Chapter 1

Introduction

Shrimp is one of the the seafood products, which have become economically important for Thailand. However, shrimp is perishable and undergoes discoloration known as 'black spot'. This phenomenon leads to the lowered acceptability since color is one of the first attributes used by consumers for quality evaluation. Apart from proteolysis, freshness deterioration of shellfish is related to enzymatic discoloration (Jiang *et al*., 1994). After catch, polyphenoloxidase (PPO) or phenoloxidase (PO) is responsible for the formation of melanins causing darkening of meat and shell. (Haard and Simpson, 2000; Adachi *et al*, 1999,2000; Simpson *et al*., 1987, 1988; William *et al*., 2000).

Enzymatic browning in many food systems generally causes the undesirable appearance. Many approaches have been used to prevent the reaction by eliminating the essential components including oxygen, copper, or substrate needed for PO activity (Kim *et al*., 2000). Currently, sulfite has been prohibited owing to its adverse health effect (Roux *et al*., 2003; Tan and Harris, 1995). Since the consumer's demand for natural food additive has been increasing, some natural agents with inhibitory activity towards PO have been focused such as cysteine (Ding *et al*., 2002), MRPs (Tan and Harris, 1995; Lee and Park, 2002; Roux *et al*., 2003; Billaud *et al*., 2004; Brune-Merimee *et al*., 2003), caramelization products (CPs) (Lee and Lee, 1997) and honey (Kim *et al*., 2000).

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 and 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 and Munari, 2000). In the early stage of the Maillard reaction, the reducing sugar condences with a free amino group of an amino acid or proteins to give a condensation product N-substituted glycosylamine via the formation of a Schiff's base and the Amadori rearrangement (Van Boekel, 1998; Friedman, 1996). The subsequent degradation of Amadori product is dependent on the pH of the system (Martins and Van Boekel, 2003a). Additionally, the velocity of reaction depended upon many factors such as temperature, time, water activity (Aw), reactant source and concentration (Jing and Kitts, 2002), the type and ratio of reducing sugar (Naranjo et al., 1998; Yeboah et al., 1999), amino acids (Morales and Jemeez-Perez, 2001; Yeboah et al., 1999), pH (Ajandous et al., 2001) and food compositions (Tanaka et al., 1994; Lerici et al., 1990).

MRPs are known for their ability to retard lipid oxidation as well as to inhibit certain oxidoreductase such as PO (Tan and Harris, 1995). Effectiveness of such agent depends on several mechanisms of action in preventing oxidation reaction, including free radical scavenging action (Morales and Jemenez-Perez, 2001), metal ion chelating property (Wijewickreme et al., 1997; O'Brein and Morrissey, 1997) and/or reducing activity (Tan and Harris, 1995). MRPs have been used to inhibit apple PPO (Roux et al., 2003; Billaud et al., 2004). So far, no information regarding the use of MRPs in inhibitory PO from crustacea has been reported. Thus, MRPs produced under the optimal condition might serve as the promising inhibitor for black

tiger prawn PO, thereby extending the shelf-life of this species during post-mortem storage. As a consequence, a loss in quality can be retarded.

Literature Review

Browning reaction

Browning of food takes place during processing and storage of meat, fish and vegetable products, especially when fresh fruits and vegetables are subjected to mechanical injury (Eskin, 1990). Browning usually impairs the sensory properties of products due to the associated changes in the color, flavor and nutritional properties (Eskin, 1990; Martinez and Whitaker, 1995). The loss of nutritional quality is attributed to the destruction of essential amino acids and a decrease in digestibility and inhibition of proteolytic and glycolytic enzymes. The production of antinutritional and toxic compounds may further reduce the nutritional value and possibly the safety of foods (Freidman, 1996).

The major groups of reaction leading to browning are enzymatic phenol oxidation and nonenzymatic browning. The latter is favored by heat treatment and includes a wide number of reactions such as Maillard reaction, caramelization and chemical oxidation of phenols (Manzocco *et al.*, 2001).

1. Enzymatic browning reaction

Food color can be influenced and determined by many compounds including naturally occurring pigments such as chlorophylls, carotenoids, anthocyanins and others or other colors formed through enzymatic and nonenzymatic browning reactions. One of the most important color reactions that affect many fruits, vegetables and seafoods especially crustaceans is enzymatic browning (Kim *et al.*,

2001), defined as an initial enzymatic oxidation of endogenous phenolic compounds by polyphenoloxidase (Gauillard and Richard-Forget, 1997).

1.1. Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO, EC. 1. 14. 18. 1) is a cupric enzyme (Goulart *et al*., 2003), whose the active site contains two copper atoms, each of which is liganded to three histidine residues (Fig. 1) (Whitaker, 1995; Kim *et al*., 2000). This copper pair is the site of interaction of PPO with both molecular oxygen and its phenolic substrates (Kim *et al*., 2001). PPO is also known as polyphenolase, phenolase, tyrosinase and cresolase (Whitaker, 1995). This enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Quinones are very reactive compounds which strongly interact with other molecules, leading to a large pigment of high molecular weight with very dark or black coloring (Fig. 2) (Goulart *et al*., 2003; Montero *et al*., 2001). Melanin pigments, that are visible as melanosis in the processed lobster (Williams *et at*., 2003), normally react further with other quinones, amino acids, peptides and proteins, thus altering not only the structural and functional properties of the protein but also its nutritive value (Escribano *et al*., 1997).
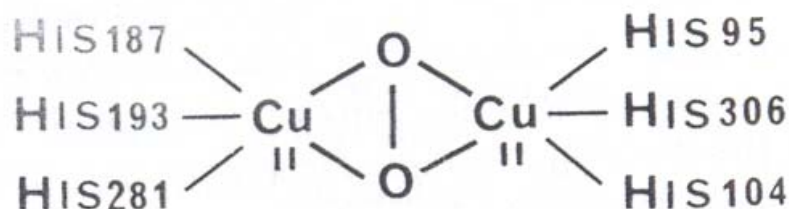

Figure 1. Coordination of copper to six histidine residues in active site of PPO
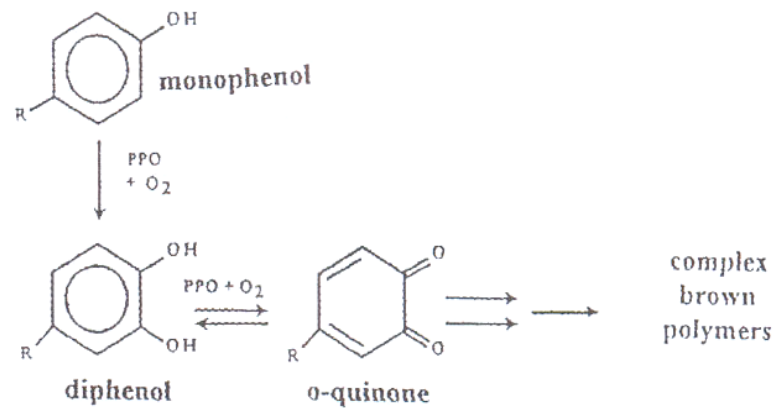
Source: Whitaker (1995)

Figure 2. Enzymatic oxidation induced by PPO

Source: Ding *et al*. (2002)

PO has been shown to play a central role in the innate immune system of crustacean (William *et al*., 2003). This enzyme is expressed from their hemocytes as its precursor, prophenoloxidase (proPO) and functions in various phases of the live organism such as sclerotization, pigmentation, wound healing on cuticles and defense reaction (Adachi *et al*., 1999; 2000; 2001). PO has been detected in the hemocyte, hemolymph, cuticle, accessory gland, egg case, salivary gland and midgut in insects (Adachi *et al*., 1999). In crustaceans, PO is localized in different parts. It is found on the exoskeleton, chiefly on the region of the pleopods' connection (Montero *et al*., 2001).

