24. Changes in formaldehyde of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in melanosis

Melanosis score of black tiger shrimp and white shrimp kept in air or under MAP with and without ascorbic acid pretreatment is depicted in Figure 25 A and 25 B, respectively. Melanosis score of the sample kept in air increased to a greater rate, compared with those kept under MAP during storage ($P<0.05$). The scores of 8 and 7 were obtained in the sample kept in air at day 6 days of storage in black tiger shrimp and white shrimp, respectively. At the end of storage (12^{th} day), melanosis developed intensively in all black tiger shrimp samples, regardless of atmosphere used. Black spots were fully developed in both the cephalothorax and tail. However, the samples kept under MAP showed the lower score in comparison with those kept in air during the first 9 days. For white shrimp, similar results were observed, but melanosis occurred to a lower extent at day 12 of storage. The result suggested that melanosis phenomenon was different between both species. From the result, MAP with greater CO_2 content could retard the melanosis more effectively than MAP having lower CO_2. Acid formed possibly lowered PPO activity, leading to the decreased melanosis. Matches and Layrisse (1985) reported the delay of melanosis in whole spotted shrimp stored 7 day under 100% CO_2. From the result, most samples with ascorbic acid pretreatment had the lower melanosis score, compared with those without pretreatment, regardless of packaging atmosphere. Ascorbic acid exhibited the inhibitory activity toward melanosis of black tiger shrimp under MAP storage, possibly owing to its oxygen scavenging property, in which of molecular oxygen in polyphenoloxidase reactions was removed (Pizzocaro *et al*., 1993). This can be attributed to the fact that the melanin-forming reaction requires sufficient amounts of oxygen. The inhibition of PPO was studied by Montero *et al*. (2001b) who found that the melanosis score of shrimp without additive reached 4 within 4 days. Ascorbic, citric, benzoic, sorbic, kojic and phytic acid, protease inhibitors, and 4-hexylresorcinol were effective for the inhibition of shrimp melanosis, which are considered to be safe compounds (Taoukis, 1990; McEvily *et al*., 1991; Chen *et al*., 1991a; Chen *et al*., 1991b; Otwell *et al*., 1992; Slattery *et al*., 1995; Yu *et al*., 1996; Kubo and Kinst-Hori, 1998). However, the samples pretreated with ascorbic acid gradually turned to red color, mainly on the shell. The color changes were most likely caused by the denaturation of astaxanthin associated proteins. As a result, astaxanthin became dominant. From the result, packaging

atmosphere having 80% CO_2 effectively extended the shelf-life of black tiger shrimp and showed inhibitory activity toward melanosis. Nevertheless, ascorbic acid was not appropriate as melanosis inhibitor due to its negative effect on color, in which red/orange colors were apparently formed. This phenomenon results in the unacceptability of resulting shrimp.

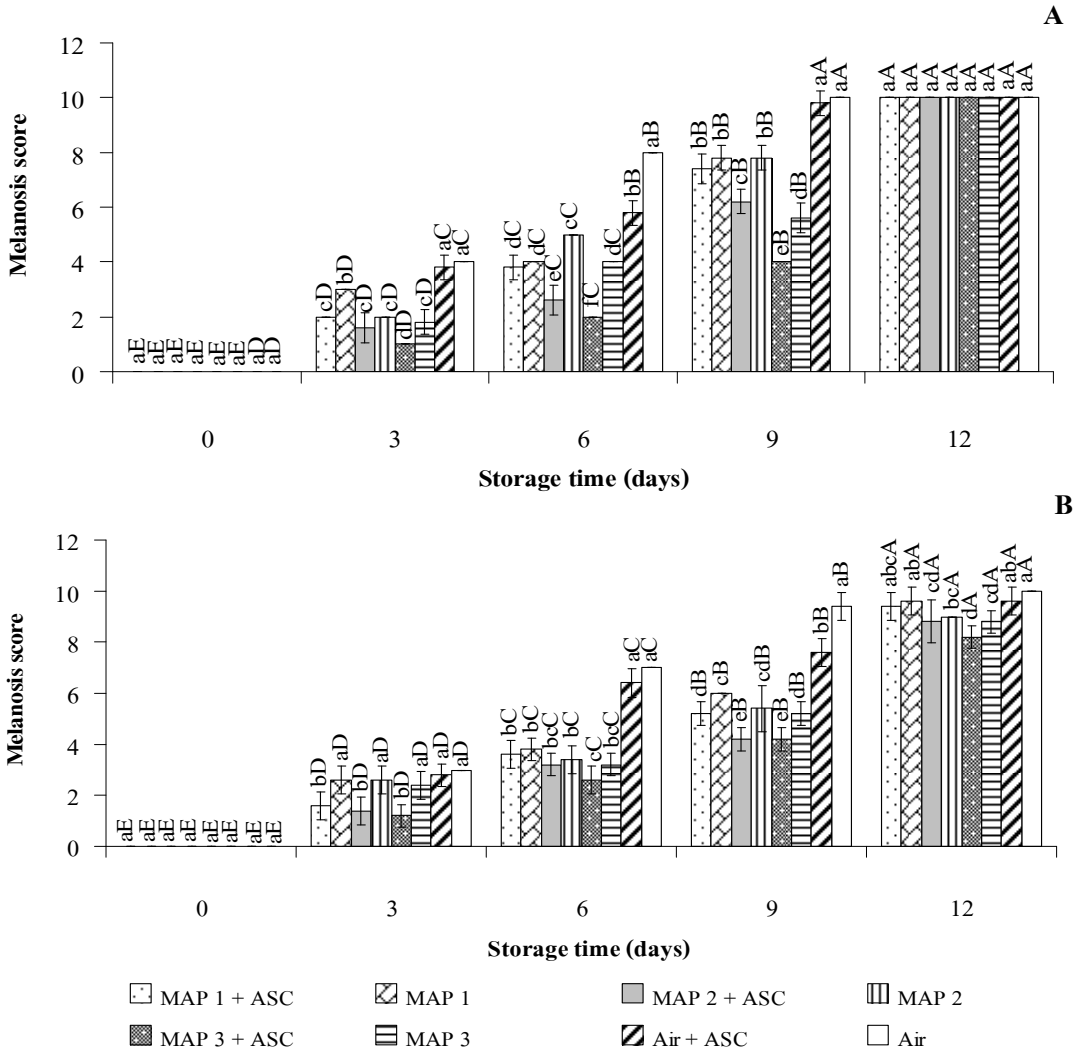

Figure 25. Changes in melanosis score of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within

the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in water holding capacity

Water holding capacity (WHC) of black tiger shrimp and white shrimp during storage in air or under MAP with and without ascorbic acid pretreatment is shown in Figure 26 A and 26 B, respectively. The continuous decreases in WHC of both species were observed throughout the storage ($P<0.05$). Both shrimps had similar WHC irrespective of atmospheric condition. Black tiger shrimp and white shrimp had WHC with a range of 81-99% during storage. Shrimps stored under MAP with a higher content of CO_2 had a slightly lower WHC than those stored in air or under MAP containing lower CO_2 ($P<0.05$). WHC from both shrimps stored under MAP were directly related to the CO_2 level. The increased CO_2 level had an adverse effect on the WHC of the shrimp tissue (Layrisse and Matches, 1984). Parkin *et al*. (1981) reported that there was little difference in amount of drip loss between rockfish samples stored in modified atmosphere (80% CO_2/ 20% air) and air. However, Boone (1982) observed greater drip loss for both Pacific Ocean perch and cod fillets stored under modified atmosphere (50% CO_2/ 50% air) than in air. A greater loss in water holding capacity of muscle protein can be found at lower pH values (Offer and Knight, 1988; Fennema, 1990; Foegeding *et al*., 1996). From the result, acid formed could result in protein denaturation, in which water could not be bound to those proteins effectively. WHC of meats is related to the amount of free water released. The main factors that affect WHC are protein content and pH (Olsson *et al*., 1994). The mean isoelectric point of the major myofibrillar proteins is about pH 5 (Foegeding *et al*., 1996). Minimum water holding capacity and swelling of meat are observed around the isoelectric point, but it increases again with either decreasing or increasing pH, which results in stronger electrostatic repulsion forces and increased space for water to be held in the muscle (Offer and Knight, 1988). Ascorbic acid pretreatment caused the slight decrease in WHC of most samples during the storage of 12 days ($P<0.05$). WHC variations are related to muscle pH and muscle protein modifications (Olssona *et al*., 2007). The results revealed that MAP could lower WHC, possibly caused by the acid formed, which was more pronounced in atmosphere possessing the higher CO_2 content.

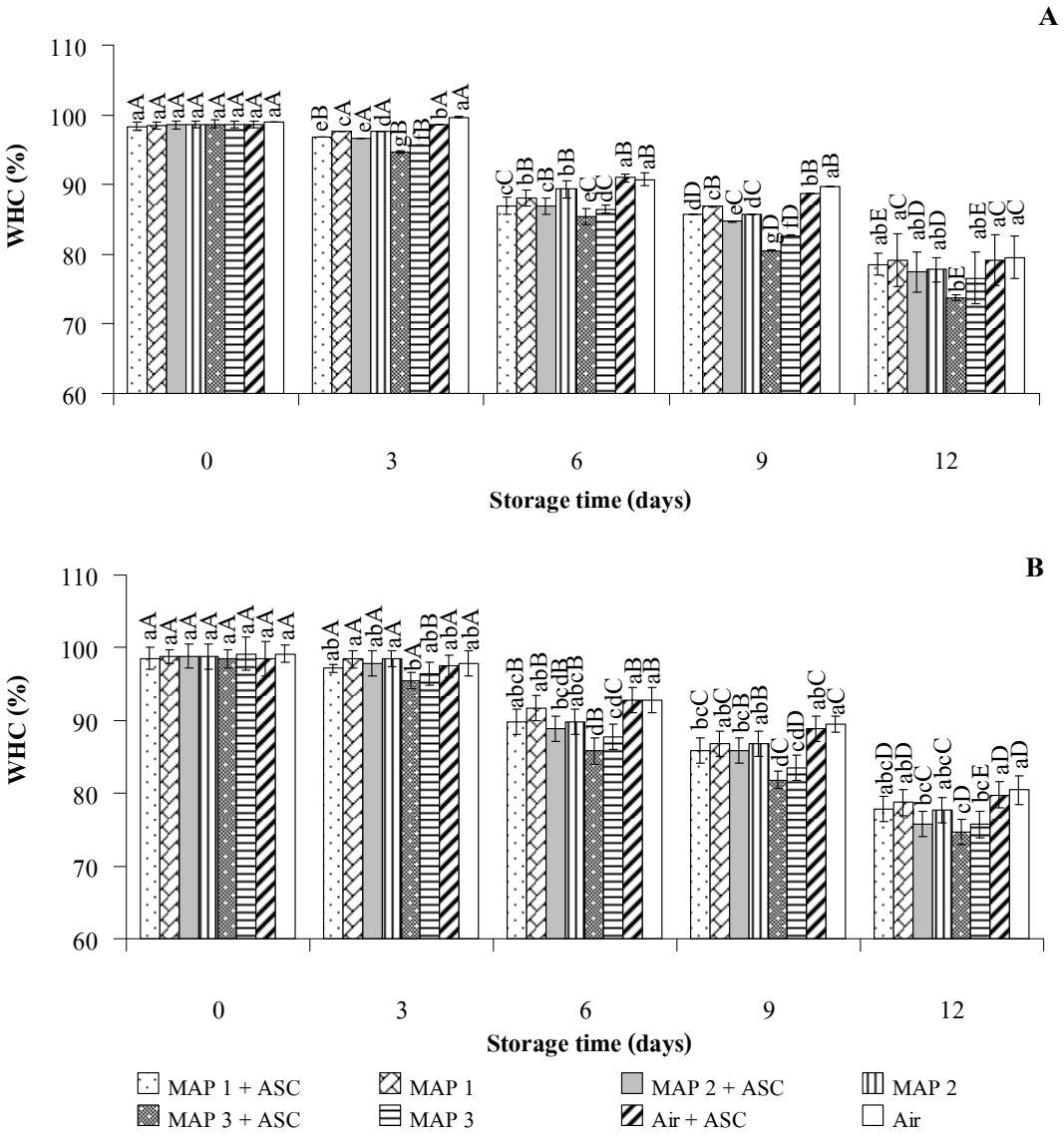

Figure 26. Changes in WHC of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in shear force

Shear force of black tiger shrimp and white shrimp kept under MAP with and without ascorbic acid pretreatment in comparison with the sample kept in air during the storage of 12 days at 4°C is shown in Figure 27 A and 27 B, respectively. Generally, shear force of black tiger shrimp kept in air decreased continuously during the storage of 12 days ($P<0.05$). However, white shrimp had the sharp decrease in shear force at day 3 of storage, followed by the gradual decreases up to 12 days. MAP with higher CO_2 contents yielded the shrimp with higher shear force than MAP having lower CO_2 and air atmosphere for black tiger shrimp. Conversely, no differences in MAP conditions on shear force were found in white shrimps, except at day 3 of storage. Protein hydrolysis of shrimp meat might occur as storage time increased. The degradation of shrimp tissue caused by hepatopancreatic enzymes started from the perimysium, endomysium, the Z line and the H zones with concurrent degradation of the connective fibers and the sarcoplasm (Nip *et al.,* 1985). Raw fish meat from most fish species softens after few days of chilled storage (Sato *et al.,* 1991). From the results, shear force of white shrimp decreased to a greater extent, compared with black tiger shrimp during the storage time of 12 days ($P<0.05$). It was most likely that the destruction of muscle fibers of white shrimp was more pronounced, compared with black tiger shrimp. At the end of the storage (12 days), shear force of both shrimps stored in air was lowest, when compared with other samples. Apart from proteinase, collagenase localized in cephalothorax might involve in hydrolysis of collagenous constituents in whole black tiger shrimp and white shrimp. This led to the softened texture. The lower microbial load might be associated with lower proteolysis caused by proteases from those microorganisms. Ascorbic acid treatment generally had no marked impact on shear force but showed some effect on some samples.

