ROSALINDA, M.M., PARK, G.L., NELSON, D.B., and McIVER, R.C. 1986. HPLC analysis of HMF in orange juice. J. Food Sci. *51*, 843-844.

WONG, D.W.S. 1989. *Mechanism and Theory in Food Chemistry*, pp. 113-120 Van Nostrand Reinhold, New York.

YAYLAYAN, V., and HUYGHUES-DESPOINTES, A. 1994. Chemistry of Amadori rearrangement products: analysis, synhesis, kinetics, reactions, and spectroscopic properties. Crit. Rev. Food Sci. Nutr., *34*, 321-369.

YAYLAYAN, V., and SPORNS P. 1987. Novel mechanisms for the decomposition of 1-(amino acid)-1-deoxy-D-fructoses (Amadori compounds): A mass spectrometric approach. Food Chem. *26*, 283-305.

YOKOTSUKA, T. 1960. Aroma and flavour of Japanese soy sauce. Adv. Food Res. *10*, 75-134.

YOKOTSUKA, T. 1986. Soy sauce biochemistry. Adv. Food Res. *30*, 195-329.

YONG, F.M., and WOOD, B.J.B. 1977. Biochemical changes in experimental soy sauce Koji. J. Food Tech., *12*, 163-175.

Output จากโครงการวิจัยฯ

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1) Publication

Sittiwat LERTSIRI, Roungdao MAUNGMA, Apinya ASSAVANIG, and Amaret BHUMIRATANA., Roles of the Maillard reaction in browning during moromi process of Thai soy sauce., *Journal of Food Processing and Preservation* (2001) 25, 149-162

1.2) Manuscript submitted to *Food Chemistry*

Sittiwat LERTSIRI, Roungdao MAUNGMA, Apinya ASSAVANIG, and Amaret BHUMIRATANA., Roles of the Maillard reaction in browning during storage of Thai soy sauce.

1.3) Manuscript to be submitted to *Food Chemistry*

Sittiwat LERTSIRI, Keittipum PHONTREE, Wanee THEPSINGHA, and Amaret BHUMIRATANA., Evidences of enzymatic browning due to laccase-like enzyme during mash fermentation in Thai soybean paste.

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1) การนำไปใช้ในเชิงพาณิชย์

สามารถนำไปประยุกต์ใช้ในอุตสาหกรรมการผลิตซีอิ๊วและเต้าเจี้ยวได้ โดยเฉพาะการควบคุมการเกิดสีน้ำตาลของผลิตภัณฑ์สำเร็จเพื่อสนองความต้องการของตลาด เนื่องจากผู้บริโภคในตลาดบ้านเราไม่นิยมผลิตภัณฑ์ที่มีสีเข้ม

2.2) ผลของโครงการฯ ในเชิงวิชาการ

ด้วยโครงการฯ นี้ สามารถผลิตนักศึกษาระดับปริญญาโท 2 คน และมีนักศึกษาระดับปีที่ 4 ร่วมทำงานวิจัยในลักษณะ senior project 1 คน

ภาคผนวก

Reprint และ Manuscript

ROLES OF THE MAILLARD REACTION IN BROWNING DURING MOROMI PROCESS OF THAI SOY SAUCE

SITTIWAT LERTSIRI[1], ROUNGDAO MAUNGMA, APINYA ASSAVANIG and AMARET BHUMIRATANA

Department of Biotechnology
Faculty of Science
Mahidol University
Bangkok, Thailand

Accepted for Publication November 7, 2000

ABSTRACT

Moromi samples of traditional Thai soy sauce from two conventional manufacturers A and B were used to investigate the browning and chemical changes related to the Maillard reaction (i.e. reactive amino compound, soluble protein, 5% trichloroacetic acid precipitated protein, reducing sugar, and 5-hydroxymethyl-2-furaldehyde). The samples were collected at specific intervals during the 60-day process of moromi fermentation. The browning (OD 420 nm; y) was expressed as powered regression with fermentation time-course (day; x), $y=0.5191 * x^{0.236}$, $r^2=0.978$ and $y=0.6188 * x^{0.159}$, $r^2=0.821$ for moromi A and B, respectively. The browning rate was high at the first 3 days and declined in the later stage, while the accumulation of 5-hydroxy-2-furaldehyde, which is the Maillard reaction intermediate, increased linearly. The size of proteins and peptides observed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the measurement of the ratio of 5% trichloroacetic acid precipitated protein to total soluble protein (PP/SP). This PP/SP ratio showed the linear correlation to browning rate with $r^2=0.921$ and 0.965 for moromi A and B, respectively. The results suggested that the rate of browning of the moromi fermentation did not entirely depend upon the Maillard reaction rate. It appeared that the browning is enhanced with the proper size of proteins and peptides.

INTRODUCTION

Nonenzymatic browning via the Maillard reaction is one of the important reactions in food chemistry, and its implication must be considered when a food

[1] Corresponding author: Dr. Sittiwat Lertsiri, Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Payathai, Bangkok 10400, Thailand. TEL: +66-2-246-1358 ext. 2700, FAX: +66-2-246-3026, E-mail: scsls@mahidol.ac.th

Journal of Food Processing Preservation **25** (2001) 149–162

process is investigated (Nursten 1980-1981). The Maillard reaction involves the condensation of carbonyls e.g. reducing sugars, aldehydes; and amine e.g. amino acids, peptides, proteins. The reaction plays a major role in food stability, flavor development, nutrition, and health. Many aspects such as types of reactants, reacting conditions, and products formed have been investigated (Bell 1997; Labuza and Massaro 1990; Ashoor and Zent 1984; Feather and Nelson 1984). Despite the large number of investigations, the detail mechanisms of this reaction are not completely elucidated.

Soy sauce is one of the foods in which Maillard reaction takes part in the occurrence of browning. The Maillard browning occurs among various kinds of important flavoring components in soy sauce and is responsible for the color and aroma changes during the manufacturing process (Yokotsuka 1960, 1986). The color and flavor of soy sauce are affected by both the aging of moromi fermentation and the pasteurization of raw soy sauce. During the brewing process, the development of browning derives mainly from nonoxidative and nonenzymatic reactions (Yokotsuka 1986).

Although there are numerous studies on Japanese soy sauce, especially kokuchi type, the results of those studies are not completely matched with Thai soy sauce. This is because of its different ingredients, such as using less wheat flour without roasting for Thai soy sauce. The processing conditions are different as well, such as longer koji fermentation period, climate temperature of morimi fermentation with shorter duration (Mongkolwai *et al.* 1997; Bhumiratana *et al.* 1988). These cause the different mechanisms of the reaction.

The aim of this work was to investigate the browning process and the roles of the Maillard browning during moromi fermentation of Thai soy sauce. This elucidation would bring about the browning control in the moromi fermentation, which affects the color of a finished product of soy sauce.

MATERIALS AND METHODS

Preparation of Soy Sauce Moromi

Koji and moromi were prepared by the manufacturers with traditional substrates, consisting of soybean and wheat flour at a ratio of 20:3 (w/w). Soybean was soaked in water for 4 h, then cooked at 116 to 121C, 1 kg/cm^2, for 3 h. The mixture of wheat flour and soybean was inoculated with 0.1% koji starter culture and kept for 36 h at 35C in a koji-making room. The koji is then mixed with salt brine containing 20% NaCl, in the ratio of 50 kg to 100 L. Two lots of the moromi from manufacturer A (moromi A) and two lots of the moromi from manufacturer B (moromi B) were fermented for 60 days in 100-L earthen jars and 2-ton fiberglass tanks, respectively.

Chemical Analyses

All chemicals were obtained from Merck (Darmstadt) unless otherwise stated. The 5-hydroxymethyl-2-furaldehyde (HMF) standard was purchased from Fluka (Buchs, Switzerland).

The moromi samples were withdrawn from fermenting vessels of the two manufacturers at various time intervals. Each sample was centrifuged at 15,000 × g and filtered through 0.45 μm membrane filter before being analyzed. The browning was analyzed by measuring the absorbance at 420 nm (OD 420 nm) in a spectrophotometer (Davies *et al.* 1998; Lertsiri *et al.* 1995). The pH of the sample was measured directly with a pH meter. Titratable organic acid was measured according to AOAC (1984). Reducing sugar (RS) and reactive amino compound (RAC) were determined by the methods of Nelson (1944) using D-glucose as standard, and 2,4,6-trinitrobenzenesulfonic acid (Habeeb 1966) using glycine as standard, respectively. Total soluble protein as total nitrogen was determined by Kjeldahl method (AOAC 1984).

For determination of HMF, the sample was first deproteinized with 5% trichloroacetic acid (TCA). The neutralized sample was submitted onto HPLC with 5 mm × 21 mm C18 column (UG 12, Shiseido Corp., Tokyo) equipped in Shimazu 6A HPLC set. The column temperature was 40C. The mobile phase was 5% acetonitrile in 0.2% phosphoric acid (pH 4.2), v/v; with the flow rate of 1.0 mL/min. Absorbance was measured at 280 nm. The HPLC conditions were described previously by Rosalinda *et al.* (1986).

The protein precipitated from deproteinization was redissolved in distilled water, and then determined by the Bradford method (Bradford 1976) using bovine serum albumin (Fraction V; Sigma, MO) as a standard.

Sizes of protein were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The portion of 500 μL moromi filtrate was precipitated with 5% TCA. The 5% TCA precipitated protein was then redissolved in 100 μL of 0.1N NaOH and denatured with 5% (v/v) 2-mercaptoethanol and 2% (w/v) sodium dodecyl sulfate by heating at 100C for 5 min. The sample (2-6 μg/lane) and a mixture of molecular weight markers (5 μg/lane) containing prestained protein standards (Keleidoscope) in the molecular weight range of 7,400 to 208,000 Daltons (BIO-RAD, CA) were loaded onto 12% gel. The gel sheet was stained with a solution of 0.25% Coomasie Brilliant Blue R-250 in water-2-propanol-acetic acid (5:5:1, v/v) and destained with 7% acetic acid containing 5% methanol (v/v).

Data Analysis

Data were analyzed using SPSS software, San Rafael, CA. ANOVA was used to describe the significance of the effects of fermentation time on browning

and chemical changes. Each value was an average of three separate determinations.

RESULTS AND DISCUSSION

Browning of the moromi from both manufacturers developed in the similar pattern. At the first 3 days of the process, the browning developed very rapidly, after that the rate of browning declined (Fig. 1). Using powered regression equation could fit the correlation of browning with fermentation time-course during 60-day fermentation. The browning equations for the moromi A and B are $y = 0.5191 * x^{0.2367}$, $r^2 = 0.978$ and $y = 0.6188 * x^{0.1596}$, $r^2 = 0.821$ where x and y stand for moromi fermentation day and OD 420 nm, respectively.

