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Effect of reactant concentrations on the Maillard reaction in a fructose–glycine model system and the inhibition of black tiger shrimp polyphenoloxidase

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Abstract

The characteristics and the inhibitory activity towards black tiger shrimp polyphenoloxidase of Maillard reaction products (MRPs), prepared by heating an equimolar mixture of fructose and glycine at various concentrations (0.75–30 mM) at 100 °C for 12 h, were investigated. Increase in the intermediate products was observed with increasing reactant concentration, as evidenced by the increase in A_{294} and fluorescence intensity. Furthermore, development of browning (A_{420}) was noticeable when the reactant concentration increased. The inhibitory activity of MRPs towards PPO gradually increased when the concentration of each reactant increased and reached a maximum at 30 mM (80% inhibition). The increase in the inhibitory activity of MRPs was coincidental with the increase in the reducing power, A_{294}, fluorescence intensity, as well as browning intensity. Generally, the development of Maillard reaction products was associated with decrease in pH and loss of reducing sugar and free amino groups, with coincidental increase in reducing power and copper-chelating property. MRPs with the reactant concentration of 4.5–30 mM were able to chelate the copper ion. Therefore, the inhibitory activity of MRPs towards browning, induced by PPO, was most likely due to their copper-chelating property as well as reducing power.

Keywords: Maillard reaction; Polyphenoloxidase; Black tiger shrimp; Inhibition; Glycine; Fructose

1. Introduction

Non-enzymatic browning reactions between amino acids and reducing sugar are the basis of the Maillard reaction, which takes place in thermally processed food (Carabasa-Giribet & Ibarz-Ribas, 2000). The formation of a complex series of compounds called, Maillard reaction products (MRPs), is associated with the development of brown pigments (Mastrocola & Munari, 2000). In the early stage of the Maillard reaction, the reducing sugar condenses with free amino group of amino acids or proteins to give a condensation product, *N*-substituted glycosylamine, via the formation of a Schiff's base and the Amadori rearrangement (Friedman, 1996; Van Boekel, 1998). The subsequent degradation of Amadori product is dependent on the pH of the system (Martins & Van Boekel, 2003a). Additionally, the velocity of reaction depends upon many factors, such as temperature, time, water activity, reactant source and concentration (Jing & Kitts, 2002), the type and ratio of reducing sugar (Naranjo, Malee, & Vigo, 1998; Yeboah, Alli, & Yaylayan, 1999), amino acids (Morales & Jimenez-Perez, 2001; Yeboah et al., 1999), pH (Ajandouz, Tchiakpe, Ore, Benajibas, & Puigserver, 2001) and food composition (Lerici, Barbanti, Manzano, & Cherubin, 1990; Tanaka, Chiba, Ishizaki, Takai, & Taguchi, 1994).

Enzymatic browning in many food systems generally causes an undesirable appearance. Many approaches have been used to prevent the reaction by eliminating the essential components, including oxygen, copper, or substrate needed for polyphenoloxidase (PPO) activity (Kim, Marshall, &

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Wei, 2000). Currently, sulfite has been prohibited, owing to its adverse health effect (Roux, Billaud, Maraschin, Brun-Merimee, & Nicolas, 2003; Tan & Harris, 1995). Since the consumer's demand for natural food additives has been increasing, natural agents with inhibitory activity towards PPO have been studied, e.g., as cysteine (Ding, Chackin, Ueda, & Wang, 2002), MRPs (Billaud, Brun-Merimee, Louarme, & Nicolas, 2004; Brun-Merimee, Billaud, Louarme, & Nicolas, 2003; Lee & Park, 2005; Roux et al., 2003; Tan & Harris, 1995), caramelisation products (CPs) (Lee & Lee, 1997) and honey (Kim et al., 2000).

