Chapter 3

Results and discussion

1. pH profile

The pH profile of PO isolated from black tiger prawn cephalothorax is shown in Figure 7A. The optimum pH for the phenolase-DOPA reaction was 6.0. A sharp decrease in activity was noticeable at pH lower than 5.5. Approximately 20% of activity was remained at pH 5.0 and activity was completely lost at pH 4.5. At the basic pH, PO activity decreased gradually, especially with higher pHs. The optimum pH of PO depends to a large extent on the physiological pH in which the enzyme activity occurs in nature (Kim *et al*., 2000; Montero *et al*., 2001). Optimum pH of PO from the carapace of cephalothorax was 7.16, while that from the abdominal cuticle was 8.76 (Montero *et al*., 2001). PO would probably present different optimum pH characteristics, depending on species (Rolle *et al*., 1991). The high activity was reported in the pH range of 6-8 in carapace extract of *Penaeus japonicus* (Montero *et al*., 2001), whereas PO from *Penaeus duodorum* was active in the pH range of 6.5-9 (Simpson *et al*., 1988). Chen *et al*. (1991) reported that the Western Australian lobster PO had optimum pH between 6 and 8 while Florida spiny lobster PO had a pH optimum of 6.5. The optimum pH of PO from *Penaeus monodon* was 6.0 (Rolle *et al*., 1991). The differences in pH activity profiles suggests that the nature of protopic groups involved in active site of different PO may be different (Whitaker, 1972).

2. Temperature profile

The optimum temperature of black tiger prawn PO was 45 °C (Fig. 7B). PO activity gradually increased with increasing temperature and reached the optimum at 45 °C. The increase of temperature produces more activity, as the reaction is accelerated by the enhanced kinetic energy (Montero *et al*., 2001). However, approximately 85% loss in activity was at the temperature higher than 50 °C, probably due to thermal denaturation of enzyme. At higher temperature, the greater loss in activity was noticeable. At 65 °C, approximately 5 % activity was remained, compared with that found at optimal temperature. This indicated that the enzyme was almost completely denatured at very higher temperature. Optimal temperature of PO can be varied with species. PO from carapace of prawn (*Penaeus japonicus*) showed the maximal activity at 30-35 and 40-45°C (Montero *et al*., 2001). Maximal activity of PO from *Penaeus setiferus* (Simpson *et al*., 1987; 1988) and from *Penaeus monodon* (Rolle *et al*., 1991) was observed at 40-45°C. The ability of enzyme to be activated by heat and to maintain activity at high temperatures is most important when considering the impacts of heat processing on processed lobster quality (Williams *et al*., 2003). The PO activity seems to be correlated with water temperature and environment condition (Bartolo and Birk, 1998).

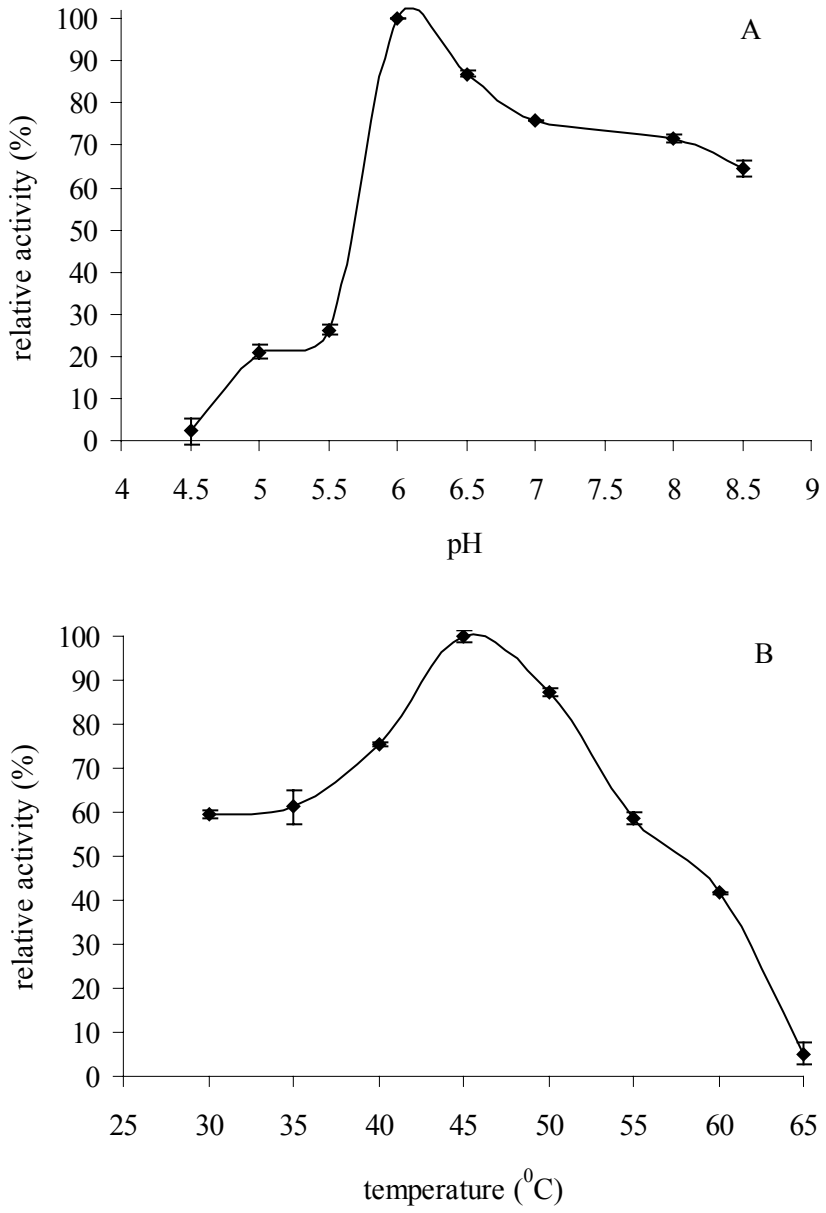


Figure 7. pH (A) and temperature (B) profile of black tiger prawn PO activity.

3. pH stability

PO from black tiger prawn cephalothorax was stable at pH 6.5 and slightly basic pH (pH 8.0) (Fig 8A). Activity tended to decrease in the acidic pHs. The instability of PO at acidic pH suggested that treatment of prawn with acid solution would inhibit melanosis to some extent. In addition, the greater loss in PO activity was observed when the incubation time increased from 10 to 30 min. The results was in agreement with Simpson *et al.* (1987) who found that the PO from *Penaeus setiferus* was not stable at acidic pH. Rolle *et al*. (1991) also observed that PPO from *Penaeus monodon* was unstable at pH below 5. PO from Florida spiny lobster and Western lobster exhibited optimum stability at pH 7 (Chen *et al*., 1991). The stability of PO varied depending on a number of factors such as temperatures, pH, substrate used, ionic strength, buffer system, and time of incubation (Kim *et al*., 2000).

4. Thermal stability

PO from black tiger prawn cephalothorax was not stable when subjected to heating at temperatures higher than 40ºC (Fig. 8B). About 35 % of original activity was destroyed after incubation at 45 ºC for 10 min and 60 % after 30 min with heating at the same temperature. Montero *et al*. (2001) reported that PO activity was considerably reduced when the PO extract from *Penaeus japonicus* was subjected to heating at 35°C. The PO from *Penaeus monodon* has been found to be unstable at temperture over 30-35°C (Simpson *et al*., 1988) and the upper limit of *Penaeus setiferus* was 50°C (Simpson *et al*., 1987).

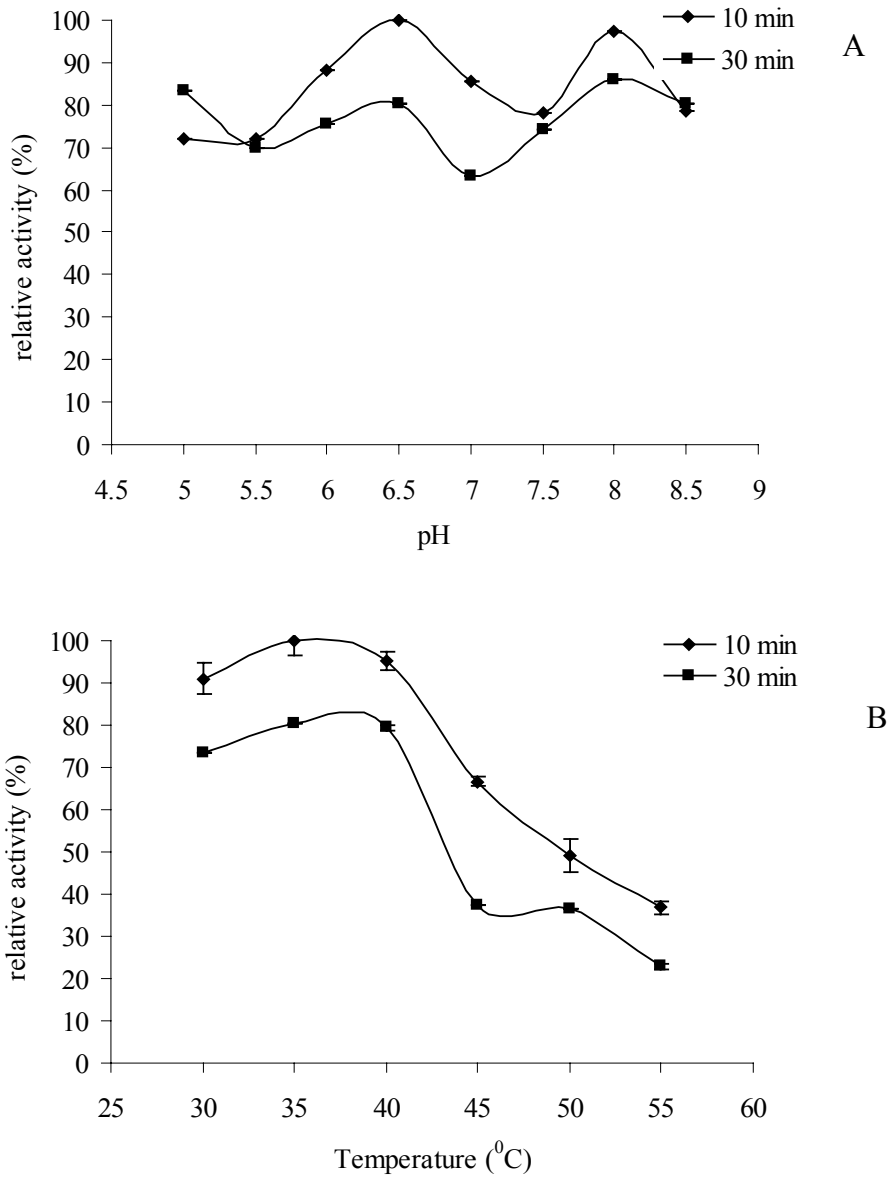


Figure 8. pH (A) and temperature (B) stability of black tiger prawn PO activity.

5. Effect of chemicals

The effects of various chemicals on PO activity from black tiger prawn cephalothorax are shown in Table 1. D- glutathione, L- cysteine or thiourea was shown to have high inhibitory activity against PO. Among all inhibitors tested in this study, L-cysteine was the most effective, followed by thiourea and glutathione, respectivly. The inhibition of PO activity was enhanced with increasing concentration of the chemical used. The browning process has been induced by PO, which catalyzes the hydroxylation of monophenols to *o*-diphenols, and the oxidation of *o*-diphenols to *o*-quinone, very reactive compounds which strongly interact with other molecules, leading to a large pigment of high molecular weight and very dark or black coloring (Montero *et al*., 2004). A number of compounds with different inhibitory mechanism have been reported as potent inhibitors of mushroom tyrosinase (i.e. L-cysteine and glutathione as couplers of quinones). Cysteine reduces PO activity due to the formation of the colorless cysteine-quinone adducts (Gerrero-Beltran *et al*. 2005; Jang *et al*., 2003; Ding *et al*., 2002; Jiang *et al*., 1999; Robert and Cadet, 1996) and the reduction of the *o*-quinone to the colorless diphenols (Kim *et al*., 2000; Robert *et al*., 1996). Copper in the active site of PO has been known to involve in activity. Met-type [Cu(II)Cu(II)] is initially reduced by reductants and subsequently interacts with molecular oxygen to form PPO oxy-type [Cu(II)Cu(II)O_2] being capable of catalyzing the reaction of mono- and diphenol (Jang *et al*., 2003). The thiol may inhibit PO activity by combining with the copper at the active site (Dawson and Magee, 1996; Ding *et al*., 2003) However, the formation of more stable colorless products was been recognized as the major effect (Robert *et al*., 1996). The use of 10 mM glutathione gave good control of browning of litchi fruit and 80-85% inhibition of PO was

observed (Jiang and Fu, 1998). Jiang (1999) showed that longan PO can be completely inhibited by 1 mM glutathione or 1 mM L-cysteine. As studied by Gomez-Lopez (2002), L- cysteine was the most effective inhibitor against PO from avocado. Kim *et al*. (2000) and Dogan and Dogan (2004) reported the inhibition modes of three inhibitors as competitive.

Activity of PO was increased in the presence of SDS or methanol (Table 2). ProPO from kuruma prawn was activated by SDS and methanol (Adachi *et al*., 1999). PO is frequently reported in a latent stage (Gauillard and Richard-Forget, 1997; Chazarra *et al*., 1997) and it can be activated by a number of different treatments such as protease (Adachi *et al*., 2003; Bartolo and Birk, 1998), low as well as high pH and detergent (Soderhall, 1995). The activation of PO by SDS suggested as process involving a conformation change in the protein that may induce the increase of enzymatic activity (Escribano *et al*., 1997). SDS at 0.69 mM was the optimum concentration to avoid undesirable effect (micelle formation) (Escribano *et al*., 1997). SDS at an optimal concentration of 0.8 mM activates the enzyme by opening or unblocking the active site of the enzyme making it accessible for the substrate (Soderhall, 1995).

Table 2. Effect of chemicals on black tiger prawn PO activity

Compounds	Relative activity (%)
Inhibitors	
Cysteine (1 mM)	0.60 ± 0.10
Cysteine (2 mM)	0.00 ± 0.50
Glutathione (1 mM)	6.8 ± 1.17
Glutathione (2 mM)	1.3 ± 3.18
Thiourea (1 mM)	3.8 ± 0.33
Thiourea (2 mM)	0.20 ± 0.67
Activators	
SDS (0.01 %)	111.4 ± 0.19
SDS (0.005 %)	102.3 ± 0.97
Methanol (40 %)	149.0 ± 1.94
Methanol (25 %)	100.5 ± 0.34

Mean ± SD from triplicate determinations.

6. Effect of L-cysteine on PO activity

Inhibition of PO activity by cysteine was in a concentration dependent manner (Fig. 9). Approximately, 10% of PO was inhibited when cysteine at 0.25 mM was used. At higher concentration, 0.5 and 0.75 mM, the activity decreased by 27 and 47 %, compared with that of control (Fig. 9). The decrease in activity might be due to the drop in the pH. Cysteine structure possesses 1COOH, 1 SH and 1 NH_2, which are responsible for lowering the pH, keeping PO away from its optimum pH. In addition, L-cysteine can directly inhibit the PO by prevention of the oxidation by removing

oxygen from the solution and/or by reacting with quinone by formation of quinone-cysteine compound (Viera and Fatibello-Filho, 1999). These results confirm those obtained by Valero *et al*. (1991) and Gauillard *et al*. (1993) who found a direct effect of cysteine on the enzyme protein. From the result, cysteine at concentration of 0.5 and 0.75 mM which caused the decrease in PO activity by 27 and 47% were chosen for further study to increase PO inhibitory activity via Maillard reaction.

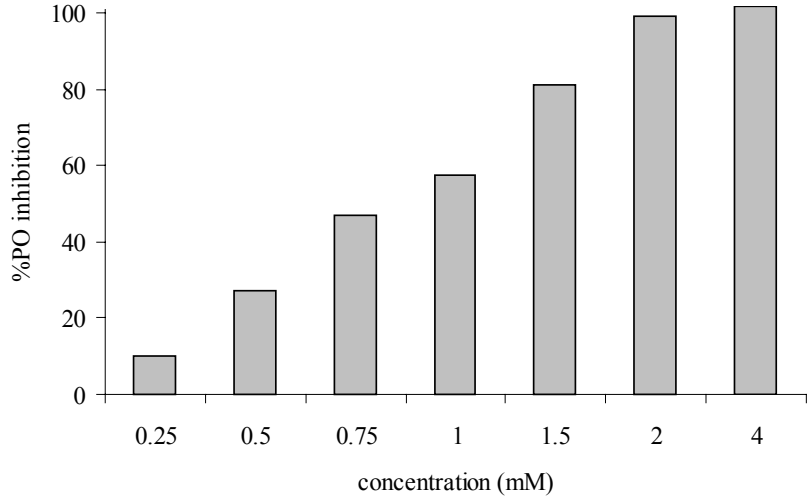


Figure 9. Inhibitory activity of cysteine with various concentrations towards PO from black tiger prawn.

7. Effect of amino acids and sugars on the characteristics and PO inhibitory activity of MRPs

Changes in pH

The pH with MRPs derived from different reducing sugars (glucose, fructose, galactose) and amino acids (cysteine, glycine) are shown in Figure 10. The reaction with an initial pH of 8.0 was allowed to proceed without pH control for 8 h at 100°C. No marked changes in pH were found in all samples tested. However, Berg

and Van Boekel (1994) reported that the decrease in pH was observed during Maillard reaction of lactose and amino acids. From the result, no decrease in pH of the samples was possibly due to the low reactant concentration.