The activation of proPO in hemocyte or hemolymph, is initiated by the minute amount of bacterial or fungal cell wall components (β-1,3-glucans, lipopolysaccharides and peptidoglycans) and intermediated by several factors including protease cascade reaction (Adachi *et al*., 1999; Williams *et al*., 2003). The

serine proteinase that exhibits trypsin-like activity cleaves the proPO between an arginine residue and a threonine residue to yield active PO (Soderhall and Cerenius, 1998).

1.2. Factors affecting PO activity

PO differs in isoforms, latency, catalytic behavior, molecular weight, isoelectric point, specificity and hydrophobicity (Ho, 1999). Additionally, optimum pH, thermal stability and kinetic parameters can vary with species, body parts, etc. (Montero *et al.*, 2001).

1.2.1. Temperature

PO shows the different optimal temperature among various animals or species. Montero *et al.* (2001) reported that the temperature profile of PO from imperial tiger prawn shows two plateaus between 30-35 and 40-45°C. The activity increased by around 20% for every 10°C. Thermal stability was considerably reduced when the enzyme extract was subjected to temperatures up to 35°C. Rolle *et al.* (1991) observed that the PPO from Taiwanese tiger shrimp (*Penaeus monodon*) exhibited a temperature optimum of 45°C and showed stability over temperature range of 30-35°C. Simpson *et al.* (1997) reported that the maximum activity occurred at 45°C for white shrimp PO (*Penaeus setiferus*) and the activity decreased at temperature above 50°C.

The activity of enzyme to be activated by heat and to maintain activity at high temperatures is most important when considering the impacts of heat processing on processed lobster quality. The determination of the temperature/time profiles required to activate the pro-enzyme and to prevent melanosis and improve

post-processing quality and acceptability is a means to regulate or control the quality of crustaceans deteriorated by melanosis (Williams *et al.*, 2003).

1.2.2. pH

The optimum pH depends largely on the physiological pH in which the enzyme activity occurs in nature. For example, the pH of the carapace of the cephalothorax was 7.16±0.07, while in the abdominal cuticle, it was 8.76±0.04. Therefore, the enzyme would probably present different optimum pH characteristics, depending on the locus of extraction. Montero *et al.* (2001) reported that the high activity occurred at pH 5 and 8 in *Penaeus japonicus.* Simpson *et al.* (1988) found that the PO from *Penaeus duorarum* was active in the pH ranging from 6.5 to 9 and stable at pH 8-9. Simpson *et al.* (1987) also reported that the PO from *Penaeus setiferus* was active over a pH range of 6.0-7.5 and stable at pH 8.0 but unstable at acidic pH.

The stability of the PO was very different depending on a number of factors such as temperatures, pH, substrate used, ionic strength, buffer system and time of incubation. The characteristics of PO from different crustaceans are summarized in Table 1. (Kim *et al.*, 2001).

Table 1. Characteristic of PO from crustaceans

Source	pH Optimum	pH Stability	Temperature Optimum (°C)	Temperature Stability (°C)
White shrimp (*Penaeus setiferus*)	7.5	8.0	45	25-50
Pink shrimp (*Penaeus duorarum*)	8.0	6.5-9.0	40	20-40
Florida spiny lobster (*Panulirus argus*)	6.5	6.5-8.0	35	30-40
Western Australian lobster (*Panulirus Cygnus*)	7.0	5.0-9.0	30	25-35
Lobster (*Homarus americanus*)	6.5	6.5-7.5	30	15-30

Source: Modified from Kim *et al*. (2000); Rolle *et al*. (1991)

1.2.3. Activators/ inhibitors

One unusual and intriguing characteristic of this enzyme is its ability to exist in an inactive or latent state. PO can be released from latency by several agents or treatments such as incubation with ammonium sulphate, anionic detergents, carboxymethylcellulose, urea, guanidine salts, short-time acid or base treatment or a limited proteolysis (Whitaker, 1995) and alcohol (methanol) (Adachi *et al*., 1999).

Several factors have been reported to be involved in PO activity. They include: (i) a proenzyme, (ii) a PO-bound inhibitor and (iii) a conformation change (Gauillard and Richard-Forget, 1997). Chazarra *et al*. (1997) reported that the

presense of SDS in the assay medium provokes a conformation change in the latent iceberg lettuce PPO, leading to the increase PO activity with increasing concentration. Thus, PO activity is stimulated by SDS (Mazzafera and Robinson, 2000). Some ion or salts affect the PO activity. $MnSO_4$ and $CaCl_2$ enhanced PPO activity (Jiang, 1999; Adachi et al., 1999). Kong et al. (2000) observed that the activity was increased by addition of 1 mM Mg^+, K^+ or Cu^{2+}. However, the activity of PO was dependent on the concentrations of divalent cations (Jiang, 1999; Adachi et al., 1999). Ascorbic acid, cysteine, glutathione, thiourea and hexylresocinol were demonstrated to control enzymatic browning induced by PO (Guerrero-Beltran et al., 2004; Kavrayan and Aydemir, 2001). Richart-Forget et al. (1992) reported that cysteine has no direct effect on PO, which allowed a permanent protection against enzymatic browning. Son et al. (2000) observed that oxalic acid has a strong antibrowning activity and the inhibitory pattern appeared to be competitive. Molnar-Perl and Friedman (1990) revealed that N-acetyl-L-cysteine and reduced glutathione were nearly as effective as sodium sulfite in preventing browning of both apple and potatoes.

1.2.4. Substrate specificity

Enzymes isolated from different sources exhibited their activity differently depending on the substrates used. The substrate specificity was studied for PO from taro (*Colocasia esculenta*), potato (Duangmal et al., 1999) and avocado were 4-methyl catechol (Gomez-Lopez, 2003). Tyrosine has been reported to be a natural substrate for PO activity in crustaceans. Tyrosine is the monohydroxyl phenol. Hydroxylation of tyrosine leads to the formation of dihydroxyphenylalanine (DOPA) (Kim et al., 2000), which then converted to dopaquinone and water and can be readily converted to dopachrome, an orange-red pigment (Simpson et al., 1987).