MRPs are known for their ability to retard lipid oxidation, as well as to inhibit certain oxidoreductases, such as PPO (Tan & Harris, 1995). Effectiveness of such agent depends on several mechanisms of action in preventing the oxidation reaction, including free radical-scavenging action (Morales & Jimenez-Perez, 2001), metal ion-chelating property (O'Brien & Morrissey, 1997; Wijewickreme, Kitts, & Durance, 1997) and/or reducing activity (Tan & Harris, 1995). So far, no information regarding the use of MRPs in inhibiting PPO from crustaceans has been reported. Our investigation aimed to study the effect of reactant concentration in a fructose–glycine system on the formation of MRPs and to study the inhibitory activity of MRPs towards PPO from black tiger shrimp.

2. Materials and methods

2.1. Chemicals

2,4,6-Trinitrobenzenesulfonic acid (TNBS), L-leucine, L-β-(3,4 dihydroxylphenyl) alanine (L-DOPA), Brij 35, potassium ferricyanide and tetramethylmurexide (TMM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trichloroacetic acid was obtained from Riedel-deHaen (Seelze, Germany). Ferric chloride, ammonium sulfate and sodium sulfite were purchased from Merck (Darmstadt, Germany). Fructose and glycine were obtained from Fluka (Buchs, Switzerland).

2.2. Preparation of MRPs

MRPs were prepared by heating equimolar fructose and glycine in 0.1 M borate buffer, pH 8 at 100 °C. Different reactant concentrations (0.75, 1.5, 4.5, 7.5, 15 and 30 mM) were used. To produce MRPs, the mixtures were transferred to screw-sealed tubes, tightly capped and heated in an oil bath (Buchi labortechnik AG, Flawil, Switzerland) at 100 °C for 12 h. The heated samples were cooled immediately in iced water. MRPs obtained were kept at 4 °C until analysed.

2.3. Extraction of black tiger shrimp PPO

Ground black tiger shrimp cephalothorax was homogenised with 0.05 M phosphate buffer containing 1 M NaCl, pH 7.2, at a ratio of 1:3 (w/v) using an IKA Labortechnik (Selangor, Malaysia) for 2 min. The homogenate was then stirred for 30 min at 4 °C. After centrifugation at 8000*g* for 30 min at 4 °C, using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA), the supernatant was fractionated with solid ammonium sulfate (0–40% saturation). The precipitate formed was collected by centrifugation at 12,000*g* for 30 min at 4 °C. The pellet was dissolved in a minimum volume of 0.05 M phosphate buffer, pH 6.5. The crude extract was dialysed against 15 volumes of cold 0.05 M phosphate buffer, pH 6.5. The dialysate was referred to as 'PPO extract'.

2.4. PPO activity assay

PPO activity was assayed using L-DOPA as a substrate, as described by Simpson, Marshall, and Otwell (1987) with a slight modification. PPO extract (150 μl) was mixed with 150 μl of distilled water and 150 μl of phosphate buffer, pH 6.0. The assay mixture was then treated with 750 μl of L-DOPA preincubated at 45 °C to initiate the reaction. The reaction was run for 3 min and the absorbance at 475 nm (A_{475}) was measured. One unit was defined as the change in 0.001 U of A_{475}/min/ml.

To study the effect of MRPs on PPO activity, 150 μl of MRPs were added instead of distilled water. The mixture was allowed to stand at room temperature for 10 min prior to the assay. The residual activity was determined and % inhibition was calculated.

2.5. Measurement of UV-absorbance and browning

The UV-absorbance and browning of MRP samples were measured according to the method of Ajandouz et al. (2001). Appropriate dilution (20-fold) was made using distilled water and the absorbance was measured at 294 and 420 nm, using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) for determining UV-absorbance and browning intensity, respectively.

2.6. Measurement of fluorescence

Fluorescence of MRP samples with an appropriate dilution (20-fold) was determined as described by Morales and Jimenez-Perez (2001) with a slight modification. The fluorescence intensity was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

2.7. Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morressey (1997). MRP samples (10-fold dilution) (500 μl) were mixed with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution was added. The solution was mixed thor-

oughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was cooled at room temperature for 15 min. The absorbance was measured at 420 nm. Free amino group content was expressed in terms of L-leucine.

