Chapter 3

RESULTS AND DISCUSSION

1. Effect of sugars and heating time on the characteristics and antioxidative activity of Maillard reaction products (MRPs)

1.1 Changes in pH

The pH of all PPP-sugar MRPs decreased gradually as the heating time increased up to 5 h (Figure 5). Different sugars and concentrations resulted in

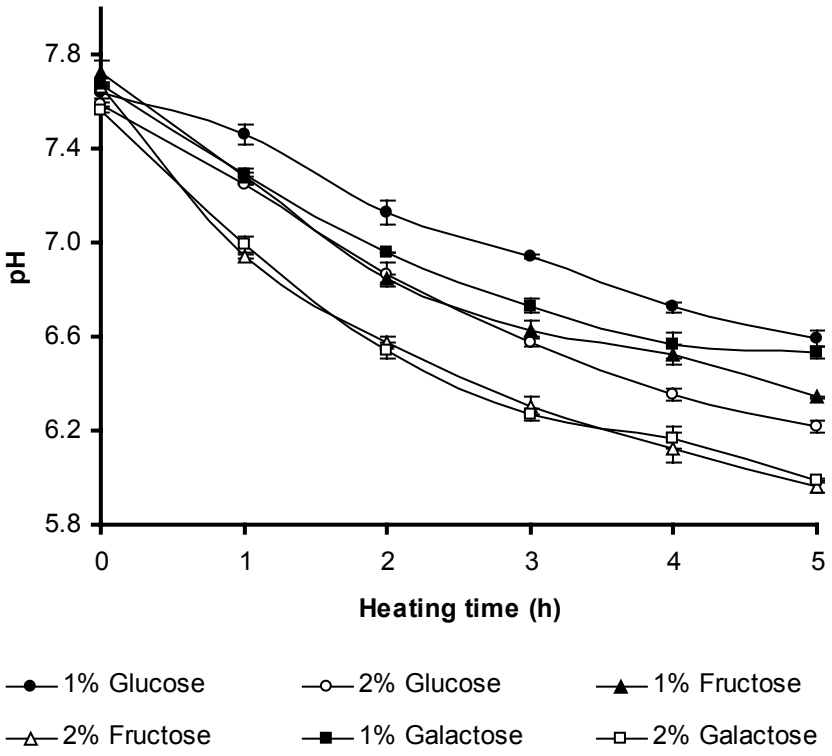

Figure 5 Changes in pH of various PPP-sugar MRPs during heating for different times. Bars indicate the standard deviation from triplicate determination.

1.2 Changes in A_{294}

Continuous increase in A_{294} was observed as the heating time of all PPP-reducing sugar mixture increased up to 5 h ($P<0.05$) (Figure 6(a)). MRPs derived from galactose showed the highest increase in A_{294}, followed by fructose and glucose, respectively. MRPs derived from 2% sugar had a greater A_{294} than did those with 1% sugar. A_{294} was used to determine the intermediate compounds of Maillard reaction (Lerici *et al.*, 1990; Ajandouz *et al.*, 2001). From the result, the increase in A_{294} suggested the formation of an uncolored compound, which could be the precursor of the Maillard reaction (Lerici *et al*., 1990; Ajandouz *et al*., 2001). This result was in agreement with Lerici *et al*. (1990) who found that heat treatment of glucose-glycine mixture caused marked increase in A_{294}.

1.3 Changes in fluorescence intensity

Fluorescence intensity of all MRPs, except MRPs derived from 1% glucose, reached a maximum at a heating time of 1 h ($P<0.05$). Thereafter, fluorescence intensity decreased sharply up to 5 h of heating (Figure 6(b)). For the MRPs derived from 1% glucose, fluorescence intensity decreased markedly after 2 h. Decrease in fluorescence was observed with the increasing heating time. Development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Morales *et al.*, 1996; Jing and Kitts, 2002). Jing and Kitts (2002) found that fluorescence of a ribose-casein mixture heated at 55ºC reached the maximum within 4 days before decreasing. Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992). Therefore, the decrease in fluorescence intensity as heating time increased, was presumably due to the decrease in precursor, which was associated with the development of browning.

The difference in pattern between fluorescence and absorbance (A_{294}) of MRPs suggested that different types of intermediate products, either fluorescent or non-fluorescent compounds, were formed and underwent the final stage of reaction at different rates. However, the fluorescent intermediate was possibly more reactive in formation of brown color than non-fluorescent compounds, as shown by the decrease in fluorescence intensity as heating time increased. Therefore, the formation of intermediate compounds varied, depending on the type of sugar and heating time. For PPP-sugar system, fructose was more reactive than galactose in forming the fluorescent intermediate compounds, whereas galactose was more reactive than fructose, generating non-fluorescent intermediates. For the system derived from glucose, fluorescent compounds were formed to the highest extent but non-fluorescent intermediates were generated at a lower level than with those from galactose and fructose.

1.4 Changes in browning intensity

The increase in browning of PPP-sugar MRPs, as measured by A_{420}, was observed as the heating time increased ($P<0.05$) (Figure 6(c)). MRP samples derived from 2% galactose showed a greater increase in browning intensity than those from 2% fructose or 2% glucose. From this result, MRPs derived from glucose showed the lowest browning ($P<0.05$). The increase in browning was found to depend on sugar concentration. The higher concentration of sugar used, the higher the increase in browning was found. The increase in A_{420} was used as an indicator for browning development in the final stage of the browning reaction (Ajandouz *et al*., 2001;

Morales and Jimenez-Perez, 2001). Browning rate is influenced by the type of reducing sugar involved in the reaction. The reactivity of reducing sugar was reported to decrease in the following order: aldopentoses> aldohexoses> ketohexoses> disaccharides (Spark, 1969). Based on browning development, galactose was most reactive in PPP-sugar system. Yeboah *et al.* (1999) found that the aldehyde group of the acyclic form of aldoses was more electrophilic than the keto group of the acyclic form of ketose. However, MRPs derived from fructose showed more browning intensity than those with glucose, presumably because fructose had a higher proportion of open chain form than glucose (Naranjo *et al*., 1998). Thus, an amino acid-sugar complex could be formed more easily. The differences among different studies were possibly due to the diversity of sugars, protein and amino acids as well as conditions used to prepare MRPs. Apart from the Maillard reaction, caramelization of sugar could occur, leading to browning of the mixture (Ajandouz *et al*., 2001; Benjakul *et al.*, 2005). Fructose, a ketose sugar, was browned more quickly than glucose, an aldose sugar, when heated at 120ºC for up to 60 min (Brands *et al.*, 2000).

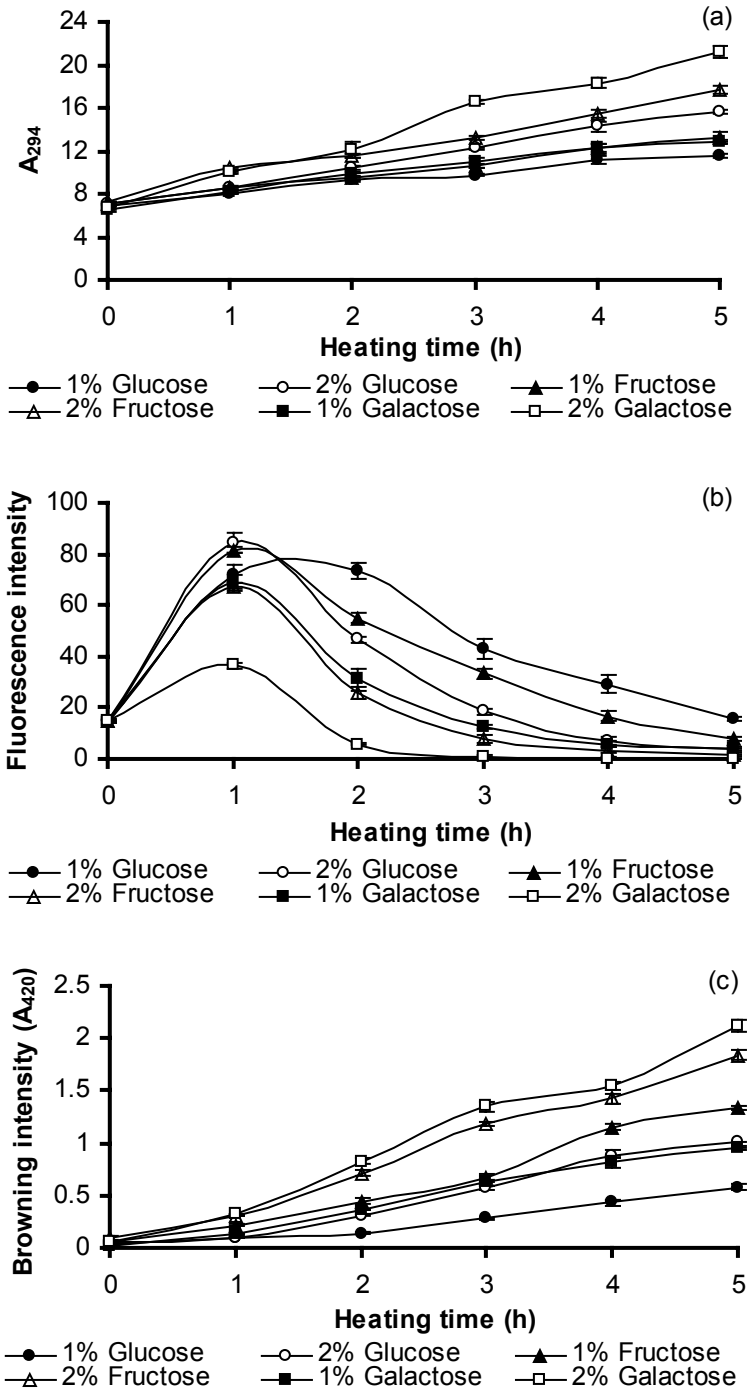

Figure 6 Changes in A_{294} (a), fluorescence intensity (b) and browning intensity (c) of various PPP-sugar MRPs during heating for different times. Bars indicate the standard deviation from triplicate determination.

1.5 Changes in free amino group content

Changes in free amino group content of MRPs during heating are depicted in Figure 7. No changes in free amino group content of MRPs were observed when heating time was 1 h ($P>0.05$). However, a continuous decrease in amino acid group content of all MRP samples was noticeable when the heating time increased ($P<0.05$). This result suggested that α- or ε-NH_2 group of amino acids or proteins covalently attached to a sugar to form glycated proteins to a greater extent, particularly when the heating time increased. The first glycation product, or Schiff base, rearranges to a more stable ketoamine or Amadori product. The Amodori products can then form cross-links between adjacent proteins or with other amino groups, resulting in polymeric aggregates called advanced glycation end products (Friedman, 1996). Our result was in agreement with Wahyuni *et al.* (1999) who reported that free amino groups of fish water-soluble protein decreased gradually via Maillard reaction with glucose-6-phosphate. Available lysine losses were observed in a casein-sugar system during extended heating (Naranjo *et al*., 1998).

From the results, the decreases in free amino group content were in accordance with the increase in browning (Figure 6(c)) and A_{294} (Figure 6(a)) and the decrease in fluorescence intensity (Figure 6(b)). This indicated that extended heating catalysed the interaction between free amino groups, such as ε-NH_2 groups of lysine, and sugar via glycation process. As a result, the intermediate products were formed and further converted to brown pigments, as observed by the increased A_{420}. In general, galactose was more reactive in forming the glycated PPP than were fructose or glucose, as shown by the greatest decrease in free amino groups with the concomitant increase in browning. The reaction rate of glycation between casein and

sugars depended on the percentage of the acyclic form and the electrophilicity of the carbonyl groups (Bunn and Higgins, 1981; Naranjo *et al*., 1998). Naranjo *et al*. (1998) found that glucose, which is more electrophilic, could react faster than fructose in casein-sugar system. The difference in reaction rate of sugar observed in many studies was possibly due to the different compositions of amino acid and conformation of protein, as well as the conditions used in different studies.

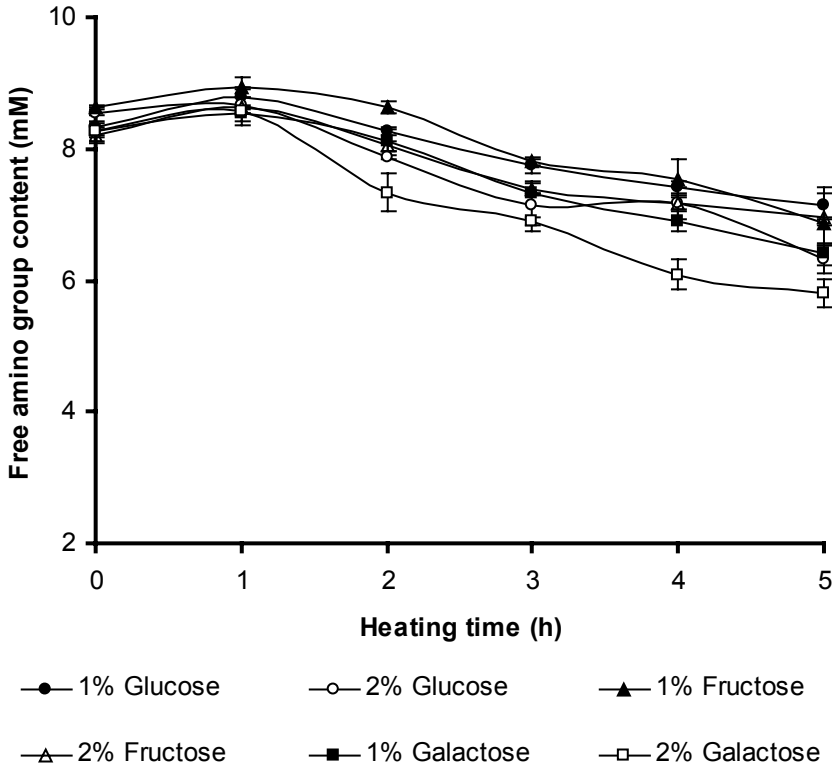

Figure 7 Changes in free amino group content of various PPP-sugar MRPs during heating for different times. Bars indicate the standard deviation from triplicate determination.

1.6 Changes in reducing power

Reducing power of MRPs increased as the heating time increased, as shown by an increase in A_{700} ($P<0.05$) (Figure 8(a)). MRP samples prepared with galactose showed the highest reducing power. MRPs derived from glucose exhibited the lowest reducing power, especially with increasing heating time. MRP samples containing a higher level of sugar had a greater reducing power than those with a lower level. The result revealed that MRPs could function as the electron donors. The result was in accordance with Yoshimura *et al*. (1997) who reported that MRPs from glucose-glycine mixture had a higher reducing power, especially when the heating time increased. Hydroxyl group of MRPs play a role in reducing activity (Yoshimura *et al*., 1997). From the results, reducing power correlated well with browning intensity and A_{294}, but not fluorescence intensity.

1.7 Changes in DPPH radical-scavenging activity

DPPH radical-scavenging activity of MRPs increased as the heating time increased ($P<0.05$) (Figure 8(b)). The DPPH radical was scavenged by MRPs by donating hydrogen to form a stable DPPH-H molecule (Matthaus, 2002). The DPPH radical had an absorbance at 515-520 nm. The color changed from purple to yellow by acceptance of a hydrogen radical from MRPs and it became a stable diamagnetic molecule. From the results, the decrease in DPPH radical indicated the radical-scavenging activity of MRP samples. At the same concentration used, MRP samples derived from glucose showed the lowest radical-scavenging activity ($P<0.05$). MRPs derived from galactose and fructose had similar activities. However, a slightly greater activity was formed with MRP derived from 2% galactose with a heating time of 3-5 h. Yen and Hsieh (1995) also found the DPPH radical-scavenging activity of

xylose-lysine MRPs. Therefore, PPP-sugar MRPs possessed hydrogen-donating ability, suggesting potency to react with free radicals.

DPPH radical-scavenging activity of the heated sugar-amino acid mixture was correlated to the changes in fluorescence (Morales and Jimenez-Perez, 2001). The present study showed no correlation between DPPH radical-scavenging activity and fluorescence intensity. However, radical-scavenging activity correlated well with browning intensity and absorbance at 294 nm. Due to the reducing power and radical-scavenging activity of MRPs from the PPP-sugar system, it can be used as an antioxidant to prevent lipid oxidation in food products.

1.8 Effect of MRP amounts on antioxidative activity

PPP-sugar MRPs were prepared by heating 2% fructose or 2% galactose with 2% PPP at 100^{o}C for 5 h. Different amounts of MRPs from both sugars showed different antioxidative activities (Figure 9). From the results, reducing power (Figure 9(a)) and DPPH radical-scavenging activity (Figure 9(b)) increased linearly with the amount of MRPs. It is suggested that MRPs in increasing amounts could donate hydrogen more effectively to the free radicals. In general, MRPs derived from 2% galactose exhibited a slightly greater reducing power and radical-scavenging activity than did those from 2% fructose. The results indicated that the PPP-galactose system gave a greater antioxidative activity than the PPP-fructose system. Chiu *et al*. (1991) reported that the antioxidative effect of MRP against sardine lipid oxidation during storage at 37^{o}C was increased with the increase of MRP amount.