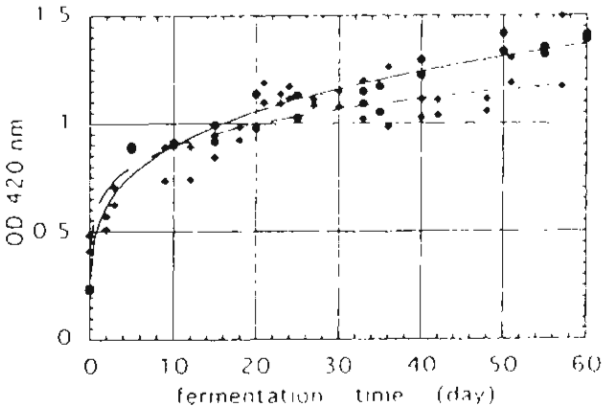


FIG. 1. TIME-COURSE FOR BROWNING DEVELOPMENT (OD 420 nm) IN MOROMI FERMENTATION

Data were from two separate lots of each manufacturer: moromi A (●···●), $y = 0.5191 * x^{0.2367}$ ($r^2 = 0.978$, $p < 0.01$); moromi B (◆···◆), $y = 0.6188 * x^{0.1596}$ ($r^2 = 0.821$, $p < 0.01$)

Figures 2a and 2b showed the amounts of reactive amino compound (RAC) and reducing sugar (RS), the reactants of the Maillard reaction. Both amounts increased sharply at the beginning of the process due to soybean protein and sugar diffused from koji solid phase and dissolved into the brine. After 3 days, RAC including soluble protein (Fig. 3a) remained increasing with low rate, conceivably because of proteolytic enzyme activities from koji digested and solubilized soybean protein (Ling and Chou 1996; Fukushima 1989; Noda

1978). RS amounts in moromi A and moromi B were almost constant. HMF, which is the intermediate of the Maillard reaction, was monitored as progressive index of the Maillard reaction (Gogus *et al.* 1997, Yaylayan and Sporns 1987). During the fermentation, the amount of HMF increased in linear manner with fermentation time (Fig. 4). This indicated that the Maillard reaction progressed through the moromi fermentation, despite the amounts of reactants (i.e. RAC and RS did not obviously decrease. These results suggested that the reactants were in excess, and the browning was partially dominated by the Maillard reaction. The overall of browning could not be predicted by the amounts of reactants of the Maillard reaction.

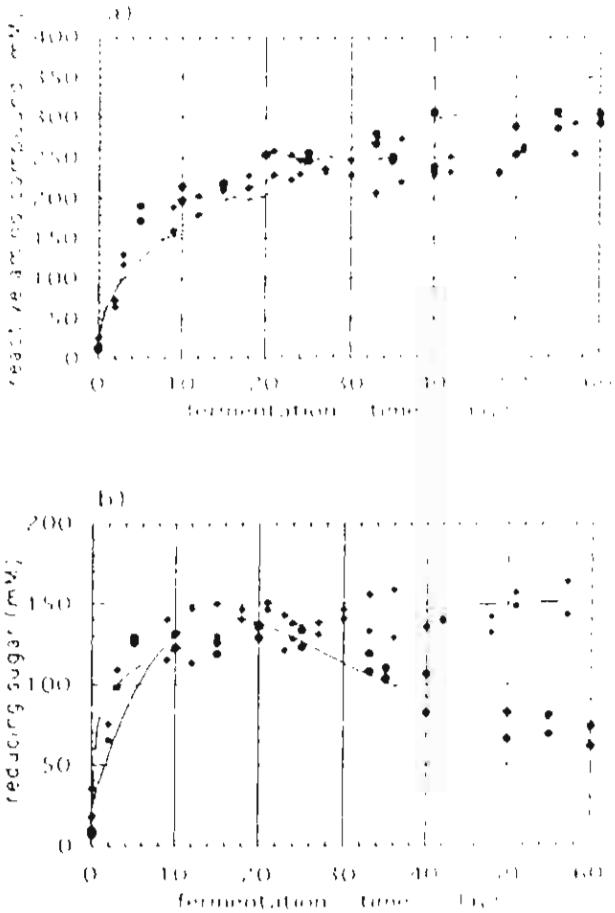


FIG. 2. THE CHANGES IN CONCENTRATIONS OF REACTIVE AMINO COMPOUND (a), AND REDUCING SUGAR (b)

Data were from two separate lots of each manufacturer: moromi A (● ●), moromi B (◆ ◆)

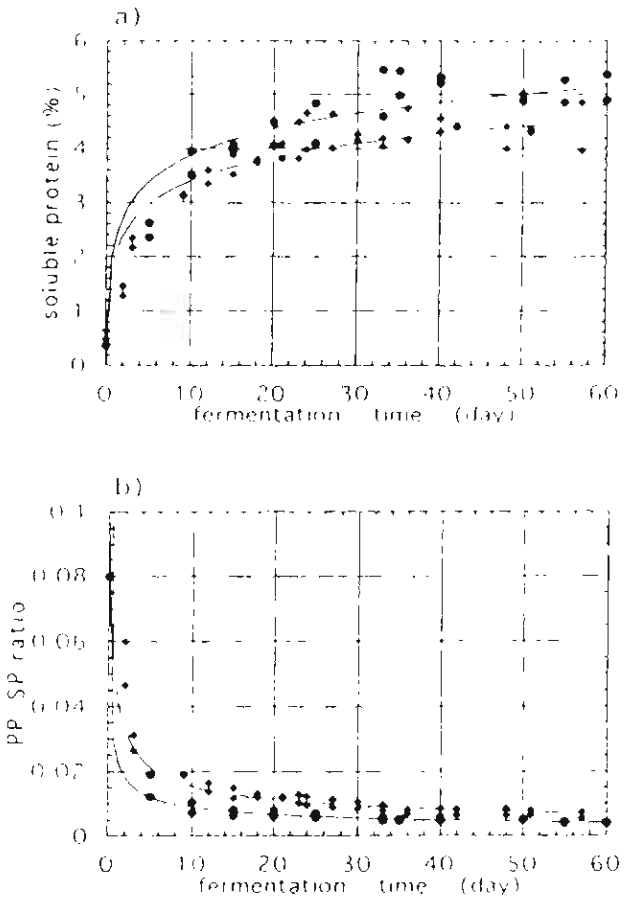


FIG. 3. THE CHANGES OF TOTAL SOLUBLE PROTEIN IN LIQUID PHASE OF MOROMI, (a), AND THE RATIO OF 5% TRICHLOROACETIC ACID PRECIPITATED PROTEIN AMOUNT TO TOTAL SOLUBLE PROTEIN AMOUNT (PP/SP), (b)
Data were from two separate lots of each manufacturer. moromi A (●···●), $y = 0.2449 * x^{(0.4933)}$, ($r^2 = 0.984$, $p < 0.01$), moromi B (◆···◆), $y = 0.4461 * x^{(0.4884)}$ ($r^2 = 0.964$, $p < 0.01$)

The early stage of moromi fermentation was also investigated in the other lot of moromi from manufacturer A. To study the changes in detail, the sampling was conducted everyday during the first 10 days of the process (Fig. 5). The results ensured that the browning rapidly developed and the chemical components (i.e. RAC and RS) vigorously changed in liquid phase of moromi during the first 3 days. Since the browning rate and the degree of chemical

changes in the liquid phase of moromi were high at the early stage of the fermentation, this should be considered as a critical stage of browning development.

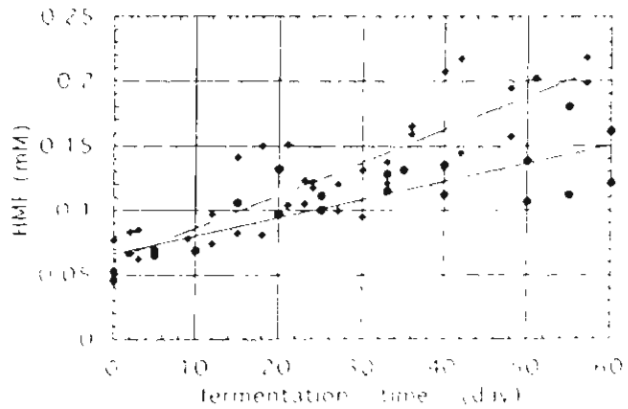


FIG. 4. HMF ACCUMULATED WITH TIME-COURSE IN MOROMI FERMENTATION
Data were from two separate lots of each manufacturer: moromi A (●···●), $y = 0.0065 + 0.0014^{*}x$ ($r^2 = 0.821$, $p < 0.05$), moromi B (◆···◆), $y = 0.0612 + 0.0025^{*}x$ ($r^2 = 0.884$, $p < 0.05$)

During first 10 days of the fermentation, the pH of each moromi dropped from 6.5 to 5.2 and dropped to 5 at the end of the 60-day process. This was due to organic acid production of lactic acid bacteria (Fukushima 1989; Yokotsuka 1986), increasing from 0% to 0.8% during 10 days to reach about 1.1% at the end of the 60-day fermentation. In general, high pH enhances the overall rate of the Maillard reaction by facilitating the tautomerization of reducing sugars to yield acyclic aldehydic form. The aldehydic sugar molecules would easily react with amino groups to undergo the Maillard reaction (Lertsiri *et al.* 1995; Wong 1989). On the other hand, low pH promotes protonation to the amino compound and increases cationic properties of the amino compound molecules. Since the amino compound attacks the aldehydic sugar in a nucleophilic manner, only unprotonated molecules of the amino compound could readily react with the reducing sugar (Labuza and Baisier 1992; Wong 1989). Therefore neutral pH at the beginning of the moromi fermentation possibly enhanced the rate of the Maillard browning in the early stages of moromi fermentation, whereas acidic pH at the latter stages could partially retard the Maillard browning.

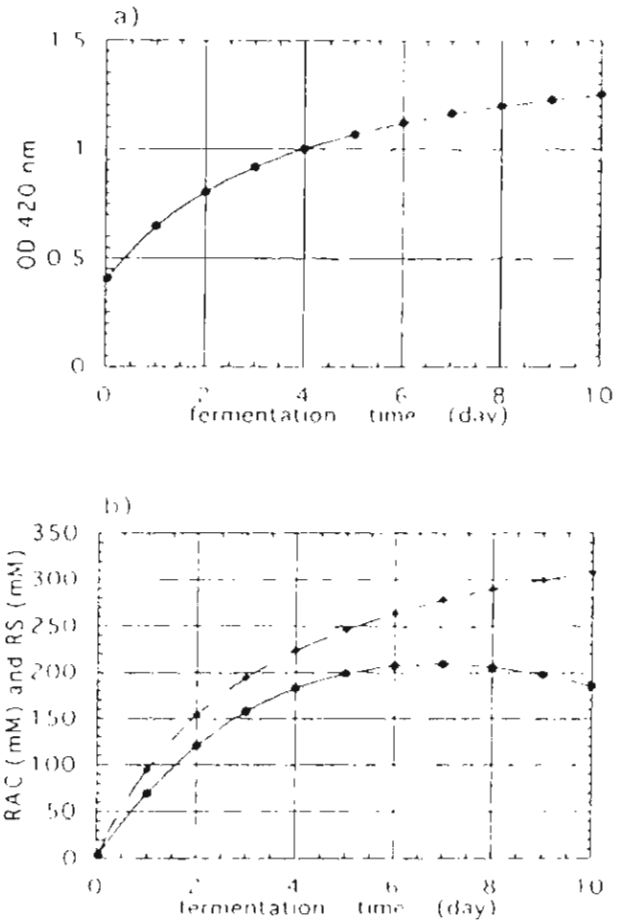


FIG. 5. TIME-COURSE FOR BROWNING DEVELOPMENT (OD 420 nm, (●---●), (a); AND THE CHANGES IN CONCENTRATIONS OF REACTIVE AMINO COMPOUND (◆---◆) AND REDUCING SUGAR (●---●), (b), DURING EARLY STAGE OF MOROMI FERMENTATION
Samples were collected from manufacturer A.

The pH effect could not completely explain the high rate of browning in the early stages. The pH did not affect the rate of the Maillard reaction in this study since HMF increased linearly with the time-course. It is previously reported that not only HMF, but also 4-hydroxy-2,5-dimethyl-3(2H)-furaldehyde is a representative intermediate of the Maillard reaction occurring in the soy sauce, whereas analogue substance of 2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone is generated by yeast fermentation during moromi aging (Hayashida *et al.* 1997; Yokotsuka *et al.* 1980). As reported by Hashiba (1981 a), larger peptides

undergo browning with reducing sugar more rapidly than small peptides and free amino acids. Soybean protein hydrolysate with low ratios of formol nitrogen/total nitrogen (34.5%) undergoes 2 X greater browning than soybean protein hydrolysate with high ratios of formol nitrogen/total nitrogen (66.0%). And in the case of free amino acids and dipeptides of glycine, glycyl glycine yields 17.6 X higher browning than free glycine. This browning is nonoxidative browning. The results in this study also reflected this phenomena.