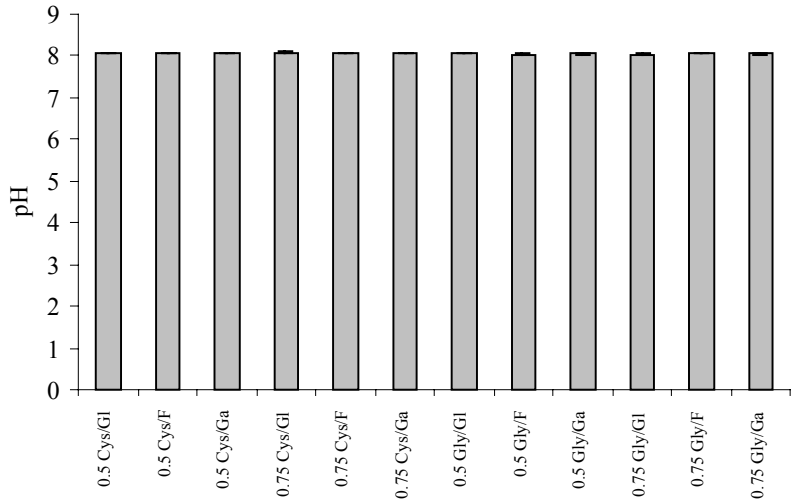


Figure 10. Changes in pH of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

Changes in fluorescence intensity, A_{294} and browning intensity

The fluorescence intensity (Fig. 11A), A_{294} (Fig. 11B) and browning intensity (Fig. 11C) generally increased with increasing reactant concentrations. The developments in these contents were more pronounced in sugar/glycine model system. The rate of intermediate formation was more intense in model system containing galactose. In the absence of sugars, cysteine exhibited the lower fluorescent intensity, A_{294} and browning intensity. Furthermore, cysteine is acidic amino acid, which is difficult to complex with sugar, resulting in lower formation of

MRPs, compared with glycine. Kwak and Lim (2004) found that the color intensity of each MRPs derived from the basic amino acids was greater due to the higher reactivity than those from acidic amino acids. From the results, the reactivity in Maillard reaction of various sugars was different, possibly due to the different ease in ring opening. For most chemical and biochemical reaction of reducing sugar, the major pathway involves only one of its tautomeric forms, usually the acyclic form (free aldehyde or ketone) (Naranjo *et al*., 1997). Generally, aldose are intrinsically more reactive than ketose. Thus, in the Maillard reaction, the concentration of open chain form might be a crucial factor in determining the rate of glycation if the interconversion rate is slow than the reaction rate (Yaylayan *et al*., 1993). Naranjo *et al*. (1998) reported that fructose has the highest proportion of open chain form when compared with glucose, but aldose would react faster than ketoses because they are more electrophilic. When the glycine was used, MRPs derived from galactose showed the highest A_{420} and A_{294}, followed by MRPs from fructose and glucose. Fructose appear to be converted into the intermediate, which reacts with 3, 4, -dideoxy-4-sulfohexosone (DSH). During reaction, the final intermediate converted to melanoidins (Mundt and Wedzicha, 2003). This could be explained by the advanced stages of reaction, brown pigments with high molecular weights are formed (Lerici *et al*., 1990) whereas the brown polymers are formed at final stages (Ajandouz *et al*., 2001).

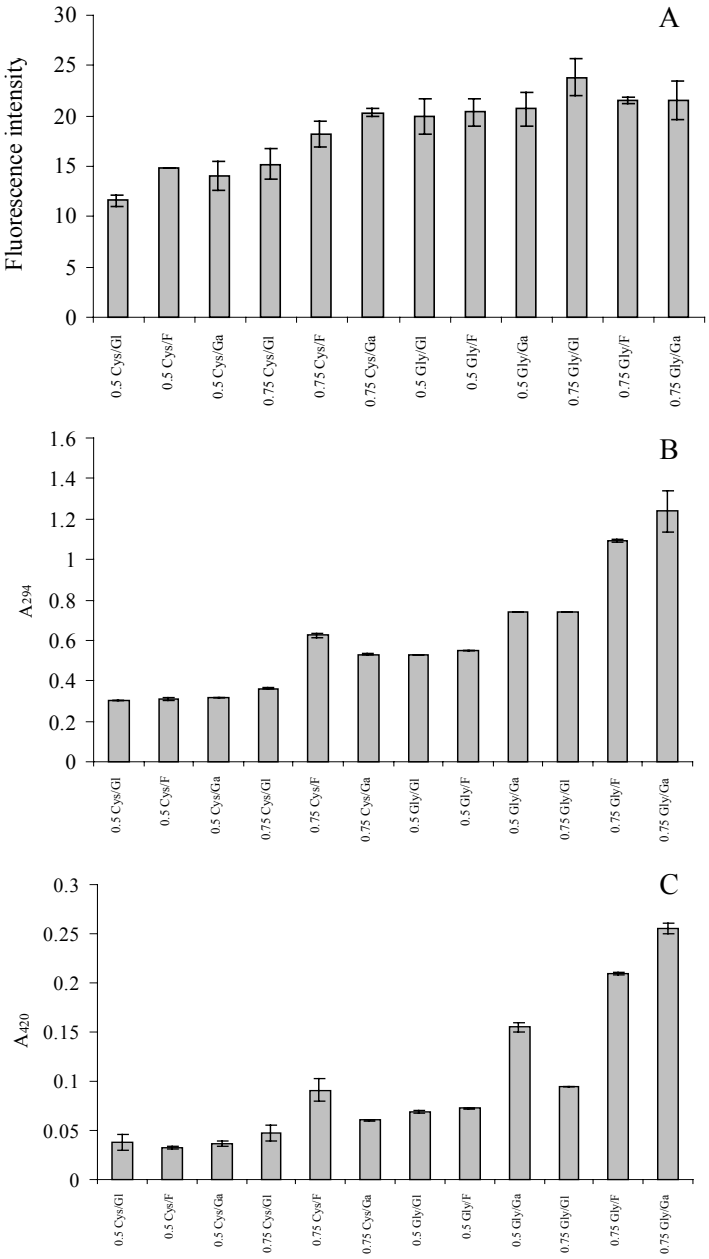


Figure 11. Changes in fluorescence intensity (A), A_{294} (B) and A_{420} (C) of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

Changes in reducing sugar and amino group contents

The reducing sugar and amino group contents were monitored for the extent of browning reaction. The greater losses in reducing sugar and amino group were found with the fructose/amino acid model system (Fig. 12). Fructose contains a high concentration of acyclic form (Ajandouz *et al*., 2001), leading to the greater formation of Schiff's base between sugar and amino acids. Kato *et al*. (1969) showed that in the presence of a low concentration of glycine, fructose underwent browning more rapidly than glucose. Swales and Wedzicha (1992) reported that the kinetics of fructose-glycine reaction were much simpler than those of glucose-glycine reaction. At pH higher than 7, the degradation of Amadori compound involve 2,3-enolisation and take part further reaction which are highly reactive compound (Van Boekel, 1998). From the result, The decreases in free amino group and reducing sugar were in accordance with the increase in fluorescence intensity (Fig. 11A), A_{294} (Fig. 11B) and browning intensity (Fig. 11C).

However, MRPs derived from galactose/amino acid model system had the lower losses in reducing sugar and free amino groups, though it had the highest A_{294} and A_{420}. Therefore, it was suggested that glycation of galactose/amino acid model system might take place at the lower rate, but the colorless intermediate could be formed and converted to brown products effectively. Therefore, the rate of reaction and characteristics of MRPs was dependent upon the types of sugars and amino acids.

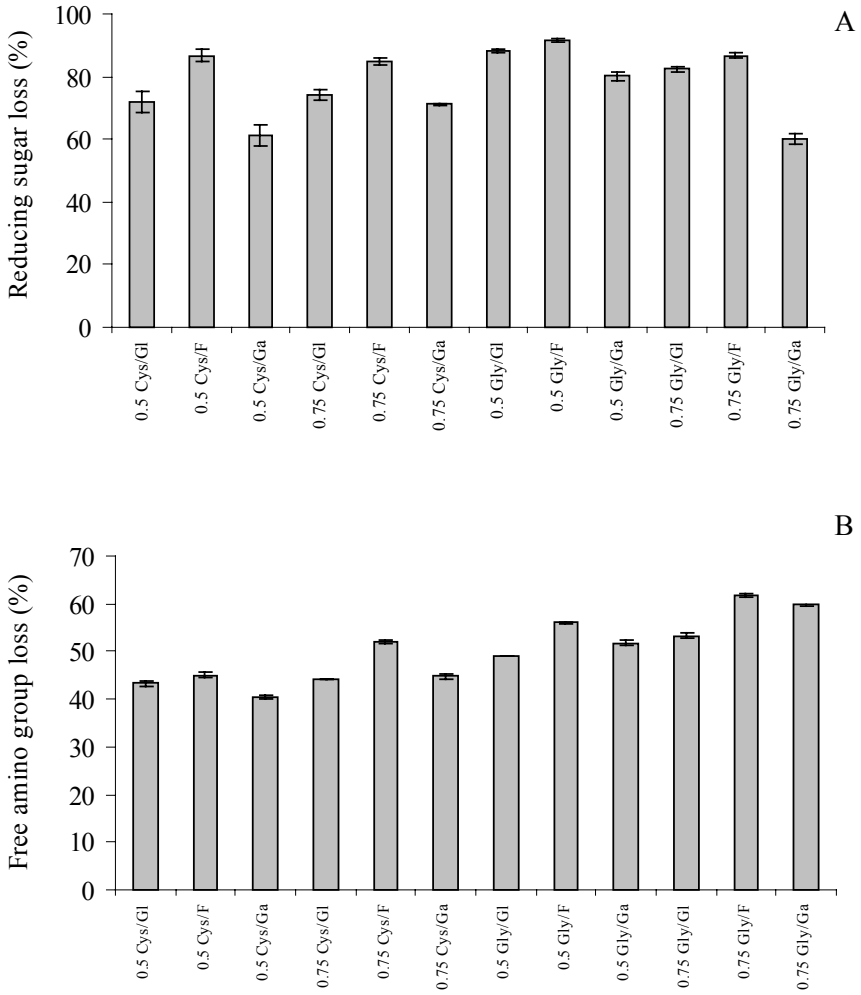


Figure 12. The loss of reducing sugar (A) and free amino group contents (B) of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

Changes in reducing power

The greater reducing power was found in MRPs derived from glycine and reducing sugar at the concentration of 0.75 mM (Fig. 13). Galactose/glycine model system with an equimolar of 0.75 mM exhibited the greatest reducing power. At 0.75 mM concentration, MRPs derived from glucose and fructose in the presence glycine had the similar reducing power. With the same sugar type and concentration used, the system derived from glycine showed the higher reducing power than that derived from cysteine. The results suggested that types of sugar is a crucial role in formation of reducing compounds via Maillard reaction. Yoshimura *et al*. (1997) reported that the increase in heating time of glucose/glycine mixture led to increase the reducing power. From the result, reducing power correlated with browning intensity (Fig. 11C) and intermediate products (Fig. 11A and 11B). However, reducing power was not related with the loss in reducing sugar and amino group content (Fig. 12). This might be because MRPs derived from galactose produced more highly reactive compound than those from fructose. Fructose can react at higher rate with amino acid and can generate the brightly colored pigments in the early stage of the Maillard reaction (Murakami *et al*., 2002).

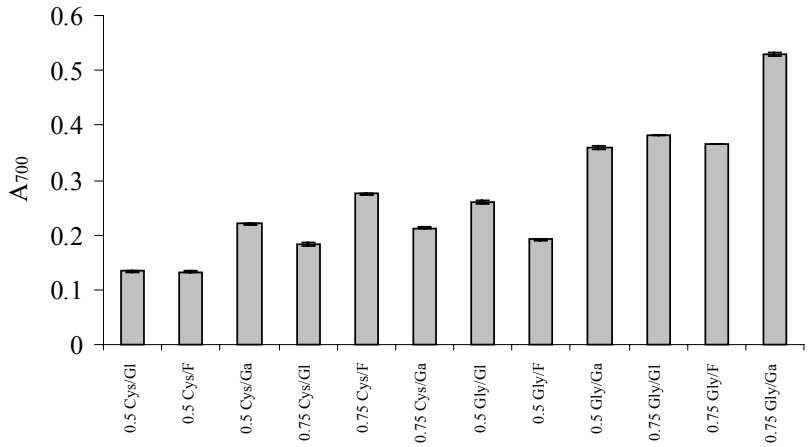


Figure 13. Reducing power of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

Changes in copper chelating property

Copper chelating property of MRPs prepared with different sugar/amino acid as well as with different reactant concentrations is depicted in Figure 14. With higher reactant concentrations (0.75 mM), MRPs exhibited higher copper chelating property. Among all systems tested, 0.75 mM galactose/glycine model system showed the greatest efficiency in copper chelating. MRPs might contain compounds such as reductones, maltol (Eichner, 1981), melanoidin (Martins and Van Boekel, 2003), which are possible to bind with metal ions (Johnson *et al*., 1983; Wijewickreme *et al*., 1997). Seifert *et al*. (2004) reported that MRPs from proteins and carbohydrate complexes strongly with Cu(II). The formation of these compounds generally occurred during heating of different sugars and amino types (Wijewikreme

et al., 1997). Similar results were obtained to those of reducing power. It was suggested that MRPs with reducing power might have the copper chelating property.

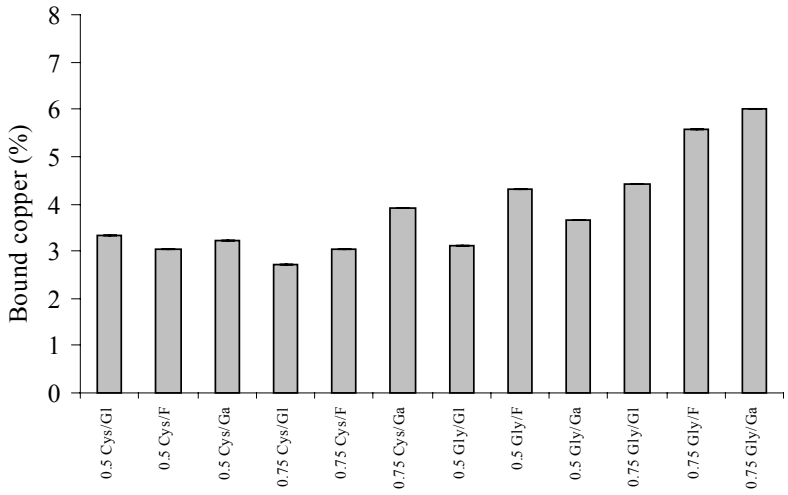


Figure 14. Copper chelating property of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

Changes in PO inhibitory activity

The inhibitory activity of MRPs prepared from different amino acids (L-cysteine, glycine) and reducing sugars (glucose fructose and galactose) heated at 100ºC for 8 h is shown in Figure 15. It was found that the MRPs, derived from equimolar of 0.75 mM galactose/glycine significantly reduced the PO activity by 39% inhibition. MRPs derived from all systems used also showed the PO inhibitory activity. This was possibly accompanied by the ability to chelate the copper ion, which is located in the active site of PO. PO was more inhibited by MRPs from galactose than fructose and glucose ($p<0.05$) when the reactant concentration of 0.75

mM was used. However, no differences in PO inhibitory activity were noticeable when glycine concentration of 0.5 mM was used. For cysteine and MRPs derived from cysteine, it was noted that cysteine showed the high PO inhibitory activity than MRPs derived from cysteine/sugar model system with the reaction concentration of 0.5 mM. No differences were obtained between inhibitory activity of cysteine and MRPs derived from cysteine/sugar at the reactant concentration of 0.75 mM. Thus, the inhibitory activity of cyteine alone was higher than MRPs prepared from cysteine/sugars. Tan and Harris (1995) found that MRPs from cysteine/glucose heated for 1 h appeared to be the most effective inhibitor and inhibitory activity of MRPs from cysteine/glucose slightly decreased after 3 h. From the result, the increase in browning intensity and intermediate compounds exhibited the increase in reducing power, copper chelating and PO inhibitory activity. Also, the concentration and ratio of amino acids/sugars mixture had the influence on the inhibitory activity of PO.

Although, the MRPs derived from galactose/glycine was more effective than fructose/glycine, it is not practical to use because of their high cost. Therefore, MRPs prepared from fructose was chosen for further study.

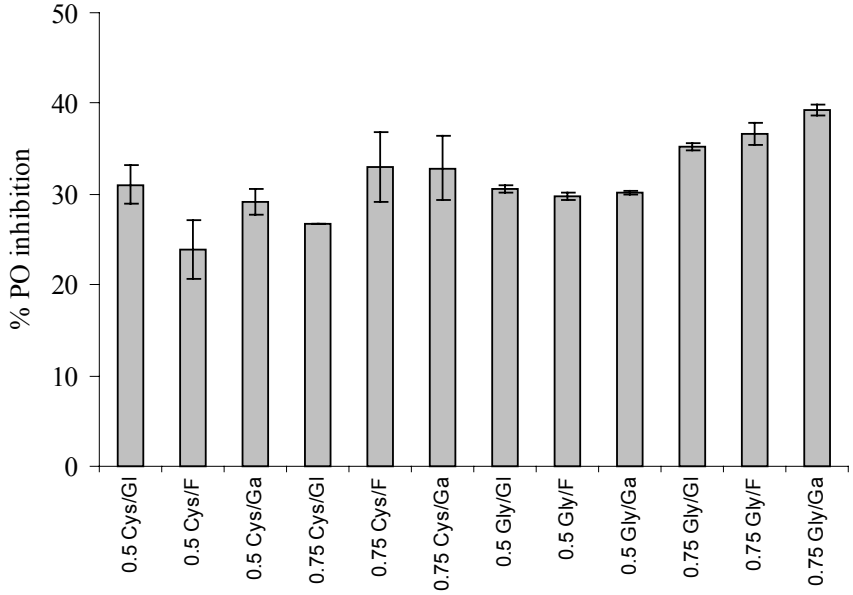


Figure 15. Inhibitory activity towards black tiger prawn PO of MRPs derived from various amino acids and reducing sugars at the concentrations of 0.5 or 0.75 mM heated at 100°C for 8 h. Cys; Cysteine, Gly; Glycine, Gl; Glucose, F; Fructose, Ga; Galactose. Bar indicated the standard deviation from triplicate determinations.

8. Effect of heating times on the characteristics and PO inhibitory activity of fructose/glycine model system

Changes in pH

No marked differences in pH of MRPs prepared by heating for different times at 100ºC were observed (Fig. 16). The pH values remained constant at pH 8 throughout the heating up to 12 h, suggesting the high buffering capacity of the system used. However, Carabasa-Giribet and Ibarz-Ribas (2000) found that MRPs derived from fructose/glycine system had the decrease in pH with increasing heating time.