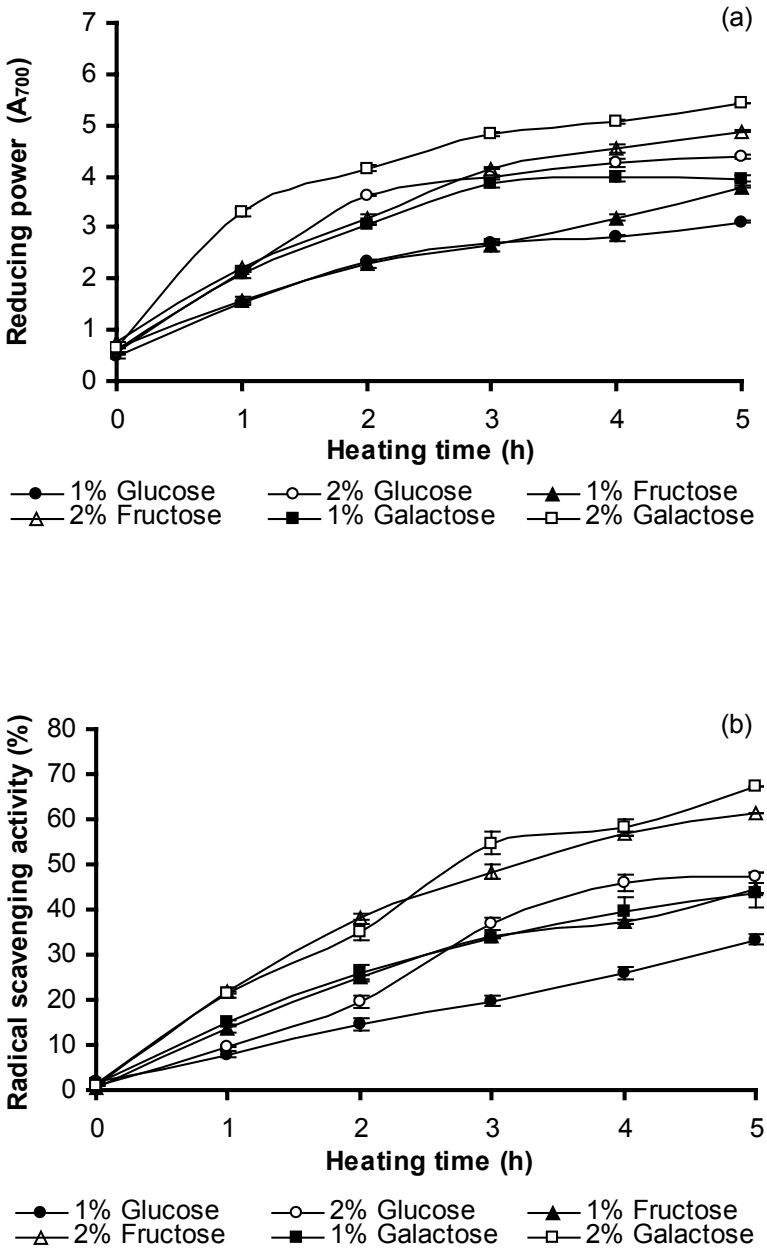

Figure 8 Reducing power (a) and DPPH radical-scavenging activity (b) of various PPP-sugar MRPs during heating for different times. Bars indicate the standard deviation from triplicate determination.

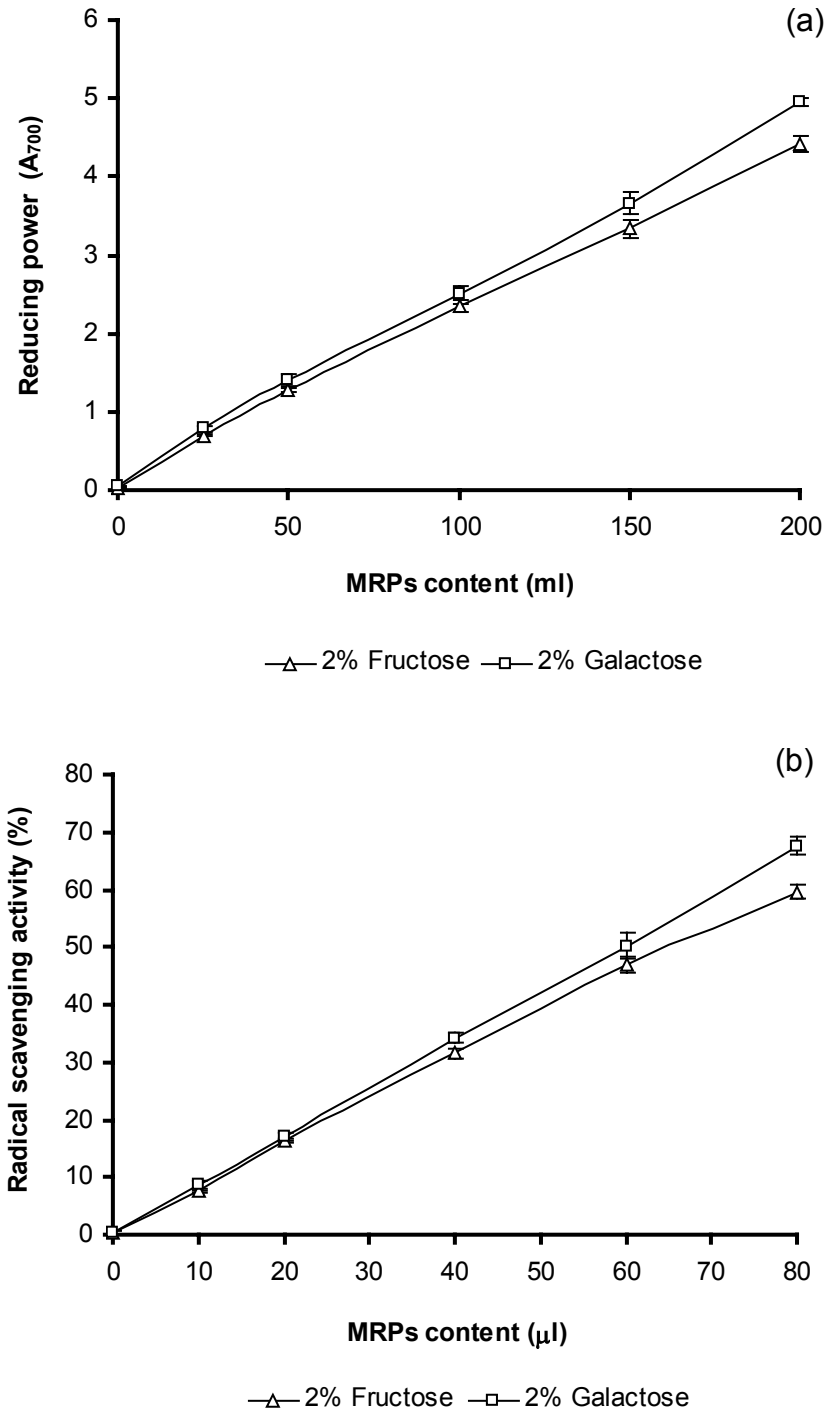

Figure 9 Reducing power (a) and DPPH radical-scavenging activity (b) of various PPP-sugar MRPs with different amounts. Bars indicate the standard deviation from triplicate determination.

2. Effect of pH on characteristics and antioxidative activity of MRPs from PPP-glucose model system

2.1 Changes in pH

The pHs of all PPP-glucose MRPs with different initial pHs decreased markedly from their initial values within the first 2 h ($P<0.05$). Thereafter, pH slightly decreased up to 8 h of heating time ($P<0.05$) (Figure 10). The reduction of pH occurred in Maillard reaction was due to the formation of organic acids, such as formic and acetic acid (Ames, 1998; Brands and Van Boekel, 2002).

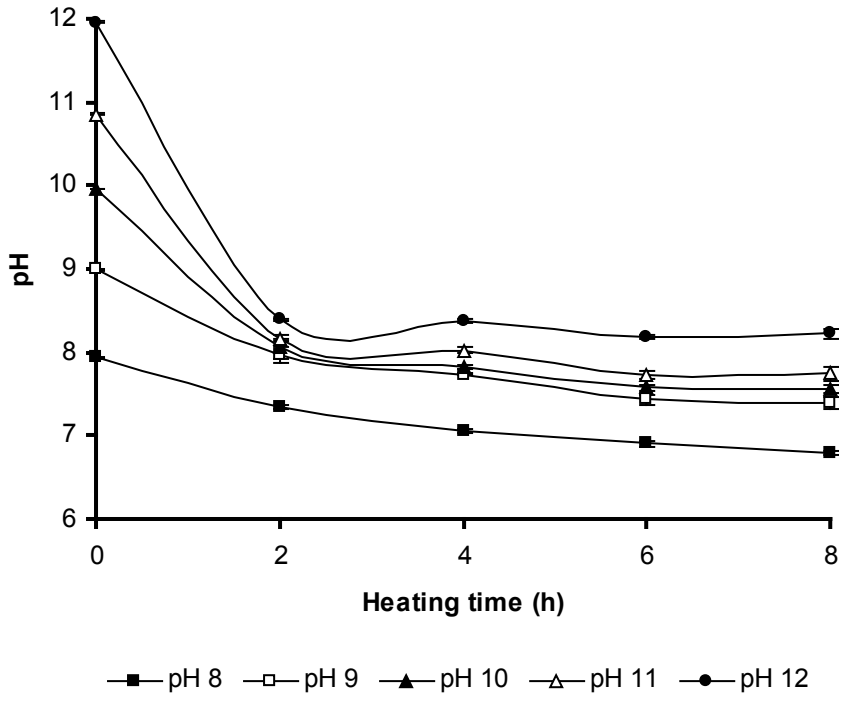

Figure 10 Changes in pH of PPP-glucose MRPs with various initial pHs during heating for different times. Bars indicate the standard deviation from triplicate determinations.

From the result, the higher initial pH rendered the greater final pH of MRPs at every heating time used. The result was in accordance with Apriyantono and Ames (1993), Molales and Jimenez-Perez (2001) and Benjakul *et al*. (2004) who found that during Maillard reaction, pH frequently decreased as the heating time increased.

2.2 Changes in A_{294}

The sharp increase in A_{294} of all MRP samples was observed at 2 h of heating ($P<0.05$) (Figure 11(a)). Nevertheless, small changes in A_{294} of all MRP samples was found with increasing heating time up to 8 h. The result suggested that intermediate products were produced to a great extent in the first 2 h. With further heating, some intermediate products might undergo the polymerization to form the brown pigments and only small amount of intermediate products were generated. The higher A_{294} observed in MRPs with higher initial pHs, suggesting that the intermediate products were flavorably formed at alkaline pH. This result was in accordance with Moreno *et al*. (2003) who found that the higher pH of glucose-lysine model system showed the higher A_{294}. Moreover, the higher pH value gave the higher A_{294} in the fructose-lysine system at 100^{o}C (Ajandouz *et al*., 2001).

2.3 Changes in fluorescence intensity

Fluorescence intensity of all MRPs with different initial pHs reached the maximum within 2 h of heating time ($P<0.05$) (Figure 11(b)). Subsequently, a gradual decrease was observed up to 8 h. Jing and Kitts (2004) found that the fluorescence intensity of sugar-lysine model systems heated at 121^{o}C and pH 9 quickly reached a maximum within 0.5 h before subsequent decrease up to 2 h of heating time. Additionally, the similar result was also found in ribose-casein system (Jing and Kitts, 2002). From the result, fluorescence intensity of MRPs with the higher initial pH

showed the lower fluorescence intensity at all heating times used. However, the fluorescence in epoxyaldehyde-lysine model system incubated at 25^{o}C for 2 h was higher at higher pHs (Hidalgo and Zamora, 1993). Generally, the increase in pH of the system influenced the Maillard reaction rate (Bates *et al*., 1998; Davidek *et al*., 2002; Martins *et al*., 2003).

The Maillard reaction is associated with the development of fluorescent compounds formed prior to the generation of brown pigments (Baisier and Labuza, 1992; Morales *et al*., 1996). This fluorescent compound may be precursors of brown pigments (Labuza and Baisier, 1992; Morales and Van Boekel, 1997). The lowest fluorescence intensity in MRPs with the highest initial pH (pH 12) was probably caused by the rapid transformation of the intermediates to brown compounds. This led to the lower remaining fluorescent intermediate products as shown by the lowest fluorescence intensity. Nevertheless, the opposite result was found, when compared with that of A_{294}. Thus, those intermediate products might be different in term of reactivity to form brown pigment. From the result, it was postulated that fluorescent compound might undergo polymerization to form brown pigment much faster than those with UV absorbing property.

2.4 Changes in browning intensity

The browning intensity as measured by A_{420} increased sharply within 2 h of heating ($P<0.05$) (Figure 11(c)). Subsequently, no differences in browning intensity of MRP samples with an initial pHs of 11 and 12 were found with increasing heating time up to 8 h ($P>0.05$). However, slight increase in browning intensity of MRP samples with initial pHs of 8, 9 and 10 was observed after 2 h of heating time ($P <0.05$). From the result, MRP samples with an initial pH of 12 had the highest

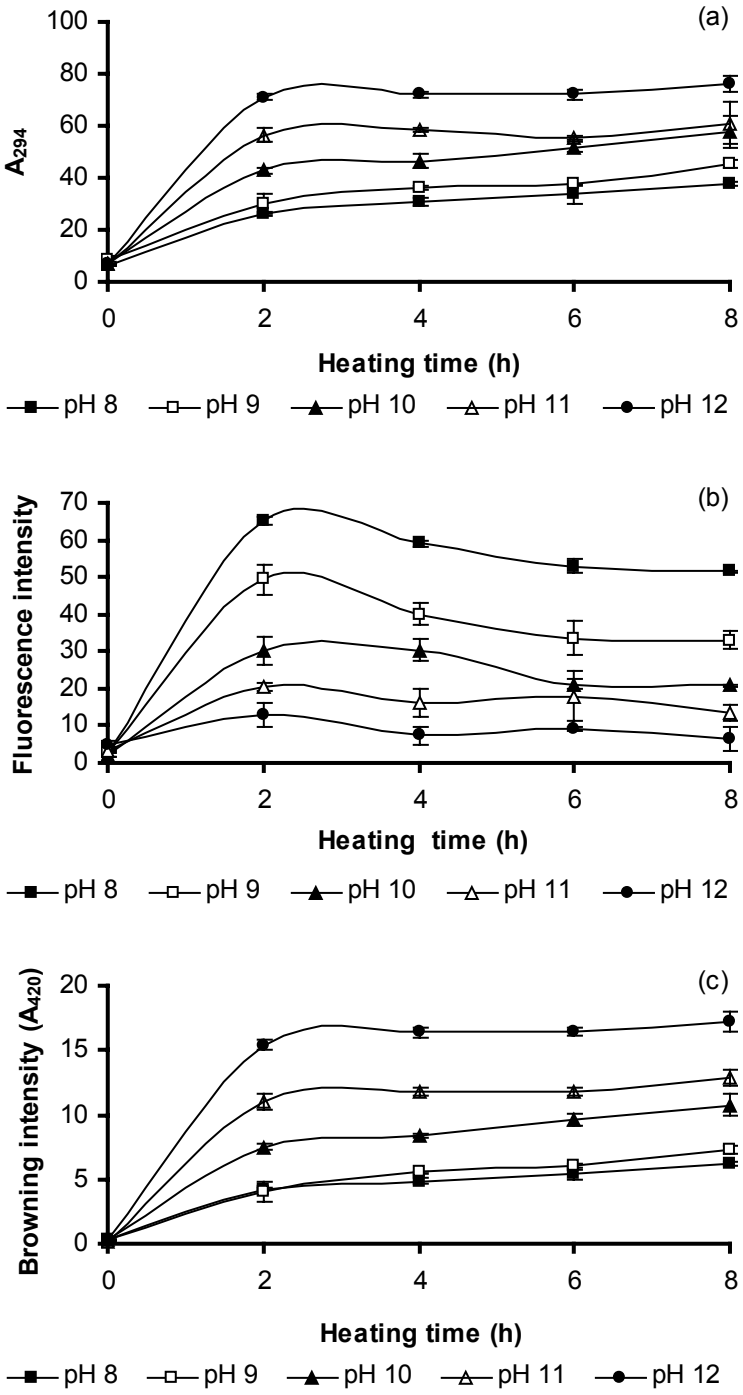

Figure 11 Changes in A_{294} (a), fluorescence intensity (b) and browning intensity (c) of PPP-glucose MRPs with various initial pHs during heating for different times. Bars indicate the standard deviation from triplicate determinations.

browning intensity, followed by those with the initial pHs of 11, 10, 9 and 8, respectively. The result was in agreement with Ajandouz *et al*. (2001) who found that the higher browning intensity of fructose-lysine system heated at 100^{o}C was observed with increasing pH values. From the result, the browning intensity of all samples, especially with high pH, increased with the concomitant increase in A_{294} (Figure 11 (a)). Nevertheless, the low fluorescence intensity was observed, suggesting the more reactivity of fluorescent compound to form brown pigments. Generally, the UV-absorbing and colorless compounds formed at intermediate stages contributed to the brown pigment formation in both Maillard and caramelization reaction (Ajandouz *et al*., 2001; Mauron, 1981; Benjakul *et al*., 2005).

2.5 Changes in free amino group content

The free amino group content in PPP-glucose system with all initial pHs sharply decreased within the first 2 h ($P<0.05$) (Figure 12(a)). No difference in free amino group content was observed with increasing heating time. However, the continuous decrease in free amino group content of MRP samples with an initial pHs of 8 and 9 were noted within 4 h of heating time ($P<0.05$). The result indicated that the Maillard reaction was taken place more effectively at high pH as evidenced by the lower amino group remained. The decrease in amino group content was coincidental with the increase in browning intensity (Figure 11(c)). The reduction of free amino group (the reactant of Maillard reaction) were also observed in sugar-amino systems (Baxter, 1995; Bell, 1997; Wahyuni *et al*., 1999; Sun *et al*., 2004). Our result was in accordance with Ajandouz *et al*. (2001) who found that free amino groups in fructose-lysine system heated at 100^{o}C were sharply decreased when high initial pH was used. Therefore, PPP might serve as a source of reactive amino group for Maillard reaction.

Since lysine has two α– and ε-amino groups, it may have higher reactivity than other amino acids (Miller *et al*., 1984). Conversely, the sulfur amino acids and peptides such as cysteine and glutathione are generally effective for inhibiting nonenzymatic browning (Kwak and Lim, 2004).

2.6 Changes in reducing sugar content

The sharp decrease in reducing sugar in PPP-glucose system with an initial pHs of 10, 11 and 12 were noticeable at 2 h of heating ($P<0.05$). The gradual decrease was found when heated up to 8 h ($P<0.05$) (Figure 12(b)). For the MRPs with the initial pHs of 8 and 9, the reducing sugar content slightly decreased as the heating time increased. The greatest decrease in reducing sugar was observed in MRPs with an initial pH of 12. This result indicated that the reducing sugar was used as the reactant in Maillard reaction and the reaction rate was faster in higher pH system than those with lower pH. Reducing sugar reduction in heated fructose-lysine (Ajandouz *et al*., 2001) and glucose-glycine (Van Boekel and Martins, 2002; Martins and Van Boekel, 2005) model systems was influenced by initial pH values. Apart from the Maillard reaction, the caramelization of glucose, which contributes to nonenzymatic browning reactions, also takes place at the same time (Benjakul *et al*., 2005). At high temperature and pH, caramelization reaction resulted in the degradation of reducing sugar (Ajandouz *et al*., 2001).