During the moromi fermentation, along with the increases of total soluble protein (SP) in moromi liquid phase (Fig. 3a), 5% TCA-precipitated protein (PP) was gradually reduced in the liquid phase of the moromi. This led to the decrease of PP/SP ratio (Fig. 3b). The amount of protein that precipitated with 5% TCA is considered as the amount of protein with high molecular weight (Hunt *et al.* 1993). Regarding SDS-PAGE study, the range of molecular weight of the proteins from 200 kDa to 7 kDa were detected on the resolution gel (Fig. 6). There were higher molecular weight proteins also stained on the stacking gel. The electrophoretic pattern on 12% gel showed the presence of two major bands at 72.2 and 40.3 kDa prior to fermentation. The ratio of PP to SP (PP/SP) reflects the average molecular weight of proteins in liquid phase of the moromi. A high PP/SP ratio indicates a greater concentration of high molecular weight proteins. During the moromi fermentation, the decrease of PP/SP (Fig. 3b), implied that the average molecular weight of soluble proteins in the liquid phase gradually became smaller due to digestion by proteolytic enzymes in the koji (Fukushima 1989; Noda 1978). This corresponded to SDS-PAGE study that proteins were degraded during prolonged fermentation (Fig.6). The small protein bands of 21.8, 18, and 7 kDa were detected after the 3rd day of fermentation and faded out along with prolonged fermentation. These bands were not detected prior to fermentation. The bands of large proteins ranging 200 kDa to 30 kDa faded out along with fermentation time. The bands with 200, 115, 80.6, 63.3, 40.3, 21.7, 18 and 7 kDa disappeared within 15 days. At the early stage of the moromi fermentation, the small molecular weight protein and the peptides might be more reactive to yield browning because of their small size.

When the rate of browning was calculated using the derivative of browning equations, the browning rate of each moromi in the 60-day fermentation process dropped from 0.1223 to 0.0054 and 0.0988 to 0.0031 for moromi A and B, respectively. Browning rate of moromi A was 1.24 to 1.74 times higher than moromi B during the entire process. Obviously, the initial browning rate of moromi A was higher than moromi B, although the initial PP/SP ratio of moromi A was two times lower than the ratio of moromi B. Since pH drop and the reactant amounts were almost the same, then such alteration of the entire browning rates might be caused by the different processing conditions. Moromi A was fermented in 100-L earthen jars as small batch, while moromi B was fermented in 2-ton fiberglass tanks. The jars or fiberglass tanks were placed

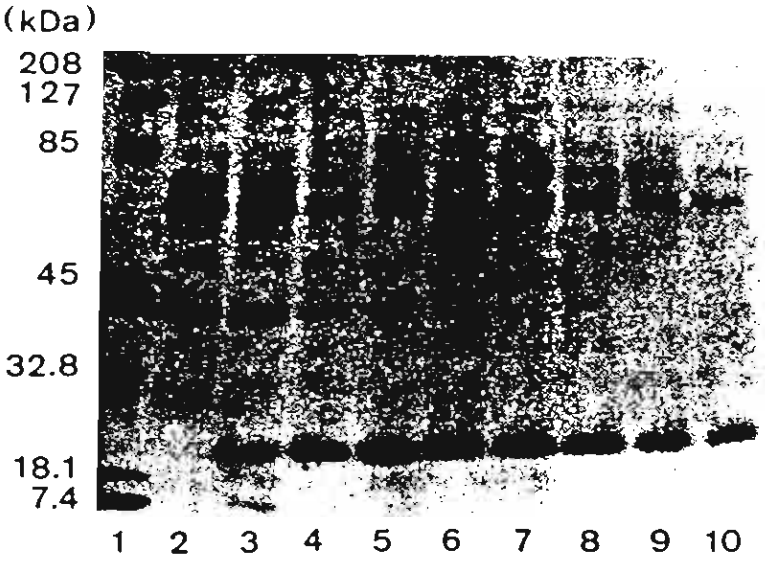


FIG. 6. SDS-PAGE PROFILES OF 5% TCA PRECIPITATED PROTEINS FROM MOROMI B

The 20-μL portion of denatured samples were loaded with 2 to 6 μg protein/lane, depending on original 5% TCA precipitated protein amount in moromi samples. Lane 2 to 10 are 0th, 3rd, 9th, 15th, 21st, 24th, 30th, 35th, 40th day samples, respectively. Lane 1 is molecular weight markers.

outside the building and exposed to the sunlight during moromi fermentation. It is possible that the average temperature of the moromi in small batches might be higher than the large batches. The temperature could bring about higher browning rates in moromi A than B. The different sizes and structures of protein or peptide molecules also conceivably affect the rates of browning. The digestion of proteolytic enzymes with different activity and specificity in both moromi might bring about various structures of the small molecular weight protein and the peptides. As reported, different dipeptide structures of leucyl glycine and glycyl leucine yields different browning rates. The former yield 1.5 X higher than the latter, and glycyl-phenylalanine yields 1.4 X higher than leucyl glycine (Hashiba 1981a). These results suggested that koji strains play an important role in browning of soy sauce, depending upon proteolytic enzyme activity and specificity. The linear regression ($r^2=0.921$ and $r^2=0.965$ for moromi A and B, respectively) between calculated browning rate and the PP/SP ratio at each point of fermentation time also confirmed the influence of large peptides on browning rates.

In Japanese soy sauce fermentation, reducing sugars in moromi normally decreases during moromi fermentation due to the conversion of sugar to alcohol by yeast (Fukushima 1989). Unlike the Japanese soy sauce, alcohol formation in Thai soy sauce is limited (Mongkolwai *et al.* 1997). Hence the reducing sugars do not obviously decrease in moromi B, probably due to the limited growth of yeast. The rate of HMF accumulated in moromi B was 1.81 X higher than in moromi A, and the overall browning of A was higher than B. This indicated that the rate of the Maillard reaction is not directly related to the entire browning rate of moromi fermentation.

The brown pigment formation in the moromi process could not be modeled as a pseudo-zero order reaction which Baisier and Labuza (1992) proposed in the Maillard reaction model system. Brown pigment formation is conceivably due to the alteration of the molecular structure of the peptides during the fermentation. The browning pigment molecules are formed from various sizes and structures of peptides with the different light absorbance. Therefore, pigment absorption at OD 420 nm did not develop in the pseudo-zero order reaction (Fig. 1), whereas HMF formation did (Fig. 4).

In the case of Japanese koikuchi soy sauce, 50% of brown color in a finished product develops during the moromi fermentation, and the remaining 50% is caused by pasteurization (Yokotsuka 1986). According to our results, 60% of browning and HMF formation were developed from the seasoning and pasteurization process of Thai soy sauce. This may be due to different raw materials, different aging duration of the moromi fermentation, and temperature of pasteurization.

In addition, it is important to study the roles of monosaccharides and dissolved oxygen in the brine during the moromi fermentation. Actually, xylose in soy sauce is much more reactive undergoing the Maillard reaction than glucose (Yokotsuka 1986; Hashiba 1981a) even though xylose is a minor monosaccharide in moromi. Dissolved oxygen also takes part in the oxidation of Maillard products, including oxidative browning in Japanese soy sauce (Hashiba 1981b).

In conclusion, the browning of the moromi fermentation of Thai soy sauce developed rapidly during the first 3 days. Powered regression equation fit the correlation of browning with the fermentation time-course. While the Maillard reaction progressed, the amount of HMF increased linearly. This indicated that the Maillard reaction rate partly affects the browning rate of moromi. The low molecular weight proteins and the large peptides underwent browning with reducing sugars at a higher rate than small peptides, yielding a greater rate of browning in the early stages of moromi fermentation.

ACKNOWLEDGMENTS

We are grateful to Thailand Research Foundation (Grant No. PDF 22/2541) and Faculty of Science, Mahidol University for providing the research funds. We thank Dr. Thongchai Suwonsichon, Department of Product Development, Faculty of Agro-industry, Kasetsart University, and Dr. Saowanee Dharmsthiti, Center of Biotechnology, Institute for Research and Development in Science and Technology, Mahidol University for their discussions.

REFERENCES

AOAC 1984. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 14th Ed. (W. Sydney, ed.) Association of Official Analytical Chemists, Washington DC.

ASHOOR, S.H. and ZENT, J.B. 1984. Maillard browning of common amino acids and sugars. J. Food Sci. *49*, 1206-1207.

BAISIER, W. and LABUZA, T.P. 1992. Kinetics of the Maillard reaction in solution. J. Agric. Food Chem. *40*, 707-713.

BELL, L.N. 1997. Maillard reaction as influenced by buffer type and concentration. Food Chem. *59*, 143-147.

BHUMIRATANA, A., FLEGEL, T.W., LOTONG, N. and SUWANARIT, P. 1988. *A Manual of Soy Sauce Production for Small Scale Manufacturers in the ASEAN Countries*. Text and Journal Corporation, Bangkok, Thailand.

BRADFORD, M.M. 1976. Protein determination by dye-binding method. Anal. Biochem. *72*, 248-254.

DAVIES, C.G.A., NETTO, F.M., GLASSENAP, N., GALLAHER, C.M., LABUZA, T.P. and GALLAHER, D.D. 1998. Indication of the Maillard reaction during storage of protein isolates. J. Agric. Food Chem. *46*, 2485-2489.

FEATHER, M.S. and NELSON, D. 1984. Maillard polymers derived from D-glucose, D-fructose, 5-(hydroxymethyl)-2-furaldehyde, and glycine and methionine. J. Agric. Food Chem. *32*, 1428-1432.

FUKUSHIMA, D. 1989. Industrialization of fermented soy sauce production centering around Japanese shoyu. In *Industrialization of Indegenous Fermented Foods*, (K.H. Steinkraus, ed.) pp. 1-88, Marcel Dekker, New York.

GOGUS, F., BOZKURT, H. and EREN, S. 1998. Nonenzymic browning reactions in multi sugar and amino acid systems. J. Food Processing Preservation *22*, 81-90.

HABEEB, A.F.S.A. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. *14*, 328-336.

HASHIBA, H. 1981a. Oxidative browning of soy sauce (Part 3): Effect of organic acids, peptides and pentoses (in Japanese). J. Japan Soy Sauce Res Inst. *7*, 19–23.

HASHIBA, H. 1981b. Oxidative browning of soy sauce (Part 4): Oxidative browning of Amadori compounds (in Japanese). J. Japan Soy Sauce Res Inst. *7*, 116–120.

HAYASHIDA, Y., NISHIMURA, K. and SLAUGHTER, J.C. 1997. The influence of mash pre-aging on the development of the flavour-active compound,4-hydroxy-2(or5)-ethyl-5(or2)-methyl-3(2H)-furanone(HEMF), during soy sauce fermentation. Intern. J. Food Sci. Technol. *32*, 11–14.

HUNT, J.V., BOTTOMS, M.A. and MITCHINSON, J. 1993. Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein-glucose adduct oxidation. Some fundamental differences in proposed mechanisms of glucose oxidation and oxidant production. Biochem. J. *291*, 529–535.

LABUZA, T.P. and BAISIER, W.M. 1992. The kinetics of nonenzymatic browning. In *Physical Chemistry of Foods*, (H.G. Schwartzberg and R.W. Hartel, eds.) pp. 595–647, Marcel Dekker, New York.