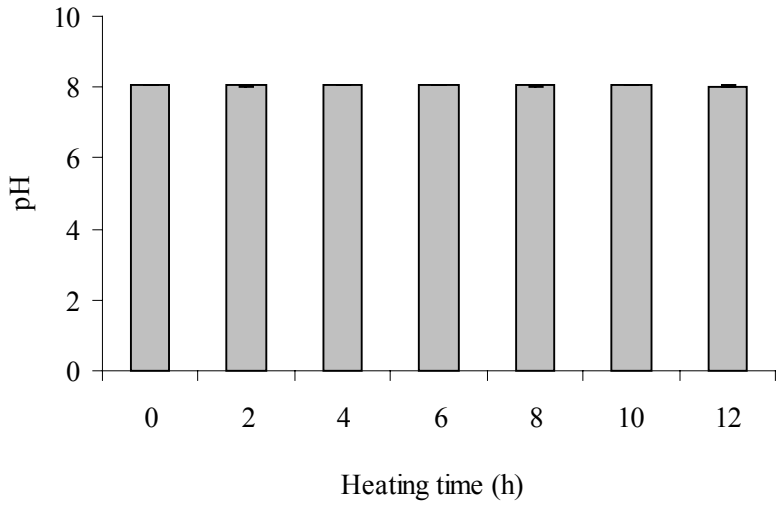


Figure 16. Changes in pH of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

Changes in fluorescence intensity

Fluorescence intensity of fructose/glycine MRPs increased continuously as heating time increased up to 10 h. With heating time of 12 h, fluorescence intensity of MRPs obtained markedly increased (Fig 17A). In stage prior to the formation of brown pigments, fluorescent compounds are formed (Morales *et al*., 1996). Baisier and Labuza (1992) stated that fluorescence accumulation in a glucose/lysine model system is due to the interaction between reactive reducing compounds and amines by an irreversible reaction. Fluorogens may be precursors of brown pigments (Baisier and Labuza, 1992). From the result, the intermediate products were formed to a higher extent with increasing heating time. With sufficient reaction time, glycation took place effectively and the intermediate products could be formed.

Changes in A_{294}

The gradual increase in A_{294} was observed as heating time increased up to 10 h. The substantial increase was noticeable when heating time of 12 h was used (Fig. 17B). The intermidiate compounds formed in the nonenzymatic browning reactions are detected by absorbance at 294 nm (Ajandenz *et al*., 2001). The increase in A_{294} of glucose/glycine system was reported (Lerici *et al*., 1990). From the result, it was noted that non fluorescent intermediates were formed increasingly with the extended heating times, especially for 12 h at 100°C.

Changes in browning intensity

The browning intensity gradually increased with heating time up to 6 h (Fig. 17C). No marked changes were noticeable during 6 and 10 h of heating ($p > 0.05$). Nevertheless, the sharp increase was observed at 12 h of heating. The increase in yellow-brown color was observed when the reaction proceeded. Color formation is likely due both to the formation of low-molecular-weight compounds and to the presence of melanoidins with high molecular weight (Ames, 1992). From the result, the development of brown color and intermediate products (fluorescent intensity and A_{294}) correlated very well. The result suggested that the formation of intermediate compounds, either fluorescent or non-fluorescent was prerequisite for brown color development. The fluorescence intensity and A_{294} were related with browning in model system containing fructose/glycine as reported by Morales and Jimnez-Perez (2001). Buera *et al*. (1987) stated that higher temperature and longer time resulted in more serious browning. However, the formation rate of intermediates was comparably higher than the browning rate.

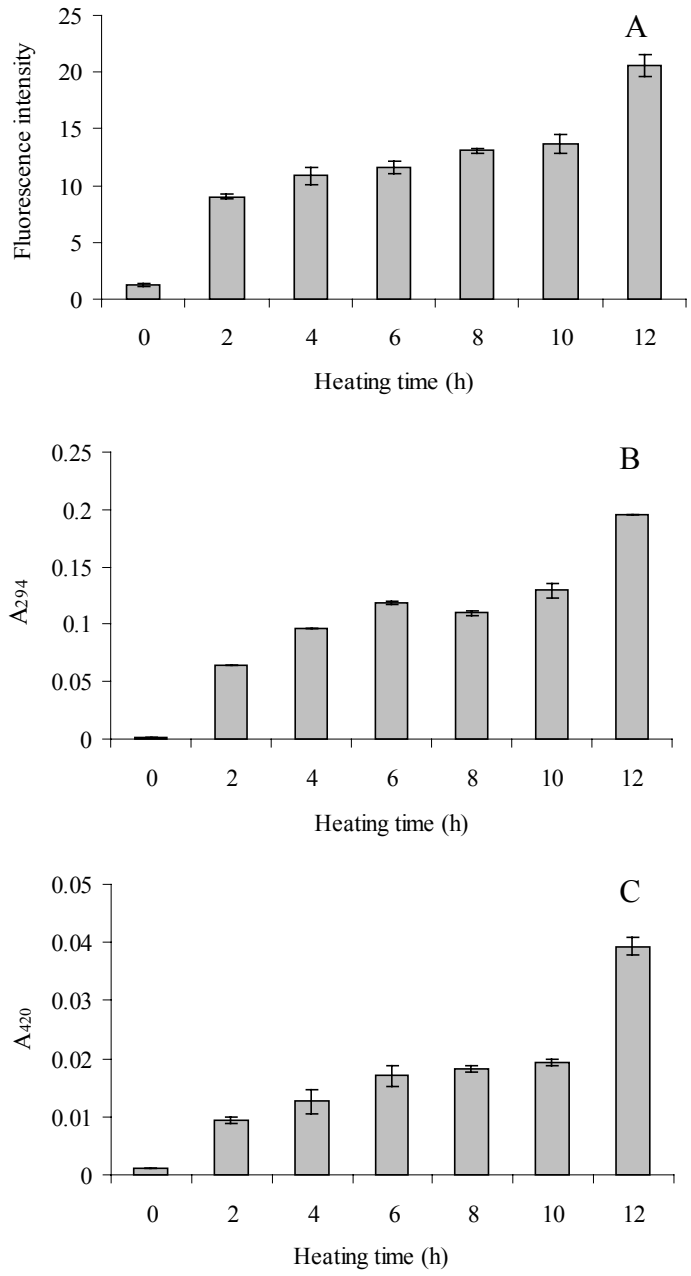


Figure 17. Changes in fluorescence intensity (A), A_{294} (B) and A_{420} (C) of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing sugar and free amino group contents

The reducing sugar loss of fructose/glycine model system was depicted in Figure.18A After 2 h of heating time, the loss of reducing sugar was 95% and increased up to 98% with heating from 4 to 12 h. However, it was noted that the loss in reducing sugar occurred at time 0. This suggested that glycation took place in the presence of glycine and fructose rapidly. The loss in free amino group in the system was observed approximately 18-20% and 26-27% at heating time 2 and 6 h, respectively (Fig. 18B). The loss of free amino group was also found at time 0, possibly caused by the interaction between the carbonyl and amino group before analysis. Glycine slowed the enhancing effect on the formation of dicarbonyl sugar fragment, leading to the degradation of sugar (Martins *et al*., 2003). Ajandauz and Puigserver (1999) reported the increase in the essential amino acid losses in glucose containing solutions heated to 100°C at pH 7.5 as the function of time.

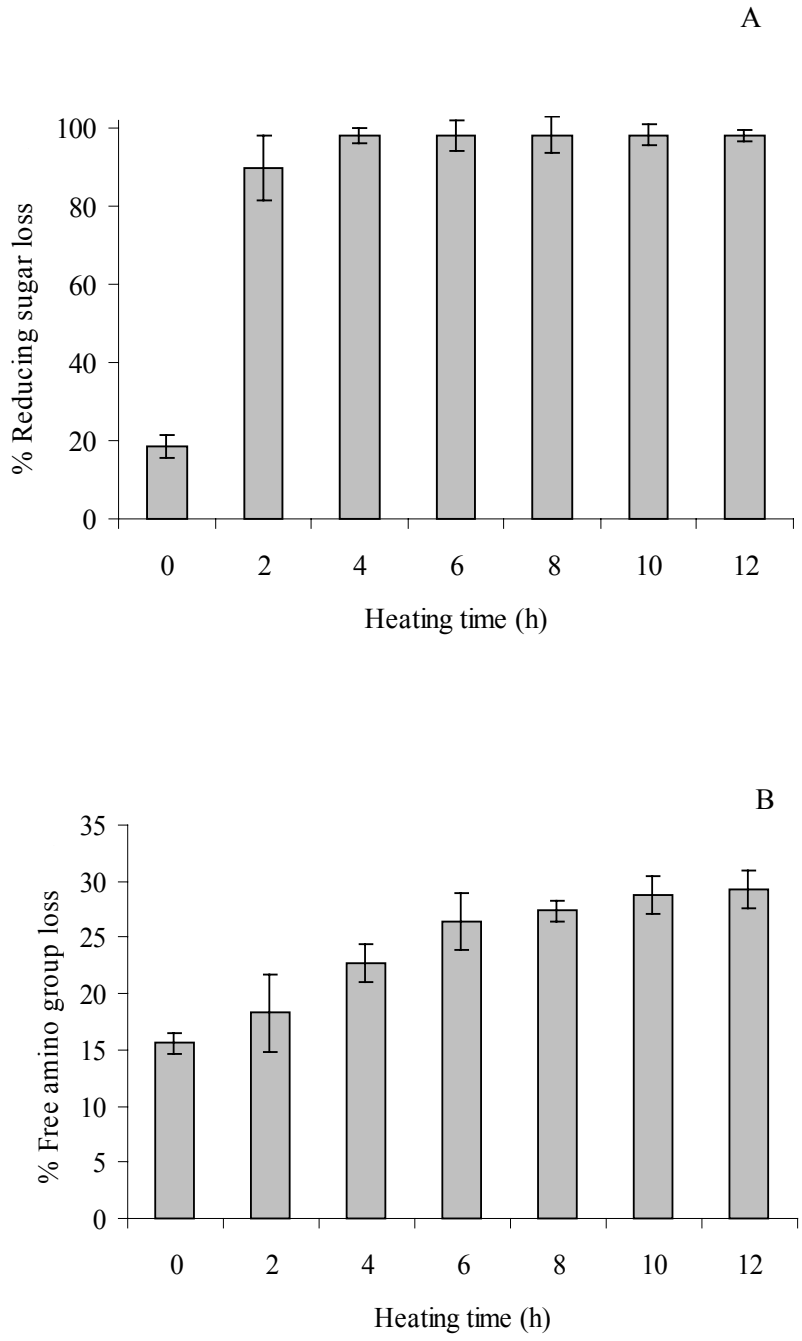


Figure 18. The loss of reducing sugar (A) and free amino group contents (B) of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing power

Reducing power of fructose/glycine model system increased with heating time (Fig 19). With a longer period of heating, a complex series of reactions took place and the products possessing the reducing power were produced. The formation of reducing compounds correlated with heating time of glucose/glycine mixture (Yoshimura *et al*., 1997). Gordon (1990) reported that the reductone is believed to break radical chains by donation of a hydrogen atom. The reducing compounds were also found in MRPs from glucose/lysine and fructose/lysine model system (Wijewickreme *et al*., 1997).

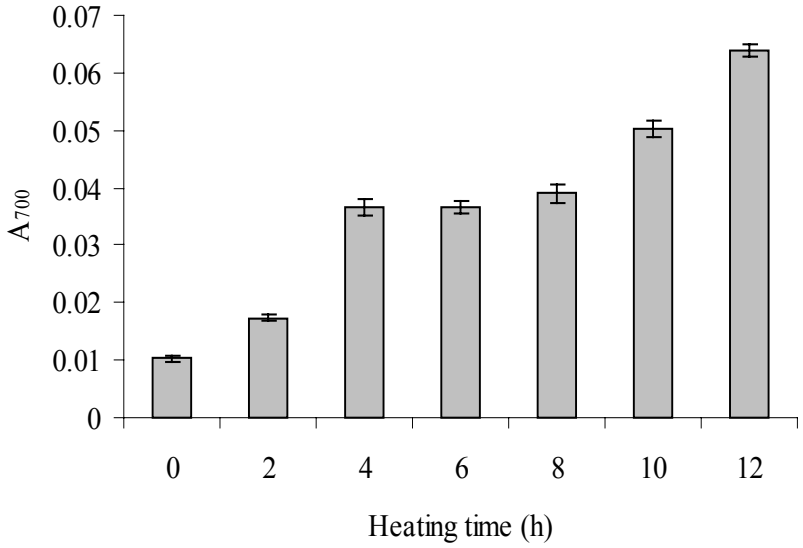


Figure 19. Reducing power of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

Changes in copper chelating property

Copper chelating activity of MRPs of fructose/glycine model system heated for various times is shown in Figure 20. Copper chelating activity of MRPs

gradually increased with increasing heating time up to 8 h. Therefore, MRPs prepared by heating for 10 and 12 had the lowered chelating activity. It was postulated that intermediate products with capability of chelating copper ion might undergo transformation to further reaction and complexed compounds formed might have the lower copper chelating activity. The Maillard reaction is not a single reaction but an extremely complex set of reaction, that produce a variety of reaction products including aroma compounds of low molecular mass, non-volatile colored compounds of intermediate molecular mass and brown substances of high molecular mass known as melanoidin polymer (Hodge, 1953). Rendleman and Inglett (1990) observed that melanoidin behave as chelating agent for polyvalent metal cation and thereby may influence the bioavailability of these ions.

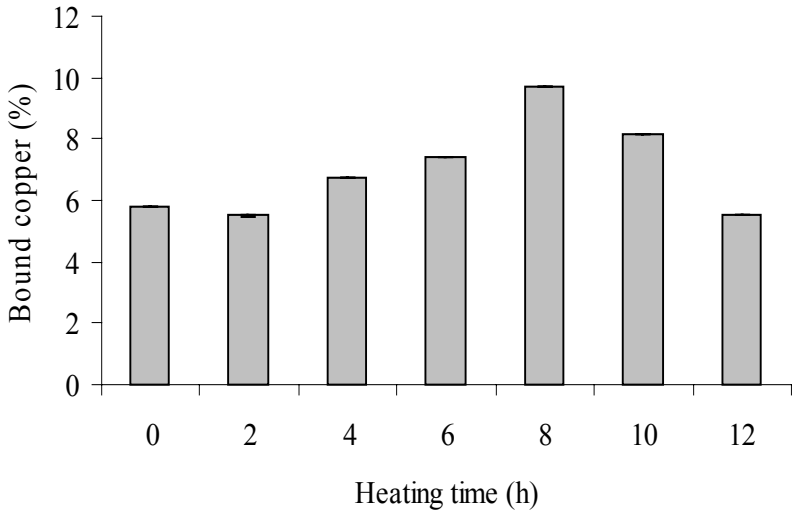


Figure 20. Copper chelating property of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

Changes in PO inhibitory activity

No changes in inhibitory activity toward PO by MRPs prepared with heating during the first 6 h were observed ($p>0.05$) (Fig. 21). The inhibitory activity increased gradually when heating time of MRPs was increased from 6 to 12 h. Approximately 35% of PO activity was inhibited by the MRPs prepared with a heating time of 12 h. MRPs has been known to be generated via the formation of heterocyclic derivatives and intermediate water-soluble compounds such as reductones, amino-reductones or pre-melanoidins (Billaud *et al*., 2003). Maillard reaction led to the formation of different chemical species having antioxidant properties, which were positively related with the development of browning (Billaud *et al*., 2003), copper chelating properties (Friedman, 1996; O'Brien and Morrissey, 1997) and donating hydrogen ion (Wijewickreme *et al*., 1997). From the result, MRPs with reducing power and copper chelating property might inhibit PO activity via reduction of *O*-quinone, resulting in the retardation of melanin formation. Since copper ion is localized in active site of PO (Jang *et al*., 2002; Zawistowski *et al*., 1991; Whitaker, 1995), complexation of MRPs with copper of PO might cause the loss in its activity.

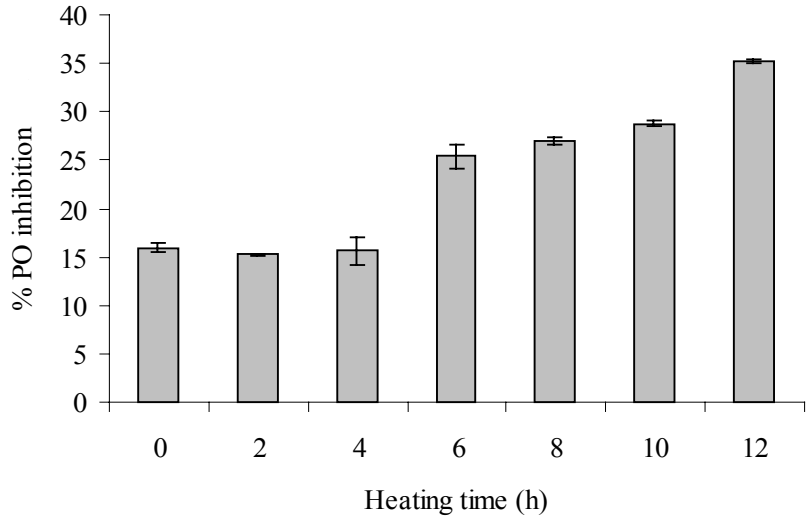


Figure 21. Inhibitory activity towards black tiger prawn PO of fructose/glycine MRPs prepared with various heating times at 100 °C. Bars indicate the standard deviation from triplicate determinations.

9. Effect of heating temperature on the characteristics and PO inhibitory activity of fructose/glycine model system

Changes in pH

The pH values of fructose/glycine system were constant regardless of temperatures used (80, 90, 100 and 110ºC) (Fig. 22). The formation of formic and acetic acid was considerably increased in Maillard reaction as the temperature increased (Morales *et al*., 1996). Martins and Van Boekel (2005) found that the formation of organic acids in the glucose/glycine increased as the temperature increased. From the result, the constant pH of MRPs might be caused by the low concentration of reactants used and high buffering capacity of system.