The decreases in free amino group content (Figure 12(a)) and reducing sugar content (Figure 12(b)) of PPP-sugar model system, especially at pH 12, were in accordance with the increases in browning intensity (Figure 11(c)), A_{294} (Figure 11 (a)) and the lowered fluorescence intensity (Figure 11(b)). This showed that the

higher pH induced the condensation reaction between free amino groups of PPP and the carbonyl groups of glucose, particularly within the first 2 h of heating time.

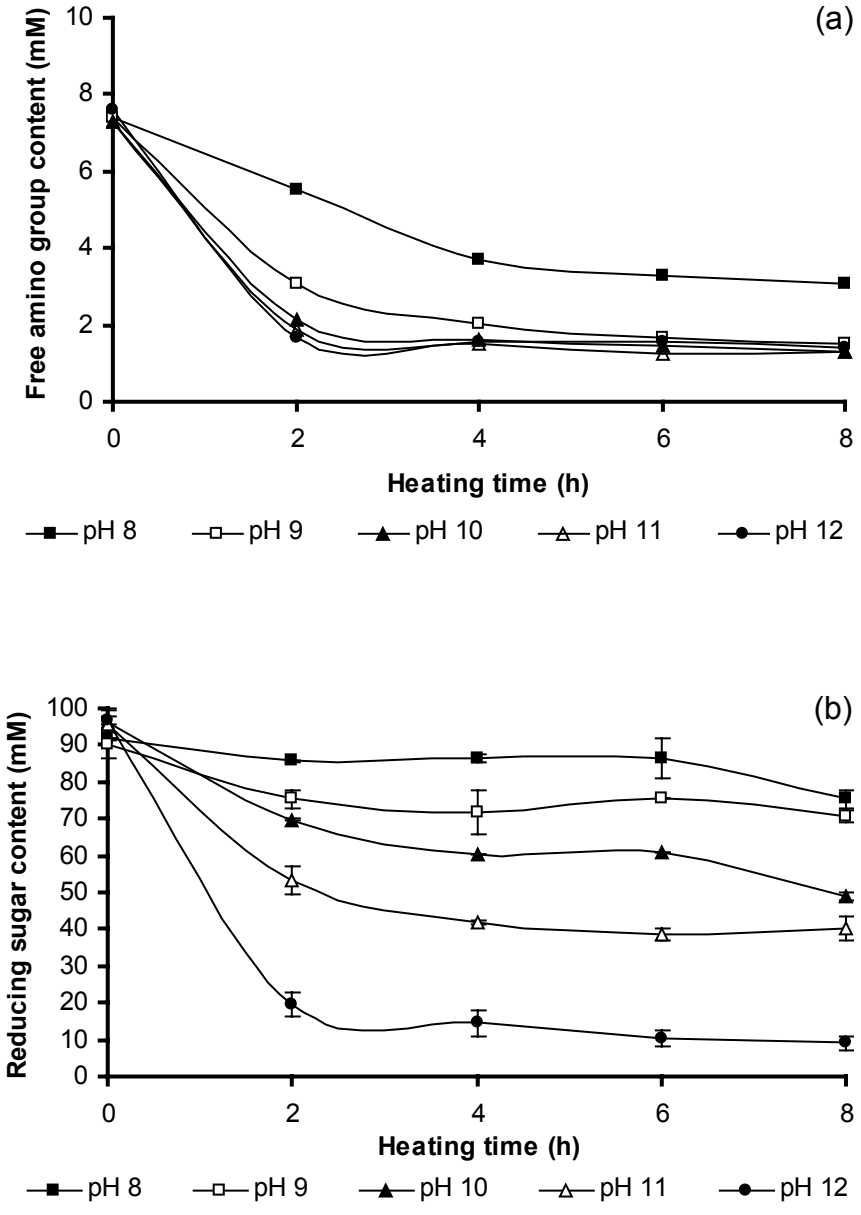

Figure 12 Changes in free amino group content (a) and reducing sugar content (b) of PPP-glucose MRPs with various initial pHs during heating for different times. Bars indicate the standard deviation from triplicate determinations.

2.7 Changes in reducing power

Reducing power of all MRPs with different initial pHs and heating times sharply increased within the first 2 h of heating ($P<0.05$) (Figure 13(a)). No differences in reducing power were observed in MRPs with the initial pHs of 10, 11 and 12 when the heating time was more than 2 h. MRPs with the initial pH of 8 showed the lowest reducing power at all heating times, compared with those with the higher pHs. MRPs prepared from PPP-glucose model systems with initial pHs of 10, 11 and 12 had the similar reducing power at all heating time used. MRPs from xylose-lysine (Yen and Hsieh, 1995), glucose-glycine (Yoshimura *et al*., 1997), sugar-lysine (Wijewickreme *et al*., 1999) and PPP-sugar (Benjakul *et al*., 2004) model systems possessed the reducing power. The result revealed that MRPs from PPP-glucose model system, especially with high initial pH, had hydrogen donating activity. The hydroxyl groups of MRPs play an important role in reducing activity (Yoshimura *et al*., 1997). Additionally, the intermediate reductone compounds of MRPs were reported to break the radical chain by donation of a hydrogen atom (Eichner, 1981). From the results, reducing power of MRPs with various initial pHs correlated well with browning intensity (Figure 11(c)).

2.8 Changes in DPPH radical-scavenging activity

The scavenging activity of PPP-glucose MRPs on DPPH radical, a molecule containing a stable free radical, is depicted in Figure 13(b). The DPPH radical-scavenging activity indicated the hydrogen donating abilities of antioxidant (Brand-Williams *et al*., 1995; Guerard and Sumaya-Martinez, 2003). DPPH radical-scavenging activity of MRPs with initial pHs of 10, 11 and 12 sharply increased at 2 h of heating (27.4-31.6% scavenging activity) ($P<0.05$) and no changes in activity were

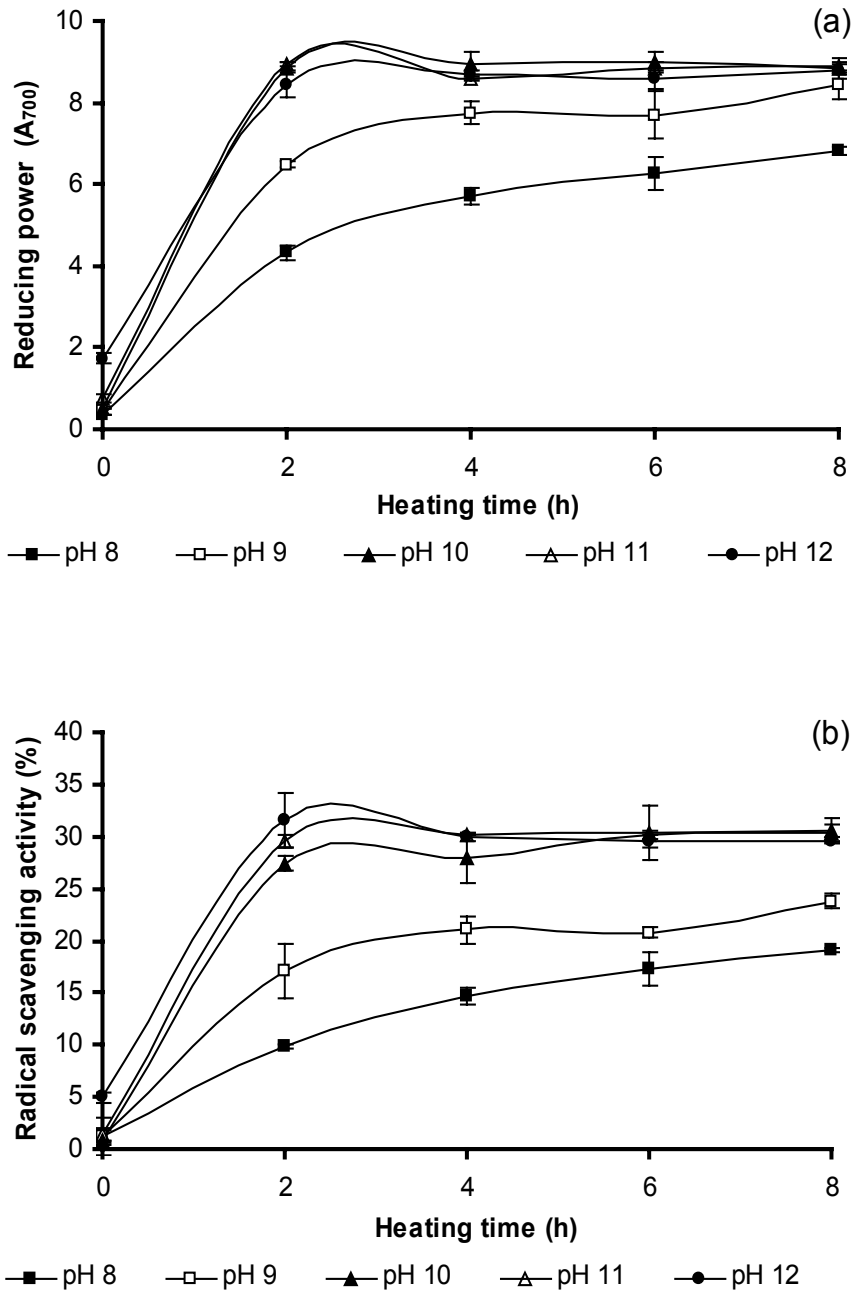

Figure 13 Reducing power (a) and DPPH radical-scavenging activity (b) of PPP-glucose MRPs with various initial pHs during heating for different times. Bars indicate the standard deviation from triplicate determinations.

found with increasing heating up to 8 h ($P>0.05$). Nevertheless, the continuous increases in radical-scavenging activity were observed in MRPs with initial pHs of 8 and 9 when heating time increased ($P<0.05$). This result revealed that MRPs derived from PPP-glucose system were free radical inhibitors, which can work as the primary antioxidant. The result was in agreement with Yen and Hsieh (1995), Murakami *et al*. (2002), Molares and Jimenez-Perez (2001) and Benjakul *et al*. (2004) who also found that MRPs had the DPPH radical-scavenging activity. The higher pH of PPP-glucose model system resulted in the greater antioxidative activity of MRPs, compared with the lower pHs used. No differences in activity were noted between MRPs with initial pHs of 10, 11 and 12. In general, the radical-scavenging activity was concomitant with the reducing power observed in MRPs. Therefore, pH was the important factor determining antioxidative activity of MRPs from PPP-glucose model system.

3. Characteristics and antioxidative activity of MRPs from PPP hydrolysate-glucose model system

Maillard reaction involves the condensation between the α-amino groups of amino acids or proteins and the carbonyl groups of reducing sugar, known as "carbonylamino" reaction (Eskin, 1990). The increase in α-amino groups by the hydrolysis of protein, either enzymatic or acid hydrolysis, would increase the reaction.

3.1 Enzymatic hydrolysis of porcine plasma protein

The enzymatic hydrolysis of PPP by alcalase was run for 4 h at pH 8.5 and 60^{o}C. The different concentrations of alcalase were used for hydrolysis. An increase in DH was observed with increasing alcalase concentration used (Figure 14). When $\log_{10}$ (alcalase amount) versus DH (%) was plotted, a linear relationship was found

with the correlation coefficient (R^2) of 0.9856 (Figure 14). The result was in agreement with Benjakul and Morrissey (1997) and Guerard *et al*. (2001) who reported the linear relationship between $\log_{10}$ (enzyme concentration) and DH for enzymatic hydrolysis of Pacific whiting solid wastes and yellowfin tuna. From this relationship, the amount of enzyme needed for PPP hydrolysis to obtain a required DH under the same condition can be calculated. In general, many parameters, such as reaction time and enzyme concentration, involved in enzymatic hydrolysis determined the DH of hydrolysate obtained (Beak and Cadwallader, 1995; Guerard *et al*., 2001). From the result, the alcalase at the concentrations of 3.01, 16.84 and 93.41 Au/L (4% PPP solution) were required to obtain the DH of 20, 40 and 60%, respectively.

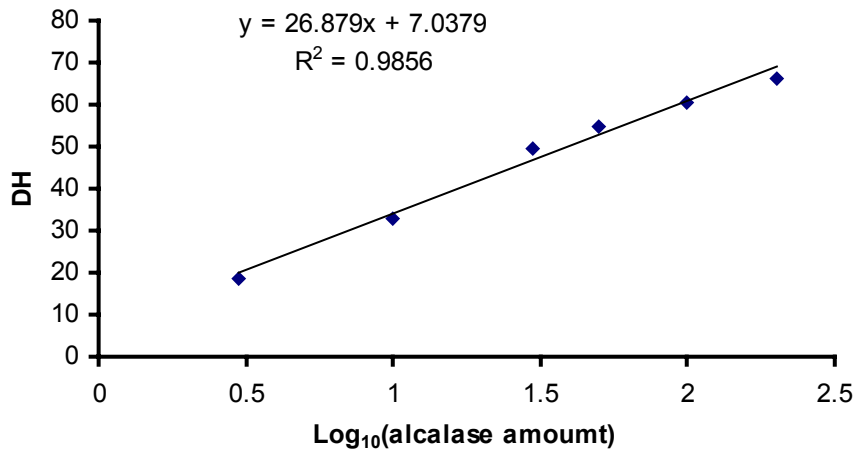

Figure 14 The relationship between $\log_{10}$ (alcalase amount) versus DH(%) in enzymatic hydrolysis of PPP for 4 h at pH 8.5 and 60^{o}C.

3.2 Acid hydrolysis of porcine plasma protein

Acid hydrolysis was carried out by refluxing the PPP in 4N HCl for different times. The DH continuously increased as heating time increased ($P<0.05$) (Figure 15). The linear relationship were observed when the plot between $\log_{10}$ (heating time) vs DH was drawn ($R^2=0.9903$) (Figure 15). From this plot, the heating time needed for required DH could be calculated. Heating time required to hydrolyze PPP to obtain the DHs of 20, 40 and 60% were 7, 20 and 62 min, respectively. In general, acid can hydrolyze the protein effectively and is used to produce the protein hydrolysate (Kristinsson and Rasco, 2000). The hydrolysis extent of egg albumin and soy protein isolate by acid treatment was greater than that by enzymatic treatment (Alfawaz *et al*., 1994).

The PPP hydrolysates produced by either acid or enzymatic process with DH of 0, 20, 40 and 60% were used as the source of amino groups for Maillard reaction. The reaction was then performed at pH 10 and 12 at 100°C for 2 h.

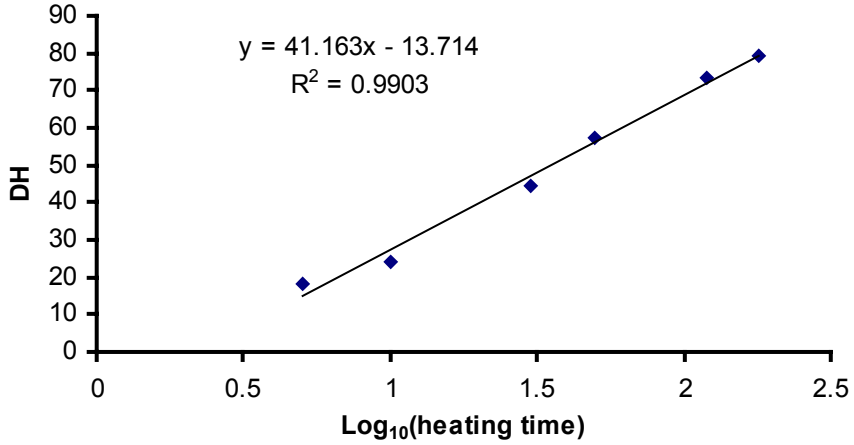

Figure 15 The relationship between $\log_{10}$ (heating time) versus DH(%) in acid hydrolysis of PPP in 4 N HCl.

3.3 Changes in pH

The final pH of all PPP hydrolysate-glucose MRPs varied with DH and pH used (Figure 16). Different hydrolysis condition and initial pHs resulted in the different final pH of MRPs. The MRPs derived from PPP acid hydrolysate-glucose at the initial pH of 12 gave the highest pH value for every DH used ($P<0.05$). From the result, the pH of all MRPs decreased from their initial pHs, 10 and 12, to 7.54-8.41 and 7.71-8.99, respectively. For PPP acid hydrolysate-glucose MRPs at every DHs, the higher final pH was observed when the higher initial pH was used ($P<0.05$).

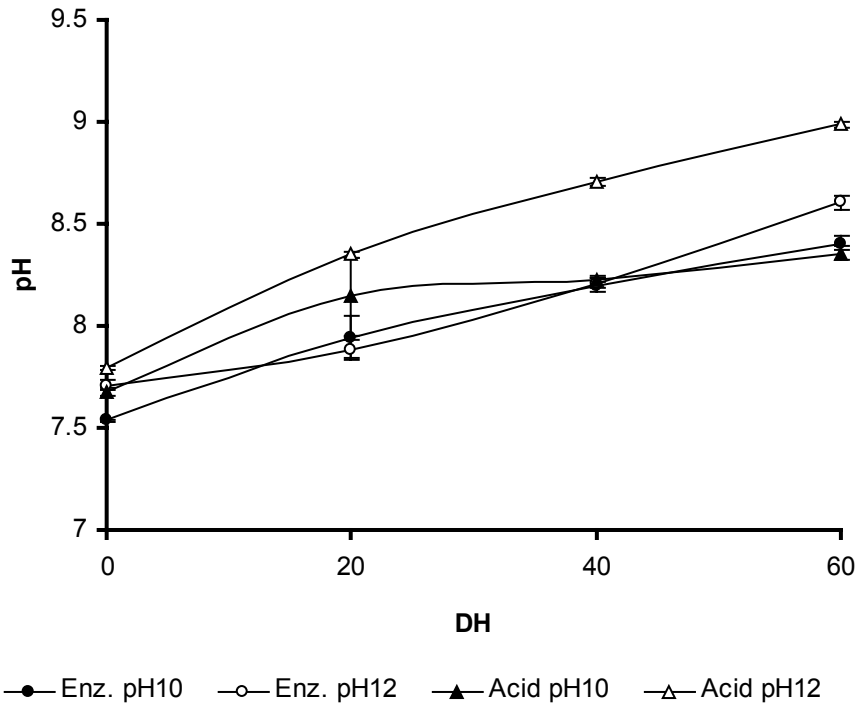

Figure 16 Changes in pH of PPP hydrolysate-glucose MRPs with various hydrolysis processes and DHs. The reaction was performed at initial pHs of 10 and 12 for 2 h at 100^{o}C. Bars indicate the standard deviation from triplicate determinations.