2.8. Determination of reducing sugar

The reducing sugar was determined according to the method of Nelson-Somogyi as modified by Chaplin and Kennedy (1994). One ml of MRP samples (10-fold dilution) was mixed with 1 ml of reagent C. The reaction mixture was heated at 100 °C for 15 min and cooled to room temperature using running water. The reaction mixture was treated with 1 ml of reagent D, followed by addition of 3.0 ml of distilled water. The absorbance at 520 nm was measured. Reducing sugar content was read from the standard curve. The loss of reducing sugar was calculated, based on the differences between initial reducing sugar content and that obtained after reaction.

2.9. Determination of reducing power

The reducing power of MRPs samples was determined according to the method of Oyaizu (1986) with a slight modification. One ml of MRP samples (5-fold dilution) was mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The reaction mixture was incubated in a temperature-controlled water bath at 50 °C for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. The mixtures were treated with 1 ml of distilled water and 200 μl of 0.1% $FeCl_3$. The absorbance at 700 nm was determined and used as the measure of reducing power.

2.10. Determination of copper chelation

The copper binding capacity of MRP samples was determined according to the method of Wijewickreme et al. (1997) with a slight modification. The MRP samples (20-fold dilution) (500 μl) were mixed with 10 mM hexamine buffer (pH 5) containing 10 mM KCl (1.5 ml). The mixture was then treated with 500 μl of 0.1 mM $CuSO_4$ and incubated for 10 min at room temperature. The reaction mixture was mixed with 100 μl of 1 mM TMM in hexamine buffer. The amount of free copper in the solutions was obtained from a standard curve, where the absorbance ratio A_{460}/A_{530}, in a solution of 500 μl of $CuSO_4$ (0.02–0.1 mM), 2.0 ml of hexamine buffer and 100 μl of TMM was plotted against the amount of $CuSO_4$ (0.02–0.1 mM). The amount of copper bound to MRP mixtures was calculated as the difference between the amount of copper added and free copper present in the solution.

2.11. Statistical analysis

All analyses were run in triplicate. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range test (Steel & Torrie, 1980). Analysis was performed using a SPSS package (SPSS 8.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Changes in pH

The pHs of a fructose–glycine model system with different reactant concentrations are shown in Fig. 1. The reaction, with an initial pH of 8.0, was allowed to proceed without pH control for 12 h at 100 °C. At low reactant concentrations (0.75–4.5 mM), only slight decrease in pH was observed. A greater pH decrease was found with the system having reactant concentrations above 4.5 mM, particularly at 30 mM ($P < 0.05$). Benjakul, Lertittikul, and Bauer (2005) also observed a decrease in pHs of MRPs from a porcine plasma protein–sugars model system. Easa et al. (1996) reported a decrease in pH of a bovine serum albumin–xylose model system. Generally, more complexity and varieties of MRPs were formed with higher concentrations of reactant, especially with a prolonged reaction time and high temperature (Carabasa-Giribet & Ibarz-Ribas, 2000; Friedman, 1996; Morales & van Boekel, 1998). Those compounds formed during the reaction include hydroxyacetone derivatives, glyceraldehydes and diketones. Acetic acid and formic acid were also detected as two major degradation products in a Maillard reaction system containing fructose or glucose (Martins & Van Boekel, 2003a, 2003b). From the results, the rate of pH decrease was greater with a higher reactant concentration. This might be due to the greater formation of acids in the 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 compounds with lower basicity

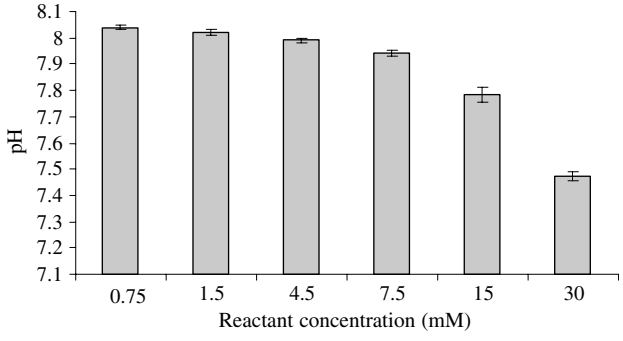

Fig. 1. Changes in pH of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

(Beck, Ledl, Seng, & Severin, 1990). Moreover, the condensation between free amino of amino acid and carbonyl groups of glucose might contribute to the lowered pH (Martins, Marcelis, & Van Boekel, 2003).