1.3. Melanoisis

Melanoisis is a process that is triggered by a chemical mechanism consisting of oxidation of phenols to quinones induced by PPO or PO (Montero *et al.*, 2001). PO is responsible for a discoloration in crustacean species such as lobster, shrimp and crab. The postmortem dark discoloration in crustaceans, called melanosis or blackspot, connotes spoilage (Kim *et al.*, 2001). Melanosis causes the large losses to the industry (William *et al.*, 2003) and reduces the market value of these foods (Simpson *et al.*, 1987).

Melanoisis or blackspot appears very frequently during the storage of crustaceans postmortem before the onset of spoilage. Melanosis starts in refrigerated crustaceans within a few hours of capture, and the susceptibility is often greater in autumn and winter, coinciding with molting and lower food availability (Montero *et al.*, 2004). Marine crustaceans form the blackspots on their tail, cephalothoraxes, and podites during storage (Adachi *et al.*, 1999). In chilled prawns and shrimps, the melanotic reaction begins at the head and then spread to the tail (Simpson *et al.*, 1997; Rolle *et al.*, 1991). The rate of spread of melanosis differs among the various species. This could be related to differences in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson *et al.*, 1987). Pink shrimp PPO oxidizes DL-β-3,4-dihydroxyphenylalanine (DOPA) 1.8-fold more rapidly than white shrimp PPO. Pink shrimp carapace has relatively higher levels of free tyrosine and phenylalanine, the natural substrates of melanosis, than white shrimp carapace (Rolle *et al.*, 1991).

The enzymatic oxidation to form melanin associated with the stages of molting cycle, sex, occurrence of injury or trauma (Ogawa *et al.*, 1984).

1.3.1. Molting cycle

Every tissue in the crustacean will be affected in some manner by molting. Four stages of molting (postmolt, intermolt, premlot, molt) are different developmental of cuticle formation. Apparently, melanosis of shell and hyperderma tissue in lobster was related to stage of molt (Ali *et al.*, 1994). Ogawa *et al.* (1984) observed that melanosis in the ventral pellicle made itself more evident in stage C (early premolt) than in stage D (late premolt), for both males and females in tail Spiny lobster (*Panulirus laevicauda*). In late premolt, the trypsin activated enzyme is present when the new cuticle is formed and needs to begin sclerotization. Due to the antiseptic nature of quinones, the increase in PO levels with a corresponding increase in quinones may prevent the penetration of microorganisms through the unfinished cuticle (Kim *et al.*, 2000. Ferrer *et al.*, 1989). Molting in crustaceans seems to be correlated with water temperature (Travis, 1954). Bartolo and Birk (1998) stated that Norway lobster in warmer season are more likely to molt and showed an increase in PO activity during the late summer. Also Ferrer *et al.* (1989) found that an increase in the levels of PO was observed during the period of warmest months of the year. Ali *et al.* (1994) observed that the Pro-PO forms were activated *in vitro* by extracts of molting fluid which is secreted into the cuticle just before molt in Florida spiny lobster (*Panulurus argus*) cuticle.

1.3.2. Sex

The black discoloration of lobsters is related with sex (ogawa *et al*, 1984ab). Females have a higher discoloration than males for the integumentary tissues of tails of spiny lobster (*Panulirus laevicaud*) (Ogawa *et al*., 1984a). Ogawa *et al*. (1984 b) also found that females showed a higher discoloration than males in junctions and bases of segments alike of *Penaeus subtilis*.

1.3.3. Traumatism

The sclerotization and melanization are two independent processes to be in fluenced by traumatism (Ogawa, 1987). Ogawa *et al*., (1984 b) state that the black discoloration is noticeable when the shrimp is injured. Whole shrimp that continue to live after being injured present a different behaviour: those in stage C are bound to recover from traumatism through cicatrization, in the first place, while those in stage D, perhaps because of forthcoming molting, are more prone to blackening (Ogawa *et al*.,1984b). Traumatism in the alive state is a principal factor of discoloration and usually happens after one day of storage. Further, the larger and the earlier the bruising, the more intensive the discoloration was obtained. Absence of air –oxygen acted as an inhibiting factor with retarding effect, even if traumatism was present in the alive state. The possibility does exist that the oxidase serves to increase the respiration of damaged tissues, by increasing their rate of oxygen uptake, which mean that the metabolism of some or all of the tissue is increased (Ogawa *et al*., 1984b). Thus, traumatism seems to trigger a defence mechanism in shellfish involving the activation of PO and resulting ultimately in increased black spot. Lobster and shrimp can be induced to form melanin by injuring (Ogawa, 1987).

2. Non-enzymatic browning reaction

The non-enzymatic browning reactions which occur in food consist of:

1. the degradation of ascorbic acid

2. lipid peroxidation

3. sugar-sugar caramelization

4. Maillard reaction

Ascorbic acid (AsA) undergoes a reaction chemically similar to that of sugar except that amino acids are not necessary for browning. Since AsA is very reactive, it degrades by two pathways, both of which lead to the formation of dicarbonyl intermediates and subsequently to form browning compound (Davies and Wedzicha, 1992).

Lipid peroxidation occurs by the action of oxygen and reactive oxygen species on the fatty acids, especially unsaturated fatty acids. These are oxidized to form aldehydes and ketones, which then react with amino acids to form browning pigments as in the Maillard reaction. It is possible that peroxidation products induce the browning reaction of the Amadori products (Hermosin, 1992).

At high temperature (>80°C), sugar-sugar interactions or the caramelization reaction occurs. This is a complex series of reactions but many of the intermediate compounds and products are similar to those observed in the Maillard reaction (Davies and Labuza, 2003).

2.1. Maillard reaction

Non-enzymatic browning reactions between amino acids and reducing sugars are the basis of the Maillard reaction, which takes place in thermally processed