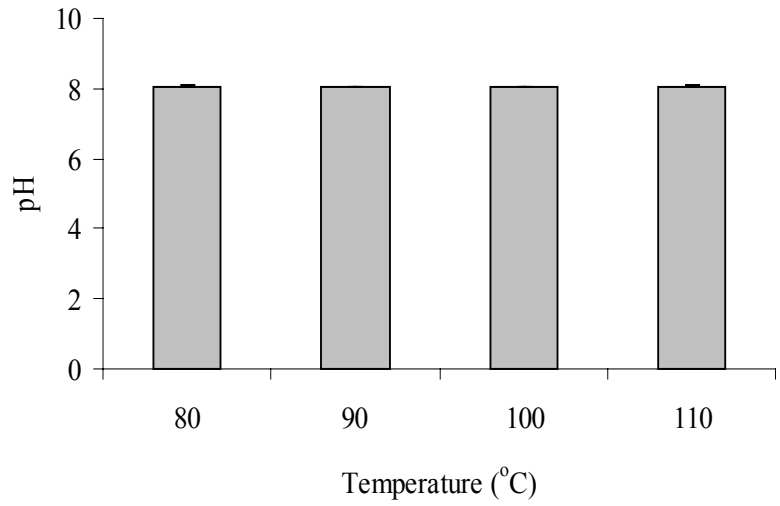


Figure 22. Changes in pH of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in fluorescence intensity

The fluorescence intensity of fructose/glycine model system increased when the heating temperature increased up to 90ºC. No marked differences in fluorescent intensity were noticeable when heating temperatures were above 90ºC ($p > 0.05$). The accumulation of fluorescent intermediary compounds was proportional to both temperatures and heating time (Fig. 23A). Morales *et al*. (1996) reported that isomerization and degradation reactions of lactose can contribute somewhat to the development of fluorescent intermediates.

Changes in A_{294}

A_{294} of fructose/glycine model system increased proportionally with increasing temperature (Fig. 23B). The result suggested that non-fluorescent intermediates were formed to a greater extent when the heating temperature increased.

Lerici *et al*. (1990) found that the kinetic rate constant (k_1) of glucose/glycine model system depended on solids concentration and temperature. In the range of 100 to 150° C, it was found that the activation energy for isomerization/degradation (about 130 kJ/mol) was higher than that for the (early) Maillard reaction (about 100 kJ/mol) (Lerici *et al*., 1990). In addition, at 130°C, the degradation of Amadori product was faster than its formation. As the temperature increased, the induction time of Maillard reaction became shorter and the kinetic constant increased (Carabasa-Giribet and Ibarz-Ribas, 2000). The differences in the formation of intermediates, both fluorescent and non-fluorescent, in the system as affected by heating temperature suggested the different intermediates were produced with different temperatures.

Changes in browning intensity

The browning intensity of fructose/glycine model system as monitored by A_{420} increased with increasing temperatures (Fig. 23C). Thus, temperature was an essential factor determining the browning development. Carabasa-Giribet and Ibarz-Ribas (2000) observed that the rates of brown pigment and colorless intermediate formation of each aspartic acid-glutamic acid and asparagine/glucose systems increased as the heating temperature became higher, and their induction times decreased with the increase in the heating temperature. From the result, browning development (A_{420}) correlated well with A_{294} rather than fluorescence intensity. It was suggested that non-fluorescent intermediates might be the important precursors for browning development, especially when the system was heated at higher temperature.

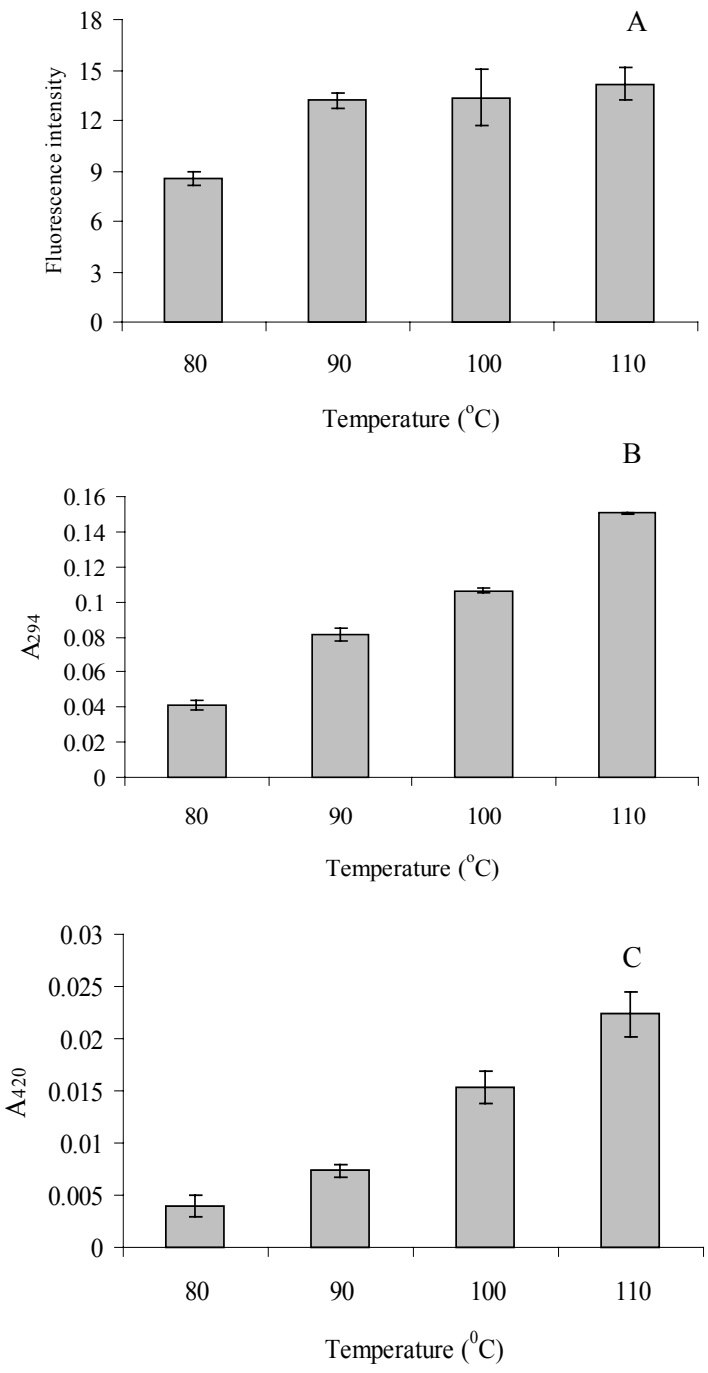


Figure 23. Changes in fluorescent intensity (A), A_{294} (B) and A_{420} (C) of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing sugar and free amino group contents

Fructose/glycine model systems obtained at different temperatures showed the increasing loss of reducing sugar and free amino group content when the heating temperature increased (Fig. 24). Nevertheless, no differences in the losses in reducing sugar and free amino group contents were found between MRPs obtained by heating at 100 and 110°C. At higher temperature, glycation might take place to a higher extent. Higher temperature might induce the chain opening of fructose and the subsequent glycation could occur more rapidly. The appearance of complex melanoidins was faster at 120° than at 100°C, which histidine and glucose model system was heated for up to 1 h (Yilmaz and Toledo, 2005).

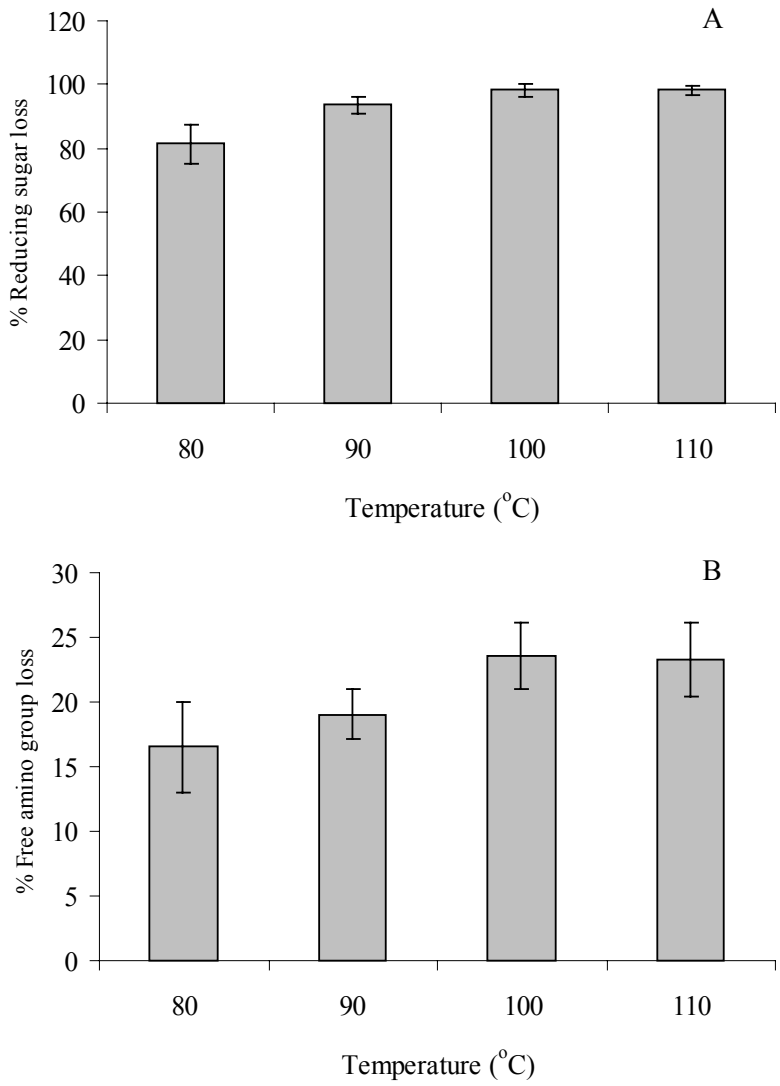


Figure 24. The loss of reducing sugar (A) and free amino group contents (B) of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing power

Reducing power of MRPs prepared with different heating temperature increased gradually as temperatures increased up to 110ºC (Fig. 25). The results indicated that reducing compounds were formed to a greater extent when the higher

temperatures were used. OH group of MRP plays a relevant role in reducing activity (Yoshimura et al., 1997). The increase in reducing power of MRPs heated at higher temperatures was in agreement with the increase in the formation of intermediates and final brown products.

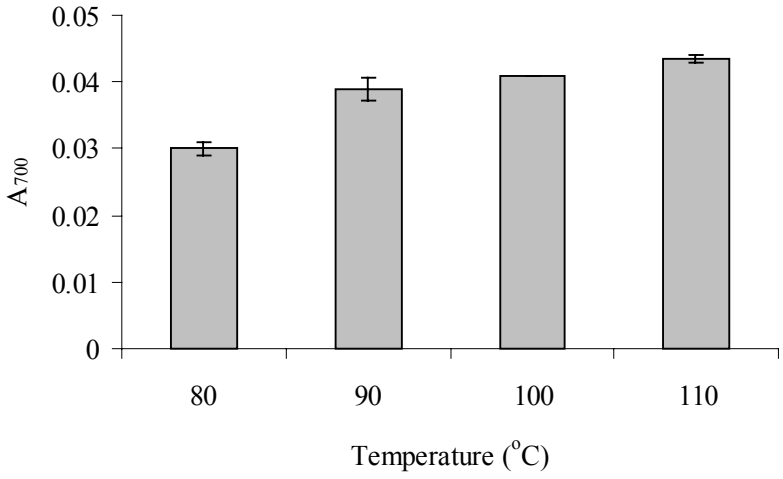


Figure 25. Reducing power of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in copper chelating property

MRPs prepared with different heating temperatures showed different copper chelating activity (Fig. 26). MRPs prepared at 100 and 110ºC exhibited the much greater copper chelating than those prepared at 80 and 90ºC. Several compounds with copper chelating activity were formed in the Maillard reaction, especially at high temperatures. Wijewickreme *et al*. (1997) found that MRPs mixture derived from glucose/lysine and fructose/lysine model systems had high metal chelating affinity. Since, intermediate compounds and melanoidin were formed to a

greater extent at 100-110ºC, those compound might function as copper chelators as evidenced by the greater binding activity (Fig. 20).

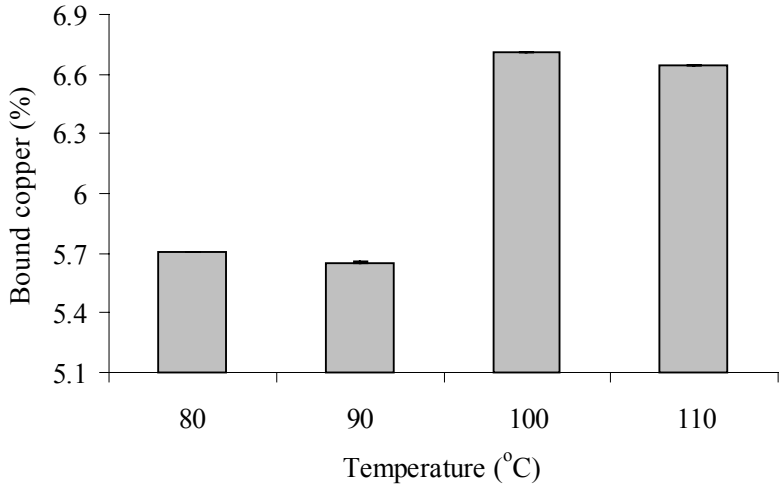


Figure 26. Copper chelating property of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in PO inhibitory activity

Inhibitory activity towards PO of MRPs increased continuously when the system was heated up to 100ºC ($p<0.05$). However, no further increase in activity was noticeable when system was heated at 110ºC ($p>0.05$). PO activity was inhbited by 40, 35 and 37% when MRPs prepared by heating at 100, 80 and 90ºC were used, respectively (Fig. 27). High inhibitory activity of MRPs prepared at 100ºC might be caused by the high copper chelating activity and high reducing power (Fig. 25). From the result, the compounds with PO inhibitory activity could be produced by the induction of sufficient energy.

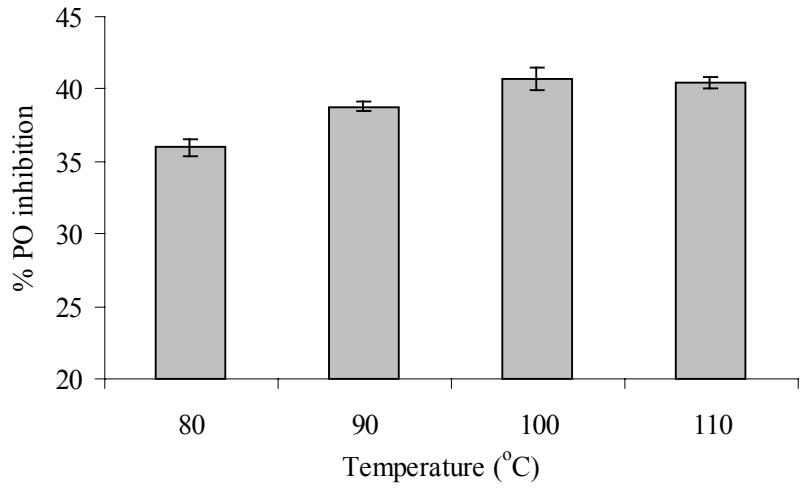


Figure 27. Inhibitory activity towards black tiger prawn PO of fructose/glycine MRPs prepared with various heating temperatures for 12 h. Bars indicate the standard deviation from triplicate determinations.

10. Effect of reactant concentration on the characteristics and PO inhibitory activity of fructose/glycine model system

Changes in pH

The pHs of fructose-glycine model system with different reactant concentrations are shown in Figure 28. The reaction with an initial pH of 8.0 was allowed to proceed without pH control for 12 h at 100 ºC. At low reactant concentrations (0.75-4.5 mM), only slight decrease in pH was observed. The greater pH decrease was found with the system having reactant concentration above 4.5 mM, particularly at 30 mM. Benjakul *et al*. (2004) also observed the decrease in pHs of MRPs from PPP-sugars model system. Easa *et al*. (1996) reported the decrease in pH of bovine serum albumin-xylose model system. Generally, the more complexity and the variety of the MRPs were formed with high concentration of reactant, especially

with the prolonged reaction time and high temperature (Carabasa-Giribet and Ibarz-Ribas, 2000; Friedman, 1996; Morales and Van Boekel, 1998). Those compounds formed during the reaction include hydroxylacetone derivatives, glyceraldehydes and diketones. Acetic acid (Martins and Van Boekel, 2003b) as well as formic acid (Martins *et al*., 2003) were also detected as two major degradation products in Maillard reaction system containing fructose or glucose. From the results, the rate of pH decrease was greater with the higher reactant concentration. This might be due to the greater formation of acids in presence of sufficient reactants. Berg and Van Boekel (1994) also reported formic acid as the main degradation reaction product from the Maillard reaction of lactose. The decrease in pH observed during the Maillard reaction could be attributed to the reaction of amines to form compound with lower basidity (Beck *et al*., 1990). Moreover, the condensation between free amino of amino acid and carbonyl groups of glucose might contribute to the lowered pH. (Martins *et al*., 2003).

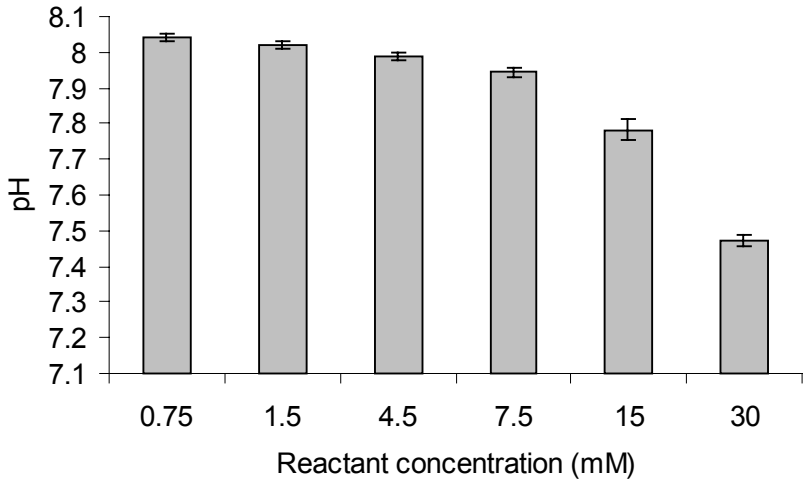


Figure 28. Changes in pH of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in fluorescence intensity and A_{294}

The fluorescence intensity of fructose/glycine model system increased with increasing reactant concentrations (Fig. 29A). However, the lowered fluorescence intensity was noticeable at a concentration of 30 mM. This might be due to transformation of intermediate to polymer compounds in presence of reactant at high concentration. The fluorescence and browning developments in Maillard reaction are generally used as an indicator of reaction rate and MRPs formation (Yeboah *et al*., 1999). The result was in agreement with Benjakul *et al*. (2004) who reported the increase in fluorescence intensity when the concentration of glucose increased from 1% to 2%. Fluorescent compounds formed prior to the generation of brown pigments are possible the intermediate precursors of brown pigments (Morales *et al*., 1996; Labuza and Baisier, 1992). The concentration and ratio of reactants also have significant impact on the reaction (Baisier and Labuza, 1992; Lerici *et al*., 1990). Consequently, in the plenty of reactants, fluorescent intermediate compounds were formed easily and effectively under such a condition.