3.2. Changes in fluorescence intensity and A_{294}

The fluorescence intensity of the fructose–glycine model system increased with increasing reactant concentrations ($P < 0.05$) (Fig. 2). However, lowered fluorescence intensity was noticeable at a concentration of 30 mM. This might be due to transformation of intermediates to polymer compounds in the presence of reactants 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. (2005) who reported an 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 possibly the intermediate precursors of brown pigments (Labuza & Baisier, 1992; Morales, Romeo, & Jimenez-Perez, 1996). The concentration and ratios of reactants also have significant impact on the reaction (Baisier & Labuza, 1992; Lerici et al., 1990). Therefore, with sufficient amounts of reactants, fluorescent intermediate compounds were formed easily and effectively under such conditions.

Continuous increase in A_{294} was observed as the concentration of reactants increased ($P < 0.05$) (Fig. 3(a)). A_{294} is used to detect the colourless compounds formed at an intermediate stage (Ajandouz et al., 2001). The result suggested that intermediate products might be formed to a greater extent with higher reactant concentrations. Benjakul et al. (2005) reported that a porcine plasma protein–sugars system with a high concentration of reactants exhibited a high A_{294}. The mechanism and the rate of the Maillard reaction depend on the type of sugar and protein used as well as the ratios (Morales et al., 1996). Different intermediate products are formed, either fluorescent or non-fluorescent compounds, during the Maillard reaction (Benjakul et al., 2005). From the result, it appears that some intermediate products might undergo conversion to the final brown compounds, while some intermediates are still being generated by both reactants.

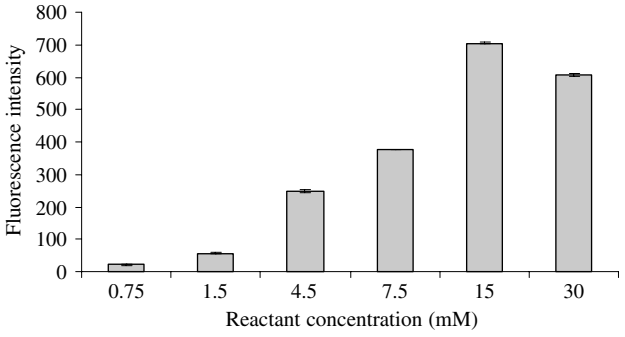

Fig. 2. Changes in fluorescence intensity of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