LABUZA, T.P. and MASSARO, S. 1990. Browning and amino acid loss in model total parenteral nutrition solutions. J. Food Sci. *55*, 821–826.

LAEMMLI, U.K. 1970. Cleavage of structures of proteins during assembly by head of bacteriophage-T4. Nature (London) *227*, 680–685.

LERTSIRI, S., FUJIMOTO, K. and MIYAZAWA, I. 1995. Pyrone hydroperoxide formation during the Maillard reaction and its implication in biological systems. Biochim. Biophys. Acta *1245*, 278–284.

LING, M.Y. and CHOU, C.C. 1996. Biochemical changes during the preparation of soy sauce koji with extruded and traditional raw materials. Intern. J. Food Sci. Technol. *31*, 510–517.

MONGKOLWAI, T., ASSAVANIG, A., AMNAJSONGSIRI, C., FLEGEL, T.W. and BHUMIRATANA, A. 1997. Technology transfer for small and medium soy sauce fermentation factories in Thailand: a consortium approach. Food Res. Intern. *30*, 555–563.

NELSON, N.A. 1944. Photometric adaptation of the Somogi method for the determination of glucose. J. Biol. Chem. *153*, 375–381.

NODA, F. 1978. Fermentation of soy sauce moromi mash (in Japanese). J. Japan Soy Sauce Res. Inst. *4*, 145–151.

NURSTEN, H.E. 1980-1981. Recent developments in studies of the Maillard reaction. Food Chem. *6*, 263–277.

ROSALINDA, M.M., PARK, G.L., NELSON, D.B. and McIVER, R.C. 1986. HPLC analysis of HMF in orange juice. J. Food Sci. *51*, 843–844.

WONG, D.W.S. 1989. *Mechanism and Theory in Food Chemistry*, pp. 113–120, Van Nostrand Reinhold, New York.

YAYLAYAN, V. and SPORNS, P. 1987. Novel mechanisms for the decomposition of 1-(amino acid)-1-deoxy-D-fructoses (Amadori compounds): A mass spectrometric approach. Food Chem. *26*, 283–305.

YOKOTSUKA, T. 1960. Aroma and flavour of Japanese soy sauce. Adv. Food Res. *10*, 75–134.

YOKOTSUKA, T. 1986. Soy sauce biochemistry. Adv. Food Res. *30*, 195–329.

YOKOTSUKA, T., SASAKI, M., NUNOMURA, N. and ASAO, Y. 1980. Aroma of soy sauce: Part 1 (in Japanese). J. Brew. Soc. Japan *75*, 517–522.

Submit to: Food Chemistry

Roles of the Maillard reaction in browning during storage of Thai soy sauce

Sittiwat Lertsiri*, Roungdao Muangma, Apinya Assavanig, and Amaret Bhumiratana

Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand

Running title: Browning during storage of Thai soy sauce

*Corresponding author

Dr. Sittiwat Lertsiri

Department of Biotechnology, Faculty of Science

Mahidol University, Rama VI Rd., Payathai,

Bangkok 10400, Thailand

Tel. +66-2-246-1358 ext. 2700

Fax. +66-2-246-3026

e-mail: scsls@mahidol.ac.th

ABSTRACT

The soy sauce products from conventional manufacturers V and T were tested for browning during storage, by incubation at 37°C for 12 weeks. Browning of each soy sauce increased in the manner of linear function, $y=10.64+3.09X$, $r^2=0.997$ for soy sauce T; and $y=3.66+0.116X$, $r^2=0.991$ for soy sauce V; where X and y stand for storage day and OD 420 nm, respectively. Along with browning, the Maillard reaction index, 5-hydroxymethyl-2-furaldehyde (HMF) increased in linear manner with storage time, $r^2=0.997$ and $r^2=0.696$ for soy sauce T and V, respectively. Similar increasing pattern and the strong correlation between HMF and browning revealed that the browning was dominated by the Maillard reaction. To explain the mechanism, the raw soy sauce and finished soy sauce T were examined the browning, HMF, and 5% trichloroacetic acid precipitated protein (PP) changes during storage test. The finishing process of heating increased the browning, HMF amount, and PP amount to 2.5, 2.7, and 2.25 folds, respectively. The rates of browning development and HMF accumulation in the finished soy sauce were higher at 3.4 and 1.7 times, respectively. The Maillard reaction predominantly reigned the browning of Thai soy sauce during storage.

INTRODUCTION

The most important non-enzymatic browning reaction occurring in food is known as the Maillard reaction. Its implication must be considered when food processing and storage are investigated. The overall of the reaction is complex series of reaction starting from the condensation of carbonyls *e.g.* reducing sugars, aldehydes; and amine *e.g.* amino acids, peptides, protein. This reaction is concerned as a causative factor for food stability, flavor formation, brown color development, as well as nutritional deterioration (Nursten, 1980-1981). Despite the large number of investigation in many aspects such as types of reactants, reacting conditions, and products occurred, the detail mechanism of this reaction are not completely elucidated (Bell, 1997; Labuza and Massaro, 1990; Ashoor and Zent, 1984; Feather and Nelson, 1984).

Soy sauce is one of the foods, which has been known that the Maillard reaction involved in the occurrence of browning. The Maillard reaction occurs among various kinds of important flavoring components such as amino acid, peptide, protein, reducing sugar, as well as other carbonyl compounds. This reaction is responsible for color changes and aroma development during the manufacturing process and storage (Yokotsuka, 1986). Although there is plenty of valuable research published to date covering different aspects of the Maillard reaction in Japanese soy sauce, those mechanisms are not completely matched with the mechanisms of Thai soy sauce due to the variation in ingredients and processing. Such differences are, for example, using less wheat flour without roasting for Thai soy sauce, longer koji fermentation for Thai soy sauce, climate temperature of moromi fermentation with shorter aging duration in Thailand (Lertsiri *et al.*, 2001; Mongkolwai *et al.*, 1997).

We previously investigated the browning during moromi fermentation and found that reaction rate of the Maillard reaction is constant, while the browning rate changes with the size of soluble protein in moromi (Lertsiri *et al.*, 2001). This indicated that the Maillard reaction partially plays roles in the mechanism of browning in the moromi fermentation of Thai soy sauce. In this study, the browning process and the roles of the Maillard reaction during storage of the Thai soy sauce was explored. The elucidation of the mechanism would lead to the understanding in browning control and extending the shelf life.

MATERIALS AND METHODS

Materials

Raw soy sauce and soy sauce products were obtained from the manufacturers (T and V), prepared with traditional substrate mixing with koji starter culture.

Chemicals were purchased from Merck (Darmstadt) unless otherwise stated. The 5-hydroxymethyl-2-furaldehyde (HMF) standard was obtained from Fluka (Buchs, Switzerland). All chemicals were of the best grade available, or HPLC grade.

Storage test

The soy sauce portion from each manufacturer (soy sauce T and soy sauce V) was incubated at 37°C for 12 weeks in a closed Erlenmeyer flask. Samples were withdrawn at time interval of 0, 2, 4, 6, 8, and 12 week, and kept at -20°C for further analyses.

Chemical analyses

The browning was analyzed by measuring the absorbance at 420 nm in a spectrophotometer (Davies *et al.*, 1998; Lertsiri *et al.*, 2001). Reducing sugar (RS) and reactive amino compound (RAC) were determined by the methods of Nelson (1944), and 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1966) using D-glucose and glycine as standards, respectively. Total soluble protein as total nitrogen were determined according to AOAC (1984).

Sugar analysis by HPLC was performed with Ionpak KS-801 column (300 mm x 8 mm, Shodex, Japan) equipped in Shimazu 6A HPLC set, and kept at 60°C. The distilled water was flowed at 1 ml/min as a mobile phase. The detector was refractive index (RI) detector (LDC Analytical, USA). The soy sauce samples were

deproteinized by centrifugal ultrafiltration (5,000 Da cut-off, Millipore, Japan) prior to submit onto HPLC. D-glucose, and sucrose were used as standards.

For determination of HMF, the sample was first deproteinised with 5% trichloroacetic acid (TCA). The neutralized sample was submitted onto HPLC with 5 mm x 21 mm C_{18} column (UG12, Shiseido Corp., Tokyo) at column temperature of 40 °C. The mobile phase was 5% acetonitrile in 0.2% phosphoric acid (pH 4.2), v/v; with the flow rate of 1.0 ml/min. Absorbance was measured at 280 nm. The HPLC conditions were described previously by Rosalinda *et al.* (1986).

The protein precipitated from deproteinisation was measured as 5% TCA precipitated protein by redissolving the precipitate residue in distilled water, and then determined by Bradford method (Bradford, 1976) using bovine serum albumin (Fraction V; Sigma, MO) as standard (Lertsiri *et al.*, 2001).

Data Analysis

Data were analyzed using SPSS software, San Rafael, CA. ANOVA was used to describe the significance of the effects of storage time on browning and chemical changes. Each value was an average of three separate determinations.

RESULTS AND DISCUSSION

Soy sauce T and V showed similar pattern of all chemical indices changes during prolonged storage test at 37°C for 3 months. Browning of each soy sauce increased in the manner of linear function (Fig.1), $y=10.64+3.09X$, $r^2=0.997$ for soy sauce T; and $y=3.66+0.116X$, $r^2=0.991$ for soy sauce V; where X and y stand for storage week and OD 420 nm, respectively. The equation indicated that the rate of browning in soy sauce T was higher than V for 26.6 folds. The results from total soluble protein analysis indicated that the quality of soy sauce T was superior to soy sauce V as observed in higher total protein content (6.19%) comparing to soy sauce V (4.27%). This led to the exceeding of browning in soy sauce T.

HMF, which is an intermediate of the Maillard reaction, was monitored as progressive index of the Maillard reaction (Gogus *et al.*, 1997; Yaylayan and Sporns, 1987). Along with browning, HMF increased in linear manner with storage time, $y=0.376+0.05X$, $r^2=0.997$ for soy sauce T; and $y=0.401+0.01X$, $r^2=0.920$ for soy sauce V; where X and y stand for storage week and HMF (mM), respectively (Fig.2). This suggested that the Maillard reaction in both soy sauces progressed during storage with the constant rates as observed in the browning. The reaction rate of HMF formation in soy sauce T was higher than in soy sauce V for 5 folds. Similar increasing pattern and the strong correlation between HMF and browning ($r^2=0.955$ and $r^2=0.916$ for soy sauce T and V, respectively) revealed that the browning was dominated by the Maillard reaction.

Since reactive amino compound (RAC) and reducing sugar (RS), which were the reactants of the Maillard reaction, did not obviously decrease (Fig. 3). Then the progress of the Maillard reaction during storage of soy sauce, which monitored by both browning (O.D. 420 nm) and HMF formation, could be modeled as a pseudo-

zero order reaction which Baisier and Labuza (1992) proposed in the Maillard reaction model system. This might be due to the reactants of the Maillard reaction presented in excess in the system. Fig.3

The 5% TCA-precipitated protein (PP) of each soy sauce increased during storage (Fig.4). Since the PP amounts increased concurrently with the increase of browning, and the correlation between PP and browning (r^2=0.765 and r^2=0.667, for soy sauce T and V, respectively) were observed. PP might occur from crosslinking of peptides or protein, which promoted by reducing sugar and carbonyls. Since, the appearance of the precipitated protein was brown. Then increase of PP reflected the formation of high molecular weight cross-link protein (Yaylalya and Huyghues-Despointes 1994), leading to the progress of the browning. Fig.4

In our previous study, we found that the browning during moromi fermentation correlates with fermentation time in the manner of powered regression with 2-stage consecutive progress. Browning develops rapidly during the first three days of fermentation, then the rate declines. This browning progress is accompanied by the decrease of PP in the manner of powered regression, along with the linear increase of HMF accumulation (Lertsiri *et al.*, 2001). From the present results, it seemed that the finishing process by heating the soy sauce product before bottling, conceivably plays roles in such changes. To explain the mechanism, the raw soy sauce and finished soy sauce (seasoned and cooked) from manufacturer T were examined the browning, HMF, and PP changes during storage test (Fig.5). Fig.5

After soy sauce was seasoned and cooked, the browning, HMF amount, and PP amount increased for 2.5, 2.7, and 2.25 folds, respectively, comparing to raw soy sauce (Fig.5). Among these soy sauce samples, the rates of browning development and HMF accumulation in the cooked soy sauce were higher at 3.4 and 1.7 times,

respectively, than in the raw soy sauce during storage test. While PP in the raw soy sauce was almost constant, the amount of PP in the cooked soy sauce obviously increased when being heated and such PP formation continued during storage test (Fig.5c). In case of Japanese koikuchi soy sauce, it is known that 50% of the total brown color in a finished product develops during the moromi fermentation, and the remaining of 50% is caused by cooking process (Yokotsuka, 1986). The similar results were observed in browning of Thai soy sauce as well.