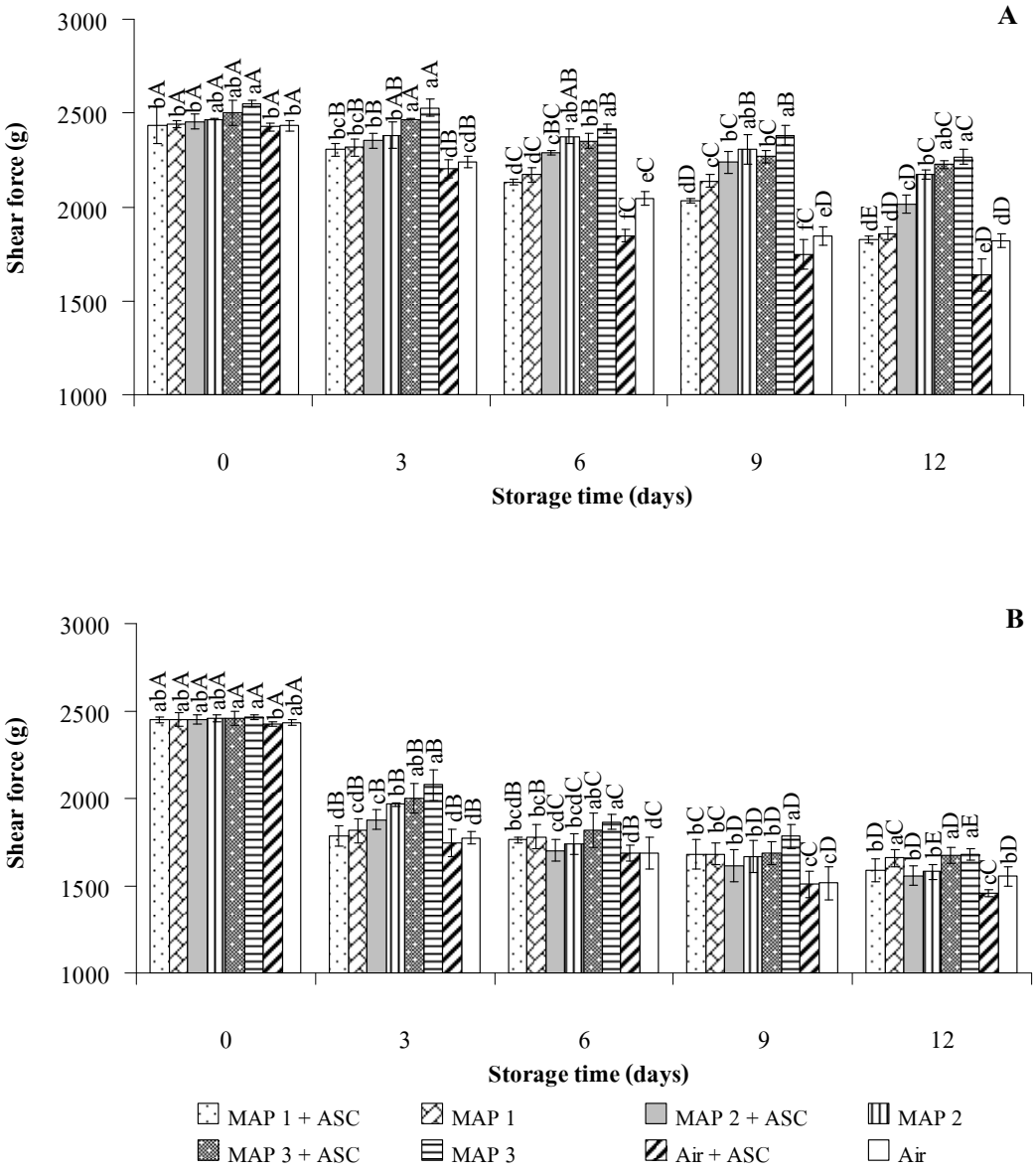

Figure 27. Changes in shear force of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within

the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in proteases, collagenase and PPO activity

Changes in proteases, collagenase and PPO activities of black tiger shrimp and white shrimp kept in air or under MAP with and without ascorbic acid pretreatment during the storage at 4°C are shown in Figure 28 A and 28 B, respectively. For proteases activities of both shrimps, activities decreased as the storage time increased ($P<0.05$). The greater decrease in protease activity was found in sample stored under MAP with higher CO_2 content and the highest activity was found in the sample stored in air ($P<0.05$). Similar results were noticeable with collagenase activity, but the decreasing rate was lower for collagenase activity. High CO_2 content (MAP3) was very effective in inhibiting both proteases and PPO. For PPO activity, CO_2 enriched atmosphere caused the sharp decrease in activity at day 0. The remaining activity also gradually decreased with increasing storage time. Activity of PPO in sample kept in air also decreased continuously during the storage of 12 days. These results were reported for shrimp by Bullard and Collins (1978) and Barnett *et al*. (1978) using CO_2-treated brine or refrigerated sea water. Thus, acidic condition of sample kept under MAP might inactivate endogenous enzymes as well as those from microorganisms. Masniyom *et al*. (2005) reported that a much lower decrease in ISC (insoluble collagen) of sea bass muscle stored under MAP was found, when compare with those kept in air. These results confirmed that CO_2 effectively inhibited the degradation of fish proteins including collagen caused by endogenous enzymes as well as those from microorganisms. Ascorbic acid pretreatment exhibited the synergistic effect on the inactivation of enzymes from both shrimps and microorganisms. Acidic pHs also contributed to lower activity of those enzymes tested. From the result, all enzymes could be affected by MAP. This effect might contribute to the shelf-life of shrimp.

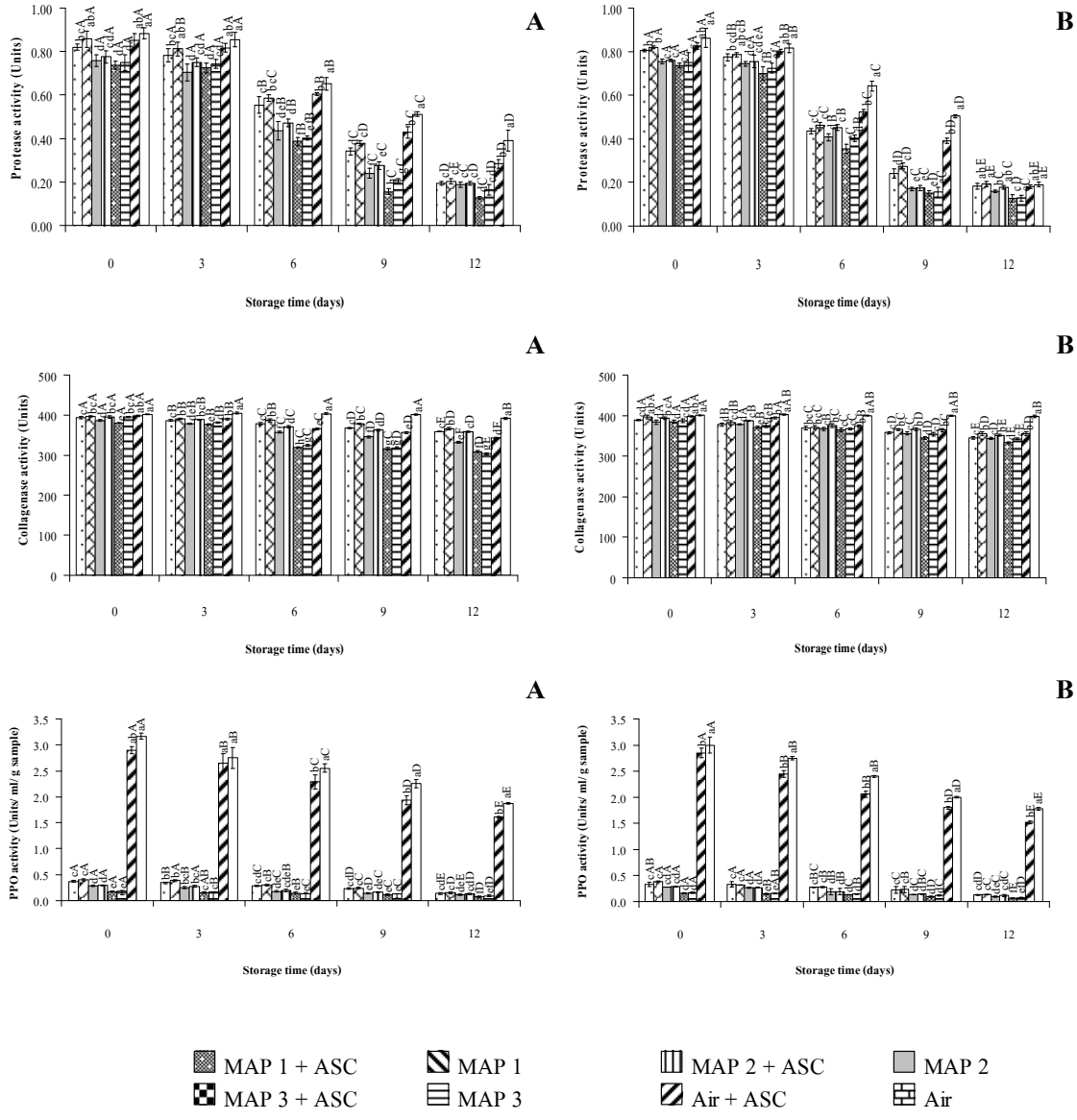

Figure 28. Changes in proteases, collagenase and PPO activity of black tiger shrimp (A) and white shrimp (B) with and without 0.5 g/l ascorbic acid pretreatment stored under different conditions at 4°C. MAP1 = 45% CO_2, 10% O_2, 45% N_2; MAP2 = 60% CO_2, 10% O_2, 30% N_2; MAP3 = 80% CO_2, 10% O_2, 10% N_2; ASC = Ascorbic acid (5.0 g/l). The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

3. Effect of phosphate and ascorbic acid pretreatment on the quality of white shrimp kept under MAP.

From previous study, MAP with 80% CO_2 showed the higher efficacy in extending the shelf-life of both black tiger shrimp and white shrimp. However, lowered water holding capacity of shrimp was a drawback of shrimps kept under MAP. Therefore, pyrophosphate at a level of 2% (w/v) was used to tackle this problem in combination with ascorbic acid, which most likely functioned as PPO inhibitor. The study was conducted only with white shrimp since black tiger shrimps have not been cultured widely due to the diseases.

Changes in total viable count

Changes in TVC of white shrimp kept in air or under MAP at 4°C with and without phosphate and/or ascorbic acid pretreatment are depicted in Figure 29. Higher TVC was found in the sample kept in air at all storage times, compared with other samples ($P<0.05$). Among all treatments, sample pretreated with PP and ascorbic acid and kept under MAP had the lowest TVC throughout the storage of 12 days ($P<0.05$). Wager and Busta (1985) reported that pyrophosphate was inhibitory toward microorganisms in a sausage. Scullen and Zaika (1994) reported growth inhibition of *Listeria monocytogenes* by sodium pyrophosphate. The effectiveness of phosphates as antimicrobial agents in meat products depends on the type of phosphate, the amount used, specific food product and conditions under which they are used (Sofos, 1986). However, the TVC of all samples were below 6 log CFU/g sample, an acceptability limit reported by Balamatsia *et al*. (2006). From the result, PP and ascorbic acid pretreatment might show the synergistic effect on the retardation of bacterial growth. The results indicated that pretreatment by phosphate solution and ascorbic acid prior to storage under MAP was more effective in reducing microbial numbers on the white shrimp, compared with the use of only MAP. Additionally, uses of both PP and ascorbic acid also showed the inhibition of microbial growth of sample stored in air. Use of ascorbic acid or PP alone was found to lower TVC, but at the lower degree than the uses of combined compounds.

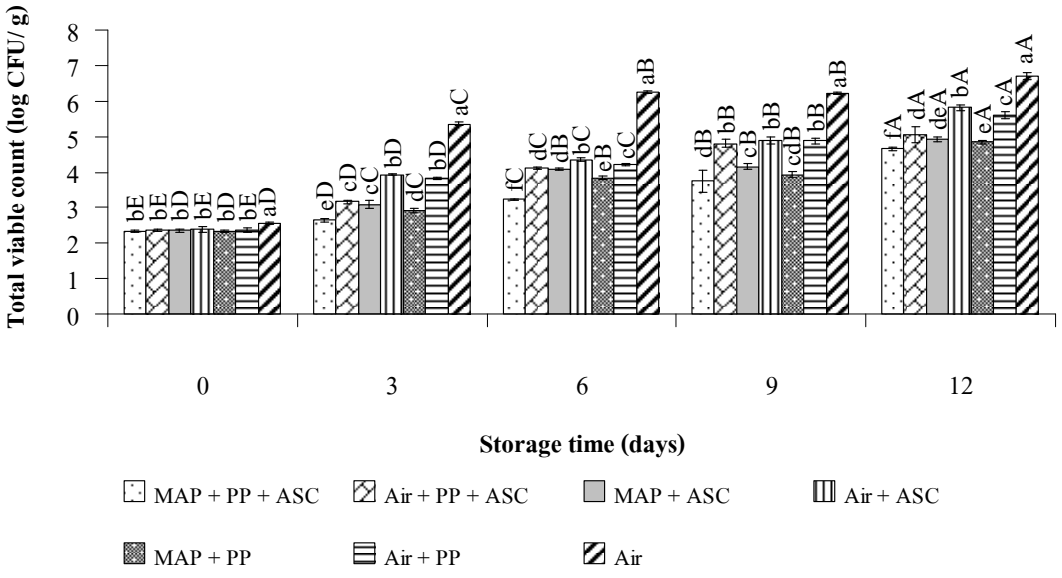

Figure 29. Changes in TVC (CFU/g) of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in lactic acid bacteria count

LAB counts in shrimps stored in air were lower than those of shrimps kept under MAP, regardless of pretreatment ($P<0.05$) (Figure 30). LAB counts of shrimp stored under MAP increased markedly within the first 6 days, followed by the gradual increase up to 12 days. Only slight increase in LAB counts was observed in sample kept in air. The microbiological characteristics of the packaged shrimp were significantly affected by storage time (Sivertsvik and Birkeland, 2006). LAB are able to compete in the packaged and refrigerated meats because of their ability to grow at refrigerated temperatures and their resistance to CO_2 (Brashears and Durre, 1999). Some bacterial groups of *Bacillus*, *Pseudomonas*, *Clostridium*, *Photobacterium,* members of *Enterobacteriaceae*, and lactic acid bacteria are capable of decarboxylating amino acids (Silla-

Santos *et al*., 1996). From the result, pretreatment with both PP and ascorbic acid could retard the growth of LAB in samples kept under MAP, while promoted LAB growth in those kept in air. Therefore, the pretreatment played a role in determining LAB growth in shrimps to some extent.