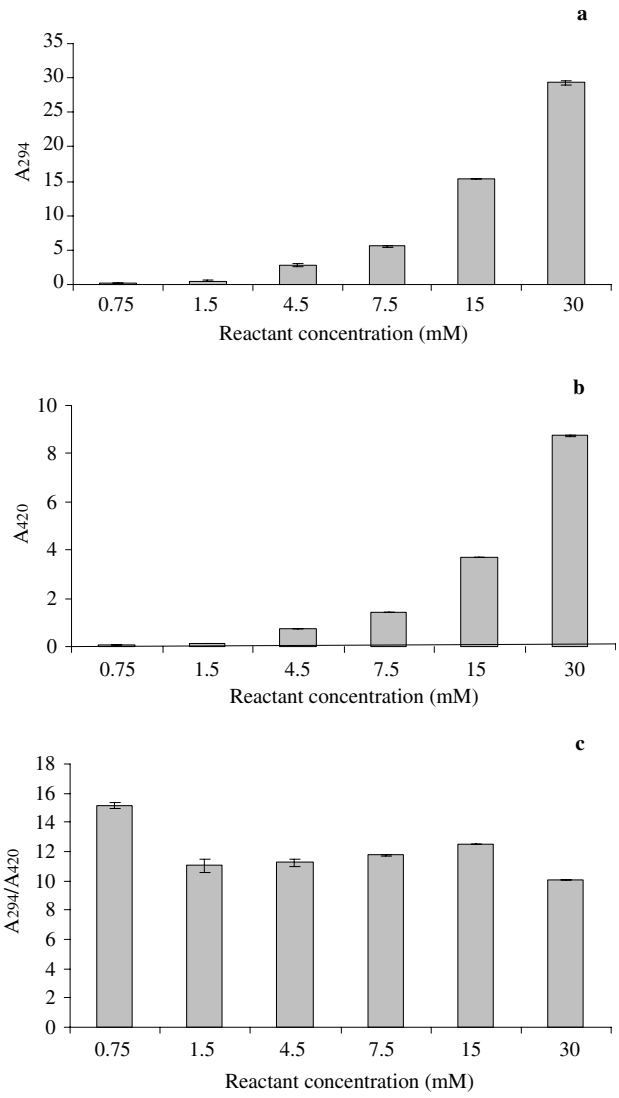

Fig. 3. Changes in A_{294} (a) A_{420} (b) and A_{294}/A_{420} (c) of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

3.3. Changes in browning intensity

A_{420} of the fructose–glycine model system increased with increasing concentration of reactants ($P < 0.05$) (Fig. 3(b)). The most commonly used indicator of the browning pigment is spectrophotometric measurement at 420 nm (Lerici et al., 1990). The increase in A_{420} indicates development of browning pigment in the final stage of Maillard reaction. The reaction consists of the condensation of amino compound and sugar fragments into polymerised protein and the brown pigment melanoidin (Van Boekel, 1998). The increase in brown pigment development was coincidental with increase in colourless intermediate formation, as evidenced by the increased in A_{294} and fluorescence intensity

(Fig. 2). This suggests that brown pigments were formed proportionally with the intermediate products generated. At 30 mM reactant concentration, fluorescent compounds might convert to the final brown pigments to a greater extent, leading to a lower fluorescent intensity observed (Fig. 2).

The A_{294}/A_{420} ratio of the fructose–glycine model system varied with the reactant concentration (Fig. 3(c)). Highest ratio was found with the system containing reactants at 0.75 mM, whereas the ratio became lowest with the highest reactant concentration (30 mM) ($P < 0.05$). It appears 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. The colour formation due to both sugar caramelisation and Maillard reaction (Ajandouz et al., 2001) was dependent on the reactant concentration.

3.4. Changes in reducing sugar and free amino group contents

The non-enzymatic browning reaction was also monitored by measuring the extent of fructose degradation. A loss in reducing sugar, in the range of 97–98%, was found with the reactant concentration of 0.75–1.5 mM (Fig. 4(a)). No differences in reducing sugar loss were observed at concentrations above 4.5 mM ($P > 0.05$). This might be due to the limited amount of sugar available 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) reported that fructose was destroyed during heating. In addition, the degradation of sugar contributed to the caramelisation, which occurs simultaneously with the Maillard reaction (Buera, Chirife, Resnik, & Wetzler, 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 occurred (Ajandouz et al., 2001).

At low concentrations (0.75–7.5 mM), a loss of free amino group in the range of 5–10% was found (Fig. 4(b)). A 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 a 32% loss of histidine in the presence of fructose after 126 weeks of storage at 50 °C.

In the early stage of the Maillard reaction, the reducing sugar condenses with a compound possessing 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).