In general, Thai soy sauce is seasoned with table sugar and molasses. With these ingredients, heating process would bring about the components *e.g.* carbonyls from sugar fragmentation and via the pathway of Amadori product degradation. As observed in the increase of HMF, it possibly came from 1,2-enolization pathway of the Amadori product degradation in the Maillard reaction (Wong, 1989). These carbonyl intermediates could promote the browning reaction. Concurrence of PP accumulation and browning development in cooked soy sauce were the outcome of carbonyls formed during heating process.

Fukushima (1989) reported the most of monosaccharide in soy sauce are glucose, xylose, galactose, arabinose, and mannose. Those hexoses and pentoses are converted from starch and other carbohydrate, which remain from the consumption of moulds during Koji fermentation (Sasaki and Nunomura, 1993). Our HPLC results confirmed that the reducing sugar amount of glucose in soy sauce T (405 mM) was higher than in soy sauce V (26.2 mM) for almost 15 folds. Whereas the dominant sugar in soy sauce V was sucrose (128.5 mM), comparing to 21 mM sucrose in soy sauce T. Sucrose might be added for seasoning. These data could explain that high concentration of the reducing sugar in soy sauce led to high browning rate during storage as observed in soy sauce T.

In conclusion, the browning of Thai soy sauce during storage developed in the linear correlation with storage time. Along with the browning, the Maillard reaction, which was monitored by HMF formation, progressed in the manner of pseudo-zero order reaction. The rate of reaction, as well as the rate of browning, was enhanced by the process of heating to finish the product. This indicated that the Maillard reaction predominantly reigned the browning of Thai soy sauce during storage.

ACKNOWLEDGEMENTS

We are grateful to Thailand Research Foundation (Grant No. PDF22/2541), and Faculty of Science, Mahidol University for funding this research.

REFERENCES

AOAC (1984). *Official Methods of Analysis of the Association of Official Analytical Chemists*. 14th edition. Ed W. Sydney. Association of Official Analytical Chemists, Washington DC.

Ashoor, S.H. & Zent, J.B. (1984). Maillard browning of common amino acids and sugars. *Journal of Food Science.*, **49**, 1206-1207.

Baisier, W.M. &Labuza, T.P. (1992). The kinetics of nonenzymatic browning. In *Physical chemistry of foods*. Ed. H.G. Schwartzberg, R.W. Hartel. Marcel Dekker, Inc., New York.

Bell, L.N. (1997). Maillard reaction as influenced by buffer type and concentration. *Food Chemistry*, **59**, 143-147.

Bradford, M.M. (1976). Protein determination by dye-binding method. *Analytical Biochemistry*, **72**, 248-254.

Davies, C.G.A., Netto, F.M., Glassenap, N., Gallaher, C.M., Labuza, T.P. & Gallaher, D.D. (1998). Indication of the Maillard reaction during storage of protein isolates. *Journal of Agriculture and Food Chemistry*, **46**, 2485-2489.

Feather, M.S. & Nelson, D. (1984). Maillard polymers derived from D-glucose, D-fructose, 5-(hydroxymethyl)-2-furaldehyde, and glycine and methionine. *Journal of Agriculture and Food Chemistry*, **32**, 1428-1432

Fukushima, D. (1989). Industrialization of fermented soy sauce production centering around Japanese shoyu. In *Industrialization of Indegenous Fermented Foods*. Ed. K.H. Steinkraus. Marcel Dekker Inc., New York.

Gogus, F., Bozkurt, H., & Eren, S. (1998). Nonenzymic browning reactions in multi sugar and amino acid systems. *Journal of Food Processing and Preservation*, **22**, 81-90.

Habeeb, A.F.S.A. (1966). Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Analytical Biochemistry*, **14**, 328-336.

Labuza, T.P. & Massaro, S. (1990). Browning and amino acid loss in model total parenteral nutrition solutions. *Journal of Food Science*, **55**, 821- 826.

Lertsiri, K., Maungma, R., Assavanig, A., & Bhumiratana, A (2001). Roles of the Maillard reaction in browning during moromi process of Thai soy sauce. *Journal of Food Processing Preservation*, **25**, 149-162.

Mongkolwai, T., Assavanig, A., Amnajsongsiri, C., Flegel, T.W. & Bhumiratana, A. (1997). Technology transfer for small and medium soy sauce fermentation factories in Thailand: a consortium approach. *Food Research International*, **30**, 555-563.

Nelson, N.A. (1944). Photometric adaptation of the Somogi method for the detemination of glucose. *Journal of Biological Chemistry*, **153**, 375-381.

Nursten, H.E. (1980-1981). Recent developments in studies of the Maillard reaction. *Food Chemistry*, **6**, 263-277.

Rosalinda, M.M., Park, G.L., Nelson, D.B., & McIver, R.C. (1986). HPLC analysis of HMF in orange juice. *Journal of Food Science*, **51**, 843-844.

Sasaki, M., & Nunomura, N (1993). Fermented foods: soy sauce. In *Encyclopaedia of Food Science, Food Technology and Nutrition, Vol 3.* Ed. R. Macrae, R.K. Robinson, M.J. Sadler. Academic press; UK

Wong, D.W.S. (1989). Carbohydrate. In *Mechanism and Theory in Food Chemistry*, Van Nostrand Reinhold, New York.

Yaylayan, V. & Sporns P. (1987). Novel mechanisms for the decomposition of 1-(amino acid)-1-deoxy-D-fructoses (Amadori compounds): A mass spectrometric approach. *Food Chemistry*, **26**, 283-305.

Yaylayan, V. & Huyghues-Despointes A. (1994). Chemistry of Amadori rearrangement products: analysis, synhesis, kinetics, reactions, and spectroscopic properties. *Critical Review in Food Science and Nutrition*, **34**, 321-369

Yokotsuka, T. (1986). Soy sauce biochemistry. *Advance in Food Research*, **30**, 195-329.

Legends for Figures

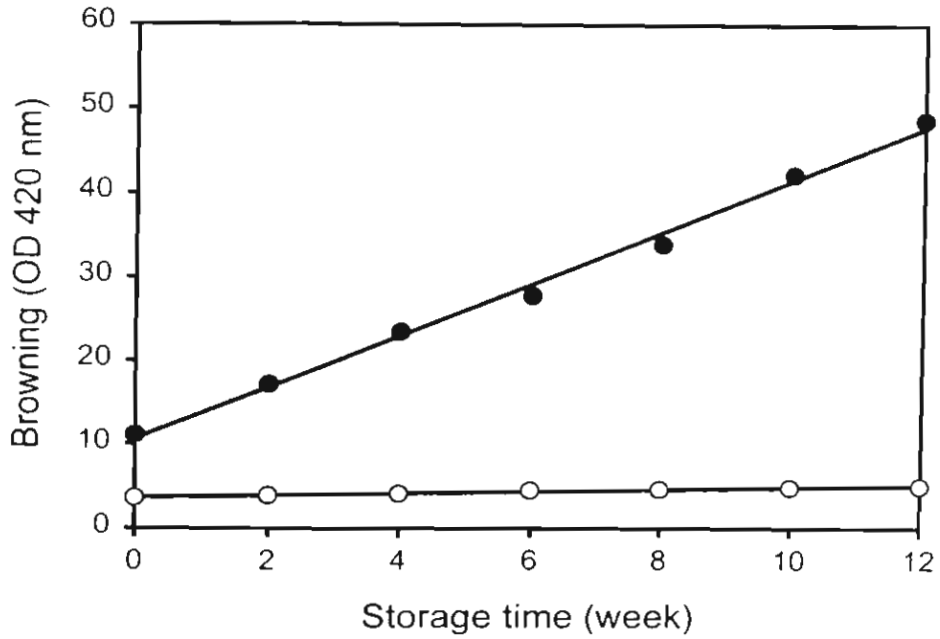
Fig.1) Browning development (OD 420 nm) during storage test of Thai soy sauce. Soy sauce T (•), $y = 10.64 + 3.09X$ ($r^2 = 0.997$, $p<0.05$); soy sauce V (o), $y = 3.66 + 0.116X$ ($r^2 = 0.991$, $p<0.05$). X and y stand for storage week and OD 420 nm, respectively.

Fig.2) 5-hydroxymethyl-2-furaldehyde (HMF) accumulation during storage test of Thai soy sauce. Soy sauce T (•), $y = 0.376 + 0.050X$ ($r^2 = 0.997$, $p<0.05$); soy sauce V (o), $y = 0.401 + 0.010X$ ($r^2 = 0.920$, $p<0.05$). X and y stand for storage week and HMF (mM), respectively.

Fig.3) Concentration changes of reactive amino compound (a), and ~~the~~ reducing sugar (b); during storage test of soy sauce T (•), and soy sauce V (o).

Fig.4) Changes of 5% trichloroacetic acid (TCA) precipitated protein amount during storage test of Thai soy sauce. Soy sauce T (•), $y = 34.48 + 1.931X$ ($r^2 = 0.973$, $p<0.05$); soy sauce V (o), $y = 6.96 + 0.471X$ ($r^2 = 0.894$, $p<0.05$). X and y stand for storage week and protein amount (mg/ml), respectively.

Fig.5) Chemical changes of finished soy sauce (o) and raw soy sauce (•) during storage test. (a) browning, $y = 1.53 + 0.371X$ ($r^2 = 0.855$) for raw soy source, $y = 9.89 + 1.804X$ ($r^2 = 0.992$) for finished soy sauce; (b) HMF, $y = 0.159 + 0.009X$ ($r^2 = 0.955$) for raw soy sauce, $y = 0.420 + 0.014X$ ($r^2 = 0.935$) for finished soy sauce; (c) 5% TCA precipitated protein, $y = 3.36 + 0.148X$ ($r^2 = 0.614$) for raw soy sauce, $y = 5.06 + 1.80X$ ($r^2 = 0.684$) for finished soy sauce.