food (Carabasa-Giribet and Ibarz-Ribas, 2000), leading to the formation of a complex series of compounds called Maillard reaction products (MRPs) (Mastrocola and Munari, 2000). In the early stage of the Maillard reaction, radical mechanisms play an important role in the formation of Maillard reaction products. These may include radical reaction pathways for the formation of brown products, which differ from the classical HODGE sequence (Fig. 3) (Cammerer and Kroh, 1996).
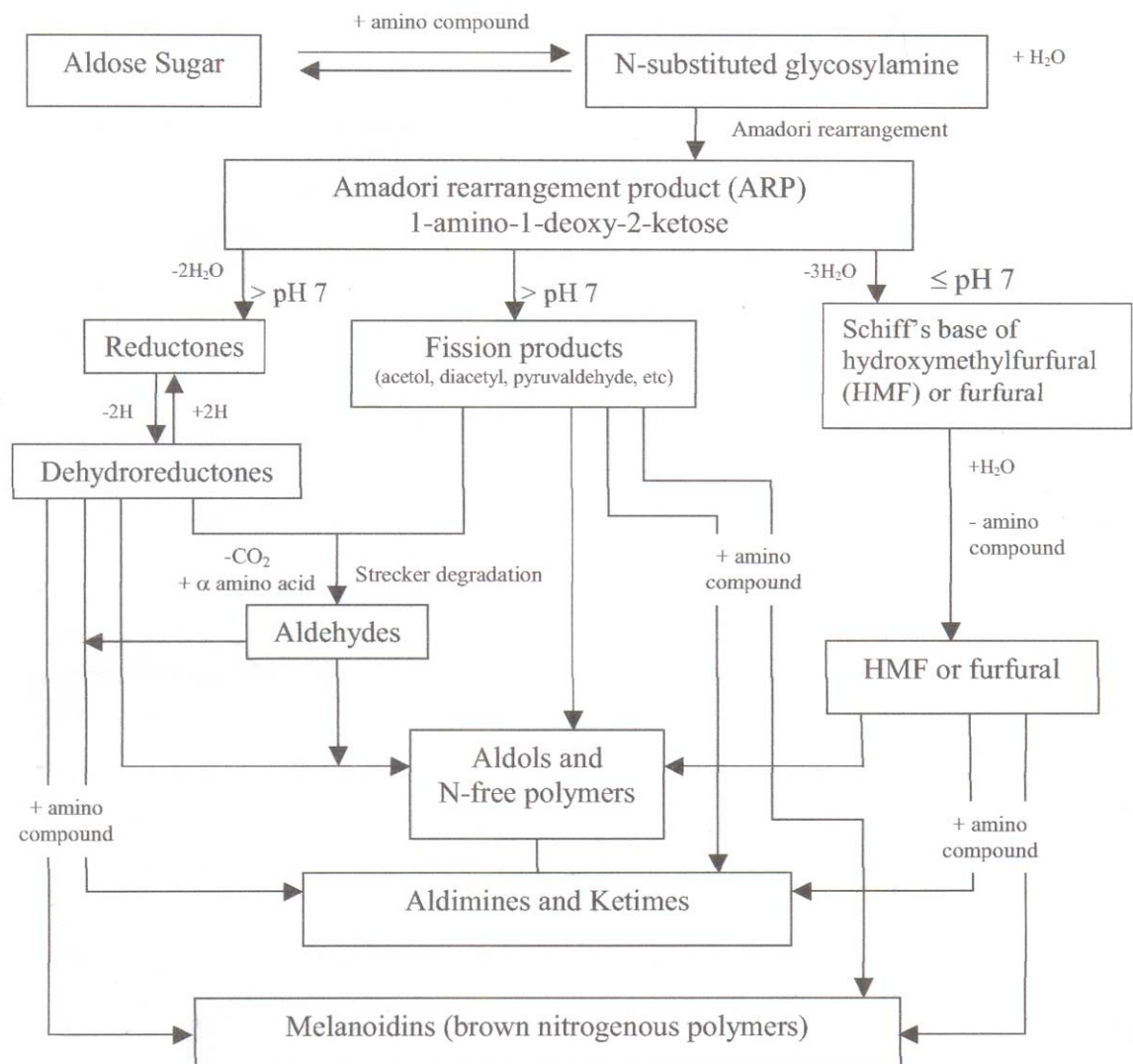

Figure 3. Scheme of Maillard reaction

Source: Hodge (1953)

2.1.1. Mechanism of the Maillard reaction

The Maillard reaction is sometimes subdivided into three stages: the early Maillard reaction, the advanced Maillard reaction and the final Maillard reaction (Van Bockel, 1998).

1. The early Maillard reaction

A reducing sugar, like glucose (as an aldehyde in open chain form), condences with a compound possessing a free amino group (of an amino acid or in proteins mainly the ε-amino group of lysine, also the α-amino groups of terminal amino acids) to give a condensation product, N-substituted glycosylamine via formation of a Schiff's base and the Amadori rearrangement, to the so-called Amadori product (Martin, 2003; Van Bockel, 1998) (Fig. 4). The degradation and dehydration of Amadori products into amine or carbonyl intermediates, and the reaction of carbonyl intermediates with other amino groups as well as subsequent rearrangement to form advanced glycosylation end-products (AGE-products) also take place (Murthy and Sun, 2000). The early stage of the Maillard reaction can be evaluated by determination of the furosine (ε-N-(furoyl-methyl)-L-lysine) amino acid formed during acid hydrolysis of the Amadori compounds. Fructosyl-lysine, lactosyl-lysine and maltulosyl-lysine are generally produced by reaction of ε-amino groups of lysine with glucose, lactose and maltose, respectively (Erversdobler and Hupe, 1991).

Figure 4. Formation of Amadori compound from glucose and amine

Source: Feather *et al*. (1995)

2. The advanced Maillard reaction

The subsequent degradation of Amadori product is dependent on the pH of the system (Fig. 4). At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF), the intermediate product in the Maillard reaction and the degradation of sugars also formed at high temperatures (Ramirez-Jimenez *et al*., 2000). At pH >7, the degradation of Amadori compound is thought to involve mainly 2, 3-enolisation, where reductones, including acetol, pyruvaldehyde and diacetyl, are formed. All these compounds are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products. Dicarbonyl compounds will react with amino acids with the formation of aldehydes and α-aminoketones. This reaction is known as the Strecker degradation (Martins, 2003).

The degradation of Amadori compound N-(1-deoxy-D-fructose-1-yl)-glycine; DFG) by 1, 2–enolisation and 2, 3-enolisation leads to the formation of 3-deoxy-2-hexosulose and 3–deoxy-2, 3-hexodiulose, respectively. The formation of