Continuous increase in A_{294} was observed as the concentration of reactants increased (Fig. 29B). A_{294} is used to detect the colorless compounds formed at intermediate stage (Ajandouz *et al*., 2001). The result suggested that intermediate products might be formed to a greater extent with the higher reactant concentrations. Benjakul *et al*. (2004) reported that sugars-PPP system containing the greater reactants exhibited the higher A_{294}. The mechanism and the rate of the Maillard reaction depends on the kind of sugar and protein used as well as the ratios (Morales *et al*., 1996). The different intermediate products were formed, either fluorescence or non-fluorescence compounds during the Maillard reaction (Benjakul *et al*., 2004).

From the result, it was postulated that some intermediate products might undergo the conversion to the final brown compounds, which some were still generated using both reactants.

Changes in browning intensity

A_{420} of fructose/glycine model system increased with increasing concentration of reactants (Fig. 29C). The most commonly used indicators of browning pigment is spectrophotometric measurement at 420 nm (Lerici *et al*., 1990). The increase in A_{420} indicates the development of browning pigment in final stage of Maillard reaction. The reaction consists of the condensation of amino compound and sugar fragments into polymerised protein and brown pigment, called the melanoidin (Van Boekel, 1998). The increase in brown pigment development was coincidental with increase in colorless intermediate formation as evidenced by the increased in A_{294} and fluorescence intensity. It suggested that brown pigments were formed proportionally with the intermediate products generated. At 30 mM reactant concentration, fluorescent compound might convert to the final brown pigments to a greater extent, leading to the lower fluorescent remained (Fig. 29A).

A_{294}/A_{420} ratio of fructose/glycine model system varied with the reactant concentration (Fig. 30). Highest ratio was found with the system containing 0.75 mM, whereas the ratio became lowest with the highest reactant concentration (30 mM). It was suggested that the intermediates were strongly polymerised at higher reactant concentration as evidenced by the lower ratio. However, no differences in ratio were observed when the reactant concentration ranges of 1.5-7.5 mM were used. Thus, the colour formation due to both sugar caramelization and Maillard reaction (Ajandouz *et al*., 2001) was dependent on the reactant concentration.

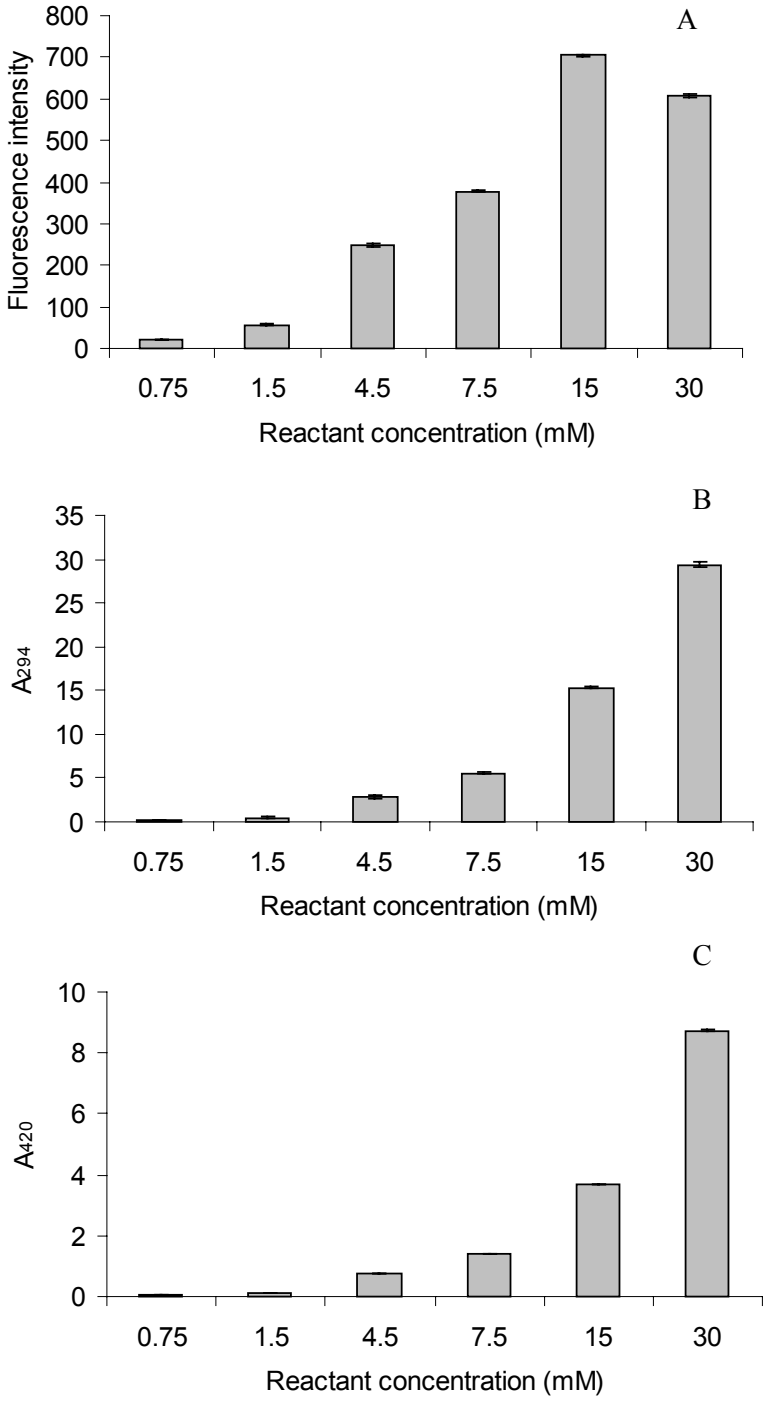


Figure 29. Changes in fluorescent intensity (A), A_{294} (B) and A_{420} (C) of fructose/glycine MRPs prepared with various concentrations for 12 h. Bars indicate the standard deviation from triplicate determinations.

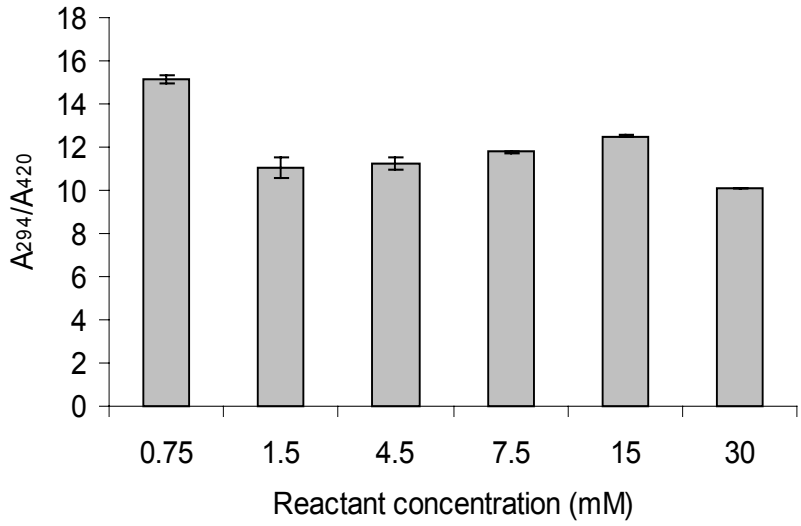


Figure 30. Changes in A_{294}/A_{420} of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing sugar and free amino group content

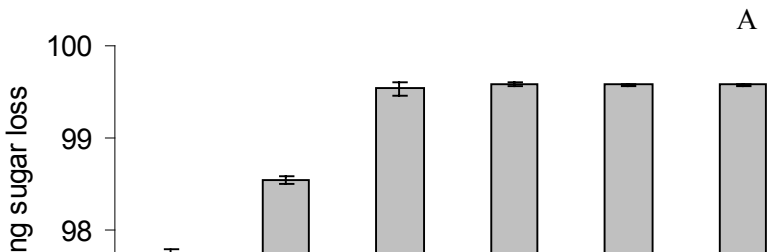
The non-enzymatic browning reaction was also monitored by measuring the extent of fructose degradation. The loss in reducing sugar in the range of 97-98% was found with the reactant concentration of 0.75-1.5 mM (Fig. 31A). No differences in reducing sugar loss were observed at concentrations above 4.5 mM. This might be due to the limited amount of sugar for the reaction. However, conversion of fructose to other compounds still occurred during heating especially at the greater fructose concentration as evidenced by the greater intermediate or browning development. Ajandouz *et al*. (2001) repoted that fructose was destroyed during heating. In addition, the degradation of sugar contributed to the caramelisation, which occurs simultaneously with the Maillard reaction (Buera et al., 1987). For

fructose, which contains a high concentration of the acyclic form, the catalytic effect of the amino acid is less important. Thus, the development of browning caused by caramelisation of reducing sugar also occured (Ajandouz *et al*., 2001).

At low concentrations (0.75-7.5 mM), the loss of free amino group in the range of 5-10 % was found (Fig. 31B).The greater loss of free amino group was noticeable with increasing reactant concentrations. The initial reaction of the Maillard process involves the formation of a shiff's base between sugar and amino acids. This reaction is reversible, however, the subsequent rearrangement is not reversible (Baxter, 1995). Baxter (1995) found 32 % loss of histidine in presence of fructose after 126 weak of storage at 50 °C.

In the early stage of the Maillard reaction, The reducing sugar condences with a compound prossessing a free amino group to give a condensation product, N-substituted glycosylamine (Friedman, 1996), via the formation of a Schiff's base and the Amadori rearrangement (Van Boekel, 1998). At pH>7, the degradation of Amadori compound is thought to involve mainly 2, 3-enolisation, where reductones, including acetol, pyruvaldehyde, and diacetyl are formed. All these compounds are highly reactive and take part in further reactions (Martins and Van Boekel, 2003b). Apparently, initial reaction products, once formed, undergo the rapid subsequent Maillard process rearrangement, leading to loss of amino groups. The small amount of remaining sugar was enough to cause considerable browning, and loss of susceptible free amino groups under stringent condition (Ajandouz *et al*., 2001). From the result, the reaction might proceed rapidly at pH higher than 7, particularly with the greater reactant concentration as shown by the increased loss in both reducing sugar and free amino groups. The reaction rate of sugar depends on the

acyclic form (Naranjo *et al*., 1998). This is considered that the concentration of open chain form might be a crucial factor to generate the other form and to condence with α- amino or ε-amino group to give further reactions. Fructose is able to form C2- and C3- fragments described as redical precursor which could be formed in the early stage of the Maillard reaction by retroaldol reaction from glycosylamines or Amadori compound. In addition, fructose can react at higher rate in basic condition known as the rapid enolisation reactions (Cammerrer and Kroh, 1996). This led to the decrease in free amino group and reducing sugar, which was in accordance with the increase in browning (Fig. 23C) A_{294} (Fig. 23B) and fluorescence intensity (Fig. 23A), especially at the higher fructose and glycine concentrations.



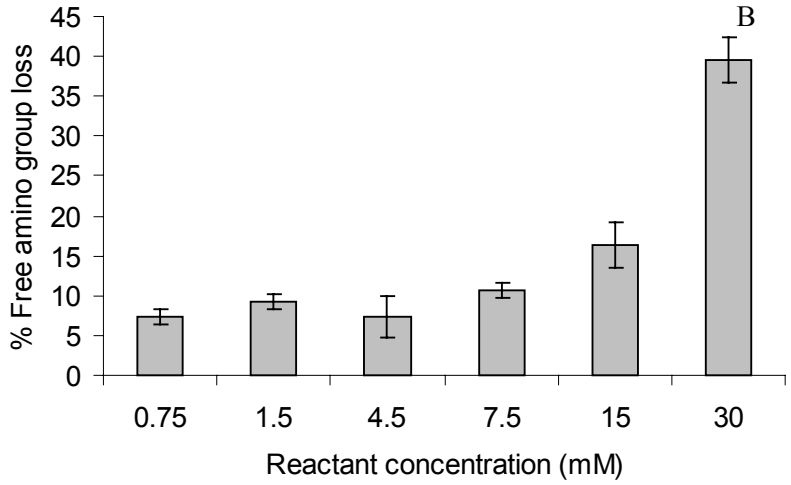


Figure 31. The loss of reducing sugar (A) and free amino group contents (B) of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing power

The reducing power of fructose/glycine system as monitored by A_{700} increased with increasing concentrations of reactants (Fig. 32), suggesting the correlation between the formation of reducing compounds and the reactant concentrations. However, no differences in reducing power were observed when reactant concentrations ranged from 0.75 to 1.5 mM. Yoshimura *et al*. (1997) found

that the reducing power increased linearly with increasing time of glucose/glycine mixture. The reaction between reducing sugars and amino acids or proteins produces the strong reducing materials such as amino reductones (Hodge, 1953), which is the key intermediate of the Maillard reaction. The intermediate compounds of MRPs were reported to be capable of donating hydrogen atom (Yen and Hsieh, 1995). In addition, the MRPs are known to possess the scavenging activity on active oxygen species (Kato, 1992). From the result, reducing power was correlated with browning intensity as well as the intermediate product formation (Fig. 29A and 29B).

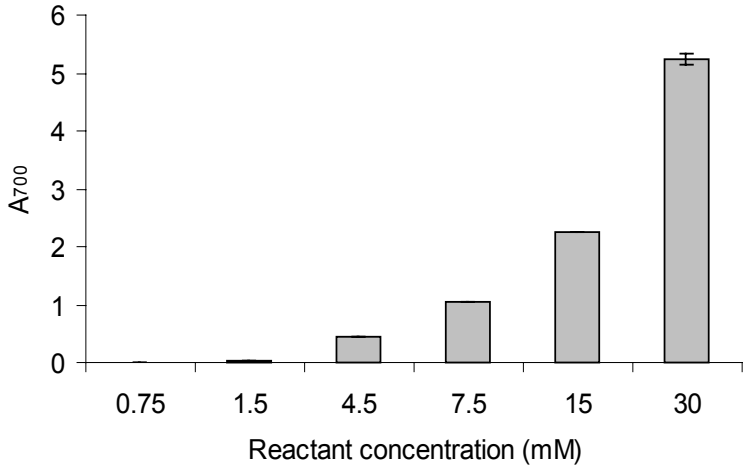


Figure 32. Reducing power of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in copper chelating

Copper chelating property of MRPs prepared with different reactant concentrations is depicted in Figure 33. With low reactant concentrations (0.75-1.5 mM), MRPs exhibited low copper chelating property. On the other hand, much

greater chelating property was observed with higher reactant concentration. Therefore, efficiency of copper binding was dependent on the reactant concentration in reaction medium. The key intermediate of the early stage of the Maillard reaction is the Amadori rearrangement product, which is a type of amino reductone that has chelating, reducing and oxygen scavenging properties (Tan and Harris, 1995; O'Brien and Morrissey, 1997; Wijewickreme *et al*., 1997). Rendleman and Inglett (1990) reported that Cu^{2+} ions have the ability to form strong complexes with various model melanoidins from glucose/glycine mixtures (Wijewickreme *et al*., 1997). Therefore, MRPs from fructose/glycine model system showed the copper chelating property, particularly when the high concentration of reactants was used.

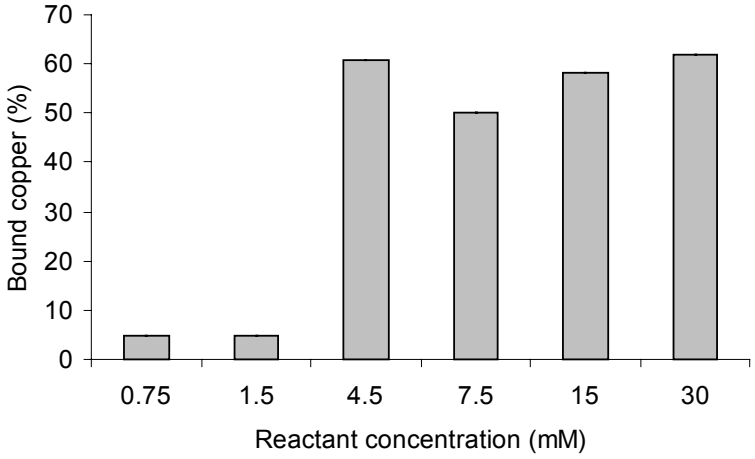


Figure 33. Copper chelating property of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

The inhibitory effect of MRPs on PO activity

MRPs with various reactant concentrations showed different inhibitory activity towards PO (Fig. 34). The inhibition of PO activity by MRPs with reactant concentration of 0.75-7.5 mM was about 35%. PO activity was inhibited by 55 and 80 % when MRPs with 15 and 30 mM reactant concentrations were used, respectively. Lee and Park (2005) found that the inhibitory activity of MRPs from glucose/glycine system against PO from potato increased as the amount of glucose increased. From the result, PO inhibitory activity was coincidental with browning development and intermediate formation (Fig. 29). The reductone moiety present in the melanoidin structure has been reported to exhibit both reducing, chelating properties and scavenging properties (Namiki, 1988).

PO from crustacean has been known to contain Cu^{2+} in the active site (Kim *et al*., 2000; Jang *et al*., 2003). The subunit folds into three domains. Domain 2 contains the binuclear copper site responsible for binding and transporting O_2 in the lobster (Gaykema *et al*., 1984). Each of the two coppers is liganded to three histidine residues. The compounds in MRPs possessing the copper binding ability might form the complex with Cu^{2+}in the active site, leading to the loss in PPO activity. Billaud *et al* (2003; 2004); Roux *et al*. (2003) demonstrated that MRPs from glucose, fructose and cysteine inhibited the apple PO activity. Also, Brun-Merimee *et al*. (2003) reported that MRPs prepared from glucose or fructose with glutathione were recognized as strong apple PO inhibitors. Additionally, Tan and Harris (1995) found that MRPs from various amino acid and glucose inhibited apple PO. Nicoli *et al*. (1991) reported that MRPs obtained by heating glucose/glycine solution caused a strong inhibiting on apple PO. A reductone generated during Maillard reaction, retards enzymatic browning by reducing Cu^{2+} to C^{+} in the PO (Tan and Harris, 1995).