60
50
40
30
20
10
0
Browning (OD 420 nm)
0
2
4
6
8
10
12
Storage time (week)

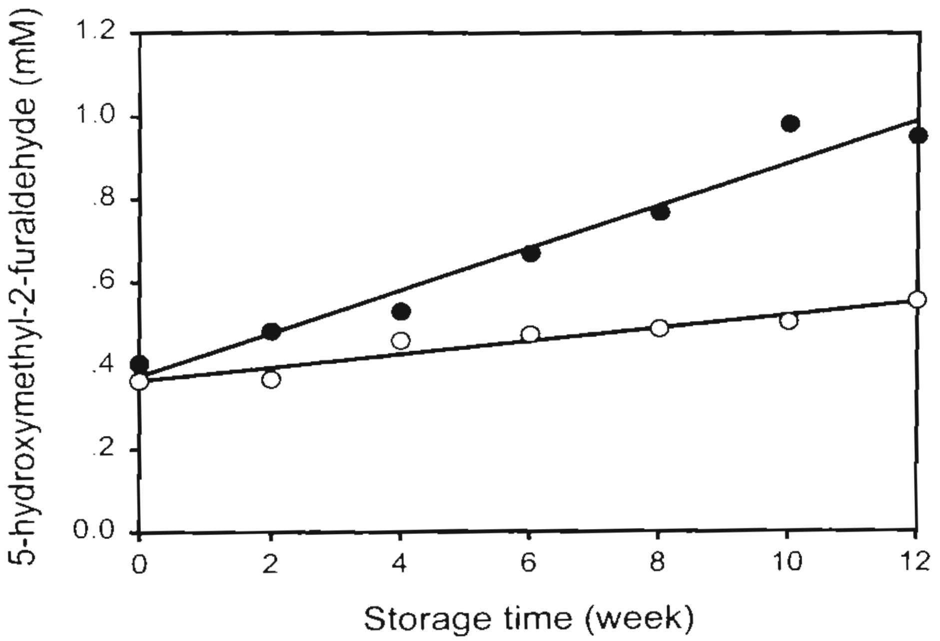
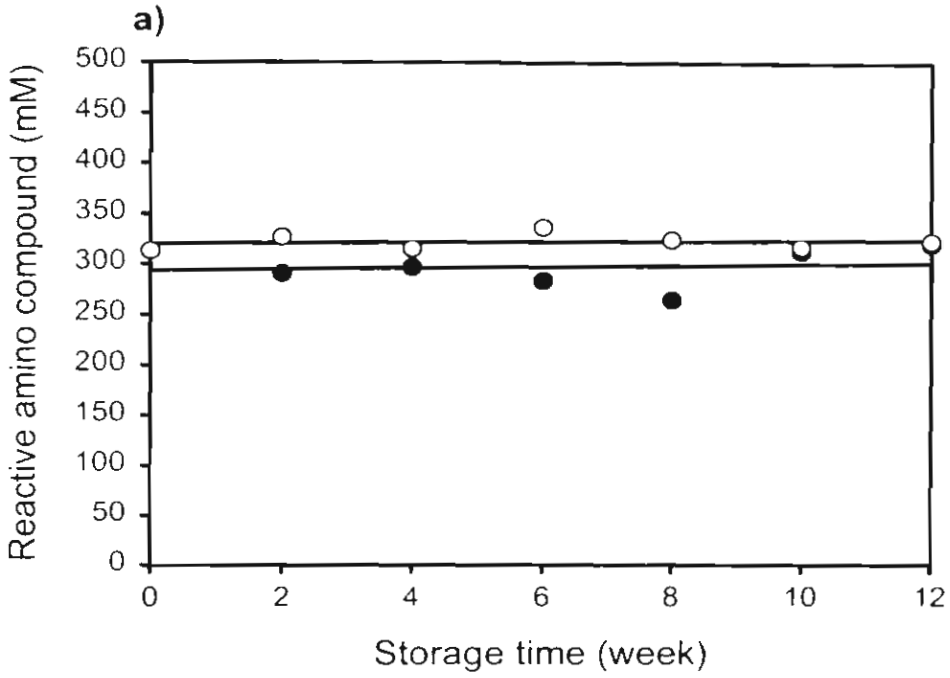


Fig. 9 Latini et al



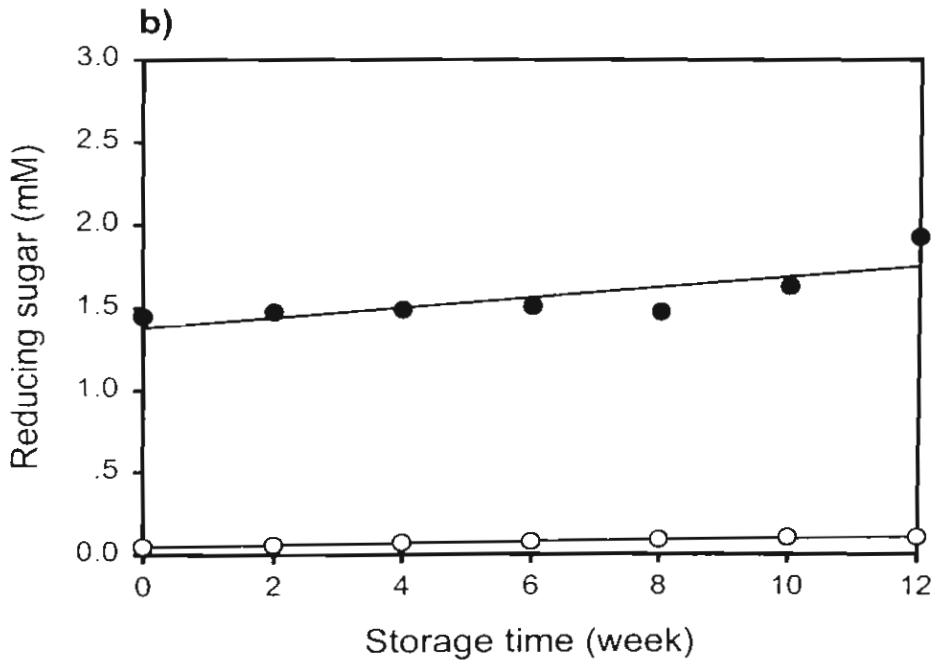


Fig. 3 Leblanc et al

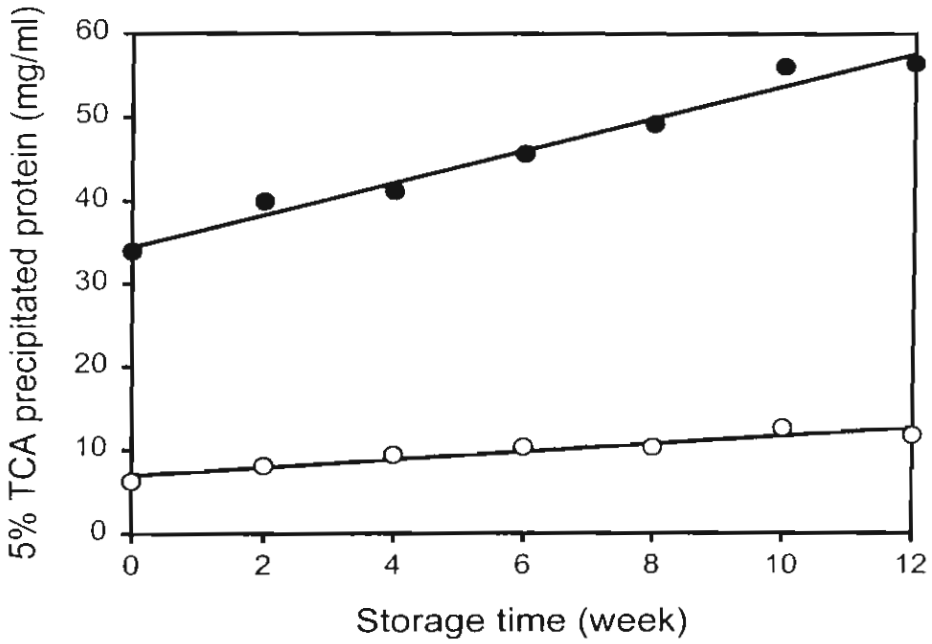
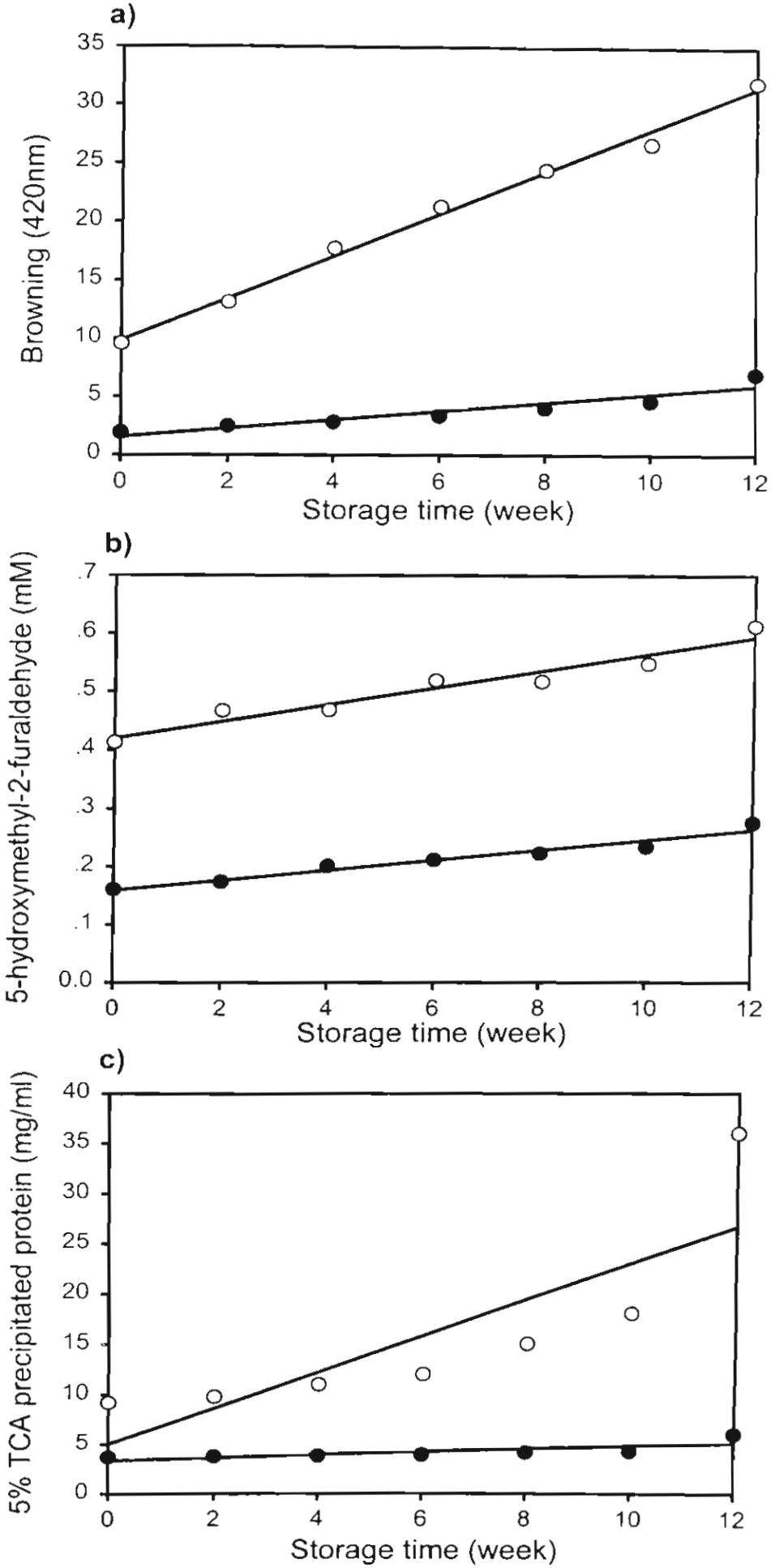

5% TCA precipitated protein (mg/ml)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Storage time (week)

Fig. 4


a)
Browning (420nm)
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
Storage time (week)
b)
5-hydroxymethyl-2-furaldehyde (mM)
.7
.6
.5
.4
.3
.2
.1
0.0
0
2
4
6
8
10
12
Storage time (week)
c)
5% TCA precipitated protein (mg/ml)
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
Storage time (week)