However, no differences in final pH of PPP enzymatic hydrolysate-glucose MRPs were observed at the DHs of 20 and 40 ($P>0.05$). From the result, the greater final pH might indicate the higher buffering capacity of free amino acid generated in PPP with greater DH. Amino acid is known as the potential buffer, which is capable of maintaining the pH of system (Van Waarde, 1988). Generally, pH drop was noticeable when Maillard reaction proceeded. The rate of decrease depended on heating time, the protein or amino acid source as well as DH of protein (Renn and Sathe, 1997; Benjakul *et al*., 2004).

3.4 Changes in A_{294}

The A_{294} of PPP acid hydrolysate-glucose MRPs markedly increased when PPP hydrolysate with DH of 20% was used ($P<0.05$) (Figure 17(a)). Lower A_{294} was observed in MRPs with DHs of 40 and 60%, compared to that with 20% DH. For PPP enzymatic hydrolysate-glucose MRPs, no changes in A_{294} were observed in MRPs with both initial pHs (10 and 12) when the DH increased up to 20% ($P>0.05$). The slight increases in A_{294} were found with increasing DH up to 60%. From the result, PPP acid hydrolysate-glucose MRPs containing PPP hydrolysate with 20% DH and the initial pH of 12 showed the highest A_{294}. At both pHs, MRPs derived from the initial pH 12 had the greater A_{294} than those with the initial pH of 10. A_{294} is used to determine the uncolored intermediate compound of the Maillard reaction (Ajandouz *et al*., 2001, Benjakul *et al*., 2004). Different conditions of PPP-hydrolysis resulted in the different amino acid sequence of the peptide as well as free amino acid available (Guerard and Sumaya-Martinez, 2003). The differences in the peptide pattern of hydrolysate used, as well as source of amino group also influenced the Maillard reaction (Alfawaz *et al*, 1994). From the result, acid hydrolysis might produce the

peptide with different reactive amino group or amino acid sequence from enzymatic hydrolysis. This is shown by the difference in initial reaction of Maillard reaction as indicated by A_{294}. It was most likely that intermediates might be formed at the greater extent when acid PPP hydrolysate was used as the reactant.

3.5 Changes in fluorescence intensity

Fluorescence intensity of all MRPs with different initial pHs, hydrolysis methods and DHs is shown in Figure 17(b). When PPP hydrolysates produced from either acid or enzymatic hydrolysis were used, MRPs with higher initial pH rendered the higher fluorescence intensity. No differences in fluorescence intensity were noted in MRPs derived from PPP hydrolysate with DHs of 0, 20 and 40% at the initial pH of 10, and MRPs derived from PPP hydrolysate with DHs of 0 and 40% at the initial pH of 12 ($P>0.05$). However, the fluorescence intensity of PPP enzymatic hydrolysate-glucose MRPs was higher than PPP-acid hydrolysate-glucose MRPs at both initial pHs when hydrolysate with 60% DH was used. The differences in fluorescent intensity was probably due to the differences in peptide patterns or amino acid sequences between hydrolysate prepared by both hydrolysis processes. This might be associated with the varying reaction rate and the formation of fluorescent compounds. The generation of fluorescent compounds occurs in the Maillard reaction before development of brown pigments (Morales *et al.*, 1996; Jing and Kitts, 2002). From the result, the increase in free amino groups from both acidic and enzymatic hydrolysis resulted in the increase in Maillard reaction rate. As a consequence, the higher fluorescence intensity was noticeable when the higher DHs of PPP-hydrolysate were used. The differences in patterns of non-fluorescent and fluorescent compounds in MRPs (Figure 17(a) and 17(b)) indicated that hydrolysis process had the influence

on the initial reaction, mostly likely “carbonylamino” reaction. This might be determined by the differences in peptide and reactive groups in peptides obtained.

3.6 Changes in browning intensity

The sharp increase in browning intensity of all PPP acid hydolysate-glucose MRPs as measured by A_{420} was observed when PPP hydrolysates with DHs of 20 and 40% were used ($P<0.05$) (Figure 17(c)). Slightly lower A_{420} was found when DH of PPP hydrolysate was 40% in comparison with that of 20% DH. Nevertheless, negligible changes in browning were found in PPP enzymatic hydrolysate-glucose MRPs at all DHs and pHs used, except with the DH of 60% and pH of 12 ($P<0.05$). From the result, MRP samples derived from the initial pH of 12 had a greater increase in browning intensity than those with the initial pH of 10. The result was in agreement with Renn and Sathe (1997) who found that the browning of MRPs prepared from leucine-glucose system increased with increasing initial pH. When comparing the effect of hydrolysis process on browning development, MRPs derived from PPP acid hydrolysate-glucose MRPs showed the greater browning than MRPs containing PPP enzymatic hydrolysate ($P<0.05$).

Generally, the profiles of browning intensity (Figure 17(c)) and A_{294} (Figure 17(a)) were similar. It was suggested that the brown products formed were correlated well with their non-fluorescent intermediates. Nevertheless, the involvement of fluorescent intermediates in browning development should be taken into consideration.

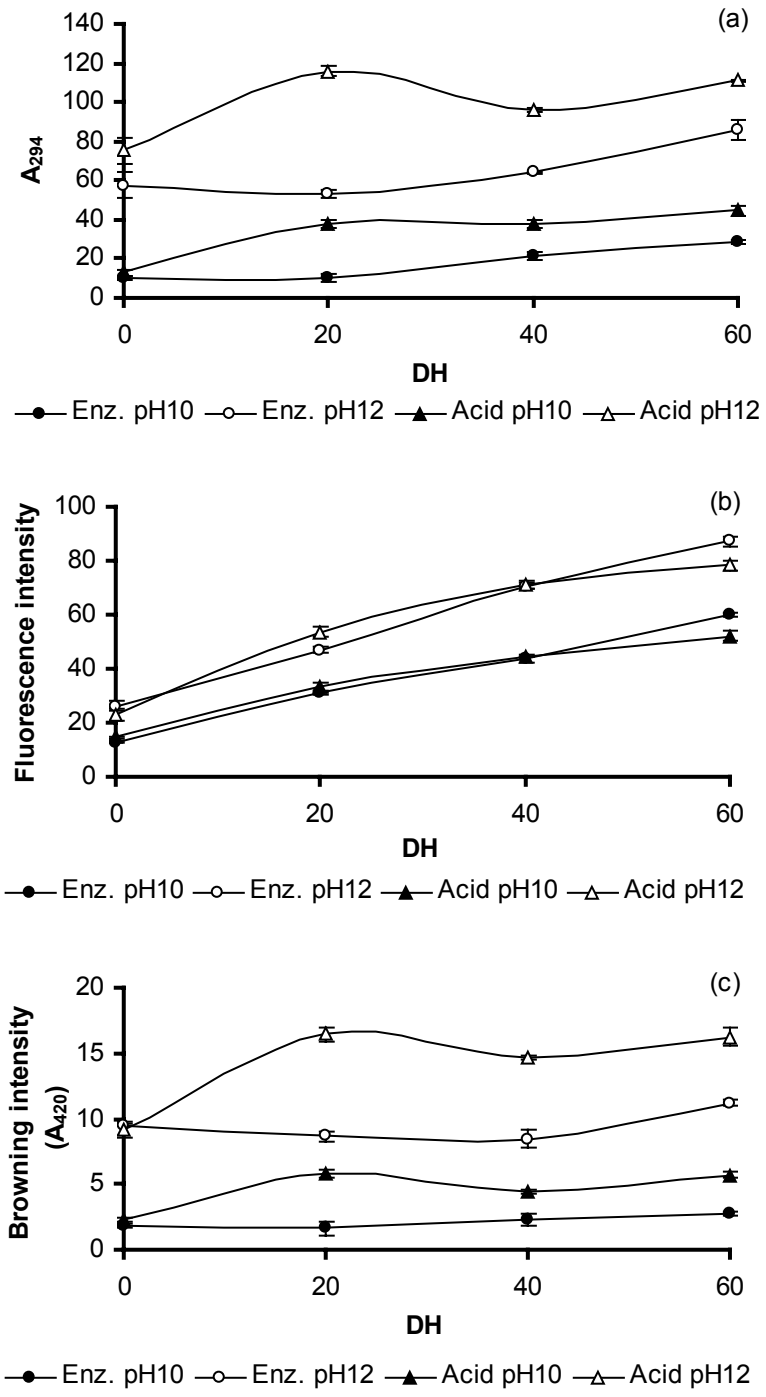

Figure 17 Changes in A_{294} (a), fluorescence intensity (b) and browning intensity (c) of PPP hydrolysate-glucose MRPs with various hydrolysis processes and DHs. The reaction was performed at initial pHs of 10 and 12 for 2 h at 100^{o}C. Bars indicate the standard deviation from triplicate determinations.

3.7 Changes in free amino group content

Free amino groups, which incorporated into carbonylamino reaction, was more intensed as the DH of all PPP hydrolysate-glucose system increased up to 40% ($P<0.05$) (Figure 18(a)). When the DH of PPP hydrolysate used was 60%, no changes in free amino groups incorporated were noted in MRPs derived from PPP acid hydrolysate-glucose, compared with that of 40% DH ($P>0.05$). The free amino group incorporated was lowered slightly in MRPs derived from PPP enzymatic hydrolysate-glucose when DH of 60% was used ($P<0.05$). Normally, the changes in the levels and compositions of free amino acids and small peptides were noted when proteins were subjected to hydrolysis (Wu *et al*., 2003). From the result, the increase in free amino group incorporated, suggested the higher formation of glycated proteins between amino acid groups of PPP hydrolysate and carbonyl group of reducing sugar were taken place. The result was in agreement with Benjakul *et al*. (2004) who found that free amino group in PPP-sugar systems heated at 100^{o}C were decreased with the concomitant increase in Maillard reaction products.

3.8 Changes in reducing sugar content

The continuous decrease in reducing sugar content of all PPP hydrolysate-glucose MRPs were observed with increasing DH up to 60% ($P<0.05$) (Figure 18(b)). Initial pHs directly influenced the reducing sugar content of MRPs. From the result, the higher initial pH rendered the greater decrease in reducing sugar at every DH used. It was suggested that the glycated proteins were formed to a higher extent at grater pH. The higher decreases in reducing sugar content were also observed in MRPs derived from PPP acid hydrolysate-glucose model systems, compared with those from system having the PPP enzymatic hydrolysate, especially at pH 12.

Generally, the decrease in reducing sugar content can be used as a nonspecific way for measuring the Maillard reactions (Van Boekel and Martins, 2002). This result revealed that the Maillard reaction, condensation between free amino acid group and carbonyl group, was enhanced in presence of sufficient free amino group particularly at high initial pH. Among all MRPs, Those derived from PPP acid hydrolysate-glucose system with initial pH of 12 and 60% DH PPP hydrolysate showed the lowest reducing sugar content (12.68 mM). Renn and Sathe (1997) reported that the higher decrease in reducing sugar content in leucine-glucose model system was observed at higher initial pH value.

From the result, the profiles of changes in free amino groups and reducing sugar contents in MRPs model systems were in accordance with that of fluorescence intensity (Figure 17(b)). This suggested that fluorescent intermediates were formed relevant to the reduction of both precursors, amino group of hydrolysate and carbonyl group of sugar. Thus, fluorescence intensity could be a good indicator to monitor the carbonylamino reaction of PPP hydrolysate-glucose model system. Since the profiles of changes in A_{294} and browning intensity were somehow deviated from those of amino group and reducing sugar content, it was most likely that non-fluorescent intermediates served as the important intermediates for browning development.

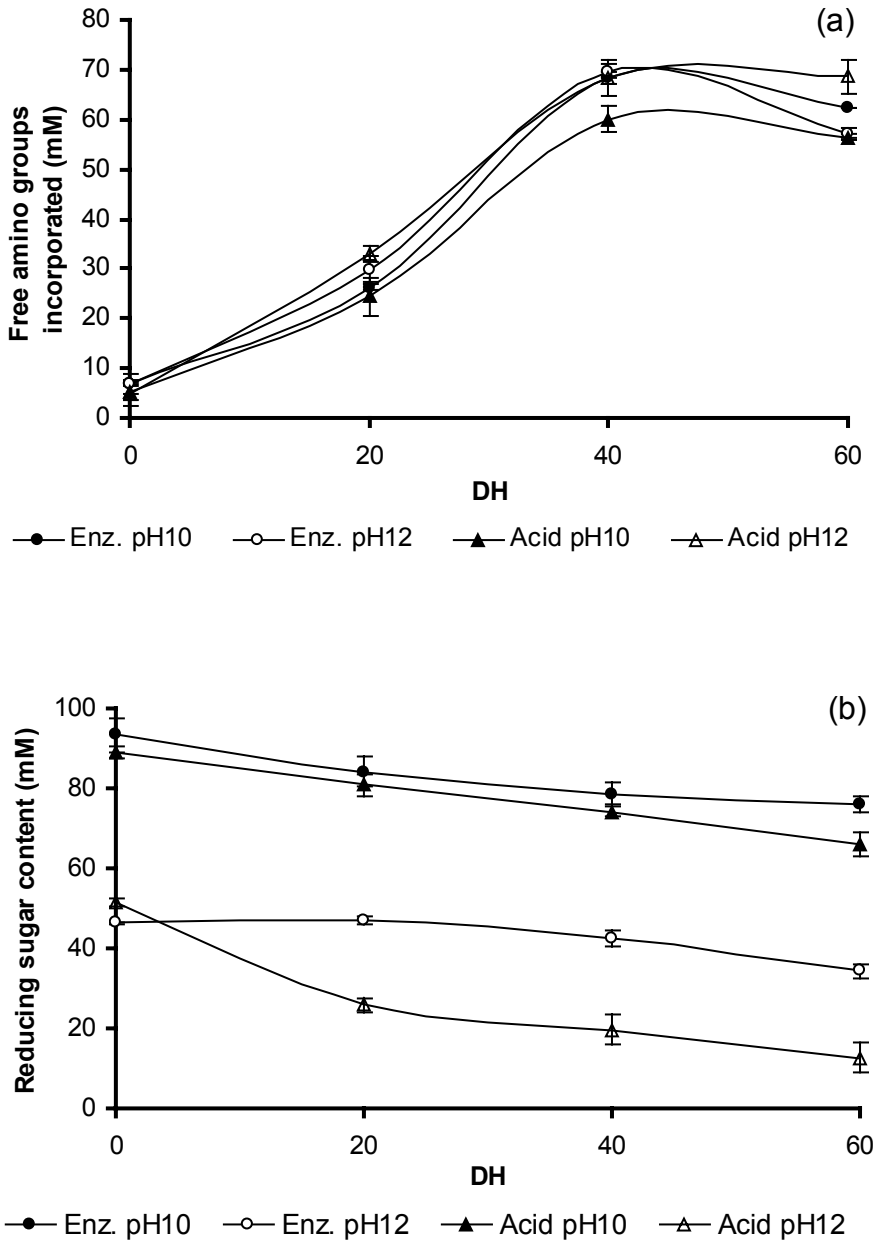

Figure 18 Changes in free amino groups incorporated (a) and reducing sugar content (b) of PPP hydrolysate-glucose MRPs with various hydrolysis processes and DHs. The reaction was performed at initial pHs of 10 and 12 for 2 h at 100^{o}C. Bars indicate the standard deviation from triplicate determinations.

3.9 Changes in reducing power

Reducing power of PPP hydrolysate-glucose MRPs with the initial pH of 10 increased as the DH of PPP hydrolysate increased ($p<0.05$) (Figure 19(a)). With initial pH of 12, no differences in reducing power of PPP enzymatic hydrolysate-glucose MRPs with DHs of 0, 20 and 40%, but the increase in reducing power was found at 60% DH. However, similar reducing power was noticeable among MRPs derived from PPP enzymatic hydrolysate with DH greater than 20%. From the result, MRPs derived from PPP acid hydrolysate-glucose system with the initial pH of 12 and 20% DH PPP hydrolysate showed the highest reducing power. From the result, it was suggested that the initial pH, degree of PPP hydolysis and hydrolysis methods had the influence on the Maillard reaction rate as well as antioxidative activity. Apart from Maillard reaction products, protein hydrolysates from herring (Sathivel *et al*., 2003) and mackerel (Wu *et al*., 2003) have been reported to exhibit an antioxidant activity**.** However, the antioxidant effect of protein hydrolysates were improved when reacting with glucose (Bishov and Henick, 1975).

Thus, peptides with different amino acid compositions and sequences were postulated to render the MRPs with different antioxidative activity. Antioxidant activity of peptide-sugar MRPs (Lingnert and Eriksson, 1981) and hydrolysate-glucose MRPs (Guerard and Sumaya-Martinez, 2003) was dependent, not only on the amino acid constitute in the peptide or protein but also on their sequence. The basic amino acids are reactive to form antioxidative product (Lingnert and Eriksson, 1981).

3.10 Changes in DPPH radical scavenging activity

DPPH radical-scavenging activity of PPP acid hydrolysate-glucose MRPs markedly increased when 20% DH hydrolysate was used ($P<0.05$) (Figure 19(b)). Slightly increased radical-scavenging activity was observed in PPP acid hydrolysate-glucose MRP systems with higher DHs used ($P<0.05$). For PPP enzymatic hydrolysate-glucose model systems, no changes in radical-scavenging activity were found at every DHs used. From the results, DPPH radical-scavenging activity of PPP hydrolysate-glucose MRPs was more pronounced at higher initial pH used. This result indicated that the higher initial pH induced the formation of MRPs with an effective radical scavenging activity. In general, the reduction of $DPPH^{\bullet}$ by antioxidant ($DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$) or by radical species ($DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$) results in a loss of absorbance at 515 nm (Fukumoto and Mazza, 2000). Guerard and Sumaya-Martinez (2003) found that MRPs derived from protein hydrolysate had the high radical-scavenging activity. Additionally, the Maillard reaction induced the formation of a high proportion of phenolic compounds, mainly for the casein peptone hydrolysate-glucose, which may be related to the increase in antioxidant/free radical scavenging properties. (Guerard and Sumaya-Martinez, 2003). From the result, DPPH radical-scavenging activity correlated well with browning intensity (Figure 17(c)) and A_{294} (Figure 17(a)). The different antioxidation activity of MRPs produced from different hydrolysis processes suggested the differences in the peptides generated, which might undergo Maillard reaction differently. As a result, products possessing antioxidative activity were formed at varying degrees. From the result, radical scavenging activity correlated well with reducing power, browning intensity and A_{294}.