these intermediates is accompanied by amino acid release (Martins *et al*., 2003). The degradation of Amadori compound take place through enolosation such as the formation of glucosones by transition-metal catalyzed oxidation of 1, 2-enaminol and 1-amino-1, 4-dideoxy-2, 3-diulose by elimination of C-4 OH group of 2, 3-enaminal (Huyghues-Despointes and Yaylayan, 1996). Another possible mechanism of degradation of Amadori compound involves a retro-aldol reaction, including that generates 1-glycine-1-deoxy-D-glyceraldehyde and glyceradehyde through a retro-aldo clevage at C-3 to C-4. The compound can react with free glycine and produce more compound that subsequently undergoes a β-elimination to form methylglyoxal and releases glycine (Martins *et al*, 2003; Thessl *et al*., 1995) (Fig. 5).
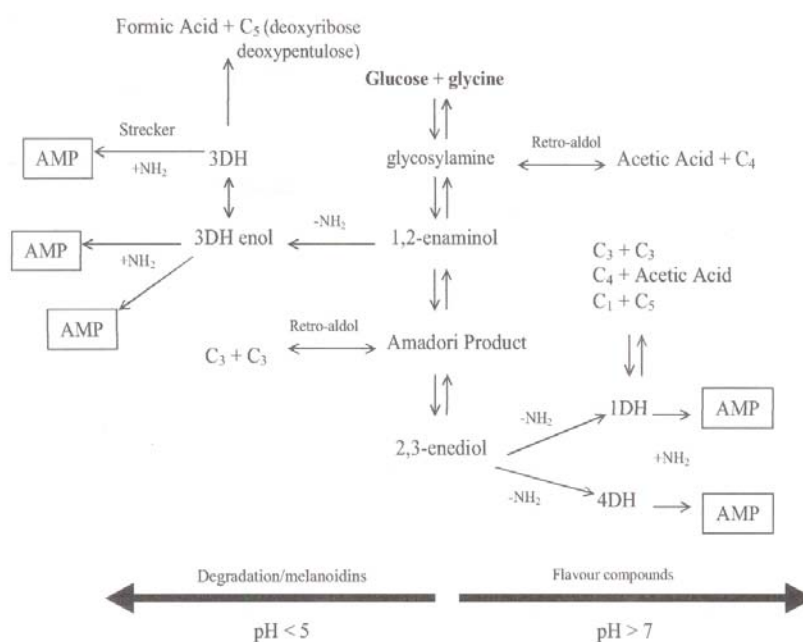

Figure 5. Scheme of advanced Maillard reaction from glucose/glycine. AMP (Advanced Maillard Products): 1-DH (1-deoxy-2, 3-diketose); 3-DH (3-deoxyaldoketose); 4-DH (4-deoxy-2, 3- diketose).

Source: Tressl *et al*. (1995)

3. The final Maillard reaction

In an advanced stage, a range of reactions including cyclization, dehydrations, retroaldolizations, rearrangements, isomerizations and further condensations take place, leading to the formation of brown nitrogenous polymers and co-polymers, known as melanoidin (Fig.3) (Martins, 2003). Melanoidins are of a high molecular weight (up to about 100,000), and they contain nitrogen by definition. However, brown pigments are also formed without nitrogen mainly, from sugar degradation. In the case of milk, it has been noted already for a long time that brown components are mainly bound to protein, but the nature of the bonds is not clear. It is expected that lysine residues are involved in melanoidins, because lysine loss also increases during the final stage (Van Boekel, 1998).

2.1.2. Color formation

The development of color is extremely important and is associated with the extent of the advanced Maillard reaction (Martins and Van Boekel, 2003a). This stage is characterized by the formation of unsaturated, brown nitrogenous polymers and copolymers. Nevertheless, nitrogen-free polymers are also formed from the condensation reactions of furfurals or dehydroreductones (Ames, 1992). The color produced range from pale yellow to very dark brown, depending on the type of food and/or the extent of the reactions. The aldehydes formed by the Strecker degradation are also a source of browning since they can condense with themselves, with sugar fragments, with furfurals, or with other dehydration products to form brown pigments. However, Strecker's pathway is not the major color-producing reaction and is better known as the source of off-flavors associated with Maillard browning (Namiki, 1988).

Moreover, the Strecker reaction possibly occurs in the absence of amino acid and is not relevant with protein (Morales and Van Bockel, 1999).

Carabasa-Giribet and Ibarz-Ribas (2003) grouped the colored compounds into two general classes: low molecular weight compounds, which typically possess two-to-four linked ring containing extended double-bond conjugation (Ames, 1992), and melanoidins which are brown polymers and possess molecular weights of several thousand daltons and discrete chromophore groups (O'Brain and Morrissey, 1989). Hofmann (1998) revealed that after an amine catalyzed ring-opening of the furan-2- carboxaldehyde, several condensation reactions between methylene-active intermediates and carbonyl compounds were involved in the formation of the chromophores.

2.1.3. Monitoring the Maillard reaction

The Maillard reaction development is generally monitored by the increase in absorbance either at 294 (early MRPs), 320-350 (soluble pre-melanoidins, advanced stage) or 420-450 (final MRPs) nm, corresponding to color intensity of the reaction medium, as well as the formation of specific compounds (Wijewikreme *et al.*, 1997; Billuad *et al.*, 2004). The formation of heterocyclic derivatives and intermediate water-soluble compounds such as reductones, amino-reductones or pre-melanoidin is detected. Generally, the low absorbance values recorded at 420 nm seemed to reveal a less proportion in brown pigments or melanoidins (Billuad *et al.*, 2004).

Prior to the formation of brown pigments, fluorescent compounds are formed. Baisier and Labuza (1992) stated that fluorescence accumulation in a model system of glucose/lysine is due to interaction between reactive reducing compounds

and amines by an irreversible reaction. The formation of major fluorescent compound was formed in the presence of casein and sugar and occurred simultaneously with browning (Morales *et al*., 1996).

Amadori products undergo further transformation to fluorescent, colored substances and cross-linked polymers (Morales *et al*., 1996; Van Boekel, 1998). Fluorescence and browning developments in Maillard reaction are generally used as indicator of the reaction rate and MRPs formation (Yeboah *et al*., 1999). Many reactive compounds are formed as intermediates, such as keto and aldoseamine compounds, 3-deoxyosones, furans, maltol, pyrrole derivatives, pyrazines, pyranones, lactones, substituted imidazoles,. etc. (Morales *et al*., 1996).

2.1.4. Factors affecting Maillard reaction

The development of the Maillard reaction shows two different and distinct phases: initially slow (lag period), and later relatively fast (post-lag period) (Lerici *et al*., 1990). Many factors influence the velocity of reaction such as temperature, time, water activity (a_w) (Buera *et al*., 1987), reactant source and concentration (Jing and Kitts, 2002), the type and ratio of reducing sugar (Naranjo *et al*., 1997), amino compounds (Morales and Jemenez-Perez, 2001; Yeboah *et al*., 1999), pH (Ajandonz *et al*., 2001) and food composition (Tanaka *et al*., 1994; Lerici *et al*., 1990).

2.1.4.1. Types of sugar and amino acid

Browning rate is significantly influenced by the type of reducing sugar involved in the reaction. The order of reactivity is follows: aldopentoses > aldohexoses > ketohexoses > disaccharides (Naranjo *et al*., 1997). A terminal pyranose group at the C-4 position of the reducing end of disaccharides retarded

further reactions of protein-disaccharide adducts (Yeboah *et al*., 1999). Generally, aldoses are intrinsically more reactive than ketoses (Yoboah *et al*., 1999). There have been conflicting reports on relative reactivity of glucose compared with fructose. Naranjo *et al*. (1998), Wijewickreem *et al*. (1997) and Yoboah *et al*. (1999) reported that glucose is more reactive than fructose but Suarez *et al*. (1995) reported that fructose is more reactive than glucose.