Submit to: Food Chemistry

Evidences of enzymatic browning due to laccase-like enzyme during mash fermentation in Thai soybean paste

Sittiwat Lertsiri*, Keittipum Phontree, Wannee Thepsingha, and Amaret Bhumiratana

Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok Thailand

Running title: Enzymatic browning in Thai soybean paste

*Corresponding author

Dr. Sittiwat Lertsiri

Department of Biotechnology, Faculty of Science

Mahidol University, Rama VI Rd., Payathai,

Bangkok 10400, Thailand

Tel. +66-2-201-5300

Fax. +66-2-246-3026

e-mail: scsls@mahidol.ac.th

ABSTRACT

Enzymatic browning reaction in food systems is due to oxidation of phenolic compounds by oxido-reductase enzymes, *e.g.* polyphenol oxidase or *ortho*-diphenol oxidase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), laccase or *para*-diphenol oxidase (EC 1.10.3.2), as well as peroxidase (EC 1.11.1.7). Since tyrosinase and laccase are prevalent among fungi, these enzymes might be responsible for enzymatic browning occurred in soy sauce and soybean paste. Diphenolic compounds, *i.e.* resorcinol, catechol, hydroquinone, guaiacol, and tyrosine, induced browning in soybean paste moromi. This browning was related to enzymatic browning, since heating at 100C for 10 min destroyed the activity due to these substrates. Moreover, enzymatic browning inhibitors, *i.e.* ascorbic acid, KI, NaCl, or Na_2SO_3, suppressed this browning. Since resorcinol, which is inhibitor of *ortho*-diphenol oxidase, also gave high browning activity, this indicated the enzyme involved might be laccase-like enzyme (*para*-diphenol oxidase) which have wide range of substrate. Moreover, such enzymatic activity was also detected in the culture of the mould used in starter culture, *i.e. A. oryzae* MUTK. This activity also showed similar substrate specificity as seen in the moromi. Supplementation of the soybean extract enhanced the enzyme activity in the cultured broth.

INTRODUCTION

Browning reaction occurring in food systems could be widely classified into non-enzymatic and enzymatic reactions. Non-enzymatic browning is resulted from oxidation, caramelization, or via the Maillard reaction. On the other hand, enzymatic browning is due to oxidation of phenolic compounds by the action of oxido-reductase enzymes, *e.g* polyphenol oxidase or *ortho*-diphenol oxidase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), laccase or *para*-diphenol oxidase (EC 1.10.3.2), as well as peroxidase (EC 1.11.1.7). Both non-enzymatic and enzymatic browning can cause destructive changes in the appearance and organoleptic attributes of food products, leading to short shelf life and lowering market value. The implication of these brownings is considered when food is processed and preserved.

Traditional fermented soybean condiments, such as soybean paste and soy sauce, are commonly consumed by people in Asian countries (Mongkolwai *et al.*, 1997). Browning in such soybean fermented products, *i.e.* soy sauce and soybean paste, plays important roles in quality attributes (Yokotsuka, 1986). Mash fermentation (moromi fermentation) and pasteurization (cooking) of raw soybean paste or raw soy sauce prior to bottling affect the browning in these products. About 50% to 60% of browning in soy sauce is developed during mash fermentation, and the remaining occurs during pasteurization (Lertsiri *et al.*, 2001; Yokotsuka, 1986). Both developments of browning are considered deriving mainly from the Maillard reaction (Yokotsuka, 1986).

Our previous study in the roles of the Maillard reaction during Thai soy sauce fermentation revealed that the rate of browning in the mash fermentation partially depends upon the Maillard reaction rate (Lertsiri *et al.*, 2001). Since polyphenol oxidase-

like enzyme, such as tyrosinase and laccase, are prevalent among fungi (Thurston, 1994), and soybean is rich in phenolic compounds (Fukutake *et al*., 1996). In this study, we hypothesized the possibility of polyphenol oxidase-like enzyme derived from the mould used in koji fermentation. This enzyme might be responsible for enzymatic browning occurred during the mash fermentation, which was concomitant to the Maillard reaction. The evidence of enzymatic browning in Thai soybean paste fermentation is first described.

MATERIALS AND METHODS

Materials

Resorcinol (*m*-dihydroxybenzene), catechol (*o*-dihydroxybenzene) and hydroquinone (*p*-dihydroxybenzene) were obtained from BDH (Poole, England). Guaiacol (1-hydroxy-2-methoxybenzene) and tyrosine were from Sigma (MO, USA). Ascorbic acid and sodium iodide were from Fluka (Switzerland). Other chemicals were of analytical grade or the best grade available supplied from Merck (Darmstadt, German). Yeast malt (YM) broth was purchased from Difco (USA). Microorganism used in the experiment, *i.e. Aspergillus oryzae* MUTK, was obtained from stock culture of Department of Biotechnology, Faculty of Science, Mahidol University.

Koji and moromi used was traditionally prepared by soybean paste manufacturer (Lertsiri *et al.*, 2001; Mongkolwai *et al.*, 1997). The substrate consisted of soybean and wheat flour at a ratio of 20:3 (w/w). Soybean was soaked in water for 4 h and cooked at 116 to 121C, 1 kg/cm^2, for 3 h. The mixture of wheat flour and soybean was inoculated with koji starter culture and kept for 36 h at 35C. The koji was then mixed with 20% NaCl brine, in the ratio of 50 kg to 50 L.

Diphenol oxidase (DPO) activity assay

DPO activity in moromi and *A. oryzae* cultured broth was determined by spectrophotometrically measuring browning at 420 nm (Ding *et al.*, 1998). The 1.5-ml portion of the filtrate of moromi or broth, was added 50 µl of various substrate solutions (0.33 mM of final concentration of resorcinol, catechol, hydroquinone, guaiacol, or tyrosine) and 50 µl distilled water, or replaced distilled water with inhibitor solutions (0.33 mM of final concentration of ascorbic acid, KI, NaCl, or Na_2SO_3). The browning

of the filtrate was determined after 2-hour incubation at 37C. The control experiment was used as the blank for measuring OD 420 nm. One unit of enzyme activity was defined as Δ OD 420 nm/hour.

Study of the substrates and inhibitors of DPO in moromi

Moromi collected on the first day of fermentation, was milled with Waring blender and filtered with membrane filter (Sartorius, 0.45 μm). The clear filtrate of the moromi was determined the activity of DPO.

Effect of NaCl on DPO activity in moromi

The fermented koji was added 0, 5, 10, 15, 20, or 25 % NaCl brine. The mixture was mixed with the Waring blender and filtered with membrane filter and assayed for the DPO activity.

DPO in A. oryzae MUTK culture

YM broth was prepared by dissolving 2.1 g of YM medium in 100 ml distilled water and supplementing with 10% v/v soybean extract (pH 6.5). Soybean extract was prepared by blending 10% cooked soybean in distilled water (w/v) in Waring blender, after that the extract was filtered and measured for phenolic compound content. The phenolic compound concentration in the soybean extract was 0.22 mg/ml. The mould was inoculated in the medium, and was incubated at 30C for 4 days without shaking. The sampling was done every day during the incubation. The filtrate of the cultured broth was determined the activity of DPO. For time-course of the browning reaction, the filtrate from 4-day cultured broth was added hydroquinone, and measured browning and phenolic compounds during prolonged incubation at 37C.

Determination of browning and phenolic compound

Browning was determined by OD 420 nm (Lertsiri *et al.*, 2001). Phenolic compound was assayed according to tannin assay method, using Folin-Ciocalteus reagent (AOAC 1984). Hydroquinone was used as a standard for calibration curve.

Data Analysis

Data were analyzed using SPSS software, San Rafael, CA. ANOVA was used to describe the significance of the enzyme activity and chemical changes. Means of three separate determinations with standard deviation are reported.

RESULTS AND DISCUSSION

When the filtrate of the moromi was added diphenolic compounds, guaiacol, or tyrosine, after 2-hour incubation, browning was observed comparing to the control treatment (Table I). The OD 420 nm of the control treatment was 0.086, measuring against distilled water. Since this browning did not occur if the filtrate was heated at 100°C for 10 min, and enzymatic browning inhibitors could suppress browning development (Table II). These results suggested that the enzymatic process involved in this browning. Diphenol oxidases, which leading to browning, are widely distributed in plants and fungi (Mayer, 1987). Such enzymes catalyze the removal of a hydrogen atom from the hydroxy group of phenolic compounds by using molecular oxygen as a terminal electron acceptor, and convert those compounds into quinones. In all cases, the quinones formed are very reactive and are precursors for brown pigment formation (Robles *et al.*, 2000; Mayer, 1987). Among phenolic compounds tested, hydroquinone, which is *p*-dihydroxy phenol, gave the highest activity. Resorcinol, which is inhibitor of o-diphenol oxidase (o-DPO) (Ferrar and Walker, 1996), also gave high activity. Considering substrate specificity of *o*-DPO and *p*-diphenol oxidase (*p*-DPO), these results indicated that the enzyme involved might be laccase-like enzyme (*p*-DPO) which have wide range of substrate. Laccase is able to oxidize methoxy-substituted monophenols, *ortho*- and *para*-diphenols, as well as aromatic amines, and a considerable range of other compounds.

When the enzymatic browning in the moromi filtrate was induced with hydroquinone, we found that all enzymatic browning inhibitors were effective, especially Na_2SO_3. Halide ions including NaCl, also exhibited inhibitory effect. This was

correspondent to the results of Ferrar and Walker (1996), those were done in the laccase enzyme from *Armillaria mellea*. Since NaCl is the main component of the brine in the moromi, the effect of NaCl concentration in moromi browning was further investigated. The moromi fermentation was simulated by immersing koji in various concentration of brine from 0 to 25% NaCl, and measured for the enzyme activity. We found that the higher percent NaCl of the brine, the browning activity decreased (Fig.1). The brine with 25% NaCl showed the lowest activity in browning. NaCl inhibited this browning, as this inhibitory effect is seen in *o*-DPO and *p*-DPO (Ferrar and Walker, 1996; Janovitz-Klapp *et al.*, 1990). These results indicated that NaCl concentration of the brine used is one of the critical factors in browning development during fermentation.

According to our knowledge, there is no report on browning due to phenol oxidase-like, laccase-like enzymes, or tyrosinase occurred during the mash fermentation of soy sauce and soybean paste. Yong and Wood (1977) reported that tyrosinase is not detected in their soybean koji fermentation, although tyrosinase is widespread among the *Aspergilli*. Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols, as well as the oxidation of the *o*-diphenols to *o*-quinones (Jimenez *et al.*, 2001). On the other hand, tyrosinase in rice koji for rice wine fermentation has been reported (Yong and Wood, 1977). This might be due to different strain of the mould used or inappropriate approach of enzyme activity assay. They conducted tyrosinase assay by incubating the koji with 3,4-dihydroxy-phenylalanine as a substrate for 5 min. Obviously, induction time for browning was much shorter than in our experiment (2 h).

Generally, soy sauce and soybean paste, are produced by similar processes (Mongkolwai *et al.*, 1997). Firstly, the koji mould (*Aspergillus oryzae* or *A. sojae*) is

grown on soybean coated with wheat flour. The koji is then immersed in brine solution for mash fermentation (moromi fermentation). The moromi of soy sauce is filtered to give a raw soy sauce, and further cooked with heating (pasteurization). In soybean paste, less amount of the brine solution is used since the solid soybean in the moromi is consumed. Unlike the soy source, the moromi of soybean paste is heated and bottling without filtration. Since koji content is high in the moromi of the soybean paste, the components including various enzymes diffused from the koji are more concentrated in soybean paste rather than in soy sauce. This might facilitate the experiment of ours to assay the activity of the DPO, which is minor enzyme. The results in this study strongly implied that the enzymatic browning occurred during soy sauce fermentation as well.