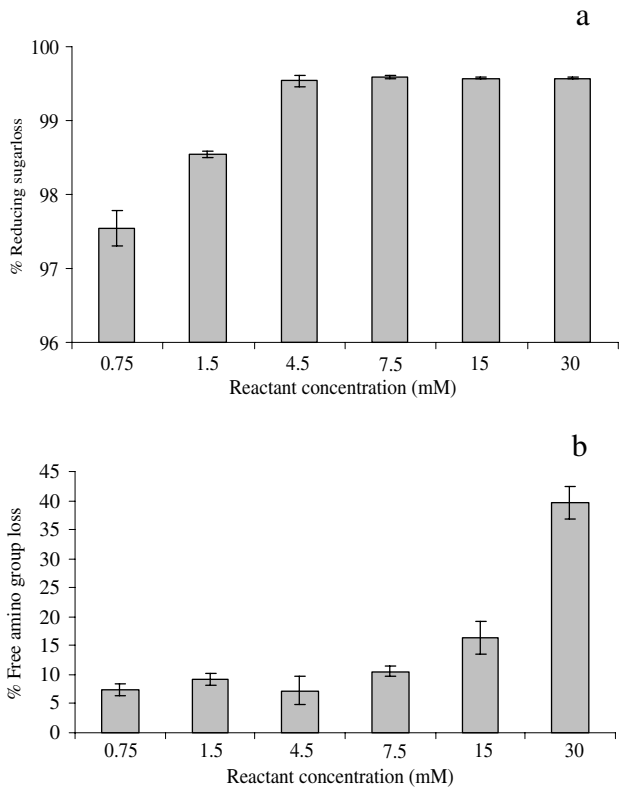

Fig. 4. The loss of reducing sugar (a) and free amino group content (b) of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

At pH > 7, the degradation of Amadori compound is thought to involve mainly 2,3-enolisation, when reductones, including acetol, pyruvaldehyde, and diacetyl, are formed. All these compounds are highly reactive and take part in further reactions (Martins & Van Boekel, 2003b). Apparently, initial reaction products, once formed, undergo 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 conditions (Ajandouz et al., 2001). From the result, the reaction might proceed rapidly at pH above 7, particularly with a 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). It is considered that the concentration of open chain form might be a crucial factor, to generate the other form and to condense with α-amino or ε-amino group to give further reactions. Fructose is able to form C2- and C3-fragments, described as radical precursors, 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 a higher rate in basic conditions known as the rapid enolisation reactions (Cammerer & Kroh, 1996). This led to the decrease in free amino group and reducing sugar, which

was in accordance with the increase in browning (Fig. 3(b)), A_{294} (Fig. 3(a)) and fluorescence intensity (Fig. 2), especially at the higher fructose and glycine concentrations.

3.5. Changes in reducing power

The reducing power of the fructose–glycine system, as monitored by A_{700}, increased with increasing concentrations of reactants ($P < 0.05$) (Fig. 5(a)), suggesting a strong positive 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 ($P > 0.05$). Yoshimura, Iijima, Watanabe, and Nakazawa (1997) found that the reducing power increased linearly with increasing heating time of glucose–glycine mixtures. The reaction between reducing sugars and amino acids or proteins produces strong reducing materials, such as amino reductones (Hodge, 1953), which are key intermediates of the Maillard reaction. The intermediate compounds of MRPs were reported to be capable of donating hydrogen atoms (Yen & Hsieh, 1995). In addition, the MRPs are known to possess 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. 2 and 3).

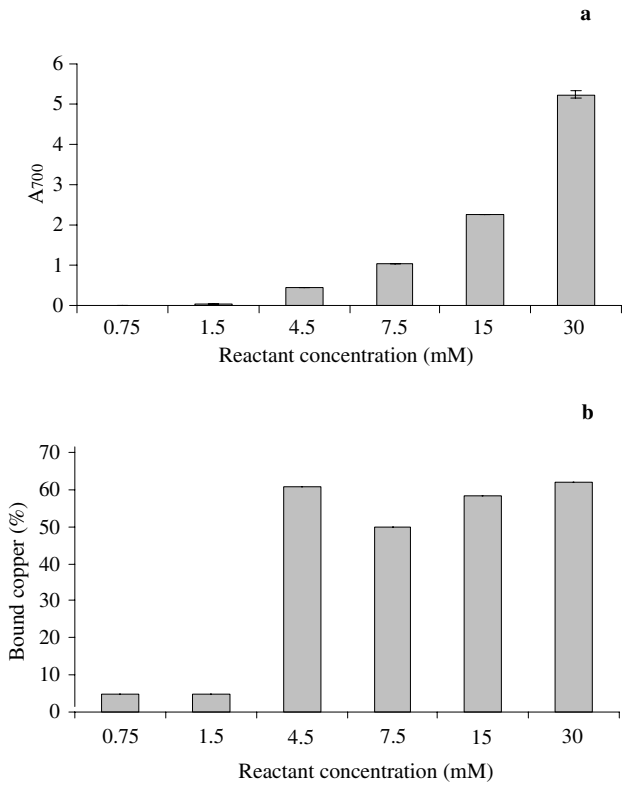

Fig. 5. Reducing power (a) and copper-chelating property (b) of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