Furthermore, the reducing power of MRPs indicated the ability to reduce quinone to DOPA, resulting in the retardation of browning development in assay mixture. Thus, MRPs from fructose/glycine system especially with greater reactant concentrations could be used as the novel PO inhibitor in the crustacean.

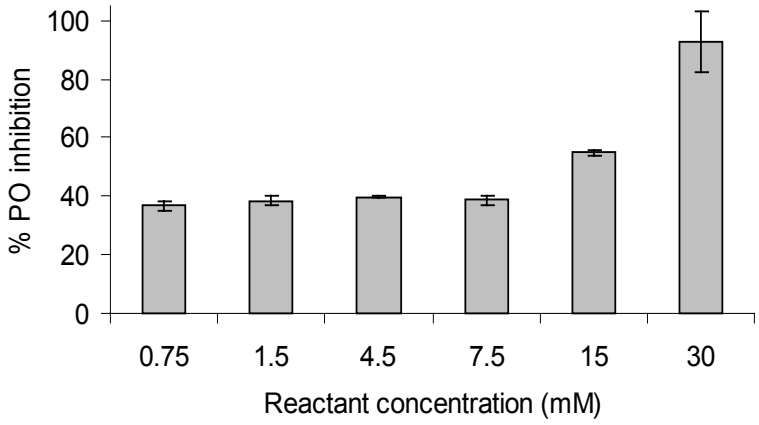


Figure 34. Inhibitory activity towards black tiger prawn PO of fructose/glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

11. Effect of pHs on the characteristics and PO inhibitory activity of fructose/glycine model system

Changes in pH

The effect of initial pHs on the final pH of fructose/glycine model system heated at 100ºC for 12 h is shown in Figure 35. Generally, the pH values of system was lower than the initial pHs. The greater decreases in pH were noticeable with system having the very alkaline pH. Van Boekel and Brands (1998) reported that formic acid and acetic acid were two main degradation products of Maillard reaction. The formation of formic and acetic acids was particularly favored at very alkaline

pHs. Regardless of reaction condition, acetic acid was always formed at a higher amounts than formic acid (Martins and Van Boekel, 2005). Martins (2003) also suggested that under alkaline conditions, Amadori products could generate acetic acid as well as formic acid, leading to the decrease in pH of system. Carabas-Giribet and Ibarz-Ribas (2000) stated that the decrease in pH observed during the Maillard reaction could be attributed to the reaction of amines to form compounds of lower basidity and to the degradation into acids. Additionally, the decrease in pH was due to the condensation between free amino group and carbonyl groups of amino acid and sugar, respectively.

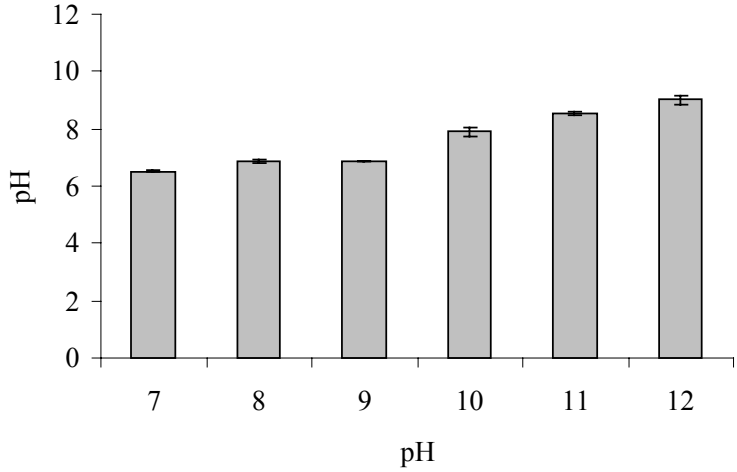


Figure 35. Changes in pH of fructose/glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in fluorescence intensity

Fluorescence intensity of MRPs decreased as the initial pHs increased (Fig. 36A). Maximal fluorescent intensity was found at pH 7. The lower in fluorescence intensity at alkaline pHs might be due to the greater formation of final

brown compounds from the fluorescent intermediates. Fluorescent compounds are intermediates in a very complex reaction (Morales *et al*., 1996). The pH of the system significantly influences the reaction rate (Ashoor and Zent, 1984). At an early stage of reaction, the protein containing free amino groups, such as the ε-NH_2 groups of lysine and arginine react with carbonyl groups of sugars to form a reversible Shiff base, which rearranges to stable, covalently bonded Amadori product. In the Advanced phase of reaction, Amadori products undergo further transformation to fluorescent substances, and cross-linked polymer (Morales *et al*., 1996; Van Boekel, 1998). However, fluorescence intensity of MRPs prepared at pH 12 was higher than MRPs prepared at pH 11, which had the lowest value.

Changes in A_{294}

The higher A_{294} was generally observed in MRPs with alkaline pH, compared with that of MRPs having the initial neutral pH (Fig. 36B). However, differences in A_{294} were found among MRPs from different systems with different initial pHs. Among all samples prepared at alkaline pHs, MRPs with the initial pH of 11 showed the highest A_{294}. MRPs with the initial pH of 12 had the lower A_{294} than those with initial pH of 11. This might be due to the greater formation of colorless intermediates from glycated proteins at alkaline pH. Martins *et al*. (2003) found that an increase in pH seems to favor the formation of 1-deoxyosone in glucose/glycine model system and pH had more influence than temperature on the reaction products formed. In general, the optimum pH for the Maillard reaction is between pH 8 and 10 (Labuza and Baisier, 1992). From the result, the increase in A_{294} was in accordance with the decrease in fluorescence intensity at alkaline pH. This suggested that the formation of non-fluorescent intermediates were favored at alkaline pHs.

Changes in browning intensity

The greater in A_{420} was observed in MRPs with initial alkaline pHs (Fig. 36C). However, browning intensity varied with pHs. From the result, the highest browning intensity was formed with MRPs having the initial pH of 11.0. Maillard reaction normally proceeds faster when the pH increases. The Amadori products undergo further transformation to fluorescent, color substances, and cross-linked polymer (Van Boekel, 1998), leading to release the H^+, which interferes Maillard reaction. Therefore, the reaction approached to the lower rate (Martins *et al*., 2003). The development of the MRPs can be affected by type of buffer (Rizzi, 2004). Davidek *et al*. (2002) stated that phosphate was shown to accelerate the degradation of Amadori compounds into an amino acid and the parent sugar. Ashoor and Zent (1984) found that browning rates in fructose/glycine and glucose/glycine systems were maximum at pH 10. In the pH range between 3.4 and 7.7, the browning in starch glucose-lysine mixture increased with the increasing pH and the browning rate between L-leucine and D-glucose was positively correlated with pH (Shen and Wu, 2004). Rizzi (2004) found that large A_{420} changes were consistently induced by phosphate at nearly constant pH, suggesting that phosphate was acting independently of hydrogen and/or hydroxide ion concentration to influence browning. For this study, 0.05 M phosphate buffer was used in the reaction medium. The increase in browning formation was not detected at the concentration of 0.05 M phosphate buffer (Bell, 1996). Bell (1996) observed that the rates of browning pigment formation and glycine loss increased with increasing phosphate buffer concentration in glucose/glycine model system at 25°C, pH 7. Therefore, pHs of model systems

directly affected the Maillard reaction as evidenced by different rate of intermediate formation and the transformation of intermediate to final products.

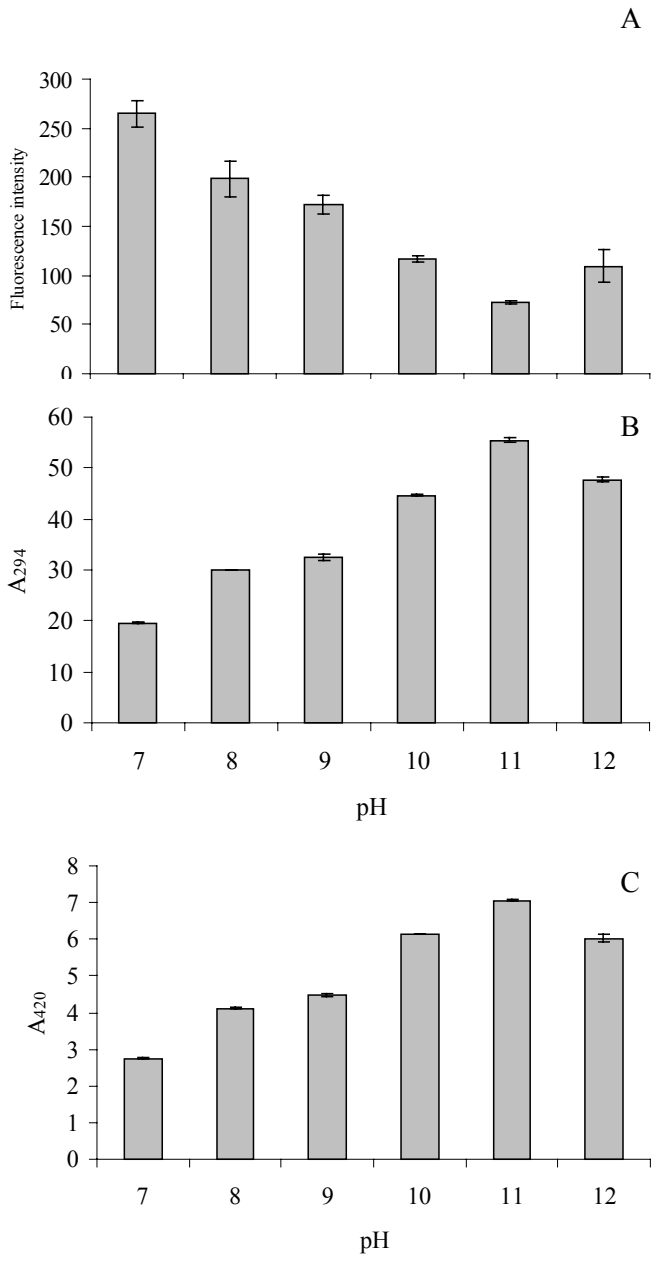


Figure 36. Changes in fluorescence intensity (A), A_{294} (B) and A_{420} (C) of fructose-glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing sugar and free amino group loss

The reducing sugar loss in fructose/glycine model system with different initial pHs ranging from 7 to 12 is given in Figure 37A. The increase in reducing sugar loss were observed with increasing pH. The high levels of destruction of sugar occurred at pH values above 9. Loss in sugar was not different at pH 11 and pH 12 ($p>0.05$). No marked differences were found among samples with initial pHs of 7 to 9. This is in agreement with Yang and Montogomery (1992) who reported that high glucose degradation at 100ºC was found under alkaline condition. The loss in free amino group as affected by initial pHs is shown in Figure 37B. The free amino group content decreased by 50% at pH 7. Generally, alkalinity favored the Maillard browning reaction. Lysine degradation was rapid in the early stage of heating, and slowed down to a no-loss period (Baisier and Labuza, 1992). Van Boekel and Martins (2002) reported the loss of glycine at higher temperature, problably due to the formation of Strecker reaction products. While the temperatures were from 80 to 100° C, the loss of glycine could account for the formation of Amadori product and melanoidins. From the result, the loss of free amino group was lower than that of sugar. The result was in agreement with Ajandouz and Puigserver (1999) who reported that hexose was destroyed more rapidly than amino acid at all of the pH values tested.

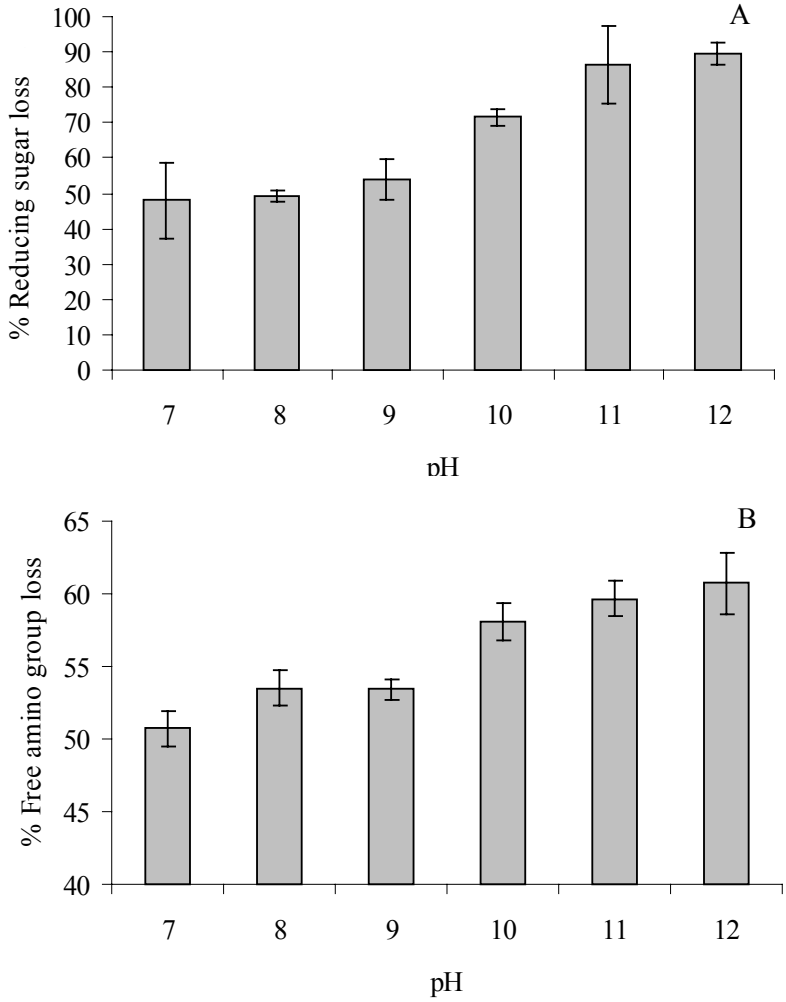


Figure 37. The loss of reducing sugar (A) and free amino group contents (B) of fructose/glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in reducing power

MRPs with different initial pHs showed the varying reducing power. MRPs with initial pHs of 11 showed the highest reducing power (Fig 38). Generally, reducing power of MRPs increased with increasing initial pHs. Nevertheless, a slight decrease in A_{700} was found at pH 12. The reducing agents such as reductone (Shimada

et al., 1992) are believed not only to react directly with peroxides but also to prevent peroxide formation by reacting with certain precursors (Mastrocola and Munari, 2000). From this result, the increase in reducing power correlated well with the increase in browning intensity and A_{294} and the decrease in fluorescence intensity. Thus, both non-fluorescent intermediate and brown compounds might have the reducing power. The result revealed that MRPs from fructose/glycine model system especially with high initial pH had hydrogen donating activity.

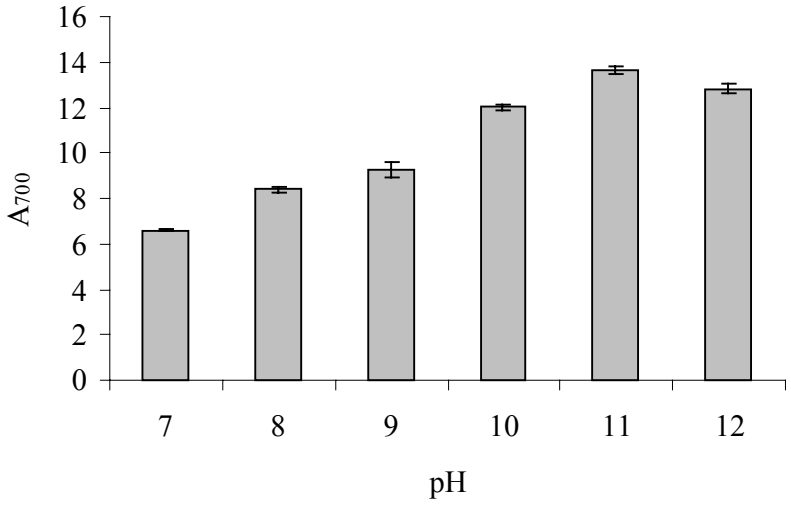


Figure 38. Reducing power of fructose/glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in copper chelating property

MRPs from fructose/glycine model system with different initial exhibited the different copper chelating as shown in Figure 39. Generally, MRPs with very alkaline pH (pHs 10-12) had the greater copper chelating property. The highest copper chelating property was observed in MRPs with initial pH of 11. Yoshimura *et*

al. (1997) reported that the high molecular weight fraction obtained from MRP were stronger in metal chelating capability than low molecular weight fraction. O'Brien and Morrissey (1997) found that MRPs were capable of binding metal ions differently (Mg^{2+} >Cu^{2+} =Ca^{2+} >Zn^{2+}). The great copper binding capacity of MRPs with the initial pH of 11 might be associated with the formation of non-fluorescent intermediates (Fig. 36B) as well as the formation of brown pigment (Fig. 36C). Therefore, the compounds with copper chelating property were formed to a greatest extent at pH 11.

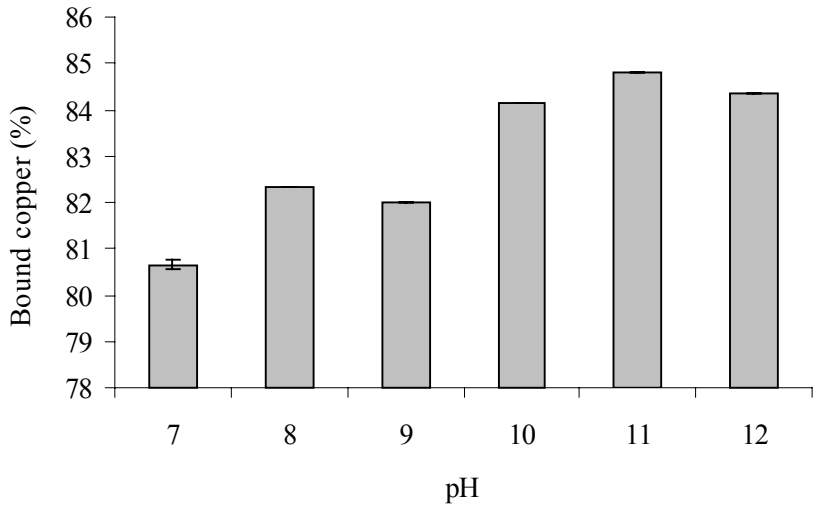


Figure 39. Copper chelating property of fructose/glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

Changes in PO inhibitory activity

PO inhibitory activity of MRPs with different initial pHs varied (Fig. 40). The highest inhibitory activity was found with MRPs having the initial pHs of 11

and 12. The inhibitory activity was in agreement with copper chelating property (Fig. 39) as well as reducing power (Fig. 38). It was most likely that the MRPs formed might inhibit PO via chelating copper ion in PO active site or the reduction of quinone.