If the reactive form reacts faster than it is generated from the other forms, the rate of tautomeric interconversion of the cyclic anomers of sugar dominates the reaction rate (Naranjo *et al*., 1997). Thus, in the Maillard reaction, the concentration of open chain form might be a crucial factor in determining the rate of glycation if the interconversion rate is slower than the reaction rate (Naranjo *et al.,* 1997). The reaction rate of each sugar depends on the percentage of acyclic form, but also on the electrophilicity of carbonyl group (Naranjo *et al*., 1997; Bunn and Higgins, 1981). Among the four sugar studied, fructose, maltose, lactose and glucose, it was observed that fructose has the highest proportion of open chain form, but aldoses would react faster than ketoses because they are more electrophilic (Naranjo *et al*., 1997).

2.1.4.2. Temperature and heating time

Temperature and duration of heating determined the rate of the reaction. An increase in temperature leads to an increase of the reactivity between the sugar and the amino group (Martins, 2003). MRPs formation is highly dependent on heating time and temperature. The rate of the Maillard reaction increased exponentially as the heating temperature increased (Billaud *et al*., 2004a).

2.1.4.3. Concentration

The rate of the Maillard reaction are governed by concentration of reactant. Carabasa–Giribet and Ibarz–Ribas (2000) reported that the increase in glucose concentration led to the decrease in induction time in the aqueous model systems containing glucose and aspartic acid, glutamic acid or asparagines. Lerici *et al*. (1990) observed that the kinetic constants (K_1) value increased with increasing solid concentration of each glucose/glycine molar ratio.

2.1.4.4. pH

In general, the reaction is inhibited by low pH values and favored by high pH values (Baxter 1995; Lerici *et al*.,). Ashoor and Zent (1984) observed that the maximum browning was obtained at a pH value of about 10 in glucose, fructose and lactose model system. Ajandouz *et al*. (2001) revealed that in Maillard reaction, highly UV – absorbing and colorless compounds and brown polymer are formed at higher pH in the fructose–lysine aqueons model system. Renn and Sathe (1997) showed that the browning rate between L–leucine and D–glucose is positively correlated with pH.

2.1.4.5. a_w

The measure of water activity (a_w) is useful to describe a thermodynamic equilibrium state and is often useful for predicting reaction rate (Sherwin and Labuza., 2003). Lievonen and Roos (2002) found that the non-enzymatic browning rates increased as the initial water activity increased in model system of lysine and xylose containing maltodextrin or polyvinylpyrrolidone. Shen and Wu (2004) propose that the reduction of a_w is not the sole major mechanism for

ethanol to accelerate HMF formation and Maillard browning in glucose/glycine solution.

2.2. Caramelization

Thermally induced caramelization also occurs during heating. Sugar, normally monosaccharide-structures, should firstly undergoes intermolecular rearrangements. Depending on the time and temperatures, yellow and brown colors are obtained when solid glucose or solutions of glucose are heated. The flavor produced varies from mild, caramel-like and sweet to burning bitter. The reaction causes the release of H^+, thus the pH of the solution undergoing caramelization falls with time, eventually into the slightly sour region of pH 4-5 (Kroh, 1994).

This caramelization reaction is proceeded by sugar isomerization and sugar degradation reactions. Monosaccharides in aqueous alkali medium undergo both reversible and irreversible transformation (De Bruijn *et al*., 1986). The sequence of reversible sugar degradation reaction by the initial enolization known as the de Bruijn van Eckenstein rearrangement (Kroh, 1994; Brand and Van Boekel, 2001), resulting in an equilibrium of neutral and ionized monosaccharides (Brand and Van Boekel, 2001). The reaction is followed by dehydration or β-elimination, dicarboxylic cleaving, retro-aldol reaction and later, aldol condensation and finally, a radical reaction. The enolisation reaction is of particular importance because it initiates the subsequent chain of events. These reactions give rise to aliphatic sugar degradation products which can react further to produce oxygen heterocyclic and carbocyclic compounds via aldol condensation (Kroh, 1994).

The decomposition of sugars leads to the formation of volatile (caramel aroma) and brown colored compound (caramel color). The colors and the

aromas depend on the sugar used and are formed mostly through deoxyosuloses, *O*-heterocyclic and carbocyclic intermediates as well as low molecular weight sugar fragment (Kroh, 1994). Glyoxal, methylglyoxal and diacetyl can also be formed from MRPs or directly from carbohydrates. These compounds can arise from the isomerization and subsequent retro-aldolization of the corresponding sugar molecule, or by cleavage of the 1-deoxyhexosulose (Ledl and Schleicher, 1990; Kroh *et al*., 1996)
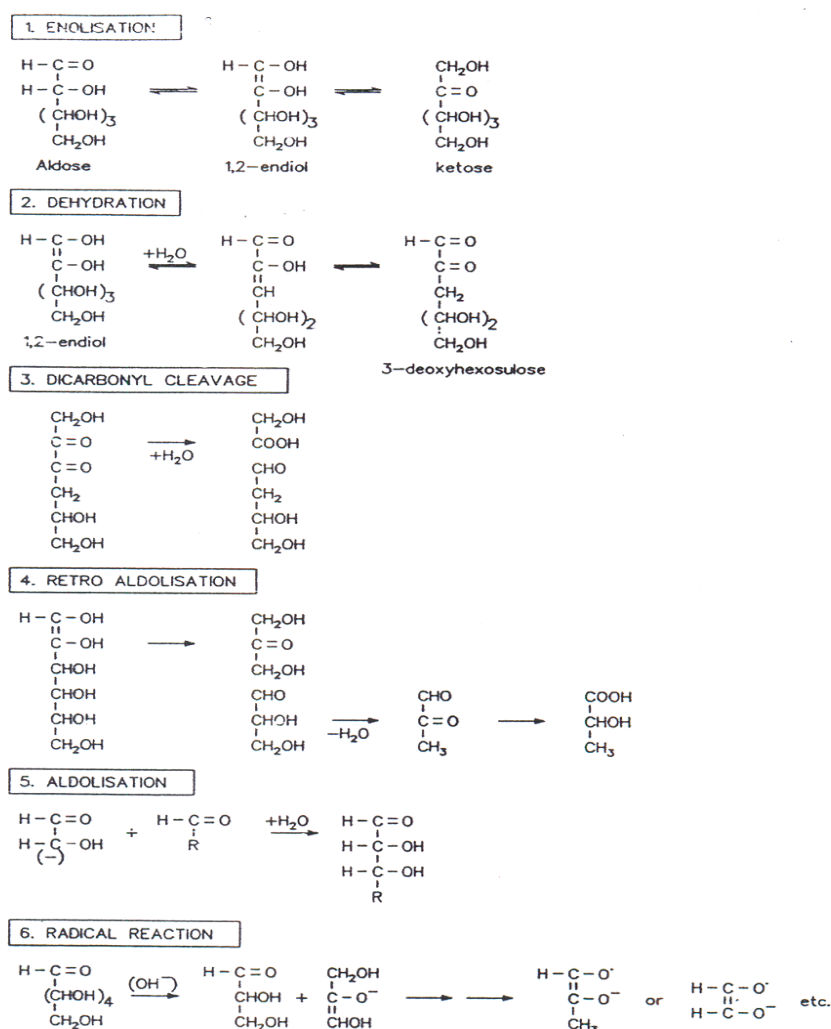