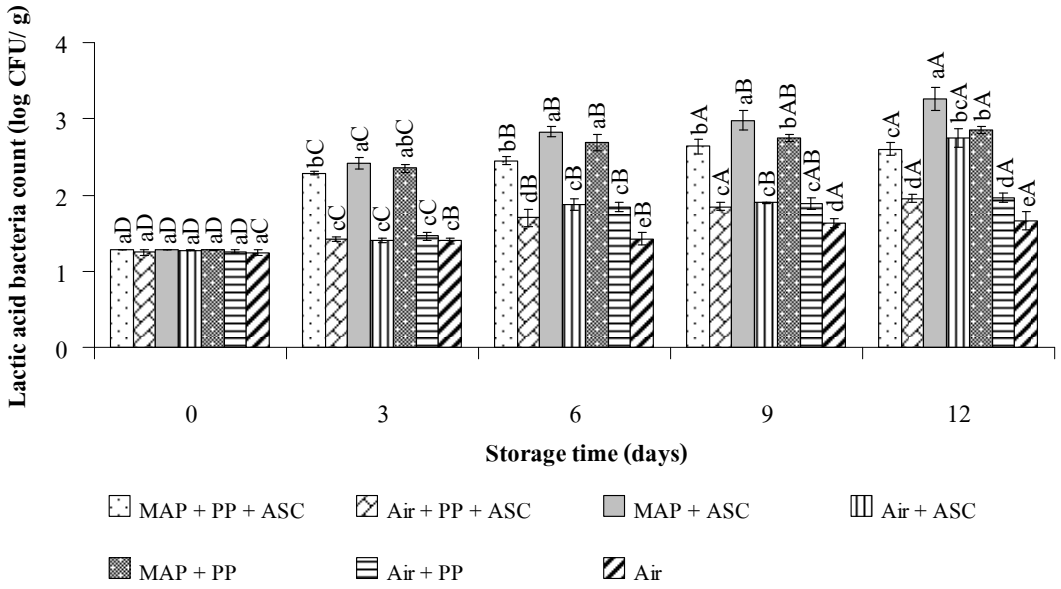

Figure 30. Changes in lactic acid bacteria (CFU/g) of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in pH

Changes in pH of white shrimp as affected by phosphate and/or ascorbic acid pretreatment during storage in air or under MAP are presented in Figure 31. The initial pH of white shrimp was 6.78 (Figure 31). During storage, pH of all white shrimp samples increased with increasing storage time ($P<0.05$). Among all samples, that stored in air had the higher increase in pH throughout storage, presumably due to the production of basic amines (Pastoriza *et*

al., 1996a; Debever and Boskou, 1996). The pH of the sample kept in air remained constant during 6-9 days (P>0.05). Unchanged pHs mostly observed in the shrimp might be due to the buffering capacity of muscle. The pH increases are related to the formation of volatile amines from microbial activity (Huss, 1995). Growth of bacteria and the generation of trimethyamine and volatile bases were inhibited when samples were stored in carbon dioxide enriched atmospheres (Ordonez *et al*., 2000). Increase in pH was lowered in sample with pretreatment using ascorbic acid and PP. Foods with a low buffering capacity have the change in pH quickly in response to acidic or alkaline compounds produced by microorganisms as they grow. Meats, in general, are more buffered than vegetables by virtue of their various proteins (Doyle *et al*., 2001).

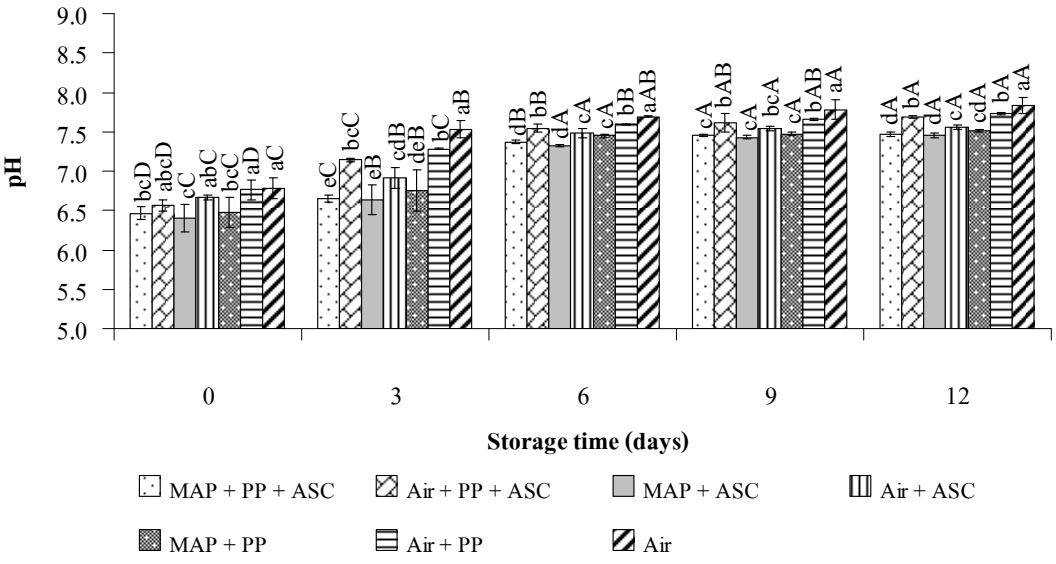

Figure 31. Changes in pH in white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively (P<0.05). Bars represent the standard deviation from triplicate determinations.

Changes in total volatile bases and trimethylamine contents

TVB and TMA contents of all samples during storage at 4°C are depicted in Figure 32 A and 32 B, respectively. The samples stored in air had the higher TVB content, compared with other samples ($P<0.05$). Regardless of packaging atmosphere, phosphate and ascorbic acid pretreatment resulted in the lower TVB and TMA contents ($P<0.05$). TVB content in the sample kept in air increased rapidly and reached 8.67 mg TVB/ 100 g sample at the end of storage. For the samples stored in air, ascorbic acid pretreatment showed the higher efficacy in retardation of TVB formation than PP pretreatment. However, both PP and ascorbic acid showed the synergistic effect on prevention of TVB formation in sample stored in air. When MAP was applied, PP pretreatment most likely showed the similar effect on preventing the TVB formation to ascorbic acid. From the result, pretreatment with both ascorbic acid and PP in combination with MAP resulted in the lowest TVB content during the extended storage.

For TMA, the pattern of changes was similar to that TVB. Samples stored in air had the highest increase in TMA, compared to other samples. For sample kept in air, pretreatment with ascorbic acid resulted in the lower TMA content than PP pretreatment. However, both ascorbic acid and PP showed the similar effect on TMA formation. TMA is produced by the reduction of trimethylamine oxide (TMAO) by TMAO reductase producing microorganisms. It is also used as a quality indicator for fish. TMA concentration is normally used to limit the acceptability of fish (Adams and Moss, 1995). Cobb *et al*. (1973) suggested that the seafood products were spoiled when the TMA concentration exceeded 5 mg TMA/ 100 g of tissue. However, even after 12 days of storage, all samples were still acceptable with TMA content lower than 5 mg TMA/ 100 g. This result was agreement with Ordonez *et al*. (2000) who reported that TMA formation was inhibited when hake steaks were stored in CO_2-enriched atmospheres. From the results, pretreatment of shrimp with both PP and ascorbic acid prior to MAP rendered the shrimp with the lowest TMA content.

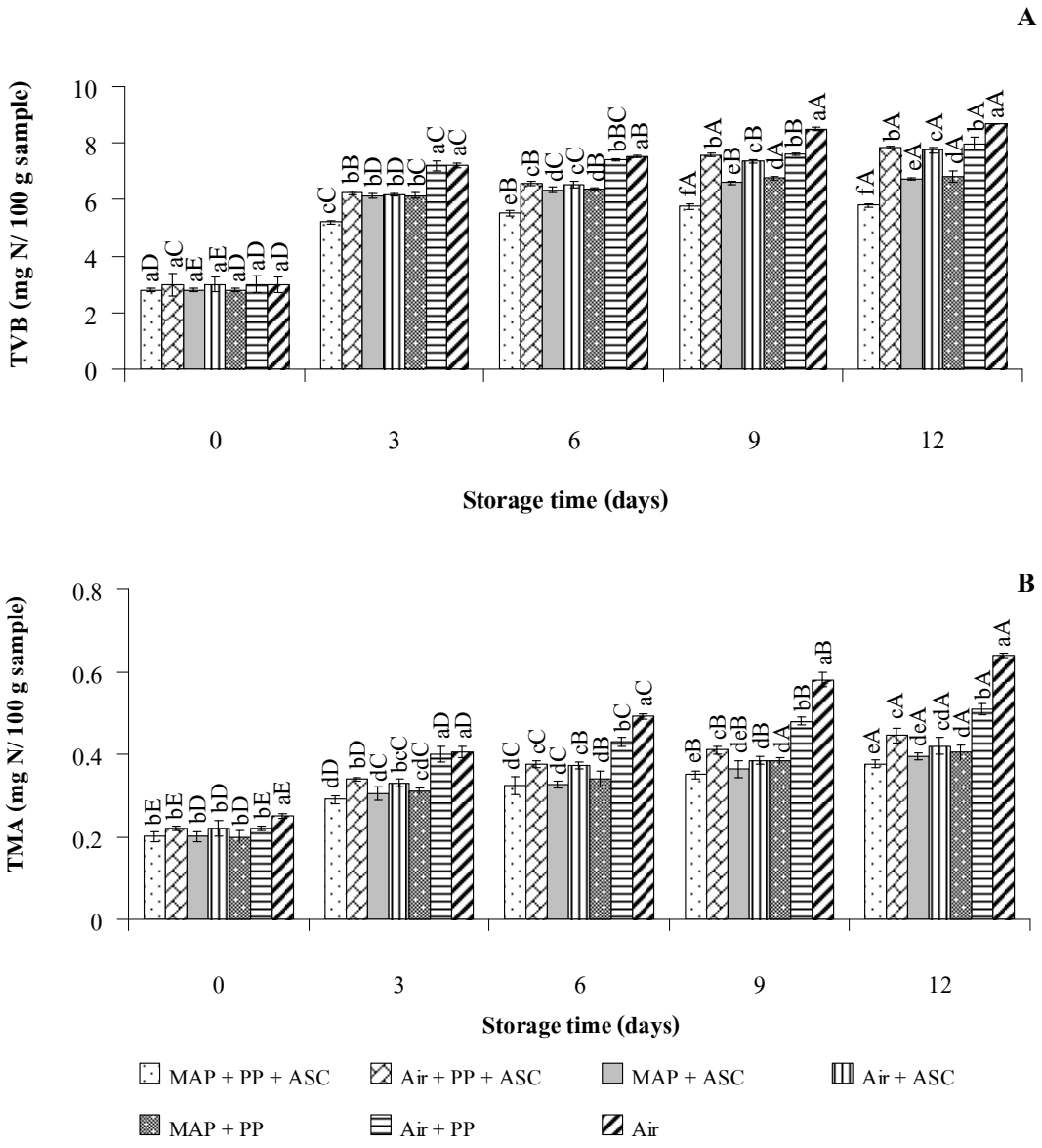

Figure 32. Changes in TVB (A) and TMA (B) contents of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TBARS

TBARS values in white shrimp stored in air or under MAP with and without phosphate and ascorbic acid pretreatment during storage at 4°C are shown in figure 33. The increase in TBARS was observed in all samples when the storage time increased ($P<0.05$). Higher increases in TBARS value were observed in the samples stored in air, compared with those stored under MAP together with PP and/ or ascorbic acid pretreatment. This was probably because the lower O_2 in the packaging and PP could function as metal chelator. As a consequence, pro-oxidants released might be sequestered. In general the samples pretreated with phosphate had lower TBARS value of the samples when the same packaging atmosphere was used. From the result, samples pretreated with PP in combination with ascorbic acid and kept under MAP had no differences in TBARS, compared with sample pretreated with PP and stored under MAP throughout the storage ($P>0.05$). Thus, phosphate retarded the oxidation more effectively than ascorbic acid in white shrimps particularly when stored under CO_2 enriched atmosphere. Additionally, sample kept under MAP with PP pretreatment had the slightly lower TBARS than sample stored under MAP together with both ascorbic acid and PP pretreatment. Ascorbic acid might induce the release of non-heme iron, which was associated with the denaturation of heme proteins caused by acids. TBARS values of samples kept in air might result from malonaldehyde and other TBARS (Branen, 1978; Rhee *et al*., 1997). From the result, all samples with different treatments and/or packaging atmosphere had TBARS below 4 mg malondialdehyde/ kg. However, some antioxidants should be applied to retard the oxidation of sample kept under MAP together with PP, which most likely acted as secondary antioxidant. TBARS value in white shrimp pretreated with ascorbic acid stored under MAP (80% CO_2, 10% O_2, 10% N_2) observed in this study was different from that found in the previous study. The lower TBARS values of samples in this study might result from direct microbial utilization of malonaldehyde and other TBARS or were caused by the reactions between these TBARS and the amine compounds produced by bacterial metabolism (Rhee *et al*., 1997).