From our previous study, we found that browning (OD 420 nm) during the mash fermentation of Thai soy sauce can be expressed as powered regression with fermentation time-course (Lertsiri *et al.*, 2001). The rate was high at the first 3 days and declined in the later stage. Considering the contradiction between the rates of the Maillard reaction and browning, the Maillard reaction rate is constant through the process while the browning rate alters from time to time, decreasing along the fermentation. Thus, in conclusion, the Maillard reaction partially reigns this browning. We assume that the enzymatic browning simultaneously occurs with the Maillard reaction and plays important roles in enhancement of the browning, particularly during the first 3 days of the mash fermentation.

To verify that such enzymatic browning was originated from koji mould, culture of the mould used in starter culture, *A. oryzae* MUTK, was assayed for enzymatic browning activity. Since such enzyme activity might relate to polyphenolic compound in

soybean, soybean extract (SE) was added in YM broth to help the induction of enzyme activity. As a result, the enzymatic browning activity was detected on the 2nd day of the culture supplemented with SE (Fig.2). On the other hand, such activity was found on the 4th day of the culture without SE. Laccase-like activity expressed in cultured broth of *A. oryzae* MUTK also showed similar substrate specificity as seen in the moromi. Hydroquinone yielded highest activity while resorcinol, guaiacol, and catechol gave 47%, tyrosine gave 31% of activity due to hydroquinone.

To investigate time course of the browning reaction, the culture (4-day) supplemented with SE was added with hydroquinone and incubated for 24 hr. The mixture was withdrawn at specific intervals to measure browning and hydroquinone amount. The browning increased sharply during first 5 hours of the incubation. This was concomitant with rapid decrement of hydroquinone amount (Fig.3). Since the amount of hydroquinone correlated with the increase in browning with strong regression (r^2=0.942), the enzymatic action on browning during incubation was confirmed.

In conclusion, enzyme activity of DPO involved in browning of the Thai soybean paste. Enzyme substrate specificity indicated that DPO found should be classified as laccase-like enzyme or *p*-DPO. This *p*-DPO activity was also detected in liquid culture of starter culture strain, *A. oryzae* MUTK. Supplementation of SE could enhance this enzyme activity. The browning due to *p*-DPO was confirmed by simulation in *A. oryzae* MUTK cultured broth by using hydroquinone as substrate. The browning occurred was correlated to hydroquinone amount remaining in the system. Since NaCl showed inhibitory effect on this enzymatic browning, therefore NaCl concentration in the fermenting mixture was also one of the critical factors in browning controls.

ACKNOWLEDGEMENTS

We thank Thailand Research Foundation (Grant No. PDF22/2541), and Faculty of Science, Mahidol University for funding this research.

REFERENCES

AOAC 1984. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 14th edition. Ed W. Sydney. Association of Official Analytical Chemists, Washington DC.

Ding, C-K., Chachin, K., Ueda, Y. & Imahori, Y. (1998). Purification and properties of polyphenol oxidase from loquat fruit. *Journal of Agricultural and Food Chemistry*, **46**, 4144-4149.

Ferrar, P.H. & Walker, J.R.L. (1996). Inhibition of Diphenol Oxidases: A Comparative Study. *Journal of Food Biochemistry*, **20**, 15-30.

Furutake, M., Takahashi, M., Ishida, K. & Kawamura, H. (1996). Quantification of genistein and genistin in soybeans and soy bean produts. *Food Chemistry and Toxicology*, **34**, 457-461.

Janovitz-Klapp, A.H., Richard, F.C., Goupy, P.M. & Nicolas, J.J. (1990). Inhibition studies on apple polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, **38**, 962-931.

Jimenez, M., Chazarra, S., Escribano, J., Cabanes, J., and Garcia-Carmona, F. (2001). Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. *Journal of Agricultural and Food Chemistry*, **49**, 4060-4063

Lertsiri, S., Maungma, R., Assavanig, A. & Bhumiratana, A. (2001). Roles of the Maillard reaction in browning during mormi process of Thai soy sauce. *Journal of Food Processing and Preservation*, **25**, 149-162.

Mayer, A.M. (1987) Polyphenol oxidase in plants. *Phytochemistry*, **26**, 11-20.

Mongkolwai, T., Assavanig, A., Amnajsongsiri, C., Flegel, T.W. & Bhumiratana, A. (1997). Technology transfer for small and medium soy sauce fermentation factories in Thailand: a consortium approach. *Food Research International*, **30**, 555-563.

Robles, A., Lucas, R., Alvarez de Cienfuegos, G. & G'Ivez, A. (2000). Phenol-oxidase (laccase) activity in strains of the hyphomycete *Chalara paradoxa* isolated from olive mill wastewater disposal ponds. *Enzyme and Microbial Technology*, **26**, 484-490.

Thurston, C.F. (1994) The structure and function of fungal laccases. *Microbiology*, 140, 19-26.

Yokotsuka, T. (1986) Soy sauce biochemistry. *Advance in Food Research*, **30**, 195-329.

Yong, F.M. & Wood, B.J.B. (1977) Biochemical changes in experimental soy sauce Koji. *Journal of Food Technology*, **12**, 163-175.

Table I Enzymatic browning activity of soybean paste moromi by different substrates.

Substrate	Enzyme activity (U)
Catechol	0.26±0.04
Resorcinol	0.32±0.05
Hydroquinone	0.41±0.02
Guaiacol	0.21±0.04
Tyrosine	0.31±0.01

Enzymatic browning activity was assayed from the moromi filtrate of soybean paste. Filtrate pH was 6.5. Values are means ± SD.

Table II Inhibitory effect of various compounds on enzymatic browning activity in soybean paste moromi.

Inhibitor	Inhibitory effect (%)
Ascorbic acid	80.3
KI	25.5
NaCl	38.4
Na_2SO_3	90.6

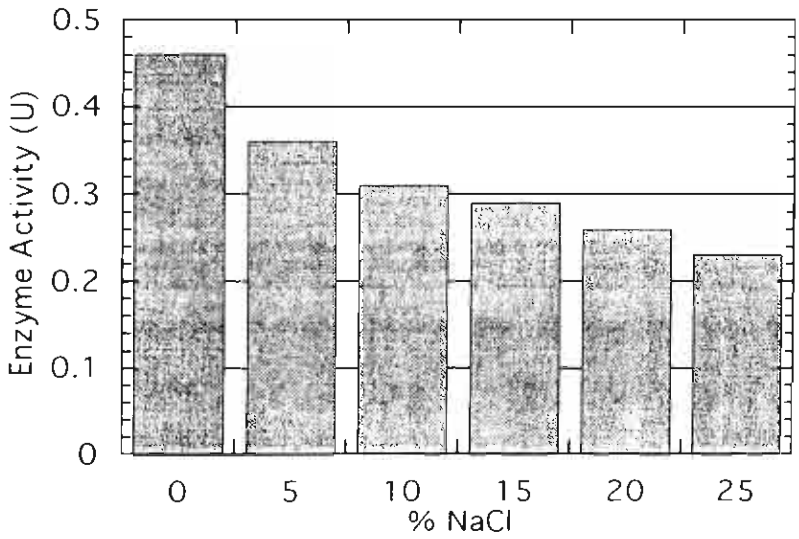
Inhibitory effect was evaluated in soybean paste moromi (pH 6.5). The enzymatic browning was induced by hydroquinone as a substrate. Inhibitory effect was compared with the control experiment. Values are means ± SD.

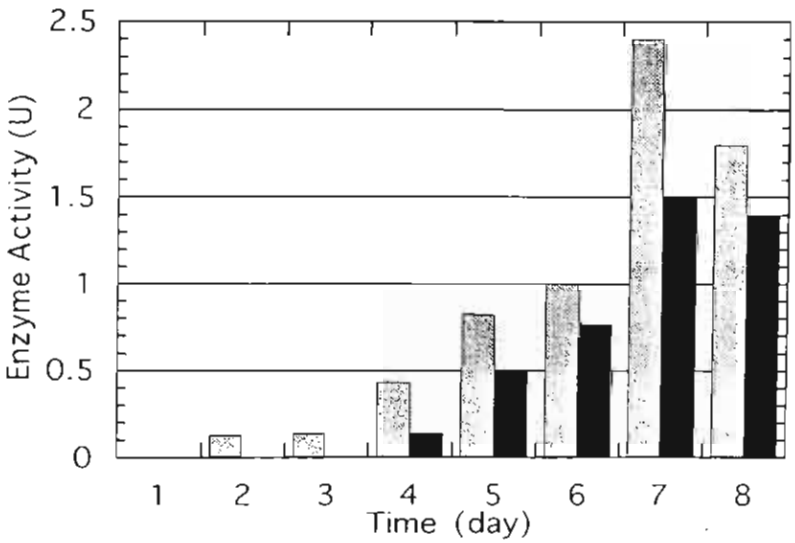
Legends for Figures

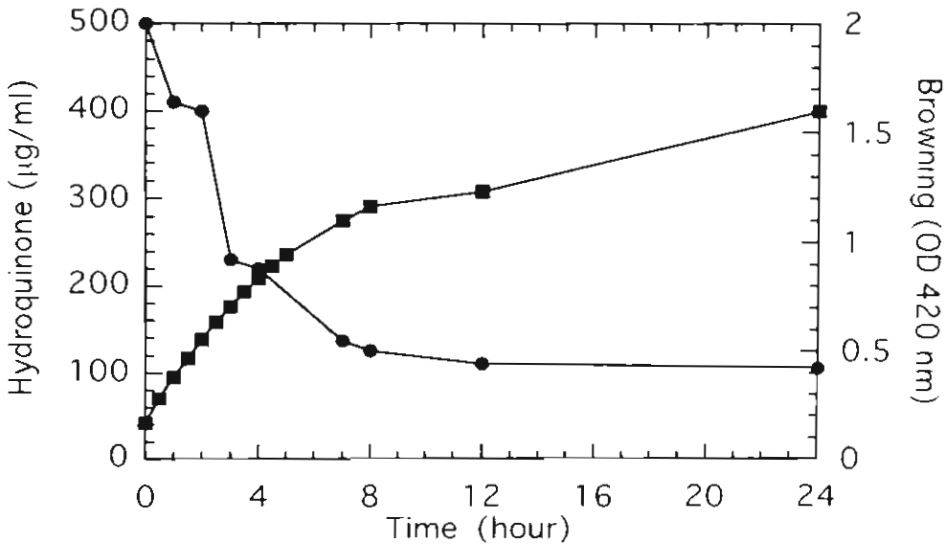
Fig.1) Effect of NaCl concentration on enzymatic browning activity in soybean paste moromi. Filtrate of the moromi was induced browning by hydroquinone as substrate. Moromi pH was 6.5.

Fig.2) Effect of soybean extract on laccase-like enzyme activity in cultured broth of *A oryzae* MUTK. The mould was inoculated in YM broth with / or withhout soybean extract supplementation. The cultured broth was withdrawn, filtered, and measured laccase-like enzyme activity. The gray bar and the black bar present the data of cultivation with and without soybean extract, respectively.

Fig.3) Time-course for browning development (OD 420 nm; ■---■) and the change of phenolic compound (μg/ml hydroquinone equivalence; ●---●) during the incubation of *A oryzae* MUTK cultured broth with hydroquinone. The aliquot of the broth was withdrawn at specific time interval for analyses.


0.5
0.4
0.3
0.2
0.1
0
Enzyme Activity (U)
0
5
10
15
20
25
% NaCl


2.5
2
1.5
1
0.5
0
Enzyme Activity (U)
1
2
3
4
5
6
7
8
Time (day)


Hydroquinone (μg/ml)
500
400
300
200
100
0
Browning (OD 420 nm)
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hour)