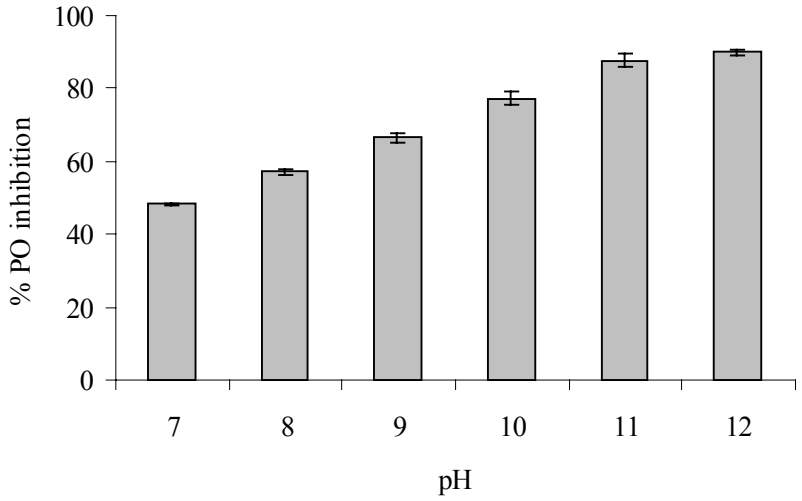


Figure 40. Inhibitory activity towards black tiger prawn PO of fructose/glycine MRPs prepared with various initial pHs at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

12. Effect of decolorization on the characteristics and PO inhibitory activity of MRPs from fructose/glycine model system

MRPs prepared by heating the equimolar of fructose/glycine (30 mM) at pH 11 and 100°C for 12 h were decolorized by Sep-Pak Cartridge C18 and activated carbon.

Color of decolorized MRPs

L*, a* and b*-values of MRPs and MRPs decolorized using activated carbon or Sep-Pak cartridge C_{18} are depicted in Figure 41. The increase in L*-value were observed in MRPs decolorized by 1%, 2% and 5% activated carbon, respectively. The L*-value of MRPs decolorized with Sep-Pak Cartridge C_{18} increased when compared with that of MRPs but was not different from that of MRPs decolorized using 1% activated carbon ($P>0.05$). Generally, the L*-value of MRPs decreased significantly during heating time, indicating the increased darkness at the advanced stage of Maillard reaction (Morales and Jimenez-Perez, 2001). The color formation is likely due both to the formation of low molecular weight compounds and to the presence of melanoidins with high molecular weight (Ames, 1992). Decolorization of MRPs generally resulted in the decrease in a*-values. The decolorization using greater amount of activated carbon resulted in the greater decrease in a*-value. The results indicated that redness of MRPs was markedly reduced after decolorization. The increase in b*-value was noticeable with MRPs decolorized using either Sep-Pak Cartridge C18 or activated carbon, suggesting the increase in yellowness of MRPs. Yen and Hsieh (1997) reported that the browning intensity of MRPs from xylose-lysine model system was lowered after decolorization with Sep-Pak Cartridge C18. Hence, binding of the brown pigments to activated carbon and Sep-Pak Cartridge C_{18} contributed to the reduced MRPs color intensity.

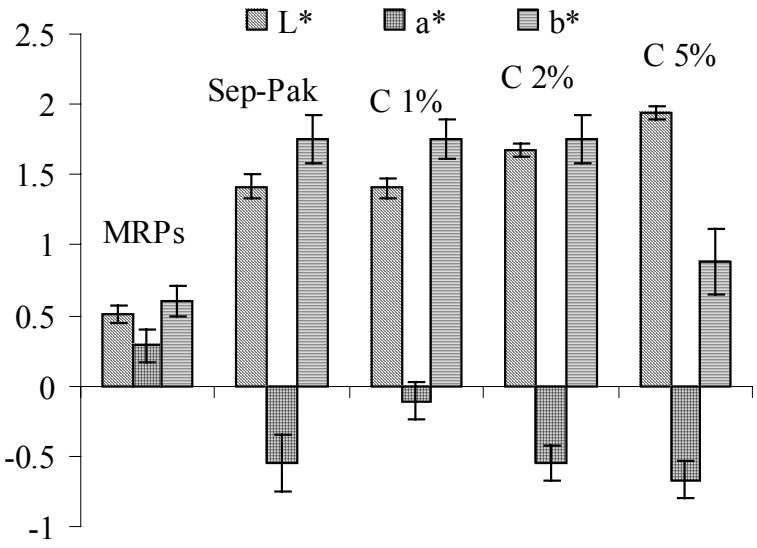


Figure 41. L*, a*, b*-values of MRPs and MRPs decolorized by activated carbon or Sep-Pak Cartridge C18. Bars represent the standard deviation from triplicate determinations.

Browning intensity of decolorized MRPs

The browning intensity (A_{420}) markedly decreased after decolorization with activated carbon or Sep-Pak Cartridge C18. The greater decrease in A_{420} was found when the higher amount of activated carbon was used (Fig. 42A). Activated carbon, a highly porous material, has a high attractive or adsorption properties that can adsorb organic molecule (Zhang and Chaung 2001; Diez *et al*., 1999). Hence, the pigment removal was found to increase with the adsorbent amount. However, very large molecular pigments might not be adsorbed on the surface of activated carbon due to its narrow pore structure. It was most likely that brown pigments or intermediate products with the smaller size than pore size of activated carbon might be adsorbed in the matrix, resulting in the decrease in A_{294} (Fig. 42B). Zhang and

Chuang (2001) reported that activated carbon (30 g) was used to achieve 95% color removal of waste water (1 kg).

Octadecyl-Siloxane (C18) is hydrophobic materials commonly used in analytical chromatography for the isolation of hydrophobic compounds from aqueous solution (Lee, 1992). Thus, hydrophobic compounds in the MRPs solution might form the complex with hydrophobic ligand, resulting in the decrease in browning intensity and A_{294} of MRPs with hydrophobic characteristics. Lee (1992) used Sep-Pak Cartridge C18 to isolate brown compounds from a temperature-controlled storage orange juice at 50°C for 15 weeks. Yen and Hsieh (1995) reported that the brown pigment from xylose-lysine MRPs was reduced after decolorization with Sep-Pak Cartridge C18.

Fluorescence intensity of decolorized MRPs increased with increasing concentration of activated carbon amount or after subjecting to Sep-Pak Cartrige C18 (Fig. 42C). For MRPs decolorized with activated carbon showed the higher fluorescence intensity compared with those decolorized using Sep-Pak Cartridge C18. The spectral characteristics of these colorants resemble conjugated Schiff bases derived from malonaldehyde and amino acids as reported by Chio and Tappel (1969). The structure of the isolated fluorophore contained a conjugated system of nitrogen and carbon in an imidazole derivertive (Baunsgaard *et al*., 2000). The exposure of the hydrophobic part due to the conformational changes may lead to increased fluorescence as stated by Kilara and Harwalkar (1996). Fom the result, the increase in fluorescence intensity of MRPs after decolorization might be owing to the removed of brown pigments or colorless intermediate (A_{294}). As a result, the greater content of fluorescent intermediates was obtained

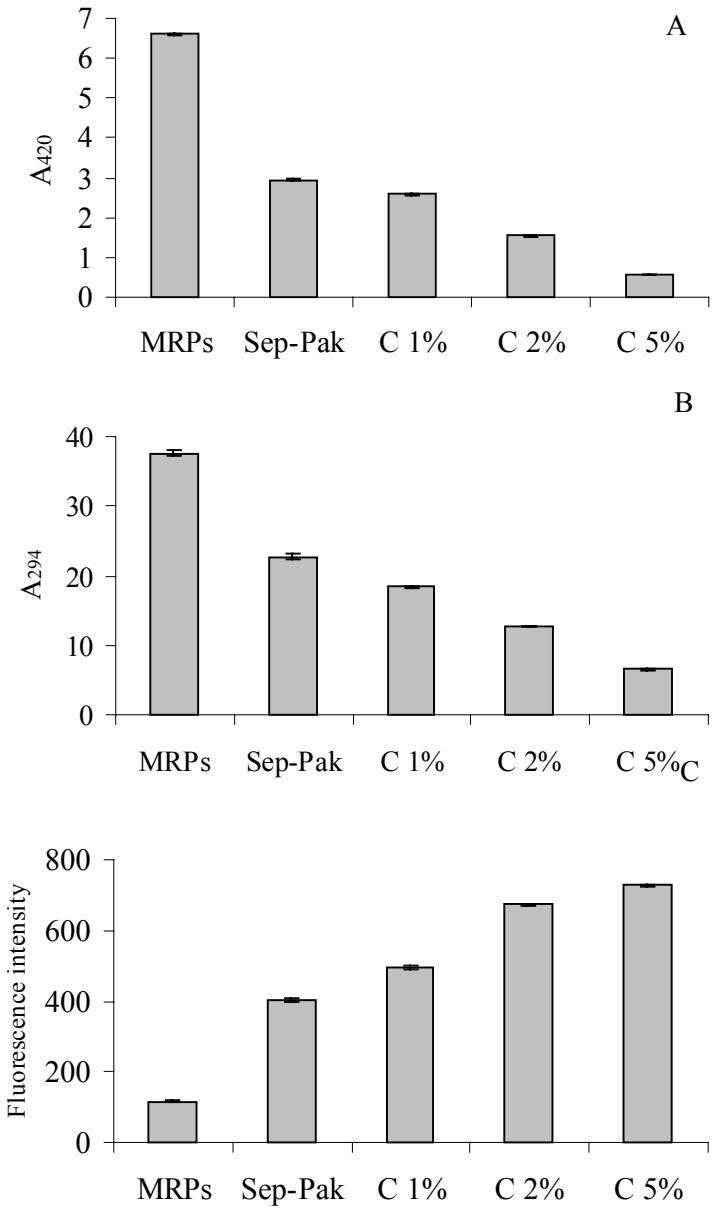


Figure 42. Browning intensity (A), A_{294} (B) and fluorescence intensity (C) of MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. C 1%, C 2% and C 5% denote MRPs decolorized by 1%, 2% and 5% activated carbon, respectively. Sep-Pak denote MRPs decolorized by Sep-Pak Cartridge C18. Bars represent the standard deviation from triplicate determinations.

Reducing power of decolorized MRPs

The reducing power of MRPs decreased after decolorization with activated carbon, especially with higher concentration used and with Sep-Pak Cartridge C18 treatment (Fig. 43). MRPs decolorized by 5% activated carbon exhibited the lowest reducing power as evidenced by the 90% reduction. For MRPs decolorized using Sep-Pak Cartridge C18, the reduction of reducing power by 30% was obtained when compared with that of original MRPs. Activated carbon, particularly at higher concentration, has a higher surface area which enhances a capacity for adsorption. Some compounds with reducing power might be adsorbed at the carbonyl oxygens on the activated carbon surface according to a donor-acceptor complexation mechanism (Mattsom *et al*., 1969). From the results, the removal of brown pigments (Fig. 42A) was in accordance with the lower reducing power (Fig. 43). Yen and Hsieh (1995) reported that the browning pigment from xylose-lysine which can donate hydrogen atoms markedly decreased after decolorization with Sep-Pak Cartridge C18. Therefore, the decolorization of MRPs might cause the decrease in reducing power of resulting MRPs. This indicated that brown pigment or some colorless intermediates could be the potential hydrogen donors.

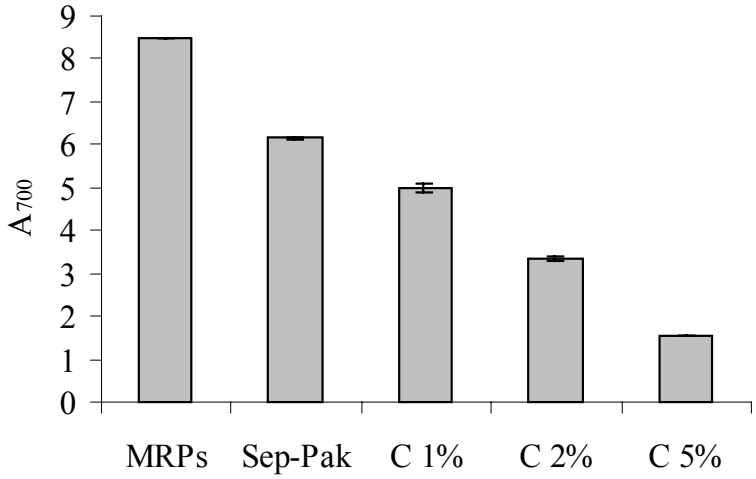


Figure 43. Reducing power of MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. C 1%, C 2% and C 5% denote MRPs decolorized by 1%, 2% and 5% activated carbon, respectively. Sep-Pak denote MRPs decolorized by Sep-Pak Cartridge C18. Bars represent the standard deviation from triplicate determinations.

Copper chelating property of decolorized MRPs

Copper chelating property as determined by %bound copper of all decolorized MRPs slightly decreased when compared with that of MRPs (Fig. 44). The increase in activated carbon used resulted in the decrease in copper chelating capacity. The higher bound copper content was found with MRPs with Sep-Pak Cartridge C18 decolorization. Generally, the reduction by 2-10% was noticeable with all decolorized MRPs. O'Brien and Morrissey (1997) stated that Amadori compound and possibly Maillard reaction products from glucose-glutamate functioned to bind metal ions. The formation of a number of ligands for metal ions in Maillard browning

reaction was reported (Bryce *et al*., 1965). Amadori compound from fructosyl glycine system may complex zinc and probably copper (Seifert *et al*., 2004). Seifert *et al*. (2004) found that a new copper-binding center is formed during the glycation of proteins. This new copper binding center forms in the event of peptide-bound N^{ε}-fructoselysine. N^{ε}-carboxymethyllysine forms 100-1000 times more stable complexes with Cu (II) compared to the well known imidazole binding site of peptide-bound histidine (Bryce *et al*., 1965). Price *et al*. (2001) reported that at millimolar concentration of the advanced glycation end-product (AGE) formation, the chelating or antioxidant activity was noticeable. Furthermore, melanoidin derived from sugar (glucose, galactose) and amino (Ala, Cys, His, Lys, Gly, Met, Phe, Asp, Arg, Trp, Tys) possessed metal chelating activity (Price *et al*., 2001). From the result, decolorization showed the negative effect on copper chelating property, possibly caused by the loss of both color and colorless compounds as well as reactive compounds. The reduction of copper chelating property correlated well with the decrease in browning intensity, A_{294} and reducing power. It was likely that intermediate compounds and brown color pigments might exhibit the copper chelating property.

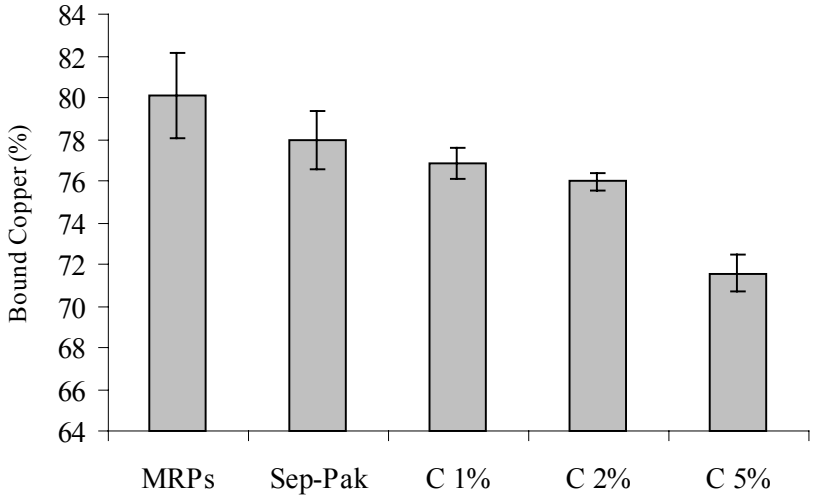


Figure 44. Copper chelating property of MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. C 1%, C 2% and C 5% denote MRPs decolorized by 1%, 2% and 5% activated carbon, respectively. Sep-Pak denote MRPs decolorized by Sep-Pak Cartridge C18. Bars represent the standard deviation from triplicate determinations.

PO inhibitory activity of decolorized MRPs

PO inhibitory activity decreased substantially after decolorization of MRPs (Fig. 45). No differences in PO inhibitory activity of MRPs decolorized by Sep-Pak Cartridge C18, 1% and 2% activated carbon were observed ($p>0.05$). MRPs decolorized by 5% activated carbon exhibited the lowest PO inhibition. In the presence of MRPs, some metal-containing enzymes are inhibited since MRPs are able to bind metal ions (Seifert *et al*., 2004). The decolorization might be associated with the removal of brown pigments or reactive compounds possessing PO inhibitory activity. From the results, the decrease in browning intensity, reducing power and copper chelating property was in agreement with the decrease in PO inhibitory activity. Lee (1992) found that MRPs from storage-aged orange juice decolorized by

Sep-Pak Cartridge C18 exhibited the lower browning intensity and scavenging activity, which correlated with the decrease in donation of hydrogen atoms or reducing power (Yen and Hsieh, 1995). From the result, the lowered reducing power, copper chelating property and browning intensity might contribute to the decrease in PO inhibition of decolorized MRPs. Thus, decolorization resulted in the loss in the capacity in inhibiting PO of MRPs.