3.11 Changes in metal chelating activity

The continuous increase in metal chelating activity in PPP hydrolysate-glucose MRPs prepare at pH 10 was observed as the DH increased up to 60% ($P < 0.05$) (Figure 19(c)). However, small changes in metal chelating activity were noticeable in PPP hydrolysate-glucose MRPs with the initial pH of 12. The higher initial pH used resulted in the higher metal chelating activity of all MRPs. The metal chelating activity of MRPs derived from PPP acid hydrolysate-glucose system containing hydrolysate with DH ranges of 20-60% are greater than that of MRPs with PPP enzymatic hydrolysate-glucose system with all DHs used. Generally, the melanoidins behave as chelating agents for polyvalent metal cations (Rendleman, 1987; Kajimoto *et al*., 1975). From the result, MRPs derived from PPP hydrolysate-glucose system exhibit more pronounced metal chelating activity when the greater initial pH value was used. Wijewickreme *et al*. (1997) found that MRP mixtures derived from lysine-glucose and lysine-fructose model systems with appropriate condition had the high metal chelating activity. Additionally, MRPs prepared from fructose-tryptophane had a capacity to chelate ferric ion (Tanaka *et al*., 1992). Therefore, MRPs from PPP-hydrolysate-glucose system could function as the antioxidant either as the primary or secondary antioxidants.

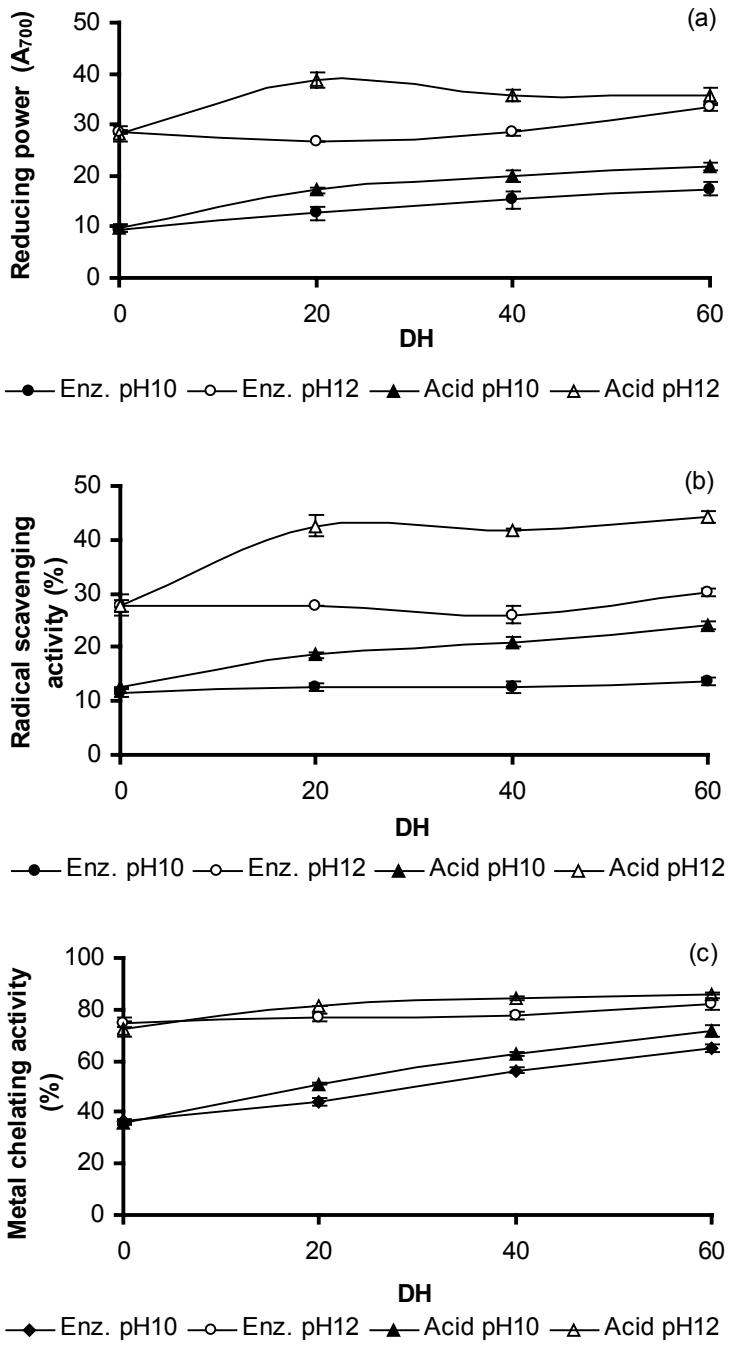

Figure 19 Reducing power (a), DPPH radical-scavenging activity (b) and metal chelating activity (c) of PPP hydrolysate-glucose MRPs with various hydrolysis processes and DHs. The reaction was performed at initial pHs of 10 and 12 for 2 h at 100^{o}C. Bars indicate the standard deviation from triplicate determinations.

4. Characteristics and antioxidative activity of PPP acid hydrolysate-glucose MRP fractions

Fractionation was applied to separate or concentrate the antioxidative compounds from MRPs. Different solvents were used for fractionation consecutively from hexane, dichloromethand to ethylacetate, respectively.

4.1 Browning intensity of various MRPs fractions

The browning intensity of all MRPs fractions obtained from different solvents as indicated by A_{420} is shown in Figure 20. Dichloromethane fraction exhibited the highest browning intensity ($P<0.05$). However, only slight differences in browning intensity of all fractions, except the residual fraction, were observed ($P<0.05$). From the result, residual fraction showed the lowest browning intensity, presumably due to the removal of lower-polarity color compounds by low-polarity solvent. The result suggested that the fractionation by solvents with different polarities could separate the different MRPs, based on the polarity. However, brown constituents were found to distribute into all fractions. Therefore, fractionation method used in this study could not remove brown pigments effectively.

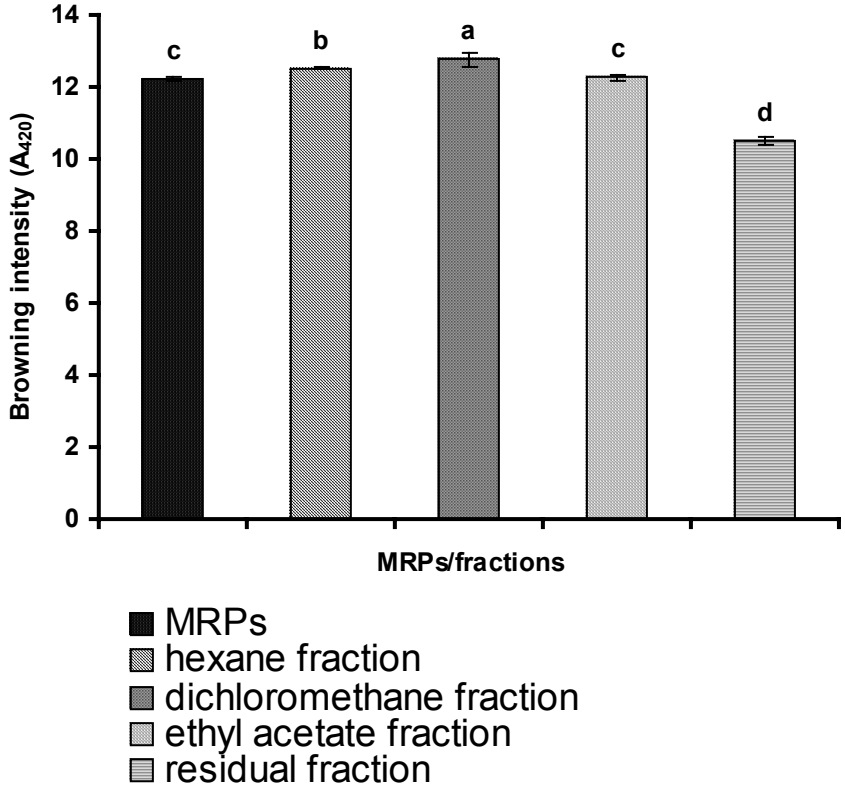

Figure 20 Browning intensity of different PPP-acid hydrolysate-glucose MRPs fractions. Bars indicate the standard deviation from triplicate determinations. Different letters on the bars indicate significant differences ($P<0.05$)

4.2 Reducing power of various MRPs fractions

Reducing power varied among MRPs and all fractions (Figure 21(a)). MRPs and fractions had the increased reducing power in a concentration dependent manner. MRPs showed the greatest reducing power at every concentration used. No differences in reducing power were observed between hexane, dichloromethane, ethylacetate and residual fractions at the lowest concentration used (2%) ($p>0.05$).

However, slight differences in reducing power were found in hexane, dichloromethane, ethylacetate and residual fractions at the highest concentration used (12%).

Stepwise fractionations of MRPs was done to isolate components which might have antioxidant activity by gradually increasing the polarity of extracting solvent. Several compounds with differing chemical structure formed during the browning reaction of PPP-acid hydrolysate-glucose system might exhibit electron donating properties with different degrees. Those compounds might work synergistically as evidenced by the highest reducing power in the original MRPs. Generally, reducing power of MRPs and fractions was found to correlate with DPPH radical scavenging activity.

4.3 Radical-scavenging activity of various MRPs fractions

Different DPPH radical-scavenging activity of MRPs and all fractions was observed. Generally, activity increased as the amount of MRPs and fractions increased ($P<0.05$) (Figure 21(b)). No differences in radical-scavenging activity were found between MRPs, hexane, dichoromethane and ethylacetate fractions at concentrations of 2 and 4% ($p>0.05$). However, small differences were noted when MRPs concentration increase up to 12%. Therefore, activity was dependent upon the concentration used. Generally, DPPH radical-scavenging activity was used to determine the hydrogen doner ability of MRPs (Benjakul *et al*., 2004; Matthus, 2002; Morales and Jimenez-Perez, 2001; Aliaz *et al*, 1999). From the result, at concentrations of 8 and 10%, MRPs showed the highest radical-scavenging activity, while the residual fraction had the lowest radical-scavenging activity. The result revealed that MRPs consisted of different antioxidative compounds with different

polarity. Those compound in MRPs might work synergistically, leading to the greatest activity in the MRPs. The lowest activity in the residual fraction suggested that more polar compounds exhibited the lower antioxidative activity, especially via the radical chain breaking.

4.4 Metal chelating activity of various MRPs fractions

The metal chelating activity of MRPs and all fractions increased continuously with increasing MRPs concentrations ($P<0.05$) (Figure 21(c)). The lowest metal chelating activity was found in MRPs compared with other fractions, particulary at the high concentrations tested. No differences in metal chelating activity of dichloromethane, ethylacetate and residual fractions were noticeable at the concentration of 12% ($P>0.05$). Nevertheless, the hexane fraction exhibited the slightly higher chelating activity when compared to other fractions. The result suggested that the fractionation using less polarity solvent might favor the partitioning the compounds with metal chelating property into those fractions. As a result, the greater chelating activity was observed with those fractions. Therefore, the chelating activity might be more pronounced when those compounds with high metal chelating activity were present as the single components.

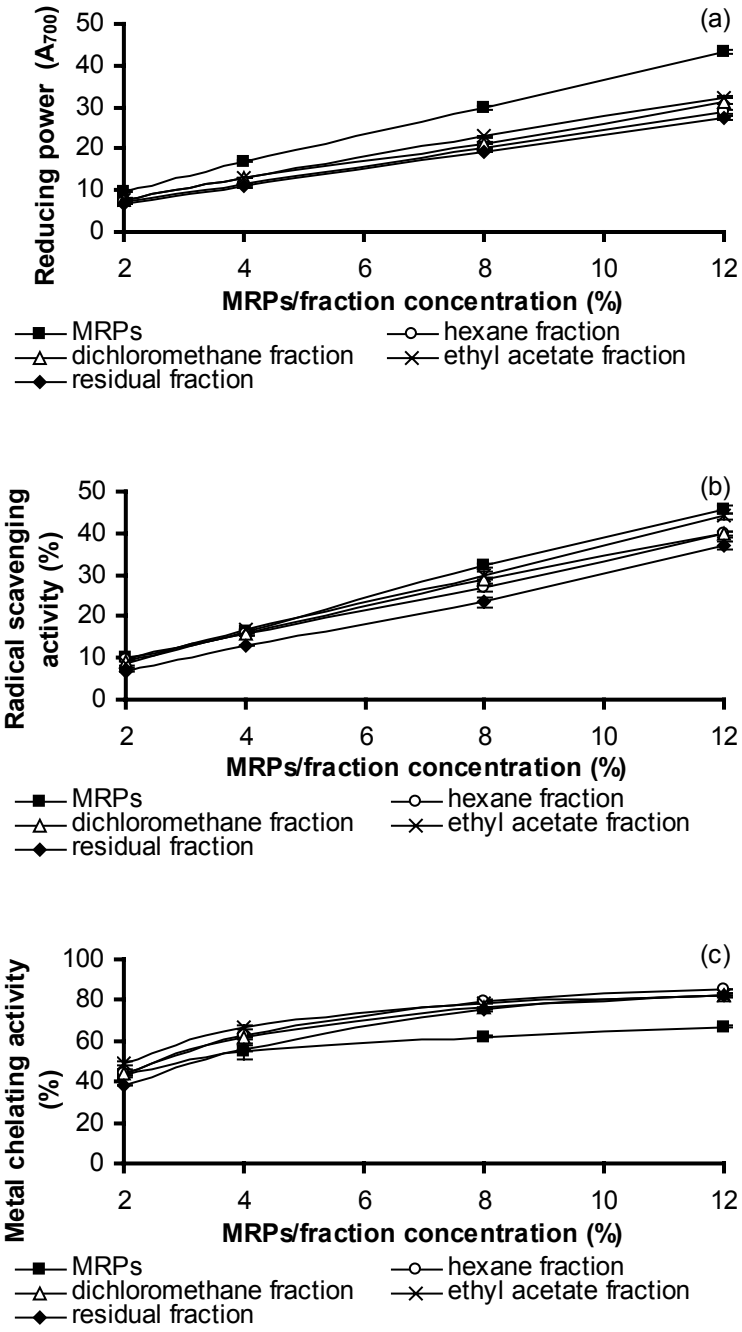

Figure 21 Reducing power (a), DPPH radical-scavenging activity (b) and metal chelating activity (c) of MRPs and different fractions at various concentrations. The solvents with various polarities were used for the stepwise fractionation. Bars indicate the standard deviation from triplicate determinations.

5. Effect of decolorization on color and antioxidative activity of MRPs from PPP acid hydrolysate-glucose model system

5.1 Color of MRPs and decolorized MRPs powder

MRPs and all MRPs decolorized using activated carbon or Sep-Pak Cartridge C_{18} were lyophilized before the color measurement. L*, a* and b*-values of MRPs and decolorized MRPs powder are shown in Table 5. The increase in L*-value were observed in MRPs decolorized by 5% activated carbon or 2 and 3 times of Sep-Pak Cartridge C_{18} treatment. The higher amount of activated carbon used and the increasing times of Sep-Pak Cartridge C_{18} treatment resulted in higher L*-value and lowered a* and b*-values. The highest L*-value was observed in the fraction with the three-times decolorization by Sep-Pak Cartridge C_{18} ($p<0.05$). In general, decreases in L*-value and hue angle indicate darker samples and a shift in color from yellow-brown to orange-brown, respectively (Bates *et al.*, 1998; Nicoli *et al*., 1997). Decolorization of MRPs generally resulted in the decrease in a* and b*-values. However, the decolorization by Sep-pak Cartridge C_{18} showed the increase in a*-value after the first decolorization and increased b*-value was also noticeable after the first and second decolorization. Calligaris *et al*. (2004) used the b*-value to indicate the browning development in heated milk. The increase in b*-value was found with the increase in browning. The result indicated that the binding of the brown pigments to activated carbon and Sep-Pak C_{18} Cartridge treatment contributed to the reduced MRPs color.

Table 5 L* (lightness), a* (redness/greeness) and b* (yellowness/blueness)-values of MRPs powder decolorized by activated carbon or Sep-Pak Cartridge C_{18}

Samples	L*	a*	b*
MRPs	49.28±0.52^{c}	9.45±0.11^{b}	24.35±0.28^{b}
1%charcoal	47.28±0.82^{d}	9.48±0.04^{b}	22.81±0.34^{d}
2%charcoal	48.78±0.21^{c}	8.95±0.06^{c}	22.82±0.10^{d}
5%charcoal	52.23±0.49^{b}	7.36±0.03^{e}	18.49±0.02^{e}
Sep-Pak 1	43.75±0.32^{e}	9.94±0.11^{a}	25.81±0.23^{a}
Sep-Pak 2	51.51±0.72^{b}	8.44±0.07^{d}	26.18±0.28^{a}
Sep-Pak 3	66.67±1.10^{a}	5.38±0.10^{f}	23.57±0.27^{c}

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

** Mean ± SD from triplicate determinations

5.2 Browning intensity of MRPs and decolorized MRPs

The A_{420} is commonly used to determine the browning intensity of Maillard reaction products (Ajandouz *et al*., 2001; Morales and Jimenez-Perez, 2001; Benjakul *et al*., 2004). The browning intensity was markedly decreased after decolorization with activated carbon or Sep-Pak Cartridge C18 ($P<0.05$) (Figure 22). Activated carbon, a highly porous material, have a high attractive or adsorptive properties that can adsorp organic molecules. (Hindarso *et al*., 2001; Stoeckli *et al*., 2001; Zhang *et al*., 2001). Thus, some brown pigments with the size smaller than the activated carbon pore size may be adsorbed in their matrix. In addition, continuous decrease in browning intensity was found as the activated carbon concentration increased up to 5%.