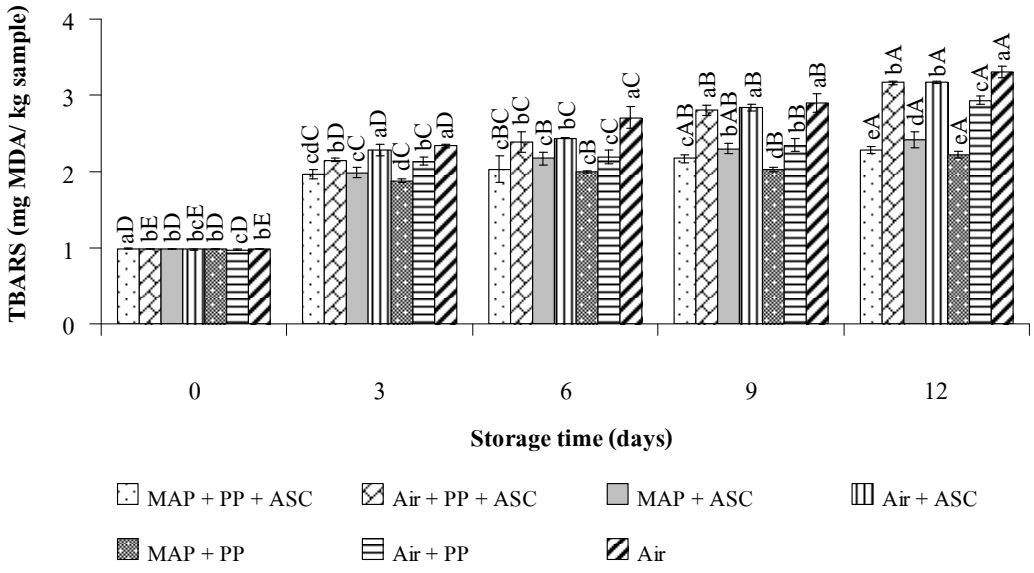

Figure 33. Changes in TBARS of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TCA-soluble peptide content

A continuous increase in TCA-soluble peptide content in all samples was observed during storage of 12 days (Figure 34). However, TCA-soluble peptides value was lower in samples stored under MAP, compared with those kept in air, irrespective of pretreatment with phosphate and ascorbic acid. From the result, the combined use of phosphate and ascorbic acid together with MAP showed the lower efficiency in lowering the degradation of muscle proteins when compared with MAP in combination with PP pretreatment. Thus, ascorbic acid used might reduce the effectiveness of PP in retardation of proteinases. PP might chelate metal ions required for proteinases or some microorganism producing proteinases. Protein degradation was observed in seabass slices caused by either endogenous or microbial proteinases during refrigerated storage

(Masniyom *et al*., 2005). Venugopal *et al*. (1983) reported that protease from *Pseudomonas marinoglutinosa* hydrolyzed actomyosin at 0-2°C. Therefore, MAP or MAP in combination phosphate and ascorbic acid pretreatment were shown to be the promising means to prevent the degradation of muscle proteins during prolonged storage. This might be associated with the inhibitory effect toward spoilage bacteria as evidenced by lowered TVC.

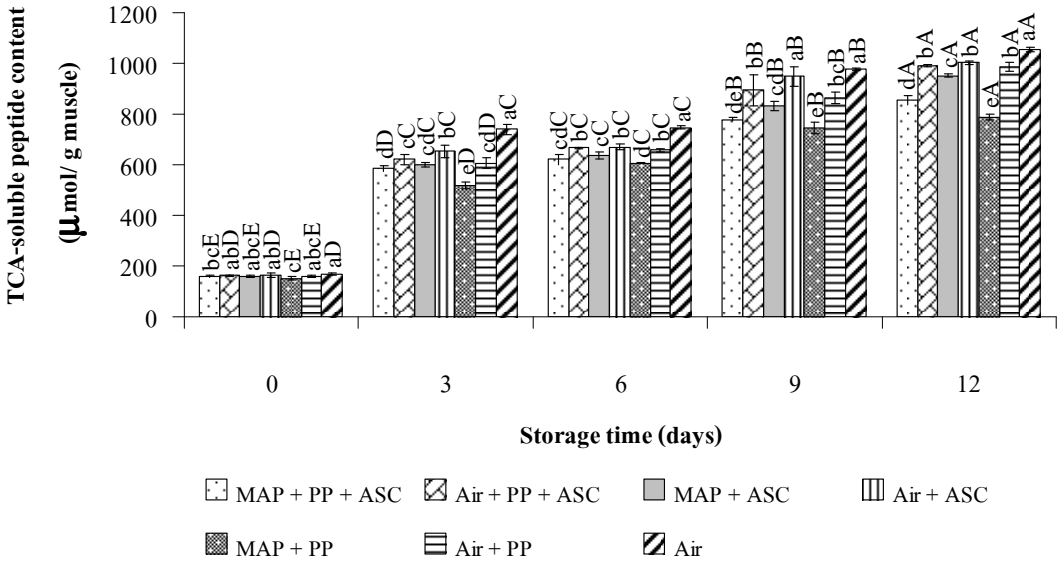

Figure 34. Changes in TCA-soluble peptide contents of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in formaldehyde content

The shrimp stored in air had the highest increase in formaldehyde content followed by sample kept in MAP together with PP pretreatment throughout the storage at 4°C (Figure 35). Conversely, the lowest formaldehyde content was observed in the samples stored in

CO_2-enriched atmosphere (80% CO_2) regardless of the pretreatment using both PP and ascorbic acid. Generally, samples stored under MAP contained lower formaldehyde content, compared to those kept in air. The result indicated that a high CO_2 concentration potentially inhibited the formation of formaldehyde. When comparing with samples pretreated with ascorbic acid and PP prior to keeping in air, the formaldehyde content was lowered in those pretreated with ascorbic acid. Ascorbic acid might affect TMAO demethylase by lowering the pH, in which the activity was decreased. Formaldehyde in marine fish and crustacean (Flores and Crawford, 1973) is caused by the demethylation of trimethylamine oxide (Amano and Yamada, 1964; Benjakul *et al*., 2003b).

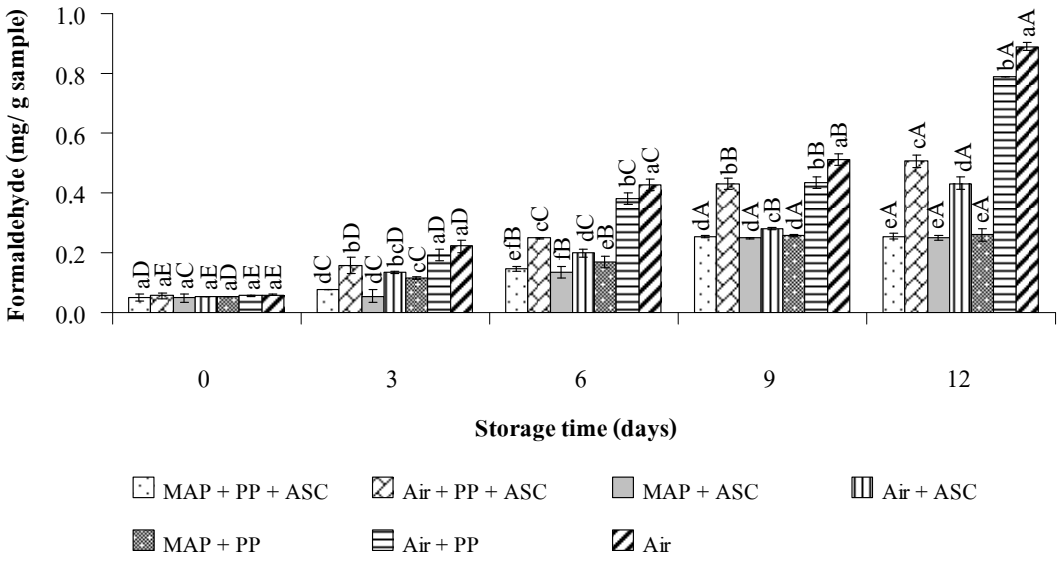

Figure 35. Changes in formaldehyde of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in melanosis

Melanosis score of white shrimp kept in air or under MAP with and without phosphate and/or ascorbic acid pretreatment is shown in Figure 36. The onset of melanosis was observed after 3 days of storage for those kept in air regardless of pretreatment. Samples pretreated with phosphate and ascorbic acid and kept under MAP exhibited the lowest melanosis score, compare to other samples, at day 12 of storage ($P<0.05$). However, sample pretreated with PP and stored under MAP showed the lowest melanosis score at day 9 of storage ($P<0.05$). Though, ascorbic acid pretreatment delayed the onset melanosis, it caused the change in color of sample. This was probably due to the denaturation of proteins associated with astaxanthin caused by an acidic pH. From the result, obvious changes in color caused by MAP and ascorbic acid were obtained. The complexes of carotenoids and proteins called carotenoproteins and carotenolipoproteins dominate in the exoskeleton. Astaxanthin appears as a red pigment, but when complexed with various proteins, the light absorbance shifts and cause crustaceans to range in color from green, yellow, blue to brown (Muriana *et al*., 1993 and Nur-E-Bordan *et al*., 1995, Britton *et al*., 1981). The red color of crustaceans is produced by the release of the individual carotenoid prosthetic group (astaxanthin) from the carotenoproteins when denatured by an acidic pH. From the result, pretreatment of shrimp with both PP and ascorbic acid prior to keeping under MAP was an effective means in lowering the melanosis of shrimp up to 12 days.

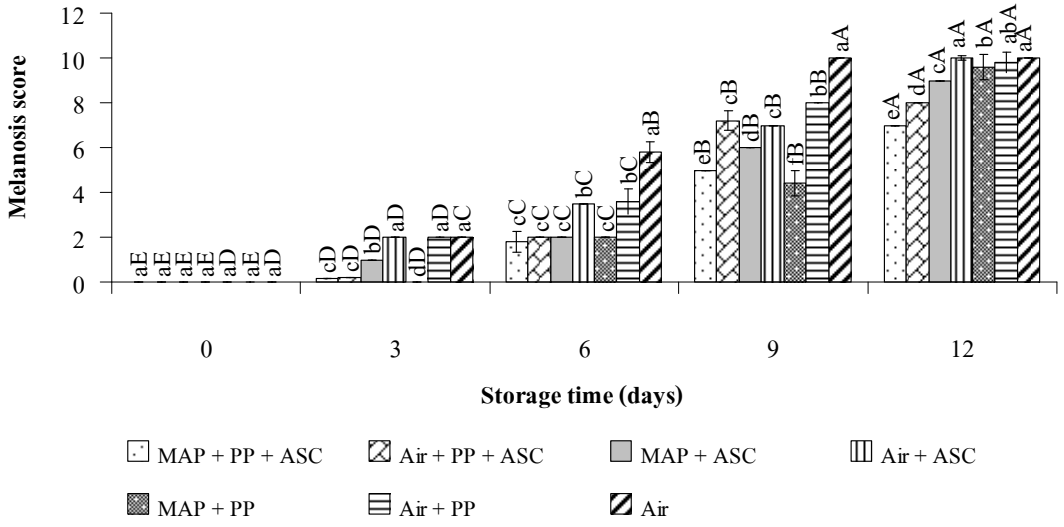

Figure 36. Changes in melanosis score of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in water holding capacity (WHC)

Water holding capacity (WHC) of white shrimp kept in air or under MAP with and without phosphate and/or ascorbic acid pretreatment for up to 12 days is shown in Figure 37. In general, water holding capacity decreased throughout the storage. Lower water holding capacity of all samples kept under MAP was observed in comparison with sample stored in air. This might be due to a greater loss of the water holding capacity of the muscle protein at lower pH values (Stammen *et al*., 1990). At low pH, proteins in muscle underwent denaturation, leading to the loss in holding the water. However, with phosphate pretreatment, water holding capacity of all samples was improved. From the result, even with PP treatment, sample stored under MAP showed the lower water holding capacity than others at day 12 of storage. Trout and Schmidt (1984) reported that pyrophosphate and tripolyphosphate were the most effective in increasing

water binding capacity in beef rolls. Thus, the combination of PP appeared to be the most effective in reducing exudate losses during storage. This result was in agreement with Alvarez *et al*. (1996) who reported that hake slices stored under MAP had the reduced drip loss, when polyphosphate pretreatment was used. The efficacy of phosphates in increasing the water-binding capacity varied with the type and concentration used (Trout and Schmidt, 1986). In general, pyrophosphate was the most effective phosphate for increasing water-binding capacity, as well as binding in restructured meat (Trout and Schmidt, 1984). Phosphates used in injected meats are typically PP having a cyclic structure. The presence of these highly charged individual or mixed compounds enables the injected meat to effectively retain water (Bendall, 1954; Hamm, 1986), thereby improving the cooking yield and textural palatability. Pyrophosphate was found to promote myofibril swelling (Parsons and Knight, 1990). The effects of PP on the myofibril morphology and myosin extraction can be explained by their interaction with the actomyosin complex (Xiong, 2005). At the ends of the A-band, myosin and actin filaments are crosslinked via the actomyosin bridges. PP has been shown to dissociate the actomyosin complex (Granicher and Portzehl, 1964). Dissociation of the actomyosin complex would also enable the myofibril lattices to expand laterally, thereby permitting further water uptake in brine-treated meat (Xiong, 2005). From the result, the enhancement in juiciness of meats proper water-binding is made possible through the use of PP. By binding to myofibrillar proteins, PP can effectively increase charge repulsions between myofilaments and facilitate removal of transverse myofibrillar proteins which may act as structural constraints to myosin extraction (Xiong, 2005). The ensuing expansion of myofilamental lattices allowing water-binding and physical entrapment was responsible for the water-holding capacity of shrimp.