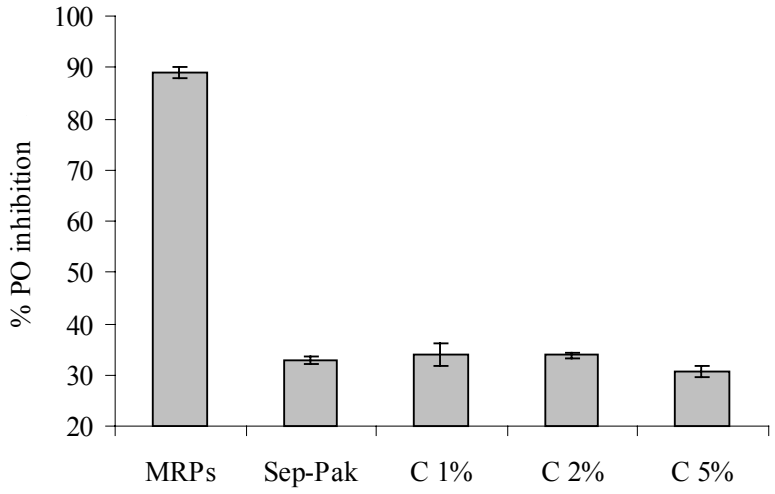


Figure 45. PO inhibitory activity of MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. C 1%, C 2% and C 5% denote MRPs decolorized by 1%, 2% and 5% activated carbon, respectively. Sep-Pak denote MRPs decolorized by Sep-Pak Cartridge C18. Bars represent the standard deviation from triplicate determinations.

13. Changes in chemical composition, melanosis and sensory property of black tiger prawn during iced storage

MRPs produced by heating the equimolar of fructose and glycine (30 mM or 1 M) at pH 11 and 100°C for 12 h were used to soak the prawn for 10 min

before storage in ice. Prawn soaked in 1.25% Na-metabisulfite was also used for iced storage.

Changes in pH

The pH values of black tiger prawn kept in ice increased progressively throughout the storage and reached the pH of 8.2 after 10 days of storage (Fig. 46). Increase in pH might be associated with the accumulation of basic substances, such as ammonia and TMA produced by prawn muscle (Campos *et al*., 2005). Campos *et al*. (2005) reported that pH of sardines stored in flake ice increased significantly with storage time. The pH of hake increased slightly during iced storage (Rudriguenz *et al*., 2004; Riuz-Capillas and Moral, 2001). However, pH changes during refrigerated storage of fish and crustacean can be varied, depending upon species and other factors. Thus, pH determination cannot be used as a reliable index of the freshness or of the onset of spoilage. Under certain restricted conditions, pH limits for definite levels of spoilage can sometimes be set. From the result, no marked differences in pH were obtained among the samples with different treatments throughout the storage of 10 days.

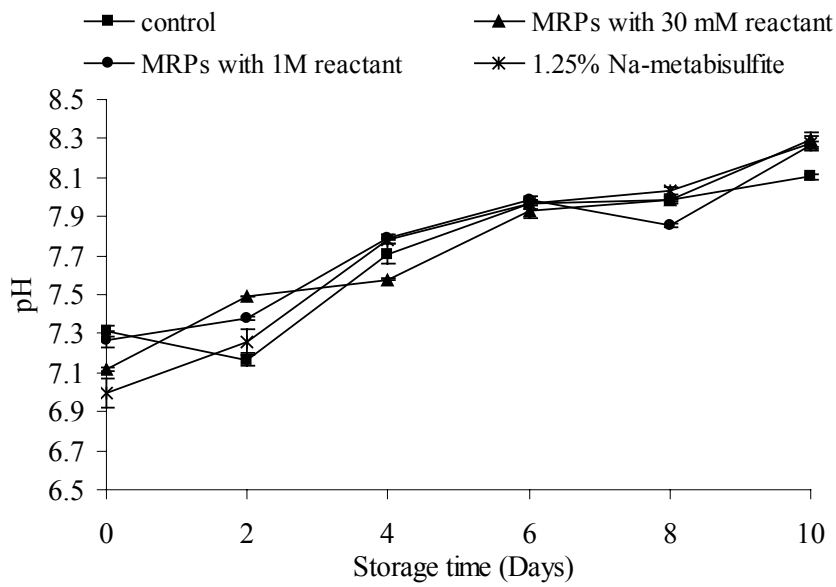


Figure 46. Changes in pH of black tiger prawn muscle treated with MRPs or Na-metabisulfite during iced storage. Bars represent the standard deviation from triplicate determinations.

Changes in TVB and TMA contents

TVB content in black tiger prawn treated with MRPs or Na-metabisulfite during iced storaged is shown in Figure 47. No significant differences in TVB content were observed among all treatments up to day 6. Thereafter, the sharp increase was found in the control and reached the level of 18 mg TVB/g after 10 days of storage. The prawn soaked in MRPs with 30 mM reactant solution or 1.25% Na-metabisulfite had the slower increasing rate of TVB, when compared with the control. At day 10, it was noticeable that TVB content lower than 12 mg/g was observed in the sample treated with 1.25% Na- metabisulfite. TVB content of samples soaked in either MRPs with 30 mM or 1 M reactant were similar; however they were lower than that found in the control sample.

The increase in TVB content indicated the breakdown of protein and the production of nitrogenous volatile material, which possibly gives rise to strong off-flavor (Ozogul and Ozogul, 2000). Several researchers observed that the TVB-N level in shrimp stored in ice increased with the time of storage (Riuz-Capillas and Moval, 2001; Baixas-Nogueras *et al*., 2002; Karungi *et al*., 2004; Campos *et al*., 2005; Sadok *et al*., 2004). From the result, it was suggested that both MRPs and metabisulfite resulted in the retardation of spoilage, especially caused by microorganisms. MRPs might inhibit or retard the growth of microorganism by chelating some ions required for microorganism. It was reported that MRPs showed the metal chelating property (Wijewickreme *et al*., 1997; Yoshimura *et al*., 1997). Bisulfite is found as the potent killer of microorganism (Di Persio *et al*., 2004; Lourila *et al*., 1998).

TMA-N was not detected in all prawn samples during 10 days of storage (data not shown). No increase in TMA over the storage period in ice possibly reflects the low level of trimethylamine oxide (TMAO) in prawn muscle. TMA is produced by the decomposition of TMAO caused by bacterial spoilage and enzymatic activity. Moreover, TMAO reducing bacteria were possibly negligible in all prawn samples. As a result, no TMA was formed during extended iced storage. Therefore, TMA formation is most likely depended upon fish species. Baixas-Nogueras *et al*. (2002) reported that the few changes in TMA content in hake during the storage in ice. Papadopoulos *et al*. (2003) reported that TMA levels of sea bass during 9 day storage was 0.07 mg/100 g sample. Additionally, Rodriguez *et al*. (2004) observed that the TMA content of hake muscle stored in either flake ice or slurry ice hardly increased during 12 days of storage.

TVB-N and TMA-N contents can be used for quality assessment of fish. In the case of prawn, limits of acceptability in some sectors of the Australian and Japanese markets are 5 mg TMA-N/100 g prawn muscle and/or 30 mg total volatile nitrogen (TVB-N) (Herbard *et al*., 1962). From the results, all samples were still acceptable during 10 days of storage.

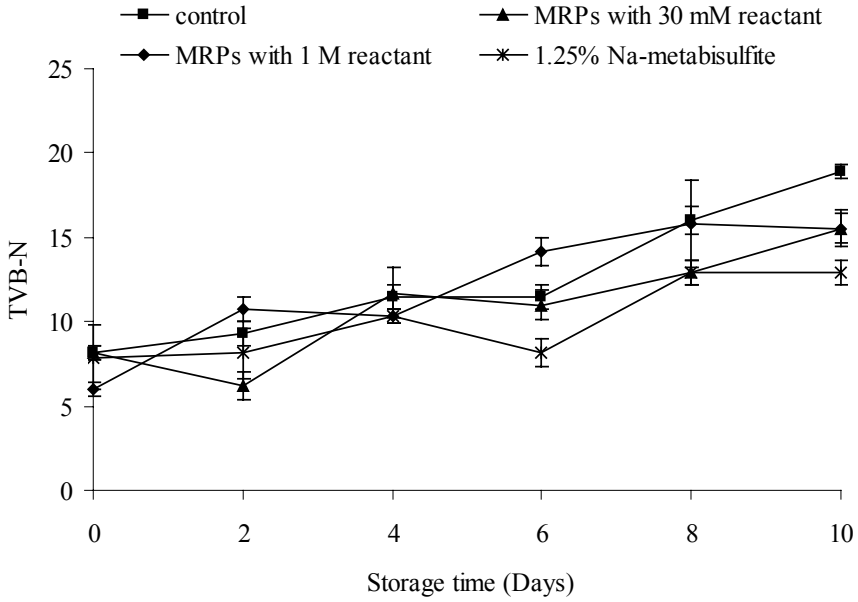


Figure 47. Changes in TVB content of black tiger prawn muscles treated with MRPs or Na-metabisulfite during iced storage. Bars represent the standard deviation from triplicate determinations.

Changes in melanosis

Melanosis of samples without and with treatment of MRPs or metabisulfite during iced storage is depicted in Figure 48. The control sample had the continuous increase in melanosis score during 10 days of iced storage. The score of 5 was obtained in the control after 4 days of storage. Otwell and Marshall (1986)

considered a score of 4 or more as indicative of product degradation, although Otwell *et al*. (1992) did not consider the product to be unacceptable until it scored 8. A melanosis score of 4 was reported for prawns of the *Penaeus genus* stored in ice for 3-4 days (Otwell and Marshall, 1986; Otwell *et al*., 1992).

The treatment of MRPs with 30 mM reactant and 1.25% Na-metabisulfite showed the inhibition of melanosis as evidenced by the lowered score in comparison with that of control. Between both treatments, 1.25% Na-metabisulfite exhibited the slightly lower melanosis inhibition efficacy than MRPs with 30 mM reactant. The treatment of MRPs with 1 mM reactant showed a high score due to the dark color of MRPs. Soaking prawn in MRPs with 1 mM reactant allowed brown pigments to penetrate into the muscles, leading to the darken color of resulting prawn. Roux *et al*. (2003) stated that MRPs with 1 M reactant (fructose or glucose/cysteine) heated at 100°C for 39 h could inhibit PPO from apple. Bartolo and Birk (1998) stated that black spot was developed on the iced Norway lobster at varying rates during storage. Because of tissue breakdown during iced storage, autolytic enzymes are allowed to come into contact with and activate the inert proenzyme. The retardation of black spot formation in prawn might be due to the reducing power of both MRPs and metabisulfite. The quinones are reduced to original phenols by MRPs and MRPs might bind with the copper at active site of PPO (Billaud *et al*., 2004; Roux *et al*., 2003). MRPs from glucose/lysine and fructose/lysine model system had the reducing power, which can vary with some factors determining the reaction (Wijewickreme *et al*., 1997; Yoshimura *et al*., 1997). The inhibition of PO was studied by Montero *et al*. (2001) who found that the melanosis score of shrimp without additive reached 4 within 4 days. Ascorbic acid or citric acid and 4-hexylresorcinol were effective for the

inhibition of shrimp melanosis. Billaud *et al*. (2004) and Roux *et al*. (2003) found that MRPs were produced from different sugar (glucose, fructose) and amino acid (cysteine, glycine) exhibited the reduction of PO activity in model system.

From the result, MRPs with 30 mM reactant exhibited the inhibitory activity toward melanosis of black tiger prawn during extended iced storage, possibly owing to its reducing power and metal chelating property.

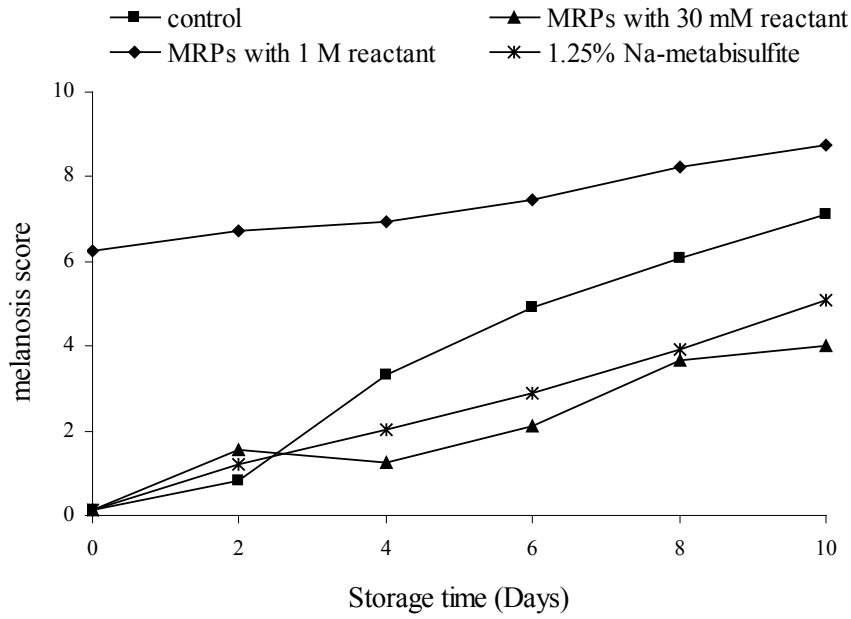


Figure 48. Changes in melanosis score of black tiger prawn treated with MRPs or Na-metabisulfite during iced storage. Bars represent the standard deviation from triplicate determinations.

Sensory evaluation

Sensory scores of different samples were generally decreased with increasing storage time ($p<0.05$), especially after 4 days of storage. By using a sensory score value below 5 as the unacceptable point, samples treated with MRPs

with 30 mM reactant and 1.25% Na-metabisulfite were still acceptable up to 10 days of iced storage. For the control, it could be stored in ice for less than 8 days (Fig. 49). However, the prawns soaked in MRPs solution with 1 M reactant had the lowest sensory quality for all attributes tested. From the results, the uses of MRPs with 30 mM reactant and 1.25% Na-metabisulfite could inhibit browning as evidenced by the greater score of color, appearance and overall likeness. As a result, the samples were judged to be accepted even after 10 day of storage.

Sadok *et al*. (2004) stated that changes of shrimp occurred after only 6 days stored at 0°C. Prawns, *Penaeus monodon* (Basavakamar *et al*., 1998) and *Penaeus merguensis* (Shamshad *et al*., 1990), had the loss in quality when the storage time in ice increased. From the result, sensory property was in a good agreement with melanosis score, which was crucial factor determining the acceptability of black tiger prawn during iced storage. However, the sensory assessment somehow correlated with TVB content (Baixas-Nogueras *et al*., 2002). According to these sensory parameter, prawn treated with MRPs having 30 mM reactant and 1.25% Na-metabisulfite was acceptable for human consumption until day 10 of iced storage.

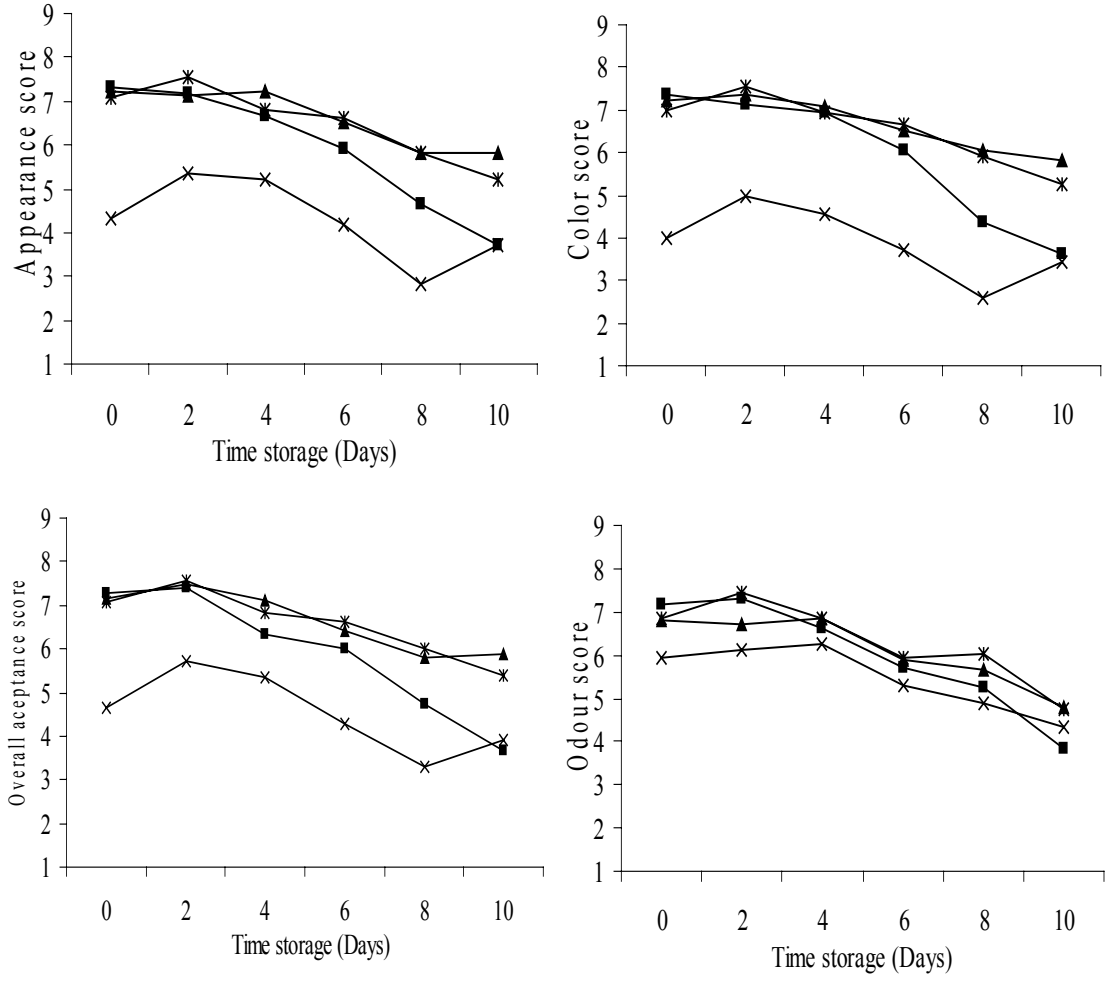


Figure 49. Changes in sensory property of black tiger prawn treated with MRPs or Na-metabisulfite during iced storage. ■ control, ▲ MRPs with 30 mM reactant, × MRPs with 1 M reactant, ✳1.25% Na-metabisulfite. Bars represent the standard deviation from triplicate determinations.