Figure 6. Selected sugar degradation reaction

Source: Kroh (1994)

3. The control of enzymatic browning

PPO or PO catalyzes the oxidation of phenols into O-quinones. The O-quinones are highly reactive compounds, which can polymerize spontaneously to form high-molecular-weight compounds or brown pigments (melanins), or react with amino acids and proteins that enhance the brown color produced (Montero *et al*., 2001; Whitaker, 1995). Preventing enzymatic browning is accomplished in many ways. The methodology is to eliminate from the reaction one or more of its essential components: oxygen, enzyme, copper, or substrate. Chemical compounds have also been used to prevent PPO action. Various techniques and mechanisms have been developed over the year to prevent PPO action in food products (Kim *et al*., 2000).

3.1. Uses of chemicals

3.1.1. Reducing agent

Reducing agent or antioxidants inhibitors prevent browning by chemically reducing the O-quiones to the colorless diphenols, or react irreversibly with the O-quinones to form stable colorless products (Kim *et al*., 2000).

3.1.1.1. Sulfiting agent

Sulfites are multifunctional agents, which can prevent enzymatic and nonenzymatic browning, control growth of microorganisms, act as bleaching agents, antioxidants or reducing agent and carry out various other technical functions. Suifite treatment can also be used in conjunction with vacuum packaging (Laurila *et al*., 1998). Currently, the use of sulfiting agents is the most efficient and cheapest chemical approach for controlling browning. Nevertheless, their use has several disadvantages, such as negative effect on the texture, development of off-flavors,

destruction of vitamin B_1 and adverse health effects in certain sensitive individuals (Roux *et al*., 2003).

3.1.1.2. Cysteine

Cysteine, an alternative reducing agent, reduces PPO activity and delays discoloration due to the formation of the colorless cysteine-quinone adducts. This compound has been proved to be competitive inhibitors of PPO (Guerrero-Beltran *et al*., 2005). However, the concentration of cysteine and other thiols necessary to achieve acceptable levels of browning inhibition have negative effects on taste (Kim *et al*., 2000). D-glutathione, L-cysteine or thiourea showed the inhibitory activity towards the partially purified longan PPO (Jiang, 1999). D-glutathione (reduced form) was the most effective, followed by L-cysteine. In addition, the use of 10 mmol/litre glutathione was found to give good control of browning of litchi fruit and 80-85% inhibition of PPO were observed (Jiang and Fu, 1997). Recently, Benjakul et al. (2005) reported the inhibitory activity of cysteine and glutathione on kuruma prawn PO.

3.1.2. Acidulants

Acidulants, such as citric, malic, or phosphoric acid, can inhibit PPO activity by reducing pH and/or chelating copper in a food product (Guerrero-Beltran *et al*., 2005). The pH of food affect ionization groups on the enzyme. These groups must be in the proper ionic form in order to maintain the conformation of active site, bind the substrates, or catalyze the reaction. However, most of these changes in ionization are reversible. Irreversible denaturation occurs usually at extreme pH values (Kim *et al*., 2000). The role of acidulants is to maintain the pH below what is needed for optimum catalytic activity of PPO. Acidulants such as citric, malic and

phosphoic acids lower the pH of the system to below 3 where PPO is inactive (Richardson and Hyslop, 1985). Furthermore, citric acid act as chelating agent and acidulant (Laurila *et al*., 1998). The inhibitor reaction mechanism differs, depending on the reducing agent employed. Inhibition by thiol compounds is attributed to either the stable colorless products formed by an addition reaction with *O*-quinones or binding to the active center of PPO. Ascorbate acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning. Ascorbic acid has also been reported to cause irreversible inhibition of PPO (Aydemir, 2004).

3.1.3. Chelators

Enzymes have metal irons at their active sites, so the removal of these ions by chelators would render the enzyme inactive. Chelating agents complex with prooxidative agents such as copper and iron ions through an unshared pair of electrons in their molecular structure which provides the complexing or chelating action (Kim *et al*., 2000). Since PPO is metalloprotein with copper as the prosthetic group, it can be inhibited by metal chelating agents such as citric acid, ethylenediamine tetraacetic acid (EDTA) (Whitaker, 1995).

3.1.4. Protease

Proteases were found to be effective browning inhibitor for apples and potato (Labuza *et al*., 1992). Taoukis *et al*. (1990) observed that ficin is presumed to be the inactivation of PPO from shrimp (*Penaeus duorarum*) by binding or hydrolysis at specific necessary for the activity of PPO. However, Zn-serine protease could

initially accelerate phenolase from Norway lobster at pH 8.2 (Zotos and Taylor, 1996).

3.1.5. MRPs

MRPs have been known to prevent oxidation reaction, via free radical scavenging action (Morales and Jamenez-Perez, 2001), metal ion chelating property (Wijewickreme and Kitts, 1998; O'Brien and Morrissey, 1997) and/or reducing activity (Tan and Harris, 1995). MRPs are known to possess the metal-chelating ability and reducing power (Yoshimura *et al*., 1997) and to inhibit certain oxidoreductase such as PPO (Tan and Harris, 1995), peroxidase, superoxide dismutase, xenobiotic enzymes, hydrolase activity (Billand *et al*., 2004). Morales *et al*. (2004) stated that the melanoidins behave as anionic hydrophilic polymers which can form stable complexes with metal ion. At pH values close to these found in most foods, melanoidins have a negative net charge and are able to bind metallic ions.

Non-enzymatic browning is commonly regarded as a series of complex reactions in which MRPs formation depends on the sugar and the amino acid used (Van Boekel, 2001). Billaud *et al*. (2004a) synthesized MRPs from equimolar glucose or fructose with L-cysteine (1 mol/l) at 90°C for 15 h and observed that glucose produced MRPs with higher PPO inhibitory power compared to counterpart fructose-cysteine MRPs. The MRPs were probably formed at a slower rate with fructose, as a result of a greater reactivity for the aldose when compared to the ketose. A similar result was also found by Billaud *et al*. (2004b). Also, Brun-Merrimee *et al*. (2003) found that the inhibitory effectiveness of MRPs from GSH/glucose system was higher than MRPs produced with fructose/cysteine, when heated at 90°C for 15-39 h. Apart from MRPs, free amino acids has been reported to exhibit the inhibitory activity

towards PPO. Vieira and Fatibllo-Filho (1999) studied PPO inhibition potency of several L-amino acids (1.3×10^{-3} mol/l) and found that only L-cysteine, L-hydroxyproline and L-proline had a inhibition of 100, 42.8 and 19.3%, respectively, whereas L-glycine had a low inhibition (2.0%).