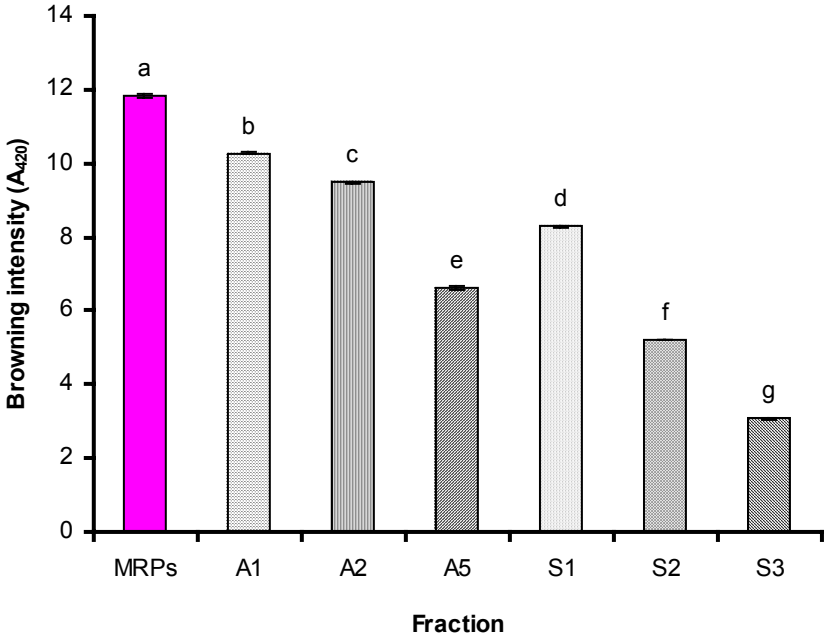

Figure 22 Browning intensity of MRPs and MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. A1, A2 and A5 donate MRPs decolorized by 1, 2 and 5% activated carbon, respectively. S1, S2 and S3 donate MRPs decolorized by Sep-Pak Cartridge C18 for 1, 2 and 3 times, respectively. Different letters on the bars indicate significant differences ($p<0.05$).

Browning intensity of MRPs treated with Sep-Pak Cartridge C18 decreased with increasing times of treatments (Figure 22). Octadecyl-siloxane (C18) is hydrophobic materials commonly used in analytical chromatography for the isolation of hydrophobic compounds from aqueous solution (Lee, 1992). Thus, the decrease in browning intensity was probably due to the decrease in hydrophobic brown color compounds. Moreover, the greater decrease in browning intensity of MRPs was observed with increasing repetition of decolorization. MRPs subjected to Sep-Pak Cartridge C18 treatment for 3 times showed the lowest browning intensity as

evidenced by the reduction of A_{420} from 11.85 to 3.07. Lee (1992) used Sep-Pak Cartridge C18 to isolate brown compounds from a temperature-controlled storage orange juices at 50^{o}C for 15 weeks. The result obtained was in accordance with Yen and Hsieh (1995) who reported that the brown pigment from xylose-lysine MRPs was reduced after decolorization with Sep-Pak Cartridge C18.

5.3 Reducing power of MRPs and decolorized MRPs

Reducing power of all decolorized MRPs was lower than original MRPs ($P < 0.05$) (Figure 23(a)). For MRPs decolorized with activated carbon, the greater decrease in reducing power was observed with increasing activated carbon amount ($P < 0.05$). Additionally, the reducing power of MRPs decolorized with Sep-Pak Cartridge C18 decreased with increasing repetition ($P<0.05$). Among the decolorized MRPs tested, the MRPs decolorized by 5% activated carbon showed the lowest reducing power. Activated carbon might adsorb the antioxidative compounds to greater extent when higher amount of activated carbon was used. For MRPs decolorized by Sep-Pak Cartridge C18, the decrease in reducing power was probably associated with the removal of antioxidative compounds, presumably brown pigments. In general, reducing power of MRPs and decolorized MRPs correlated well with radical-scavenging activity. However, the decrease in browning intensity by decolorization treatment was coincidental with the reduction of antioxidant activity. The result suggested that the brown pigment in MRPs exhibited the primary antioxidant activity (Yoshimura *et al*., 1997; Wijewickreme *et al*., 1999; Benjakul *et al*., 2004). The result was in agreement with Yen and Hsieh (1995) who found that the brown pigments, the final products in the advanced stage of the Maillard reaction,

have a high reducing power. Therefore, the decrease in brown color generally reduced the antioxidative activity of MRPs.

5.4 Radical-scavenging activity of MRPs and decolorized MRPs

DPPH radical scavenging activity of MRPs decreased after decolorization with activated carbon at concentrations of 2% and 5% and with Sep-Pak Cartridge C18 treatment for 2 and 3 times ($P<0.05$) (Figure 23(b)). However, no change in radical-scavenging activity of MRPs decolorized by 1% activated carbon was observed. Interestingly, MRPs decolorized by Sep-Pak Cartridge C18 for 1 time had the increase in radical-scavenging activity, compared with original MRPs. The removal of some hydrophobic compounds by the first decolorization might promote the antioxidative activity of decolorized MRPs. Higher concentration of activated carbon as well as the repetition of decolorization by Sep-Pak Cartridge C18 resulted in the lower radical-scavenging activity.

MRPs decolorizing by 5% activated carbon exhibited the lowest radical-scavenging activity. The result revealed that Sep-Pak Cartridge C18 treatment not only decolorized the MRPs as shown by the low browning intensity but also remained a high radical-scavenging activity. Consequently, Sep-Pak Cartridge C18 is more suitable for decolorization of PPP acid hydrolysate-gluose MRPs than activated canbon. When MRPs were decolorized by Sep-Pak Cartridge C18 for 2 and 3 times, radical scavenging activity was decreased. This might be associated with the removal of brown pigments processing antioxidative activity. Murakami *et al*. (2002) reported that the bright color pigment from histidine-xylose MRPs heated at 30^{o}C up to 120 h showed the DPPH radical scavenging activity. Lee (1992) found that the browning reaction products, isolated from storage-aged orange juice, and decolorized by Sep-

Pak Cartridge C18, exhibited the low browning intensity with high antioxidant activity. However, the brown melanoidin also showed the effective antioxidant activity.

5.5 Metal chelating activity of MRPs and decolorized MRPs

The metal chelating activity of all decolorized MRPs was slightly greater than that of MRPs ($P<0.05$) (Figure 23(c)). The increase in activated carbon amount resulted in the increased chelating activity. For MRPs decolorized by Sep-Pak Cartridge C18, the increase in chelating activity was found with increasing repetition. No differences in metal chelating activity were observed between MRPs decolorized by 1% activated carbon and MRPs decolorized by Sep-Pak Cartridge C18 for 2 times ($P > 0.05$). Since the decolorized MRPs exhibited the greater metal chelating property, it was most likely that colorless compounds in MRPs might possess the greater activity than color constituents. However, MRPs with brown color also showed metal chelating property. Morales *et al*. (2005) reported that the melanoidin derived from sugar (glucose, galactose) and amino acid (Ala, Cys, His, Lys, Gly, Met, Phe, Asp, Arg, Typ, Tyr) exhibited the strong iron-binding ability. Yoshimura *et al*. (1997) reported that the high-molecular-weight fraction of MRPs derived from glucose-glycine system processed greater metal chelating activity than the low-molecular-weight fraction. It is well known that ferrous ion, the most powerful prooxidant, can initiate the lipid oxidation by Fenton reaction (Narla and Nao, 1995). So, the complexation of MRPs with ferrous ion resulted in the complexes which had no prooxidant activity. The melanoidins have a negative net charge and are able to bind metallic ions (Migo *et al*., 1997; Gomyo and Horikoshi, 1976). From the result, decolorization showed the positive effect on metal chelating property of MRPs.

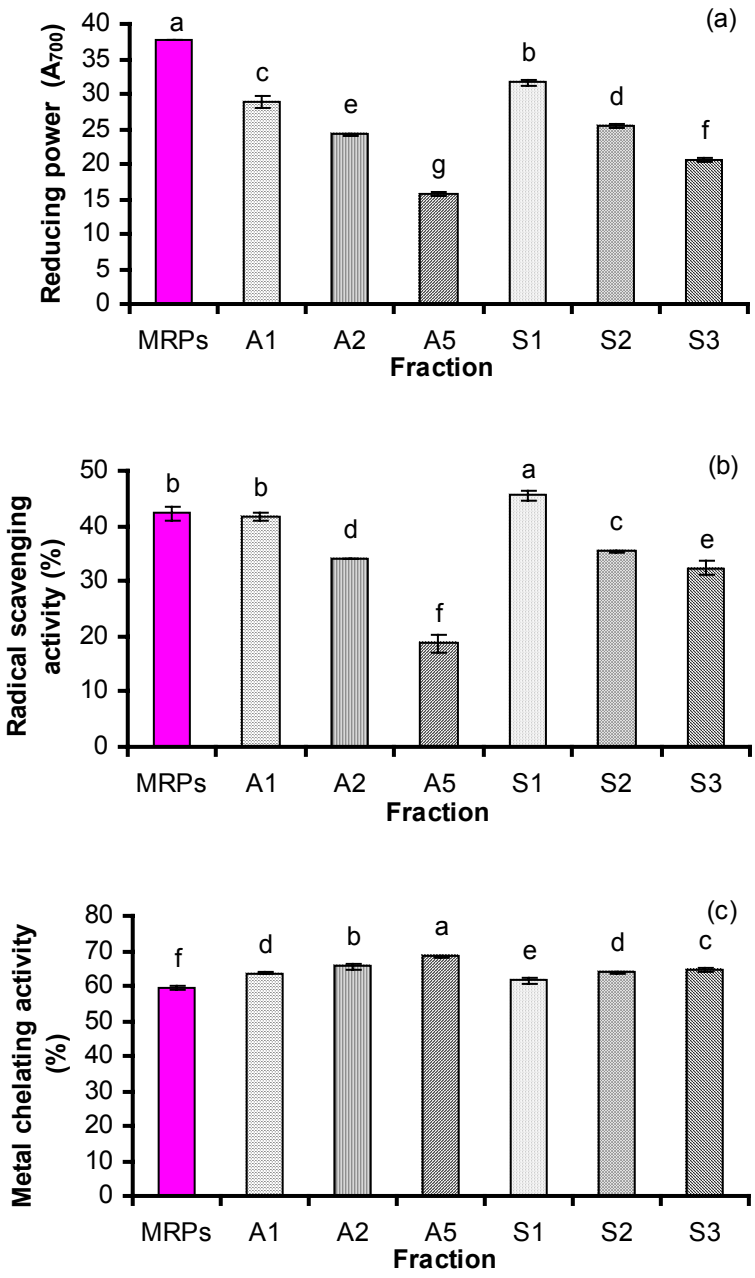

Figure 23 Reducing power (a), DPPH radical-scavenging activity (b) and metal chelating activity (c) of MRPs and MRPs decolorized by activated carbon and Sep-Pak Cartridge C18. A1, A2 and A5 donate MRPs decolorized by 1, 2 and 5% activated carbon, respectively. S1, S2 and S3 donate MRPs decolorized by Sep-Pak Cartridge C18 for 1, 2 and 3 times, respectively. Different letters on the bars indicate significant differences ($p<0.05$).

Nevertheless, MRPs without decolorization also showed this property caused by both color and colorless compounds. To reduce the brown color intensity of MRPs, decolorization was carried out using Sep-Pak Cartridge C18 for 1 time since it still possessed the high antioxidative activity.

6. Characteristic and antioxidative activity of MRPs and decolorized MRPs

6.1 Color of MRPs and decolorized MRPs solution

L*, a* and b*-values of 6% (w/v) solution of MRPs and decolorized MRPs are shown in Figure 24. The decolorized MRPs solution exhibited the higher L* and b* than MRPs ($P<0.05$), suggesting that the decolorized MRPs were more yellowish than MRPs. Decolorized MRPs solution had the lower a*-value than MRPs solution. This indicated that redness of MRPs was reduced by decolorization using Sep-Pak Cartridge C18. The decrease in redness correlated well with the decrease in browning intensity (Figure 22) and the lighter color of dry MRPs powder (Table 5).

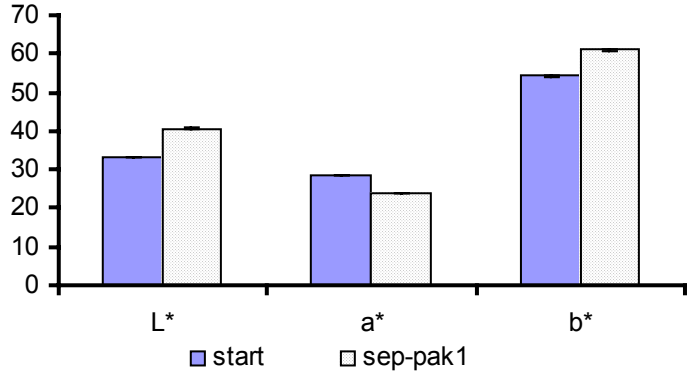

Figure 24 L* (lightness), a* (redness/greeness) and b* (yellowness/blueness)-values of MRPs and decolorized MRPs solution (6%, w/v). Bars indicate the standard deviation from triplicate determinations.

6.2 The effect of MRPs and decolorized MRPs amount on antioxidative activity

PPP acid hydrolysate-glucose MRPs were prepared by heating 2% glucose with 2% PPP acid hydrolysate (20% DH) at 100^{o}C for 2 h. Different amounts of MRPs and decolorized MRPs were tested for antioxidative activity. MRPs and decolorized MRPs with different amounts exhibited different antioxidative activity ($P < 0.05$) (Figure 25). From the result, the reducing power (Figure 25(a)), DPPH radical-scavenging activity (Figure 25(b)) and metal chelating activity (Figure 25(c)) increased with increasing MRP amounts up to 12%. The result suggested that the greater MRP amounts were more effective in donating electron or hydrogen to the free radicals. In other word, it worked as primary antioxidant more effectively when higher amount was used. Moreover, the chelating activity as the secondary function increased with increasing MRP amount ($P<0.05$). Chiu *et al*. (1991) reported that the antioxidative effect of MRP against sardine lipid oxidation during storage at 37^{o}C increased with the increase of MRP amount. Generally, MRPs exhibited a higher reducing power than decolorized MRPs at all amounts tested. Greater radical-scavenging activity of MRPs was noted when the amount of MRPs increased up to 8%, compared with decolorized MRPs. However, no differences in radical-scavenging activity of both original and decolorized MRPs were observed when 12% was used. The result was in accordance with Benjakul *et al*. (2004) who found that reducing power and DPPH radical-scavenging activity increased as the amount of PPP-sugar MRPs increased. For the metal chelating activity, the decolorized MRPs showed the slightly lower in metal chelating activity than MRPs at the low amounts

(2-4%). Nevertheless, decolorized MRPs showed the slightly greater metal chelating activity than MRPs when 12% was used.

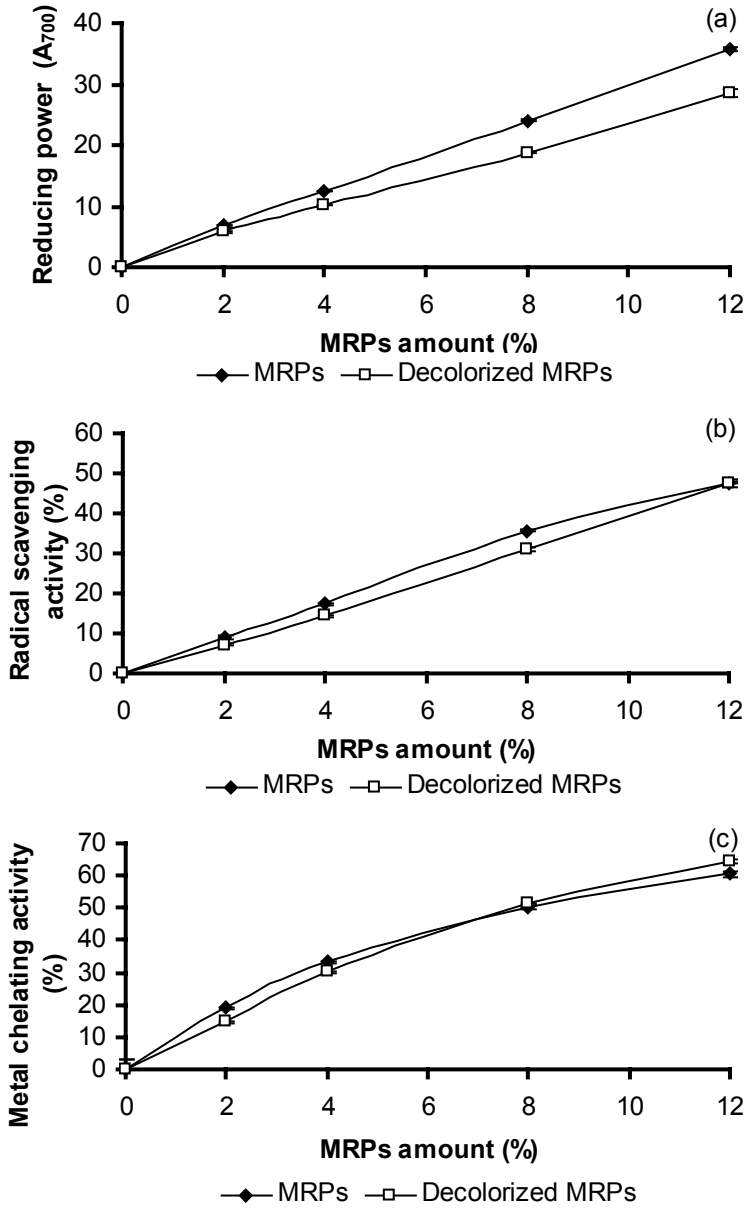

Figure 25 Reducing power (a), DPPH radical-scavenging activity (b) and metal chelating activity (c) of MRPs and decolorized MRPs with different amounts. Bars indicate the standard deviation from triplicate determinations.

6.3 Hydroxyl radical-scavenging activity of MRPs and decolorized MRPs

The hydroxyl radical-scavenging activity of MRPs and decolorized MRPs at different concentrations is shown in Figure 26. The system containing ferric-ascorbate-EDTA-H_2O_2 can generate hydroxyl radical by Fenton reaction (Fe^{2+}+ $H_2O_2 \rightarrow Fe^{3+}$+$OH^-$+OH) at a rapid rate (Narla and Nao, 1995). The hydroxyl radical generated in the system will react with deoxyribose and DNA, as targets of hydroxyl radical induced peroxidation (Aruoma, 1994; Wijewickreme and Kitts, 1997; Jing and Kitts, 2004).