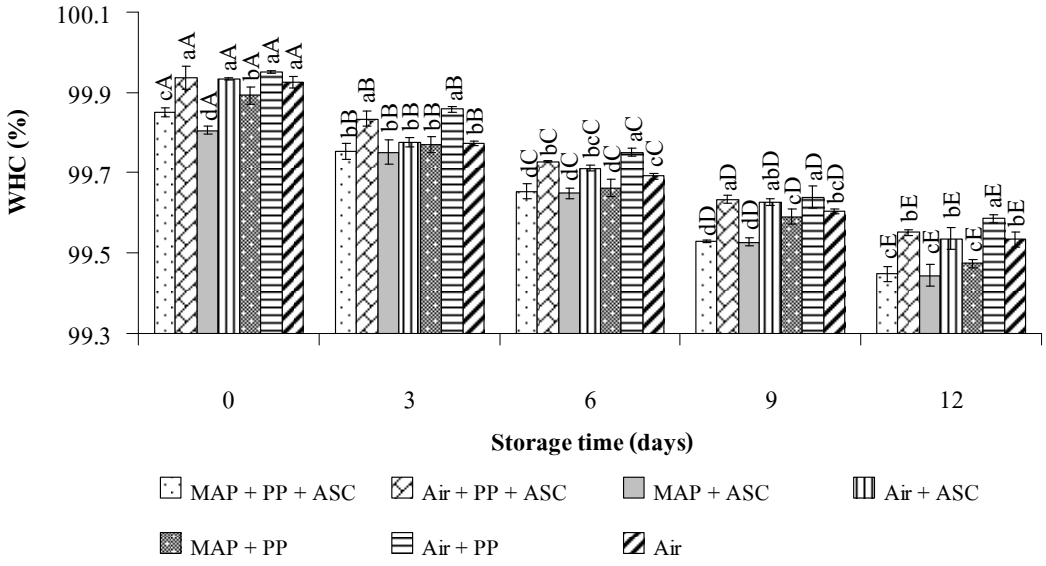

Figure 37. Changes in WHC of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in shear force

Shear force of white shrimp kept in air or under MAP either with or without PP and/or ascorbic acid pretreatment during storage at 4°C is shown in Figure 38. Shear force of all samples gradually decreased as the storage time increased ($P<0.05$). During 9-12 days of storage, samples pretreated with ascorbic acid, both stored in air and under MAP, showed the lowest shear force ($P<0.05$). Ascorbic acid might enhance the solubilization or swelling of collagen in shrimp muscle, in which collagenases from both shrimp and microorganism could hydrolyze collagen effectively. With PP pretreatment, pH of shrimp could be controlled, caused by its buffering capacity. As a result, collagen was present in the nature complex form. Furthermore, PP might inhibit growth of some spoilage microorganism. Molins (1991) reported that chelating property of

phosphate might have an important effect on metal ions essential in bacterial metabolism and growth. Additionally, PP might suppress the activity of metallocollagenase or protease by complexing the metal ion needed for full activity. Konno and Fukazawa (1993) reported that autolysis of squid mantle muscle was inhibited by sodium PP, which is a metal chelator. Ando *et al*. (1992) reported that the postmortem tenderization of rainbow trout muscle was mainly due to the weakening of pericellular connective tissue caused by the disintergration of collagen fibrils. Cathepsin L and serine proteases are capable of hydrolyzing major muscle structure proteins including collagen (Sato *et al*., 1994 and Yamashita and Konakaya, 1991) established by Yamashita and Konagaya (1990a) reported that cathepsin L degrades the nonhelical region of collagen in salmon muscle during spawning migration. The nonhelical region is more susceptible to protease action than the helical region (Etherington, 1984). After 6 days storage, no marked differences in shear force were found between the shrimp kept in air and other samples, except those pretreated with ascorbic acid. Thus, it was most likely that microbial growth did not have much impact on the softening of shrimp, but endogenases enzyme could play a major role in softening, particularly at acidic pH caused by pretreatment with ascorbic acid. Aktas and Kaya (2001) pointed out that collagen cross-links which are labile under acid condition may be broken down by the action of lactic acid. From the result it was suggested that the carbonic acid and lactic acid formed in the sample kept in MAP combined with ascorbic acid treatment induced the weakening of collagen.

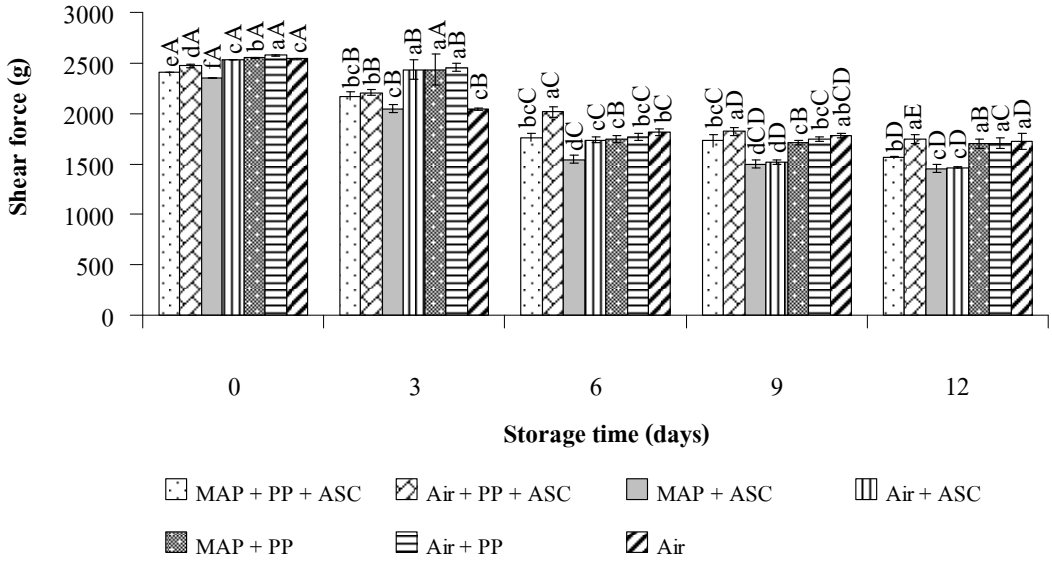

Figure 38. Changes in shear force of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in proteases, collagenase and PPO activities

Changes in proteases, collagenase and PPO activities of white shrimp kept in air or under MAP with and without phosphate and/or ascorbic acid pretreatment at 4°C are depicted in Figure 39. At day 0, the samples kept in air had the highest activities of proteases and PPO. Samples subjected to pretreatment showed the lowered activities, especially for proteases and PPO, regardless of packaging atmosphere. Therefore, both PP and ascorbic acid could inhibit the enzyme activity to some extent. Among all samples, that stored under MAP with the pretreatment of both PP and ascorbic acid had the lowest activity of proteases. For PPO, marked decrease in activity was observed with the samples stored under MAP. Slight decrease in PPO activity was also found when samples were pretreated with both PP and ascorbic acid and kept under MAP.

The higher proteases and collagenase activities were found in sample pretreated with ascorbic acid and stored in air. This was coincidental with the decrease in shear force of sample subjected to this treatment (Figure 41). The lower activity of both proteases and collagenase were in accordance with the lower TVC, especially the sample pretreated with both ascorbic acid and PP prior to keeping under MAP. This might be because the lower microorganisms would release less proteolytic enzymes. With increasing storage time, the gradual decreases in activities of all enzymes were observed. Enzyme might undergo denatuation and inactivation during the prolonged storage. Decreased PPO activity during the storage period could be related to PPO degradation by proteases, especially thiol proteases, as proposed by Wang *et al*. (1994) for Norway lobster. Besides proteases action, the final decrease in PPO activity is associated with an irreversible inactivation of the enzyme (Ramírez *et al*., 2003). The samples treated with PP and ascorbic acid showed a remarkable inhibitory effect on all enzymes tested throughout the storage of 12 days at 4°C. Ascorbic acid might act as PPO inhibition (Montero *et al*., 2001b) and PP could act as copper chelator (McEvily *et al*., 1991). Even though ascorbic acid could lower the melanosis and retard the microbial growth, it caused the orange/red discoloration. This led to unacceptability of the resulting shrimps. Therefore, other effective and promising inhibition should be used instead. Additionally, decapitation of cephalothorax contain high amount of PP could be preformed.

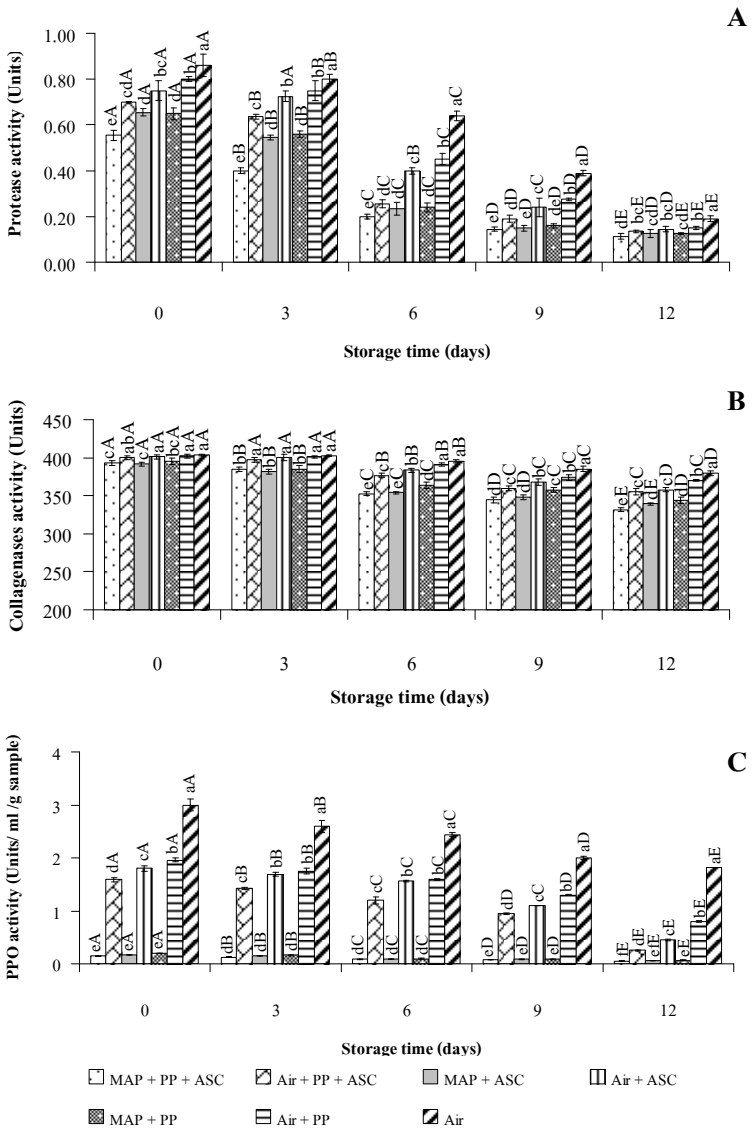

Figure 39. Changes in proteases, collagenase and PPO activity of white shrimp with and without pretreatment using 2% (w/v) pyrophosphate pretreatment and/ or 5.0 g/l ascorbic acid and stored under different packaging atmospheres at 4°C. MAP = 80% CO_2, 10% O_2, 10% N_2; PP = Pyrophosphate; and ASC = Ascorbic acid. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

4. Effect of phosphate and 4-hexylresorsinol pretreatment on quality of white shrimp kept under MAP

Changes in microbiological

Mesophilic bacterial counts of white shrimp kept in air or under MAP at 4°C with and without phosphate and/or 4-hexylresorcinol pretreatment are depicted in Figure 40 (A). All samples had the increased mesophilic bacterial counts with increasing storage time at 4°C ($P<0.05$). Mesophilic bacterial counts of white shrimp stored in air increased rapidly within 12 days and were generally higher than other treatments ($P<0.05$). Under air atmosphere, whole sample contained higher count than decapitated counterpart. The result indicated that MAP was effective in retarding the growth of bacteria. CO_2 used for MAP played a role in inhibiting the growth of microorganisms (Sivertsvik *et al*., 2002). Mesophilic bacterial counts of samples pretreated with pyrophosphate and kept under MAP increased more slowly than those of samples stored under MAP without pyrophosphate and 4-hexylresorcinol pretreatment. The results indicated that pyrophosphate might show synergistic effect with MAP on the retardation of bacteria growth. Growth inhibition induced by pyrophosphate was accompanied by changes in cellular morphology (Zaika *et al*., 1991). López-caballero *et al*. (2000) reported that 4-hexylresorcinol had no antimicrobial effect on prawns (*Penaues japonicus*) during storage. According to the European Commission (2003), a solution of 0.05% 4-hexylresocinol is not effective to prevent microbial growth.

Higher counts of psychrophilic bacteria were also observed in samples kept in air, compared with those stored under MAP, regardless of pretreatments (Figure 40 (B)). For the samples kept in air, decapitation resulted in the lower psychrophilic bacteria count. It was suggested that the removal of cephalothorax could reduce microbial load to some extent. Among all samples, psychrophilic bacteria counts in samples pretreated with pyrophosphate and 4-hexylresorcinol and kept under MAP 1 (80% CO_2, 10% O_2, 10% N_2) were lower than those of other samples at day 12 of storage. Thus, it was most likely that pyrophosphate might show the synergistic effect with MAP on retarding the microbial growth. Marshall and Jindal (1997) reported that trisodium phosphate reduced aerobic plate count and total coliform count in the meat from catfish frames. Moreover, Skandamis *et al*. (2002) reported that storage of fresh meat at increasing CO_2 concentrations caused the higher inhibition of psychrotrophic aerobes and an

extended shelf-life. It was noted that psychrophilic count of sample pretreated with pyrophosphate and 4-hexylresorcinol and stored under MAP 2 drastically increased at day 12 ($P<0.05$).

Changes in LAB of white shrimp kept in air or under MAP at 4°C with and without phosphate and/or 4-hexylresorcinol pretreatment during storage are depicted in Figure 40 (C). From the result, LAB counts in samples with pyrophosphate and 4-hexylresorcinol pretreatment in combination with MAP 2 (80% CO_2, 20% N_2) were lower than other samples throughout the storage ($P<0.05$). The lower LAB count of this sample was coincidental with the marked increase in psychrophilic count at day 12. LAB has been known to produce bacteriocin, which showed inhibitory activity towards some microorganisms (Adams and Marteau, 1995). However, bacteriocins not only inhibited the growth of spoilage bacteria, but also LAB (Gill and Penney, 1986).

Additionally, it was found that the pretreatment with pyrophosphate resulted in the lowered LAB count when the same MAP condition was used. Thus, pyrophosphate might affect the growth of LAB, possibly via its metal chelating property. As a consequence, essential metal ions required for LAB were chelated and not available for LAB. Gram-positive bacteria are more susceptible to inhibition by various pyro- and polyphosphates than Gram-negative bacteria. Polyphosphates may suppress the growth of bacteria by complexing metal ion essential for cell division (Davidson and Juneja, 1990). From the result, whole shrimp packaged under MAP 1 without pyrophosphate or 4-hexylresorcinol pretreatment had the highest LAB count throughout the storage ($P<0.05$).

From the result, whole shrimp had the higher mesophilic bacterial counts, psychrophilic bacteria and LAB than the decapitated counterpart. Cephalothorax might be the important source of microorganisms as well as various enzymes. Therefore, decapitation could be an important pretreatment to reduce the microbial load. The gradual increases in microorganisms, especially psychrophilic microorganisms, most likely contributed to the spoilage of those shrimps during refrigerated storage.