3.6. Changes in copper chelation

Copper chelating of MRPs, prepared with different reactant concentrations, is depicted in Fig. 5(b). With low reactant concentrations (0.75–1.5 mM), MRPs exhibited low copper-chelating property. On the other hand, much greater chelating power was observed with higher reactant concentration ($P < 0.05$). Therefore, efficiency of copper binding was dependent on the reactant concentration in the 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 (O'Brien & Morrissey, 1997; Tan & Harris, 1995; 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 the fructose–glycine model system showed the copper-chelating property, particularly when high concentrations of reactants were used.

3.7. The inhibitory effect of MRPs on PPO activity

MRPs with various reactant concentrations showed different inhibitory activities towards PPO (Fig. 6). The inhibition of PPO activity by MRPs with reactant concentrations of 0.75–7.5 mM was about 35%. PPO 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 a glucose–glycine system, against PPO from potato, increased as the amount of glucose increased. From the result, PPO inhibitory activity was coincidental with browning development and intermediate formation (Figs. 2 and 3). The reductone moiety present in the melanoidin structure has been reported to exhibit reducing, chelating properties and scavenging properties (Namiki, 1988).

PPO from crustaceans has been known to contain Cu^{2+} in the active site (Jang, Sanada, Ushio, Tanaka, & Ohshima, 2003; Kim et al., 2000). The subunit folds into three domains. Domain 2 contains the binuclear copper site

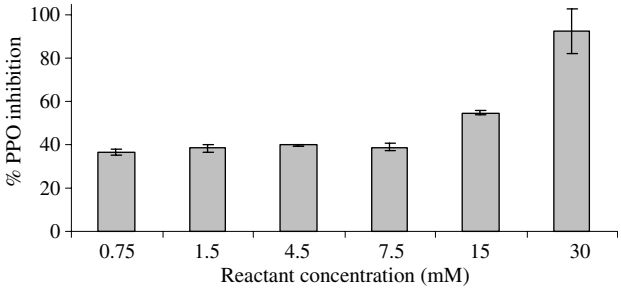

Fig. 6. Inhibitory activity towards black tiger shrimp polyphenoloxidase of fructose–glycine MRPs prepared with various reactant concentrations at 100 °C for 12 h. Bars indicate the standard deviation from triplicate determinations.

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 a complex with Cu^{2+} in the active site, leading to the loss in PPO activity. Billaud, Roux, Brun-Merimee, Maraschin, and Nicolas (2003, 2004) and Roux et al. (2003) demonstrated that MRPs from glucose, fructose and cysteine inhibited apple PPO activity. Also, Brun-Merimee et al. (2003) reported that MRPs prepared from glucose or fructose with glutathione were recognised as strong apple PPO inhibitors. Additionally, Tan and Harris (1995) found that MRPs, from various amino acids and glucose, inhibited apple PPO. Nicoli, Elizalde, Pitotti, and Lerici (1991) reported that MRPs obtained by heating a glucose–glycine solution caused a strong inhibiting effect on apple PPO. A reductone generated during the Maillard reaction retards enzymatic browning by reducing Cu^{2+} to Cu^{+} in the PPO (Tan & Harris, 1995). Furthermore, the reducing power of MRPs indicated the ability to reduce quinone to DOPA, resulting in the retardation of browning development in the assay mixture. Thus, MRPs from the fructose–glycine system, especially with greater reactant concentrations, could be used as a novel PPO inhibitor in crustaceans.

4. Conclusion

The development of MRPs and the inhibitory activity towards PPO were dependent upon the reactant concentrations. The increase in the inhibitory activity of MRPs toward PPO was coincidental with the increase in the reducing power, intermediate formation as well as browning development. The effectiveness of MRPs in black tiger shrimp PPO inhibition was most likely due to their copper-chelating property.

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