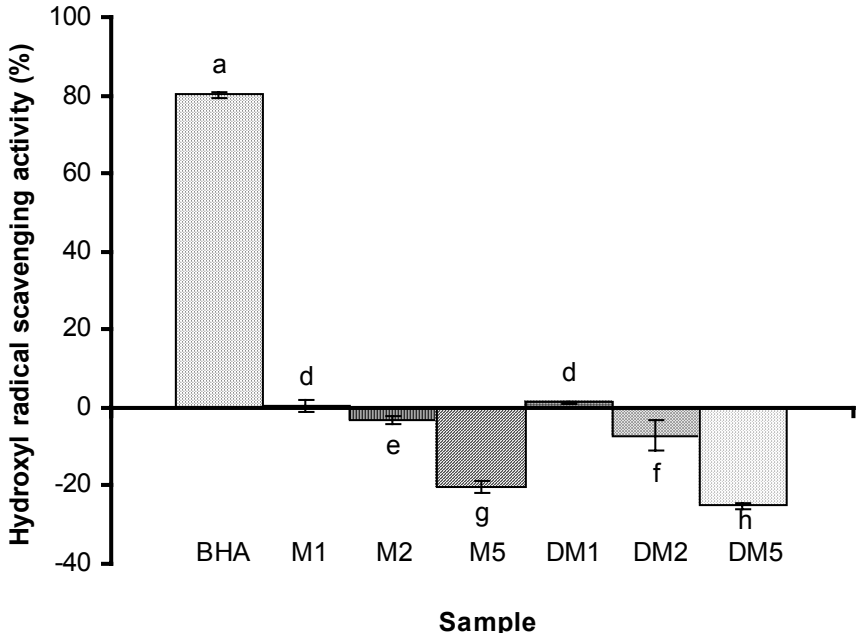

Figure 26 Hydroxyl radical-scavenging activity of MRPs and decolorized MRPs. M1, M2 and M5 denote MRPs at levels of 100, 200 and 500 ppm, respectively. DM1, DM2 and DM5 denote MRPs decolorized by Sep-Pak Cartridge C18 at levels of 100, 200 and 500 ppm, respectively. BHA denotes BHA at a leval of 200 ppm. Bars indicate the standard deviation from triplicate determinations.

The prooxidant activity was observed for both MRPs and decolorized MRPs at the levels of 200 and 500 ppm. However, no hydroxyl radical-scavenging activity was found when 100 ppm MRPs was used. The result revealed that the prooxidant products which promote the oxidation possibly occurred during Maillard reaction. Generally, hydrophilic radical (such as hydroxyl radical) can interact with MRPs in aqueous solution (Rival *et al*., 2001). MRPs derived from glucose-casein, fructose-casein and ribose-casein (Jing and Kitts, 2002) glucose-glycine (Yoshimura *et al.*, 1997) and sugar-lysine (Jing and Kitts, 2004) showed the hydroxyl radical-scavenging activity. Source of reaction sugar and protein as well as reaction condition affected the antioxidative or prooxidative activities of MRPs (Wijewickreme *et al.*, 1999). From the result, prooxidation activity might be due to the presence of some compounds or radicals which enhanced the damage of deoxyribose in the assay system.

6.4 H_2O_2 scavenging activity of MRPs and decolorized MRPs at different levels

Hydrogen peroxide scavenging activity of MRPs and decolorized MRPs is depicted in Figure 27. The increase in scavenging activity of both MRPs and decolorized MRPs were noted when the amounts used increased ($P<0.05$). However, the hydrogen peroxide scavenging activity of MRPs at a level of 100 ppm was greater than that of 200 ppm ($P<0.05$). From the result, MRPs and decolorized MRPs at a level of 500 ppm exhibited the highest hydrogen peroxide scavenging activity. Generally, hydrogen peroxide can be used as the precursor for transition metal ion-dependent $OH^{\bullet}$ formation by Fenton reaction (Narla and Nao, 1995). Spencer *et al*. (1996) reported that the addition of hydrogen peroxide to cells can lead to $OH^{\bullet}$ mediated oxidative DNA damage. Our results suggested that MRPs, an electron

donor, might accelerate the conversion of H_2O_2 to H_2O (Ruch *et al*., 1984). The result was in agreement with Ide *et al*. (1999) who reported that MRPs derived from fructose-arginine have an hydrogen peroxide scavenging activity in a dose-dependent manner.

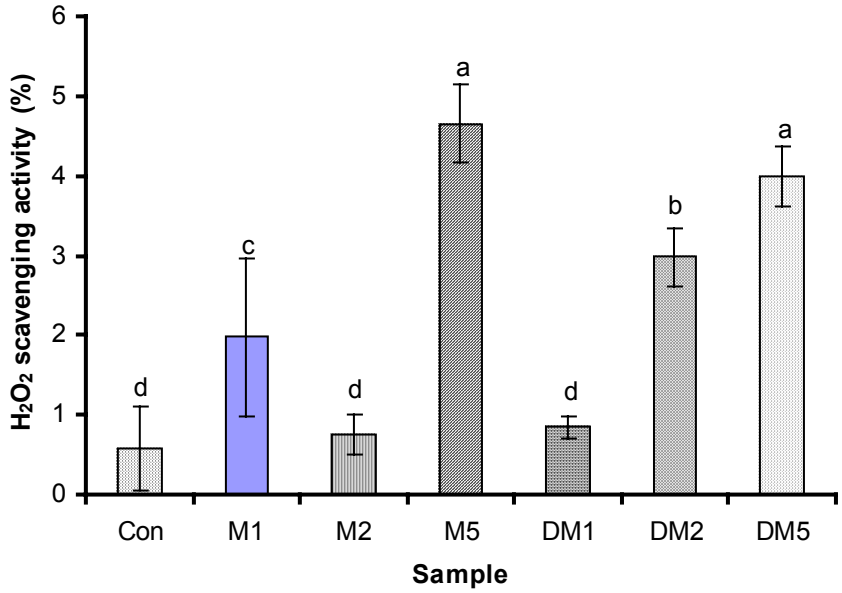

Figure 27 Hydrogen peroxide scavenging activity of MRPs and decolorized MRPs. M1, M2 and M5 denote MRPs at levels of 100, 200 and 500 ppm, respectively. DM1, DM2 and DM5 denote MRPs decolorized by Sep-Pak Cartridge C18 at levels of 100, 200 and 500 ppm, respectively. Con denotes the deionized water was added instead of MRPs. Bars indicate the standard deviation from triplicate determinations.

6.5 Antioxidative activity of MRPs and decolorized MRPs in different systems

6.5.1 Lecithin liposome system

The antioxidant activities of the MRPs and decolorized MRPs were estimated in soybean liposome system by determining TBARS (Figure 28(a)) and conjugated diene (Figure 28(b)). Both MRPs and MRPs decolorized by Sep-Pak Cartridge C_{18} showed the prooxidant activity at every concentration used, as evidenced by the greater TBARS ($P<0.05$). The prooxidant activity of MRPs was also found in liposome system on the basis of conjugated diene formation (Figure 28(b)). Generally, the increase in liposome oxidation was observed when incubation time increased. However, no changes in TBARS in liposome system added with 200 ppm BHT throughout the storage times of 30 h ($p>0.05$). Base on TBARS, MRPs at levels of 200 and 500 ppm exhibited the highest prooxidant activity at 30 h. On the basis of conjugated diene formation, both MRPs and decolorized MRPs at a level of 500 ppm showed the highest prooxidant activity at 30 h. From the result, it was suggested that some products from Maillard reaction might function as prooxidants in liposome system. Frankel *et al*. (1997) found that Chinese green tea at a level of 20 ppm and green tea catechin powder at a level of 112 ppm exhibited prooxidant activity in liposome system containing copper ion. Liposome may be an appropriate lipid models to evaluate antioxidant for food containing phospholipids (Frankel *et al*., 1997). Calligaris *et al*. (2004) reported that the heat treatment of milk can promote an increase in its prooxidant activity, probably as a consequence of the loss of natural antioxidants. However, the formation of novel oxidative molecules in the early stages of the Maillard reaction can be formed.

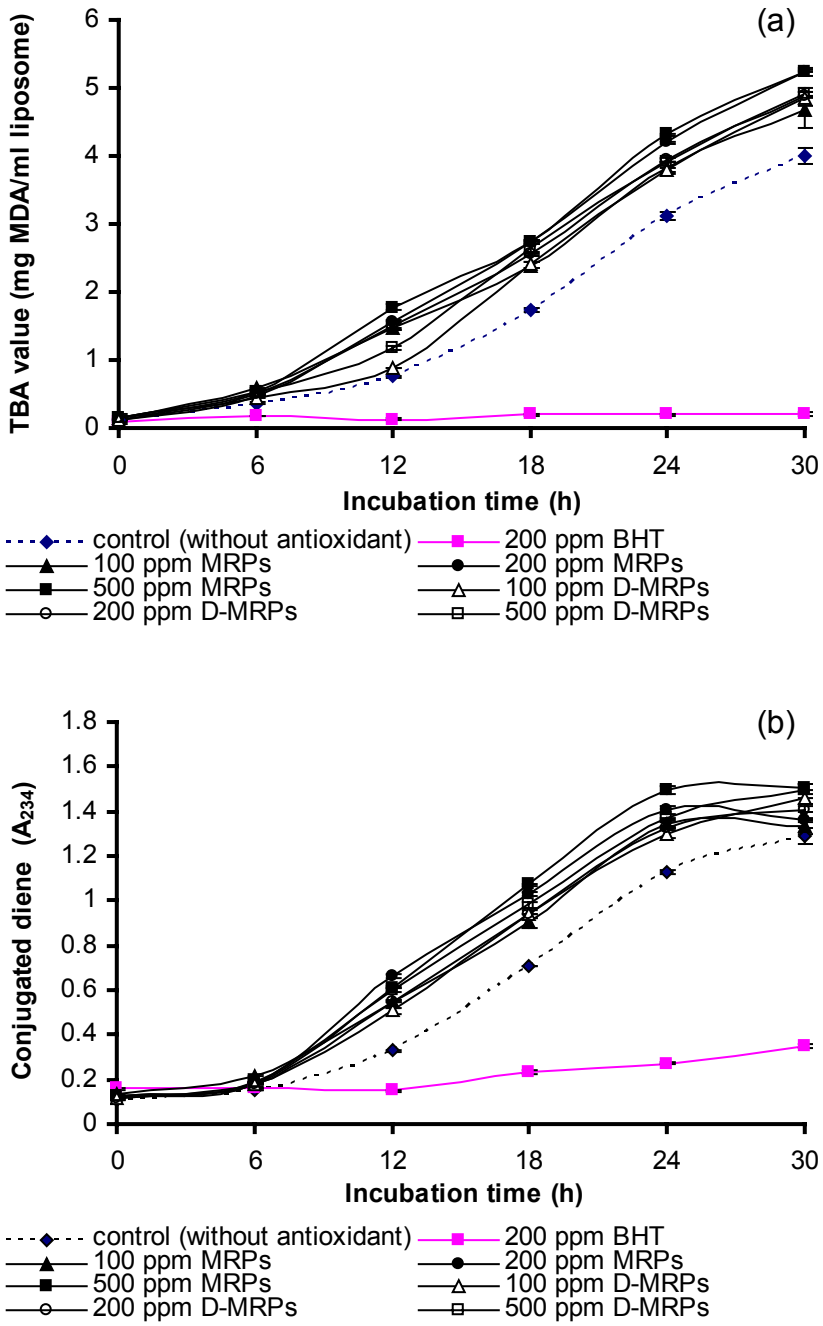

Figure 28 Changes in TBARS (a) and conjugated diene (b) in soybean lecithin liposome system in the absence or presence of MRPs and decolorized MRPs at different levels. Bars indicate the standard deviation from triplicate determinations.

The differences in antioxidative/prooxidative activity between MRP samples and BHT might be due to the differences in their polarity. Frankel *et al*. (1997) found that the differences in antioxidative activity between the hydrophilic catechins and the lipophilic α-tocopherol may be due to their relative partition between the water phase and the surface environment of the phospholipid bilayers. From the result, prooxidative activity of MRPs and decolorized MRPs might be caused by the presence of heme iron in plasma hydrolysate. Moreover, partition between water phase and surface of liposome was presumed to be prior, possibly due to their molecular constraint of MRPs to function at the interface. Incorporation of metal chelator should be a means to reduce the prooxidative effect of MRPs and decolorized MRPs.

6.5.2 β-carotene/linolenic acid system

The decrease in absorbance of β-carotene in the presence of MRPs and decolorized MRPs at different amounts is shown in Figure 29. The decrease in A_{470} indicates the oxidation of β-carotene/linoleic acid. A decrease in A_{470} was effectively retarded when the synthetic antioxidants, BHA and BHT (20 ppm), were used. MRPs and decolorized MRPs at a level of 500 ppm showed the highest antioxidant activity at 40 min of incubation period. No significant difference in A_{470} was noticeable between the control and the system with decolorized MRPs at levels of 100 and 200 ppm. Moreover, the β-carotene/linoleic system added with MRPs at levels of 100 and 200 ppm exhibited the prooxidant activity at the incubation period of 40 min as shown by the higher decrease in A_{470} in comparison with the control. In general, a model system made of β-carotene and linoleic acid in absence of an antioxidant undergoes a

rapid discoloration (Matthaus, 2002). The result indicated that both MRPs and decolorized MRPs showed the antioxidative activity, as indicated by the retardation of β-carotene blenching in β-carotene/linoleic system, at high concentration (500 ppm). However, the prooxidant activity was observed when the low concentration of MRPs was used. The result was in accordance with Wijewickreme *et al.* (1999) who found that MRPs derived from heated fructose-lysine system pH 8.51 at 157 °C exhibited the antioxidant activity in linoleic emulsion system when 0.2 mg/ml MRPs were used. However, the prooxidant activity was observed when the same MRPs at the lower concentration (0.002 and 0.02 mg/ml) were used. A prooxidative activity also observed in refluxed honey-lysine at a level of 1% in linolenic emulsion. Nevertheless, the antioxidant activity was found when a level of 8% was used (Antony *et al*., 2000).

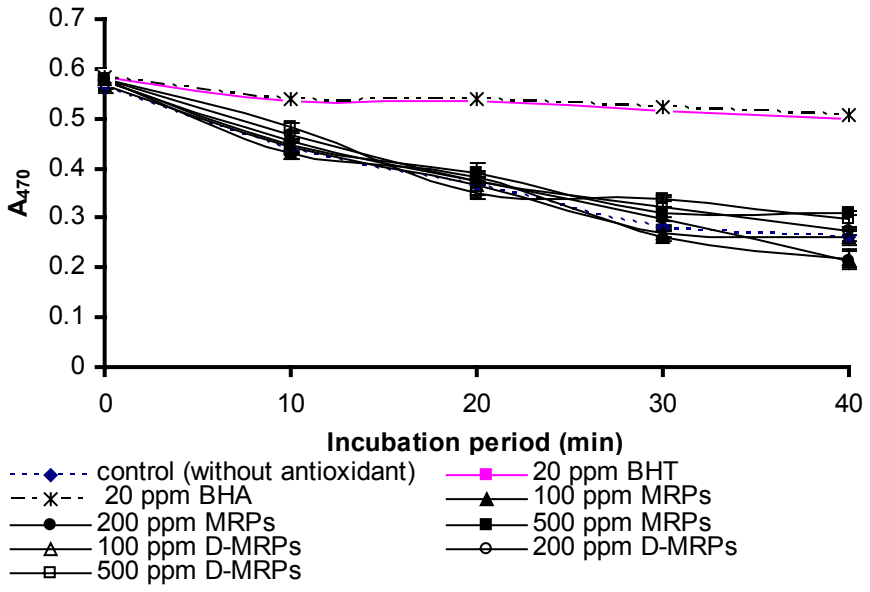

Figure 29 Changes in β-carotene bleaching in the absence or presence of MRPs and decolorized MRPs at different levels. Bars indicate the standard deviation from triplicate determinations.

6.6 The changes in antioxidative activity of MRPs and decolorized MRPs during storage at 4°C and 25°C

The stability in antioxidative activity of MRPs and decolorized MRPs powders during storage at 4°C and 25°C is depicted in Figure 30. No changes in reducing power (Figure 30(a)), DPPH radical-scavenging activity (Figure 30(b)) and metal chelating activity (Figure 30(c)) of all MRPs under both storage conditions were observed up to 6 weeks of storage ($P>0.05$). The slight decreases in reducing power, radical-scavenging activity and metal chelating activity were found at week 8 of storage ($P<0.05$). This might be due to the destruction of antioxidative compounds as the storage time increased.

The result indicated that antioxidative activities of freeze dried MRP samples, both original and decolorized, were stable for 6 weeks. No differences in antioxidant stability between both storage temperatures ($P<0.05$). The result was in agreement with Mastrocola and Munari (2000) who reported that the antioxidative activity of MRPs from mixtures (50% gelatinized starch, 30% water, 16% glucose and 4% lysine) heated at 100°C for 90 min were decreased after 40 days of storage at 25^{o}C.