Changes in coliforms of white shrimp during storage are shown in Table 7. The gradual increases in coliforms during ice storage of shrimps kept in air were observed after 3 days of storage, irrespective of decapitation ($P<0.05$). However, the higher coliform was found in

whole sample, compared with decapitated counterpart during 6-12 days ($P<0.05$). Keeping fish at low temperatures reduced bacterial growth (Sasi *et al*., 2003). During the storage, no coliforms were found in all samples with pyrophosphate pretreatment, suggesting the important role of pyrophosphate in coliform inhibition. Pyrophosphate might chelate the ions required for the growth of coliforms. Coliforms were found in whole and decapitated shrimps stored under MAP after 6 days of storage. Coliforms are the bacteria used as the sanitation index for fish and shellfish (Feng and Hartman, 1982). Buchanan and Klawitter (1991) reported that coliforms could be used as a better indicator of process integrity than total viable count. From the result, pretreatment with pyrophosphate in combination with MAP could prevent the growth of coliforms in shrimps.

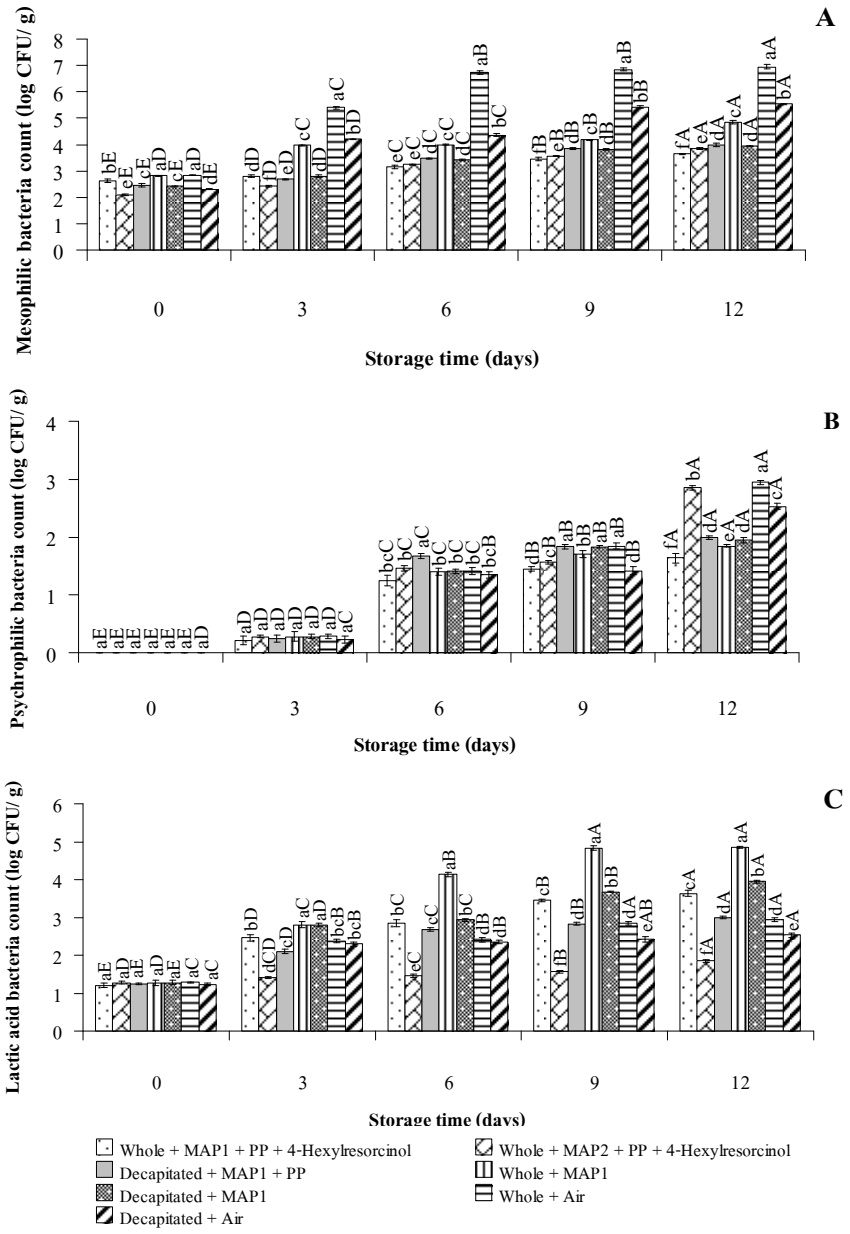

Figure 40. Changes in mesophilic (A), psychrophilic (B) bacteria and lactic acid bacteria (C) counts (log CFU/g) of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10%O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Table 7. Changes in coliforms (MPN/g) of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C.

Treatments*	Storage time (days)				
	0	3	6	9	12
Whole + MAP1 + PP + Hexylresorcinol	< 3	< 3	< 3	< 3	< 3
Whole + MAP2 + PP + Hexylresorcinol	< 3	< 3	< 3	< 3	< 3
Decapitated + MAP1 + PP	< 3	< 3	< 3	< 3	< 3
Whole + MAP1	< 3	< 3	< 3	15	30
Decapitated + MAP1	< 3	< 3	< 3	25	40
Whole + Air	< 3	< 3	23	90	200
Decapitated + Air	< 3	< 3	15	40	150

*MAP1 = 80% CO_2, 10%O_2, 10%N_2; MAP2 = 80% CO_2, 20%N_2; PP = Pyrophosphate

Changes in pH

The pH of white shrimp store under MAP and kept in air increased with increasing storage time ($P<0.05$) (Figure 41). The increase in pH was associated with the formation of basic compounds (TMA, ammonia, etc.) (Ruiz-Capillas and Moral, 2002). For samples stored in CO_2-enriched atmosphere, the pH increase was slower than those stored in air throughout the storage, particularly during the first 6 days of storage. Furthermore, the lower pHs were generally observed in sample stored under MAP ($P<0.05$). Carbon dioxide was absorbed into shrimp muscle, acidifying it via the formation of carbonic acid (Banks *et al*., 1980). Whole and decapitated shrimps stored in air showed the higher pH at day 12 of storage ($P<0.05$). From the result, MAP was effective in lowering the increase in pH of the shrimps.

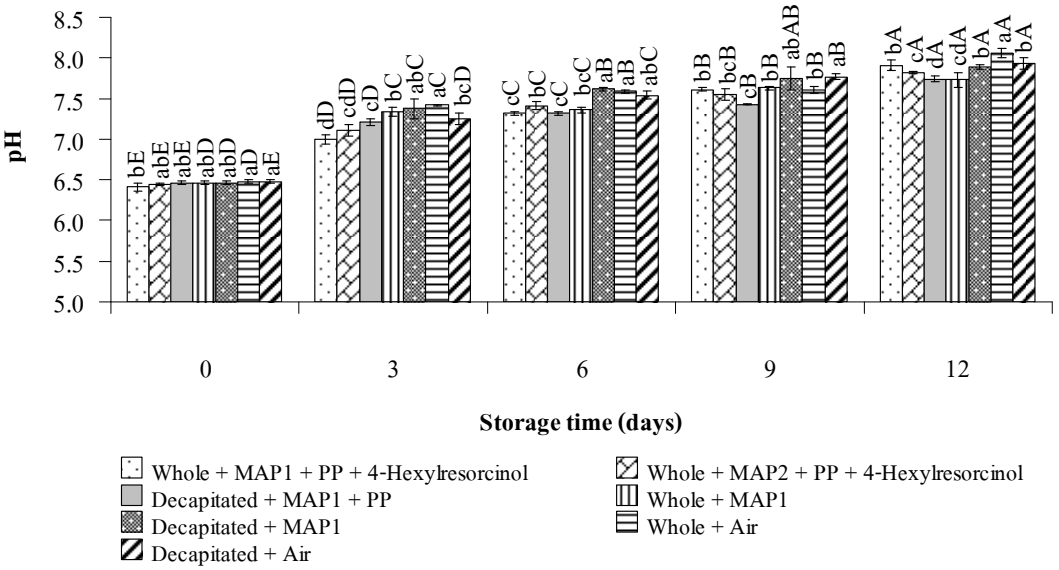

Figure 41. Changes in pH of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TVB and TMA contents

Changes in TVB and TMA contents of white shrimp as affected by pyrophosphate and 4-hexylresorcinol pretreatment, packaging atmosphere as well as decapitation during storage are presented in Figure 42. From the result, decapitated shrimp pretreated with pyrophosphate prior to MAP showed the lowest TVB content at day 9 ($P<0.05$). This indicated that pyrophosphate might show the synergistic effect with MAP on retarding the growth of TVB producing bacteria. During storage, TVB content of all samples increased slightly up to 6 days. Thereafter, samples stored in air had the sharp increase in TVB content ($P<0.05$). However, only slight increase in TVB content was observed in samples kept under MAP throughout the storage.

For the samples kept in air, the increases in TVB of whole samples occurred at a faster rate than decapitated shrimps within the first 6 days ($P<0.05$). Since digestive organs, which are the major sources of enzymes, are localized in the cephalothorax portion, the removal of cephalothorax presumably resulted in less hydrolysis of proteins. Free amino acids generated could be decomposed to various basic products by microorganisms. Coincidentally, the microbial load could be lowered due to the lower content of low molecular weight compounds, normally used for microbial growth. During extended storage in ice, microbial spoilage was more pronounced as evidenced by the increase in TVB contents. Nevertheless, the higher TVB content was observed in the decapitated shrimp after 6 days of storage ($P<0.05$). Deheading might cause the ease for microbial contamination or invasion into the meat, which was rich in nutrients for microorganisms. Cobb *et al*., (1976) found TVB content in shrimp (*Penaeus seniferus* and *Penaeus aztecus*) at levels of 26.1-38.2 mg TVB-N/100 g after 12 days and 15.3 mg TVB-N/100 in shrimp (*Metapenaeopsis barbata*) after 9 days of storage (Ming-Lang *et al*., 1986). At day 12 of storage, TVB contents in samples kept in air from a previous study was lower than that observed in this study. This was possibly cause by the differences in microorganisms present in the sample. Enterobacteria or microorganisms (normal flora) such as pseudomonads or lactic acid bacteria, are unable to produce TMA, but are capable of increasing TVB (Gram and Huss, 1996).

Changes in TMA content were similar to those observed for TVB content (Figure 42 B). Production of TMA in muscle during cold storage could be used as an indicator of bacterial activity (Gokodlu *et al*., 1998). TMA content in samples kept under MAP was lower (0-0.6 mg N/100 g sample) than that found in sample stored in air. Low contents of TMA might be associated with the bacteriostatic effect of MAP and pyrophosphate used, which could retard the growth of spoilage bacteria. However, no changes in TMA content were noticeable in white shrimp during the first 3 days of storage ($P<0.05$). Trimethylamine oxide (TMAO) is broken down to TMA by psychrotropic bacterial enzymes during iced storage (Yamagata and Low, 1995). The result suggested that the spoilage of shrimp, caused by bacteria with TMAO reducing activity, occurred when the storage time increased. The formation of TVB and TMA is generally associated with the growth of specific spoilage bacteria such as *Shewanella putrefaciens*, *Photobacterium phosphoreum*, and *Vibrioaceae* (Gram and Huss, 1996). When comparing TMA contents between whole and decapitated shrimps stored under MAP, whole white shrimp showed

the higher contents of TMA during the storage ($P<0.05$). From the results, pretreatment of shrimp by decapitation could retard the spoilage of shrimp kept under MAP during iced storage, most likely associated with the removal of enzymes or microorganisms involving in TMA formation.

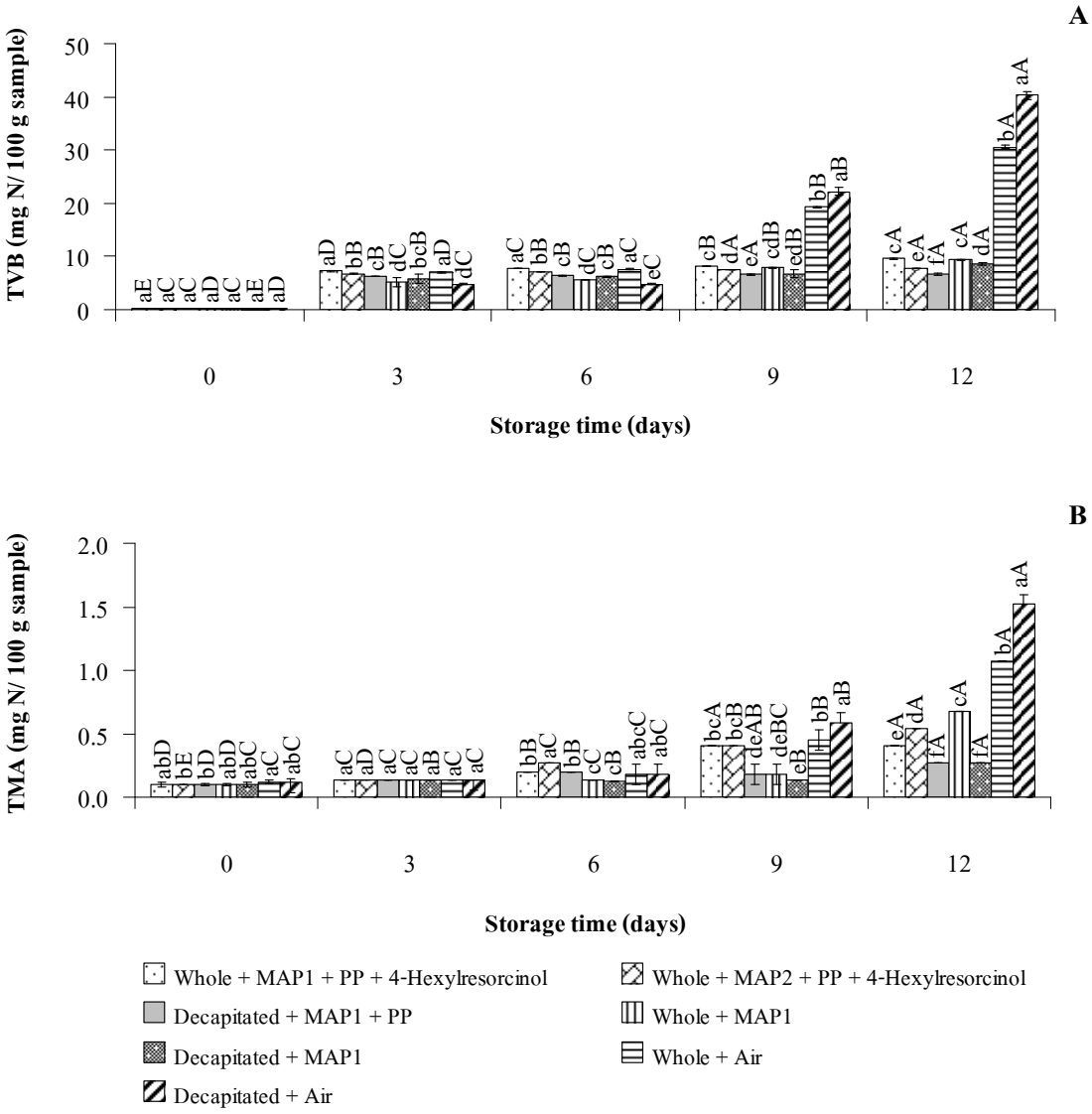

Figure 42. Changes in TVB (A) and TMA (B) contents of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TBARS