Billaud *et al*. (2004a) observed that inhibitory potency of the amino acid/sugar mixtures increased with increasing temperature 80-120°C and heating time (0-48 h). Billaud *et al*. (2003) stated that PPO inhibitory potency of an equimolar (0.25 M) hexose (fructose/glucose)/GSH mixture increased parallelly with increasing time of heating at 90°C from 2 to 39 h. Furthermore, the extent of inhibition increased with temperature in the range of 80-110°C. Roux *et al*. (2003) also found that PPO activity remaining after incorporation of MRPs from either glucose/cysteine or fructose/cysteine mixtures was lower as the time of heating increased. The glucose/cysteine mixture showed the superior inhibitory activity to the fructose/cysteine mixture prepared under the same conditions.

Increasing concentration in reaction medium resulted in a gradual loss of enzyme activity (Billaud *et al*., 2004b). High concentrations of hexose (1-2.7 M) were needed to develop the inhibiting effect on PPO activity (Billaud *et al*., 2003). Furthermore, Lee *et al*. (2004b) found that the inhibitory effect of MRPs increased as glycine amount increased (0, 0.05, 0.1, 0.5, 1.0, 1.5 M glycine) with a constant glucose amout (1.5 M) at 90°C for 7 h. Additionally, the inhibitory effect of MRPs increased as the amount of glucose increased with a constant glycine amout (1.5 M) at 90°C for 7 h.

Billaud *et al*. (2004a) mentioned that MRPs derived from either glucose or fructose heated at 90°C for 15 h in an acidic medium (pH<6) comprised

potent inhibitory compounds, especially in the range of pH 2-4. Tan and Harris (1995) found that the MRPs synthesized at lower pH levels (pH 3 and 5) were more effective PPO inhibitors than MPRs prepared at higher pH levels (pH 11). Under acidic conditions, the main products produced in the MRPs system are furfural and hydroxymethylfurfural (HMF). Nitrogen atom in the Amadori compounds is nearly completely protonized, thus enhancing the 1, 2 enolisation. Pyrole-like structures, resulting from the interaction of the amino compounds and furfural, have strong oxygen scavenging effect (Namiki, 1988). The reductone moiety present in the melanoidin structure was reported to exhibit both reducing and chelating properties and oxygen scavenging properties (Namiki, 1988).

3.2. Uses of appropriate technology

Various techniques have been developed over the years to prevent PPO action in food products such as heating, refrigeration, freezing, irradiation and high-pressure treatment (Kim *et al*., 2000).

3.2.1. High-pressure

The use of technologies like high pressure, which can slow or inhibit not only the melanosis process but also the growth of microorganisms, could lead to the extension of crustaceans shelf-life (Montero *et al*., 2001). Lopez-Caballero *et al*. (2000) observed that the combination of vacuum packaging and high pressure prevented the appearance of black spot as well as the growth of microorganisms in prawns kept longer than 20 days. Montero *et al*. (2001) found that the pressure induced inactivation of PPO was evident, particularly at 300-400 Mpa.

3.2.2. Modified atmosphere

An atmosphere modified with carbon dioxide (CO_2) has been used as an application to influence PPO activity (Chen *et al.*, 1993). Chen *et al.* (1993) observed that purified Florida spiny lobster, brown shrimp and potato PPO exhibited a time-related decline in activity following treatment at 43°C with high-pressure CO_2 at 58 atm. Bak *et al.* (1999) found that shrimp were packed in modified atmosphere during frozen storage resulted in overall better quality in relation to color fading of rancid flavor and toughening of the meat.

3.2.3. Irradiation

Food irradiation is increasingly recognized as a method for reducing postharvest food losses, ensuring hygienic quality and faciliting wider trade in food products. The unique feature of food irradiation is the use of ionizing radiation to improve the shelf-life or wholsomeness of product. Microorganisms present in the food can be inactivated to secure long-term preservation. (Kim *et al.*, 2000). Sun and Song (2003) found that the treatment at 5 kGy decreased the PPO mushroom activity by 93 %. Low-dose Ɣ-irradiation of fresh shrimp reduced melanosis and resulted in a significant extension of shelf life when stored on ice (Novak *et al.*, 1967).

3.2.4. Chilling storage of shrimp

Chilled or iced preservation during storage, distribution and retailing are necessary to prevent browning in shrimp. Refrigerated temperature is effective in reducing enzyme activity. The rate of enzyme-catalyzed reactions is controlled to a great extent by temperature. It has been found experimentally that increasing the temperature from 0ºC to 10ºC at least doubles the rate of spoilage of fish flesh and the controlling of temperature and time is of prime importance in reducing deterioration

of raw material (Haard and Simpson, 2000). The products of autolysis are also used as substrates by bacteria for metabolism and growth generating the unpleasant flavors as spoilage progresses, leading eventually to putrefaction. Melanosis discoloration formed by the oxidation of diphenolics during storage on ice. An intense blackening of epidermis occurred with the dark pigment migrating into the muscle tissue (Ferrer *et al.*, 1989). Ogawa *et al*, (1983) investigated the preservation of Lobster tails in ice storage on board found that black spots appeared in the broken and bruised, hard and soft segments of the ventral cuticle. Bartolo and Birk (1998) stated that black spot developed on the iced Norway lobsters at varying rates during storage on ice and rapid development of black spot, which showed the relatively low initial PPO activity. In contrast, the hypothesis of shell fish did not show a direct relationship between PPO activity and black spot development (Ogawa *et al.*, 1983; 1984 b). In crustacean PPO have various locations. It is found on the Ferrer *et al*, (1989) observed that PO from lobster cuticle existed as a large inert enzyme which became active (15 – fold increase) upon incubation at 4°C and 3 days storage. In the absence of a clear relationship between the rate of black spot development and initial PPO activity in ice storage. Because of other biochemical events, possibly including PPO pro-enzyme activation, initial PPO activity is not a good predictor of melanosis. To predict melanosis, the concentration of inactive of co-factors may have to be taken into account . Tissue breakdown during iced storage may allow enzymes to come into contact with and activate the inert proenzyme (Bartolo and Birk, 1998).

Refrigeration or storage on ice slows down but does not prevent this because PPO enzyme systems remain active under this condition. Raw, untreated pink shrimp develop melanosis in loss than 3 days with refrigerated storage, progressing to

a severe product detect within 7 days (Otwell and Marshall, 1986). Black spot decrease the perceived quality of the shrimp by consumers and reduce its commercial value (Cooper *et al.*, 1986).

Objectives

1. To study some parameters affecting the formation of Maillard reaction and the inhibitory activity towards PO for black tiger prawn.

2. To investigate the effects of decolorization on the characteristics and PO inhibitory activity of MRPs.

3. To study the uses of MRPs to retard the melanosis of black tiger prawn during iced storage.