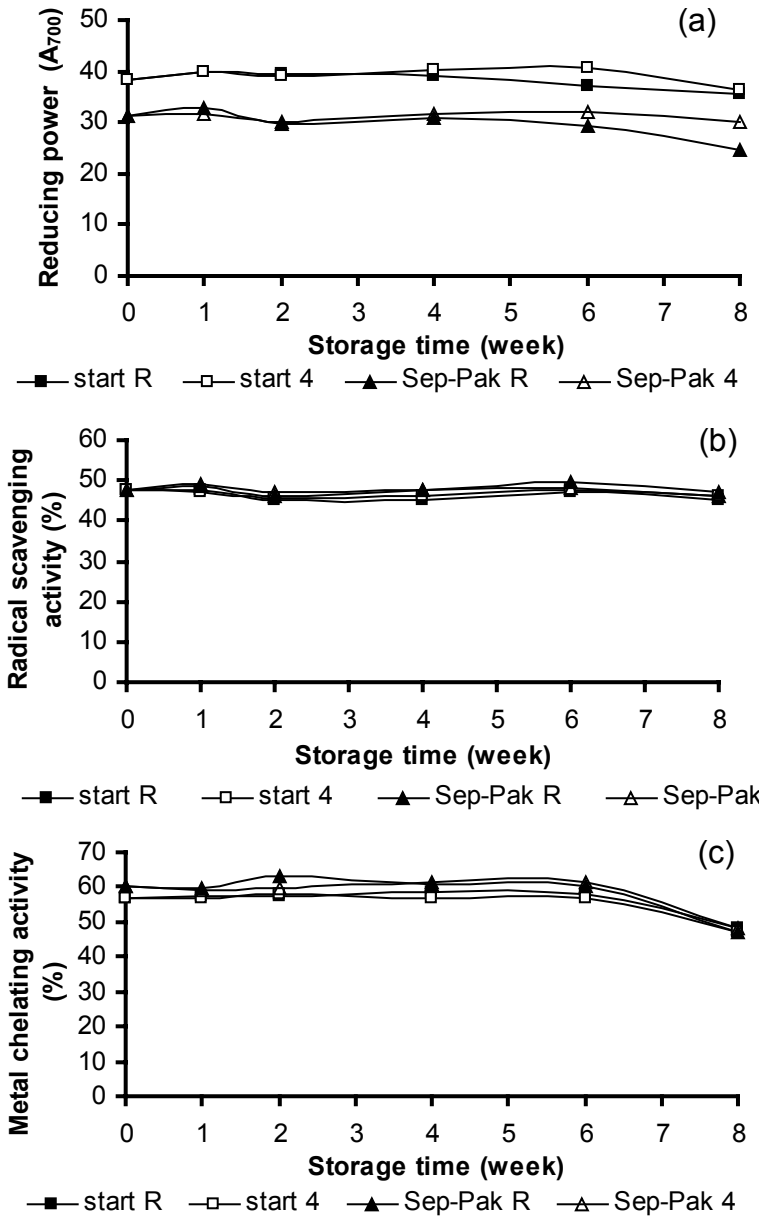

Figure 30 Changes in reducing power (a), DPPH radical-scavenging activity (b) and metal chelating activity (c) of MRPs and decolorized MRPs during storage at 4°C and 25°C for 8 weeks. Start R and start 4 denote MRPs kept at room temperature and 4°C, respectively. Sep-Pak R and Sep-Pak 4 denote MRPs decolorized using Sep-Pak kept at room temperature and 4°C, respectively. Bars indicate the standard deviation from triplicate determinations.

7. Application of MRPs and decolorized MRPs to retard the lipid oxidation in sardine mince and sardine sausage

7.1 Uses of MRPs and decolorized MRPs in sardine mince

The oxidative changes in sardine mince added with PPP acid hydrolysate-glucose MRPs, both original and decolorized, at different levels (100, 200 and 500 ppm) was monitored during storage in ice (Figure 31). No significant changes in TBARS for all treatments were observed on day 3 of storage time ($P>0.05$). Nevertheless, the sharp increases in TBARS were found in sardine mince up to 9 days ($P<0.05$). The result obviously showed that antioxidant activity of both MRPs and decolorized MRPs depended on their concentration. The higher MRPs concentration used, the higher antioxidant activity in sardine mince was observed. At the same levels added, decolorized MRPs showed the slightly lower antioxidative activity than MRPs as evidenced by the higher TBARS found in sardine mince, particularly when the storage time was extended. The decrease in TBARS after 9 days of storage might be due to the loss of volatile secondary products from lipid oxidation (Stahnke, 1994; Stahnke, 1995) and also could be attributed to reaction of malondialdehyde with protein and sugars (Janero, 1990; Gordon, 2001)

The result was in agreement with Smith and Alfawaz (1995) who reported that 0.5 or 1% MRPs derived from the egg albumin hydrolysate-glucose have an antioxidative effect in cooked ground beef stored at 4°C for 8 days. The addition of MRPs prepared from histidine-glucose or enzymatic hemoglobin hydrolysate-glucose improved the oxidative stability of sausage during frozen storage (Lingnert and Lundgren, 1980). Chiu *et al*. (1991) also reported that the fructose-tryptophan MRPs

exhibited the antioxidative effect in kamaboko-type sardine product during storage at 5°C for 15 days.

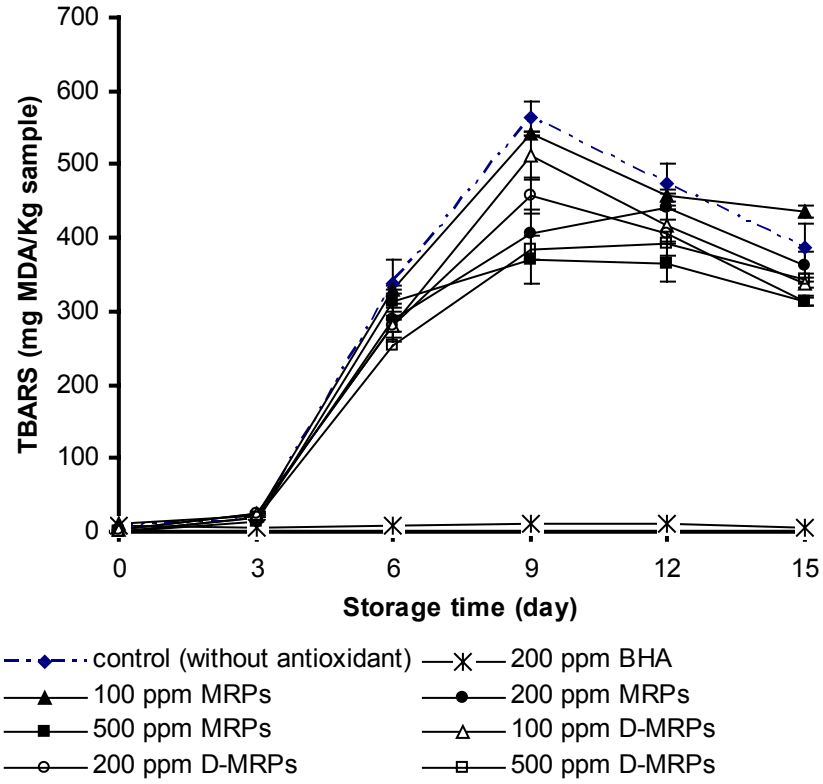

Figure 31 Changes in TBARS of sardine mince added with MRPs or decolorized MRPs (D-MRPs) at different levels during iced storage for 15 days. Bars indicate the standard deviation from triplicate determinations.

7.2 **Uses of MRPs in sardine emulsion sausage**

7.2.1 Changes in TBARS

TBARS of sardine emulsion sausage with and without MRPs at different levels during storage at 4^{o}C are shown in Figure 32. The TBARS of all treatments increased continuously up to 6 days of storage ($P<0.05$). However, no increase in TBARS was found in the control during day 6 and 9 ($P>0.05$). In general, TBARS assay is a commonly used method for the detection of lipid peroxidation (Jardine *et al*., 2002). The decrease in TBARS of all treatments were observed after 6 days of storage. This might be due to the loss of some volatile compounds, particularly aldehyde compounds. Furthermore, lipid peroxidation product, malondialdehydes, is mostly bound to proteins in foods (Giron-Calle, 2002). After 12 days, an increase in TBARS was noticeable in all samples, except sample with 0.05% MRPs addition. The lowest TBARS was noted in the treatment with highest MRPs concentration (0.1%, w/w). However, no differences in TBARS were observed in sardine emulsion sausage added with 0.02 and 0.05% MRPs. The result revealed that MRPs were able to retard the lipid oxidation of sardine emulsion sausage during the 15 days of storage at 4^{o}C. The result was in accordance with Alfawaz *et al*. (1994) who found that Maillard reaction products obtained by autoclaving glucose with acid or enzymatic protein hydrolysate of egg albumin or soy protein isolate can inhibit the lipid oxidation in cooked ground beef over 8 days of refrigerated storage. Antony *et al*. (2002) reported that honey-lysine MRPs possess antioxidative activity in turkey meat and antioxidative effect increased with increasing levels of MRPs. From the result, the marked differences in TBARS among samples at day 0 might be due to the different antioxidative effect of additives added. The oxidation occurred in the control

sample (without additive) might be due to heat-induced oxidation. Heat can induce the release of heam iron in sardine muscle, dark fleshed fish. Those free iron could function as the prooxidant. In presence of BHA, a primary antioxidant, or MRPs, primary/secondary antioxidant, the lipid oxidation was retarded. It was shown that BHA could totally inhibit oxidation, whereas the highest oxidation was found in the control.

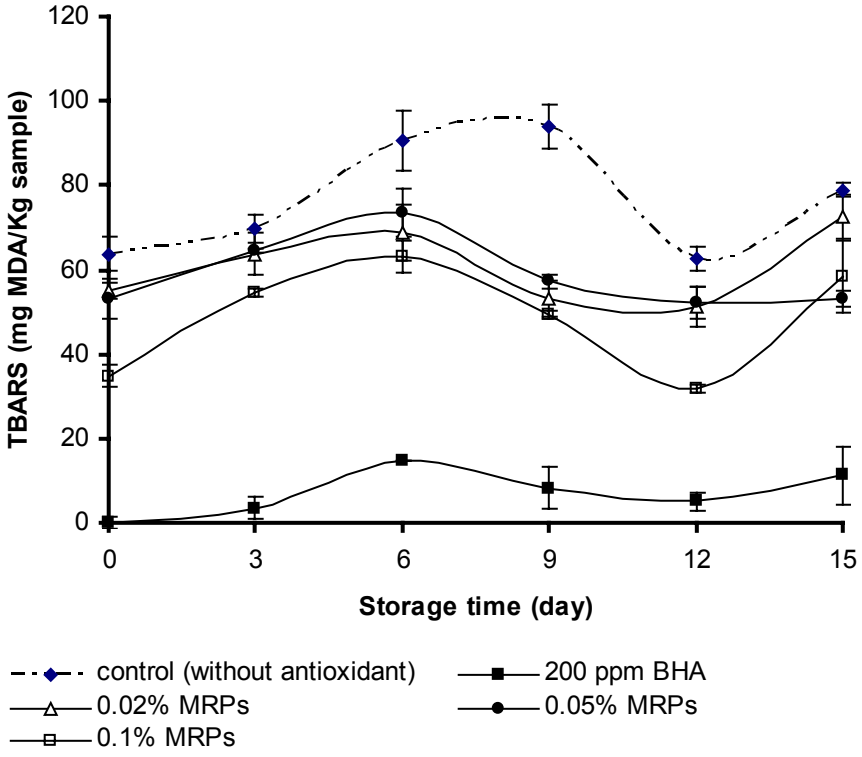

Figure 32 Changes in TBARS of sardine emulsion sausage without and with MRPs at different levels during storage at 4°C for 15 days. Bars indicate the standard deviation from triplicate determinations.

7.2.2 Changes in peroxide values

Peroxide value was also used to monitor the changes in oxidation of sardine emulsion sausage (Figure 33). The slight changes in peroxide value were observed during the first 9 day of storage. Thereafter, the peroxide value of all treatments sharply increased to their maximum at day 12 of storage. The decrease in peroxide value might be due to degradation of peroxide to secondary products (Boselli *et al*., 2005). Among all samples, the sardine emulsion sausage added with 0.1% MRPs possess the highest antioxidant activity as shown by the lowest peroxide values ($P<0.05$). However, no differences in peroxide value was found when 0.05% and 0.1% MRPs were added in sardine emulsion sausage for 3 and 9 days of storage. At day 0, the peroxide value was detected. Cooking for sausage preparations initiated the rapid development of oxidative rancidity by liberating iron from various heam protein and disrupting tissue membranes (Asghar *et al*., 1988; Decker and Xu, 1998). The result suggested that MRPs had an activity to retard the oxidation in sardine emulsion sausage. With the higher MRPs amount added, the higher inhibition of lipid oxidation was found. The result was in agreement with Chiu *et al*. (1991) who reported that the oxidation of sardine lipid and kamaboko-type sardine product was prevented by the addition of fructose-tryptophan MRPs. The prevention properties of those MRPs also increased with increasing MRPs dose. Bailey *et al*. (1987) found that MRPs prepared from histidine-glucose model system were effective inhibitors of oxidative rancidity in cooked ground beef. Obretenov *et al*. (1986) reported that MRPs prepared from acid bovine blood hydrolysate with hydrolyzed starch provide a strong antioxidative effect in lard. Bedinghaus and Ockerman (1995) reported that MRPs derived from xylose-lysine, xylose-tryptophan, dihydroxyacetone-histidine and

dihydroxyacetone-tryptophan were effective inhibitors of lipid oxidation in ground pork patties. At day 0, the similar result was observed, compared with the result of TBARS, and the same explanation was given.

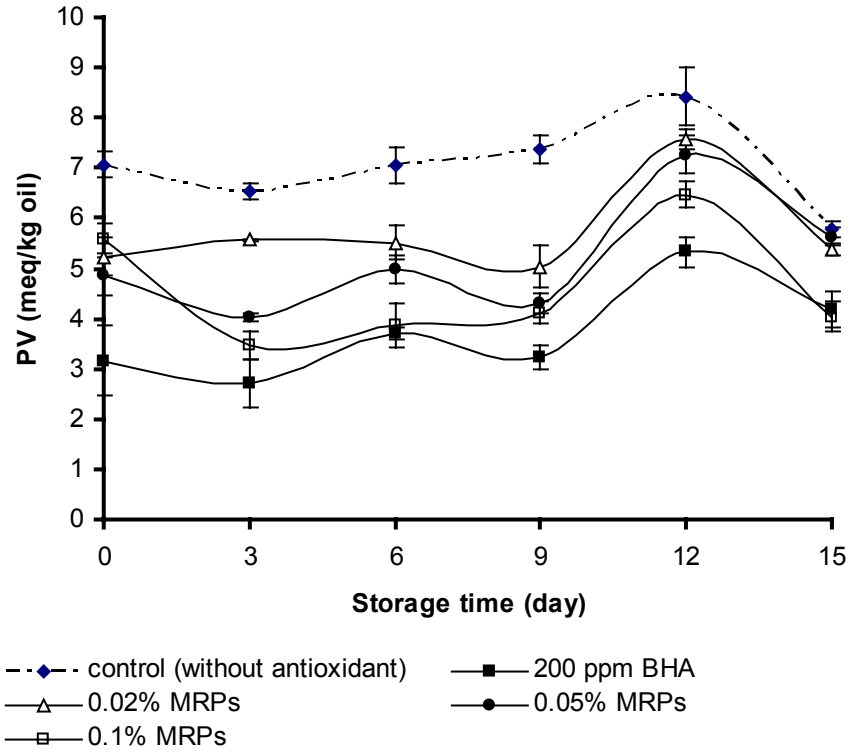

Figure 33 Changes in peroxide values of sardine emulsion sausage without and with MRPs at different levels during storage at 4^{o}C for 15 days. Bars indicate the standard deviation from triplicate determinations.

7.2.3 Changes in conjugated diene

Fluctuated changes in conjugated diene of all samples were observed throughout the storage (Figure 34). However, the sardine emulsion sausage added with 0.1% MRPs showed lower conjugated diene than other samples during the storage. From the result, the formation and decomposition of conjugated diene varied throughout the storage. In general, conjugated diene is used to monitor the early stage of lipid oxidation (Rena-Ramos and Xiong, 2003). The formation of hydroperoxide from polyunsaturated fatty acid (PUFA) leads to conjugation of the pentadiene structure which can absorb at 234 nm (Logani and Davies, 1980). Nevertheless, the conjugated diene method is less specific than peroxide value measurement (Gordon, 2001). The high conjugated diene was observed at 0 day, suggesting that the high myoglobin in sardine (Chaijan *et al*., 2004) might induce oxidation in sausage during processing. Moreover, heat treatment denatured the iron-containing protein, particilary myoglobin, causing the release of iron into the catalytic pool (Decker and Welch, 1990)

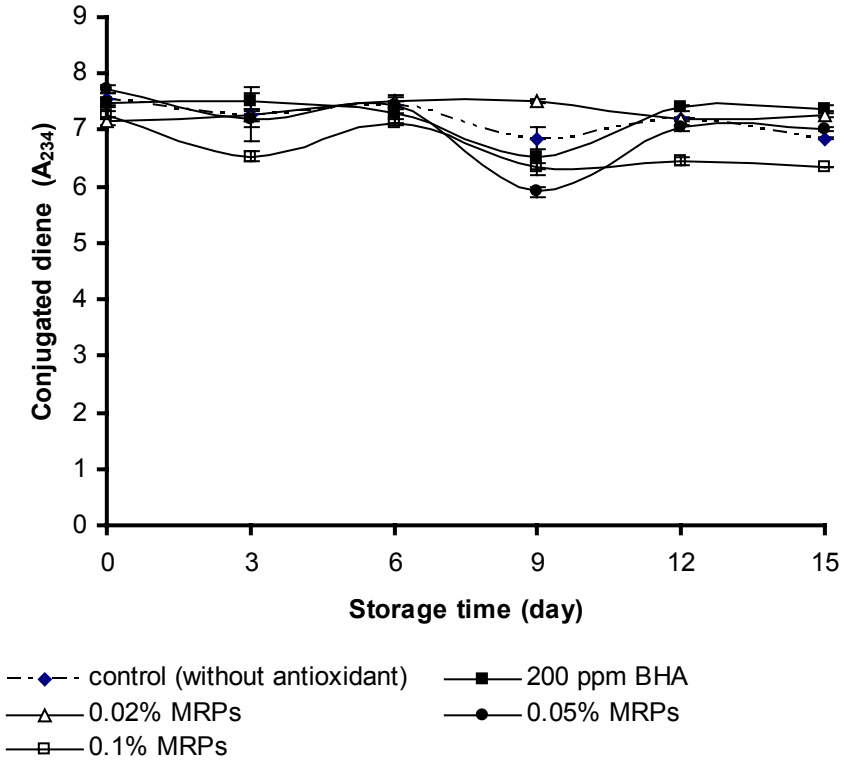

Figure 34 Changes in conjugated diene of sardine emulsion sausage without and with MRPs at different levels during storage at 4^{o}C for 15 days. Bars indicate the standard deviation from triplicate determinations.