TBARS values in white shrimp stored in air or under MAP with and without pyrophosphate and/or 4-hexylresorcinol pretreatment during storage at 4°C are shown in Figure 43. TBARS value in all shrimp samples increased as the storage time increased ($P<0.05$), suggesting that lipid oxidation occurred during extended storage. TBARS has been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). The increase in TBARS indicated the formation of secondary lipid oxidation products (Kolakowska *et al*., 1992). TBARS value was higher in the whole samples kept under MAP 1 (80% CO_2, 10% O_2, 10% N_2) than other samples including samples kept under MAP 2 (80% CO_2, 20% N_2). This result confirmed that oxygen concentration in the gas mixtures had a definite influence on the oxidation level of MAP (Gimenez *et al*., 2004). Oxygen is essential for lipid oxidation, and initiation of autoxidation might be decisive for the other quality losses of the shrimps such as color stability, lipid oxidation and meat toughening. Thus, the higher the oxygen concentration, the higher TBARS values and the greater rates of increase were observed. Due to a high content of phospholipids, possibly from the skin and subdermal fat layer, the oxidation of unsaturated fatty acid in phospholipids could take place rapidly (Schnitzer *et al*., 2007). From the result, lipid oxidation could be retarded to some extent, when the cephalothorax was removed. It was found that TBARS value was lower in decapitated sample, compared with whole sample, when MAP 1 was used. Nevertheless, decapitation did not have the impact on lipid oxidation of sample stored in air. Lipase and lipoxygynase in the cephalothorax, causing the hydrolysis and oxidation, were postulated to be removed by decapitation. Shrimp generally has low levels of fatty acids but high levels of cholesterol (Schnitzer *et al*., 2007). The oxidation of cholesterol in shrimp is favored by the presence of unsaturated fatty acids, which are easily oxidized. Connor and Lin (1982) described the crustaceans as good sources of polyunsaturated fatty acids (n3), presenting low levels of saturated fatty acids and high cholesterol levels (Schnitzer *et al*., 2007). However, shrimps had TBARS value more than 5.0 mg MDA/kg sample within 12 days of storage. Nishimoto (1985) proposed the value of 3.0 mg malonaldehyde/kg sample for good quality fish.

With pyrophosphate pretreatment, whole sample stored under MAP had the lower TBARS values, compared with the same sample without pyrophosphate pretreatment

except at day 6 of storage ($P<0.05$). Therefore, decapitation, pretreatment and packaging atmosphere affected the lipid oxidation of shrimp during the storage.

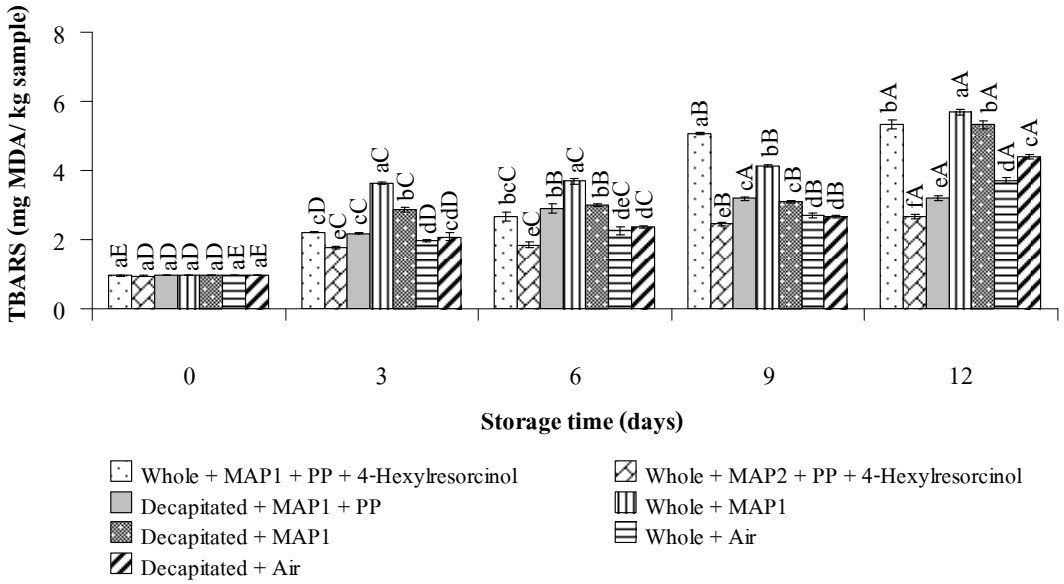

Figure 43. Changes in TBARS of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in TCA-soluble peptides

A continuous increase in TCA-soluble peptides in all samples was observed throughout the storage ($P<0.05$) (Figure 44). Among all samples, the lower TCA-soluble peptide content was found in the decapitated samples kept under MAP, indicating that the degradation caused by either endogenous or microbial proteinases was retarded during storage at 4°C. However, TCA-soluble peptide content of decapitated shrimps kept in air was generally higher than those of decapitated shrimps stored under MAP throughout the storage ($P<0.05$). The result

suggested that MAP played a role in inhibiting the growth of microorganisms with proteolytic activity. Therefore, decapitated shrimp had the lower protein degradation caused by digestive and microbial proteinases. At the beginning of storage in ice, endogenous enzymes are mainly involved in the gradual loss of fish freshness. Thereafter, bacterial metabolism predominates and leads to final spoilage (Pacheco-Aguolar *et al*., 2000). From the result, gas compositions had no pronounced effect on TCA-soluble peptide content in shrimp stored under MAP. Also, pyrophosphate and/or hexylresorcinol did not show the marked impact on TCA-soluble peptide content. The results suggested that the decapitation of shrimp and storage under MAP at low temperature could lower the activity of endogenous as well as microbial proteinases. The hepatopancreas and digestive tract of crustaceans are very rich in protelytic and collagenolytic enzyme (Brauer *et al*., 2003).

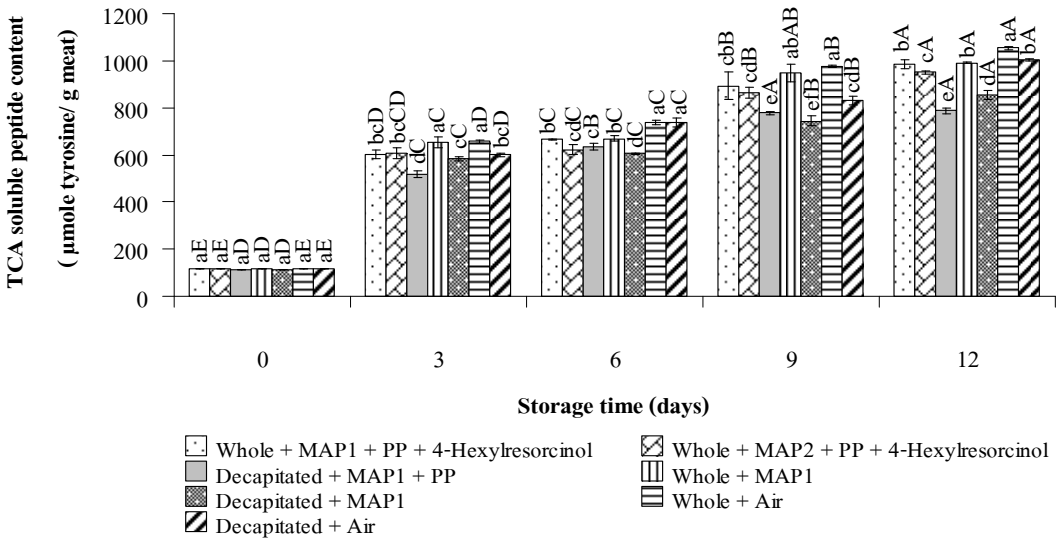

Figure 44. Changes in TCA-soluble peptides of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in formaldehyde

The formaldehyde content in all samples increased with increasing storage time (Figure 45). Among all samples, whole shrimps stored in air had the highest formaldehyde content throughout the storage ($P<0.05$), followed by decapitated shrimp kept under the same atmosphere. Under MAP 1, whole shrimps contained the higher formaldehyde content than decapitate shrimps throughout the storage ($P<0.05$). When the whole samples were treated with PP and hexylresorcinol and packaged under MAP, the lower formaldehyde was found. PP might play a role in chelating metal ions required for microbial growth or TMAO demethylase. Furthermore, MAP could prevent the formation of formaldehyde to some extent. When comparing the formaldehyde content between shrimp with and without decapitation, formaldehyde content could be lowered for the decapitated sample. Therefore, decapitation together with PP pretreatment and the appropriate packaging condition could reduce the formation of formaldehydes in the shrimp samples.

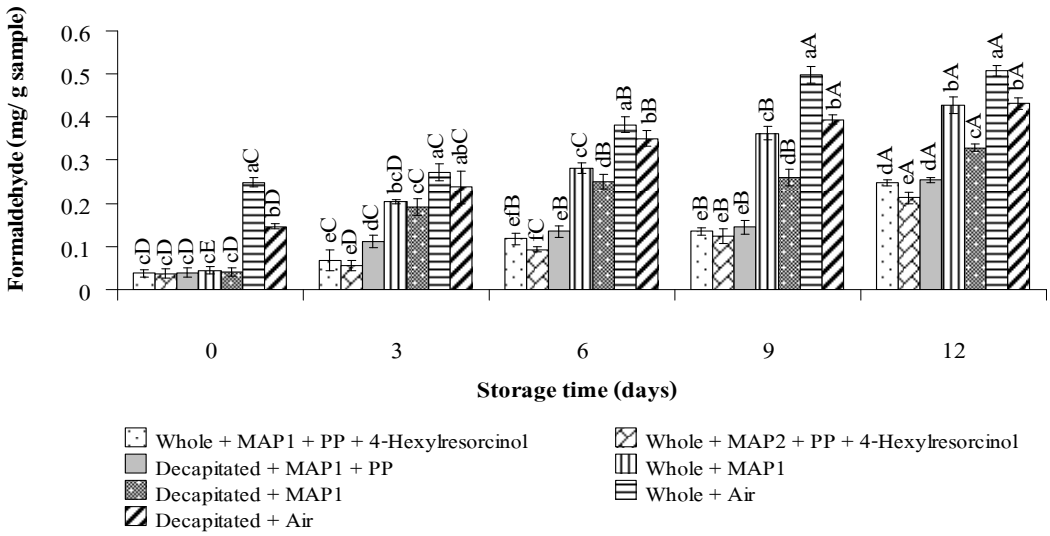

Figure 45. Changes in formaldehyde content of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in melanosis

The development of melanosis of white shrimp without and with pyrophosphate and/or 4-hexylresorcinol pretreatment stored in air or under MAP was monitored during storage (Figure 46). Degree of melanosis varied with treatments. Montero *et al*. (2001a) reported that the melanosis development starts by the enzymatic action of PPO, which oxidizes naturally occurring phenols, mainly tyrosine, into quinones. Subsequent polymerization of the colorless quinones by a non-enzymatic mechanism gives rise to the accumulation of black high-molecular-weight pigments.

For whole samples, those treated with pyrophosphate and 4-hexylresorcinol and kept under MAP, either MAP 1 or MAP 2, had the lower melanosis score throughout the storage of 12 days ($P<0.05$). However, samples treated with pyrophosphate and 4-hexylresorcinol and

kept under MAP 2 (80% CO_2, 20% N_2) showed the lower score of malenosis at the end of storage. The lowered melanosis in this sample could be related to PPO inhibition as well as the absence of O_2, which involved in melanosis formation. According to Martinez-Alvarez *et al*. (2005b), prawns treated with 4-hexylresorcinol showed a slower melanosis development. Montero *et al*. (2004) reported that melanosis inhibition of deepwater pink shrimp (*Parapeaneus longirostris*) increased with increasing 4-hexylresorcinol concentration. 4-hexylresorcinol at 0.25% was effective in extending shelf-life by lowering the melanosis of prawns (Montero *et al*., 2006). Nevertheless, the effective doses of 4-hexylresorcinol may differ, depending on several factors, such as species, physiological state, method of additive uses, etc. (Montero *et al*., 2006).

For the decapitated samples, much lower melanosis score was obtained, compared with that of whole samples, throughout the storage, regardless of atmosphere or pyrophosphate treatment ($P<0.05$). This result indicated that the cephalothorax of white shrimp was the major source of PPO. Ogawa *et al*. (1984) reported that the highest PPO activity was found in the carapace. Therefore, the removal of the cephalothorax combined with pretreatment with pyrophosphate showed the synergistic effect to reduce the PPO activity.

From the result, the use of MAP at low temperature could delay the onset of melanosis to some extent. However, the combined uses of pyrophosphate and 4-hexylresorcinol could enhance the inhibitory effect on PPO in conjunction of the MAP, leading to the retarded melanosis.

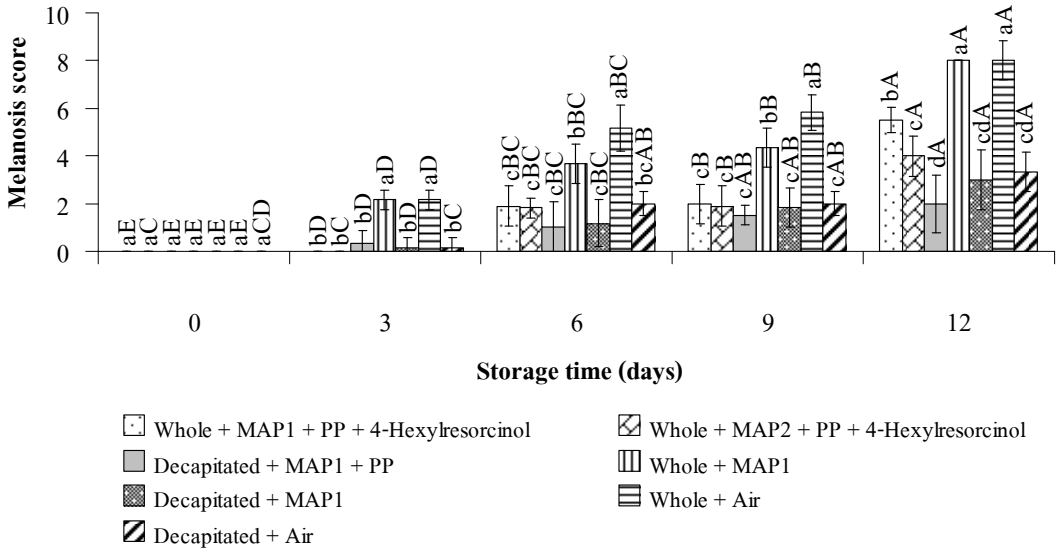

Figure 46. Changes in melanosis score of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in water holding capacity (WHC)

Water holding capacity (WHC) of white shrimp during storage in air or under MAP with and without pyrophosphate and/or 4-hexylresorcinol pretreatment is depicted in Figure 47. After 3 days of storage, lower WHC was observed with all samples. Lower WHC was found in whole sample kept in air, compared with other samples, throughout the storage ($P<0.05$). This might be associated with the higher degradation of muscle proteins. Hydrolyzed samples might loss the capacity of holding water in the muscle. Decapitated samples pretreated with pyrophosphate generally had higher WHC than the samples without phosphate pretreatment. Pyrophosphate most likely improved the WHC of muscle. WHC has been reported to be influenced by a number of factors including ultimate pH, protein denaturation, intra- and

interfascicular spacing and sarcomere lengths (Offer and Knight, 1988). From the result, the drastic decrease in WHC was observed at day 12 of storage. At day 12, the lowest WHC was noticeable in sample kept under MAP 2 with pretreatment of pyrophosphate and 4-hexylresorcinol. However, no marked differences in WHC were observed between shrimp kept under MAP and in air. This suggested that carbonic acid formed could not penetrate through the shell effectively. As a result, muscle proteins were not denatured greatly and WHC of the muscle could be maintained.

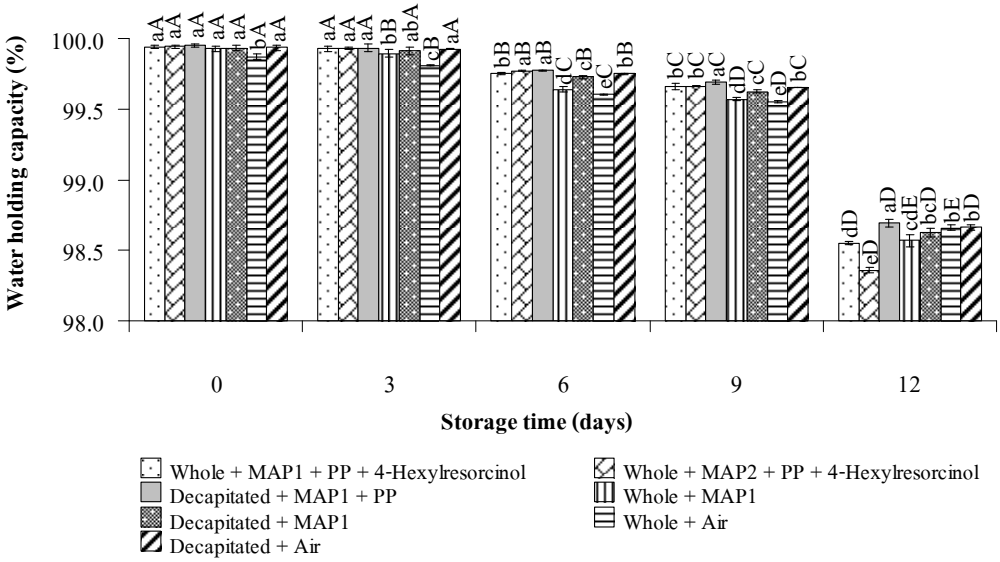

Figure 47. Changes water holding capacity of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in shear force

Shear force of white shrimp kept in air or under MAP either with or without pyrophosphate and/or 4-hexylresorcinol pretreatment during storage at 4°C is shown in Figure

48. For all samples, shear force decreased when storage time increased up to 12 days ($P<0.05$). For the samples stored in air, whole samples had the higher shear force than did decapitated counterpart within the first 9 days of storage ($P<0.05$). For samples kept under MAP, the highest shear force was found in whole samples pretreated with pyrophosphate and 4-hexylresorcinol and packaged under MAP 2 within the first 6 days of storage ($P<0.05$). Changes in shear force are associated with protein denaturation, which caused protein aggregation and loss of water-holding capacity (Shenouda, 1980). From the result, decapitation resulted in the retardation of the decrease in shear force of white shrimp stored under MAP or in air. The removal of cephalothorax could reduce the proteolytic activity found in this portion. As a result, lower degradation of proteins, especially collagen, was postulated.

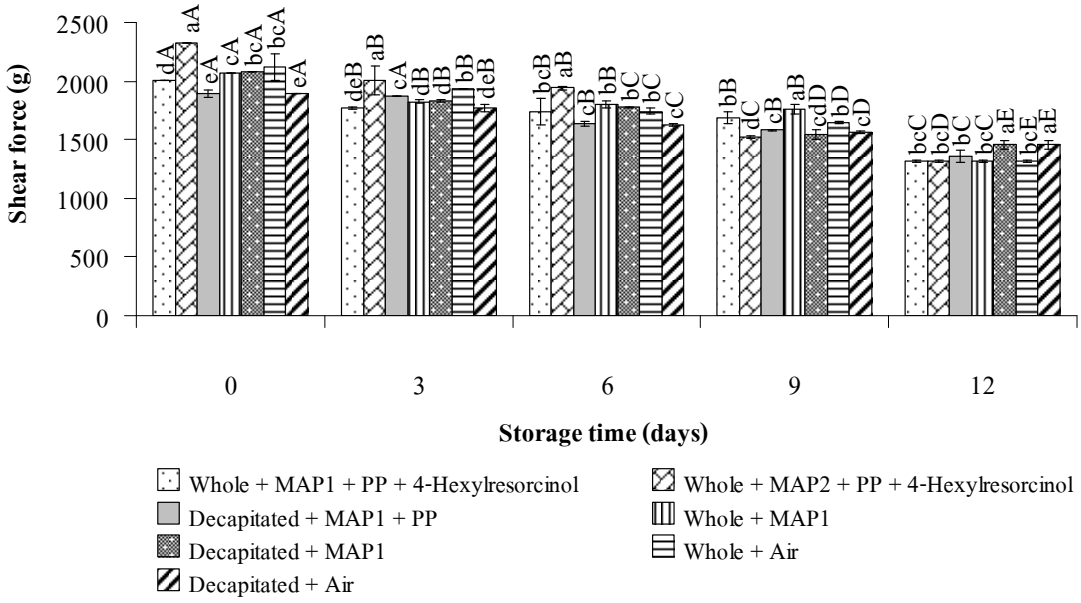

Figure 48. Changes shear force of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Changes in proteases, collagenase and PPO activities

Proteases, collagenase and PPO activities of white shrimp with different treatments kept in air or under MAP during the storage at 4°C are depicted in Figure 49. For protease activity, much lower protease activity was found in decapitated samples, especially with pyrophosphate pretreatment, compared with other samples. When comparing the proteases activity between samples kept in air and stored under MAP, it was noted that lower protease activity was found in sample kept in MAP, compared with sample stored in air, after 3 days of storage ($P<0.05$). Protease was localized mainly in cephalothorax of white shrimp. Proteolytic degradation of white shrimp proteins may therefore lead to severe textural changes during the storage. Highest collagenases activity was found in whole shrimp without pyrophosphate treatments. Collagenolytic enzymes are Zn^{2+}-containing metalloendopeptidases stimulated by Ca^{2+} ions and thiol reagents (Ashie *et al*., 1996). Lower activity was obtained in sample with pyrophosphate pretreatment. It was suggested that pyrophosphate might affect collagenases activity via its chelating property toward Zn^{2+} in active site of collagenases. For the sample without pyrophosphate treatment, slightly higher collagenase activity was found in whole sample, compared with decapitated sample. Collagenolytic enzymes have been detected in shrimp hepatopancreas and muscle from several marine organisms (Lindner *et al*., 1988). Therefore, the control of collagenases of white shrimp could maintain the textural property of shrimp during extended storage.

A marked decrease in PPO activities in whole sample was observed after treatment with pyrophosphate together with 4-hexylresorcinol. Highest PPO activity was found in whole shrimp without treatments. The inhibitory effect of 4-hexylresorcinol on PPO activity has been demonstrated in crustaceans by several authors (Otwell *et al*., 1992; Slattery *et al*., 1995). For the sample without treatment, much lower PPO activity was found in decapitated sample, compared with whole sample. From the study, PPO activity was determined in cephalothorax of whole sample, which contained very high activity. For decapitated sample, PPO was measured from the meat and shell. Therefore, PPO was localized mainly in cephalothorax of white shrimp and contributed to melanosis of shrimp during extended storage. When comparing the PPO activity between decapitated sample with and without pyrophosphate pretreatment, it was noted that lower activity was obtained in sample with pyrophosphate pretreatment. It was suggested that

pyrophosphate might affect PPO activity via its chelating property toward Cu^{2+} in active site of PPO. Martinez-Alvarez *et al*. (2005a) found that the samples treated with 4% citric acid completely inhibited PPO activity during 6 days of storage, whereas shrimps treated with 4-hexylresorcinol showed slightly higher activity. Slattery *et al*. (1995) reported that tiger prawns (*Penaeus esculentus*) dipped in 0.2% Everfresh® (commercial compound based on 4-hexylresorcinol) for 2 min had the lower melanosis during the storage. The result reconfirmed that the treatment with pyrophosphate and 4-hexylresorcinol most likely inhibited PPO. During the storage, PPO activity decreased throughout the storage for all samples. The loss in activity might be associated with the denaturation of PPO.

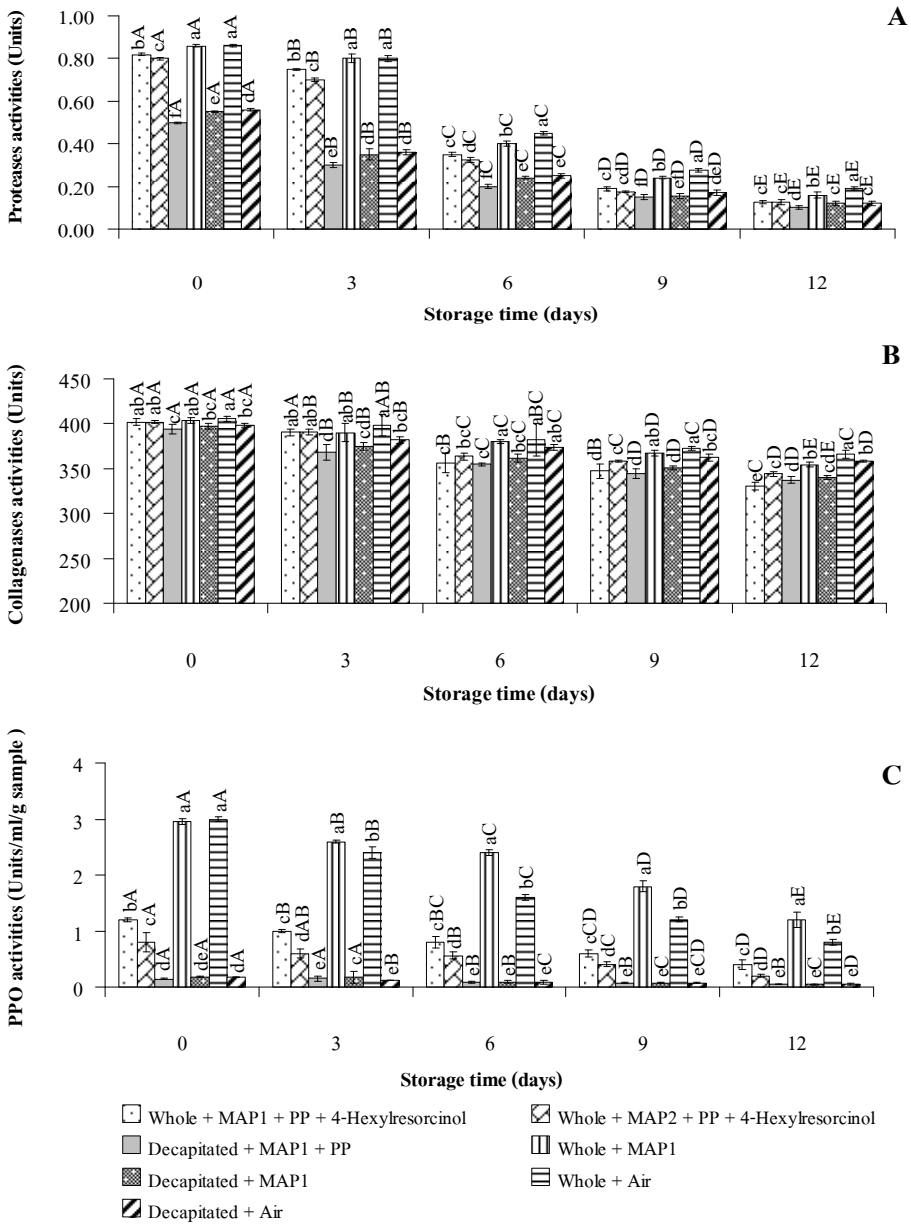

Figure 49. Changes in proteases, collagenase and PPO activity of whole or decapitated white shrimp with and without 2% pyrophosphate and/ or 0.25% 4-hexylresorcinol pretreatment stored under different conditions at 4°C. MAP1 = 80% CO_2, 10% O_2, 10% N_2; MAP2 = 80% CO_2, 20% N_2; PP = Pyrophosphate. The different letter cases within the same pretreatment/ packaging atmosphere and the different letters within the same storage time indicate the significant differences, respectively ($P<0.05$). Bars represent the standard deviation from triplicate determinations.