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

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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 occurring 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 100 °C 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 an inhibitor of *ortho*-diphenol oxidase, also gave high browning activity, this indicated that the enzyme involved might be a laccase-like enzyme (*para*-diphenol oxidase) with a wide range of substrates. Moreover, such enzymatic activity was also detected in the culture of the mould used in the starter culture, i.e. *Aspergillus 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.

Keywords: Enzymatic browning; Soybean paste; Laccase; Diphenol oxidase

1. Introduction

Browning reactions occurring in food systems may be broadly classified into non-enzymatic and enzymatic reactions. Non-enzymatic browning results from oxidation, caramelisation, or the Maillard reaction. Enzymatic browning is due to oxidation of phenolic compounds by the action of oxido-reductase enzymes, e.g. polyphenol oxidase or *ortho*-dihpenol 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 lower market value. Browning is important 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, Assavanig, Amnajsongsiri, Flegel, & Bhumiratana, 1997). Browning in such soybean fermented products, i.e. soy sauce and soybean paste, affects 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 50–60% of browning in soy sauce is developed during mash fermentation, and the remaining occurs during pasteurization (Lertsiri, Maungma, Assavanig, & Bhumiratana, 2001; Yokotsuka, 1986). Both developments of browning are considered to stem from the Maillard reaction (Yokotsuka, 1986).

Our previous study of the Maillard reaction during Thai soy sauce fermentation revealed that the rate of browning of the mash fermentation partially depends upon the Maillard reaction rate (Lertsiri et al., 2001). Polyphenol oxidase-like enzymes, such as tyrosinase and laccase, are prevalent among fungi (Thurston, 1994), and soybean is rich in phenolic compounds

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(Fukutake, Takahashi, Ishida, & Kawamura, 1996). In this study, we investigated polyphenol oxidase-like or laccase-like enzymes in koji fermentation, derived from the mould used. Enzymes might be responsible for enzymatic browning occurring during the mash fermentation, concomitant with the Maillard reaction.

2. Materials and methods

2.1. 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 potassium iodide were from Fluka (Switzerland). Other chemicals were of analytical grade or the best grade available supplied from Merck (Darmstadt, Germany). Yeast malt broth was purchased from Difco (USA). Microorganism used in the experiment, i.e. *Aspergillus oryzae* MUTK, was obtained from stock culture of the Department of Biotechnology, Faculty of Science, Mahidol University.

Koji and moromi used were traditionally prepared by a 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 121 °C, 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 35 °C. The koji was then mixed with 20% NaCl brine, in the ratio of 50 kg to 50 l.

2.2. Diphenol oxidase (DPO) activity assay

DPO activity in moromi and *A. oryzae* cultured broth was determined by spectrophotometrically measuring browning at 420 nm (Ding, Chachin, Ueda, & Imahori, 1998). A 1.5-ml portion of the filtrate of moromi or broth, was treated with 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 the distilled water replaced 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 a 2-h incubation at 37 °C. The control experiment was used as the blank for measuring OD at 420 nm. One unit of enzyme activity was defined as ΔOD 420 nm/h.

2.3. Study of the substrates and inhibitors of DPO in moromi

Moromi collected on the first day of fermentation, was milled with a Waring blender and passed through a membrane filter (Sartorius, 0.45 μm). The clear filtrate of the moromi was used to determine the activity of DPO.

2.4. Effect of NaCl on DPO activity in moromi

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

2.5. 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 a 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 into the medium, and was incubated at 30 °C for 4 days without shaking. The sampling was done every day during the incubation. The filtrate of the cultured broth was used to determine the activity of DPO. For time-course of the reaction, 4-day cultured broth was treated with hydroquinone, and browning of phenolic compounds measured during prolonged incubation at 37 °C.

2.6. Determination of browning, and phenolic compound

Browning was determined by OD at 420 nm (Lertsiri et al., 2001). Phenolic compound was assayed by the tannin assay method, using Folin-Ciocalteau's reagent (AOAC, 1984). Hydroquinone was used as a standard for the calibration curve.

2.7. Data analysis

Data were analysed 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.

3. Results and discussion

When the filtrate of the moromi was treated with diphenolic compounds, guaiacol, or tyrosine, after 2-h incubation, browning was observed compared to the control treatment (Table 1). The OD at 420 nm of the control treatment was 0.086, measured 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 2), these results suggested that an enzymatic process was involved in the browning. Diphenol oxidases, which lead to browning, are widely distributed in plants and fungi (Mayer, 1987). Such enzymes catalyse 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 (Mayer, 1987; Robles, Lucas, de Cienfuegos, & G'Ivez, 2000). Among phenolic compounds tested, hydroquinone, which is *p*-dihydroxy phenol, gave the highest activity. Resorcinol, which is an inhibitor for *o*-diphenol oxidase (*o*-DPO) (Ferrar & Walker, 1996), also gave high activity. Considering the substrate specificity of *o*-DPO and *p*-diphenol oxidase (*p*-DPO), these results indicated that the enzyme involved might be a laccase-like enzyme (*p*-DPO) which have wide ranges of substrates. Laccase is able to oxidise methoxy-substituted monophenols, *ortho*- and *para*-diphenols, as well as aromatic amines, and many 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, as well as NaCl, also exhibited inhibitory effects. This corresponded to the results of Ferrar and Walker (1996). Their study was 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 was simulated by immersing koji in various concentrations of brine from 0 to 25% NaCl, and measuring enzyme activity. We found that the higher the percent of NaCl in the brine, the more browning activity decreased (Fig. 1). The brine with 25% NaCl showed the least browning. NaCl inhibited this browning, as seen with *o*-DPO and *p*-DPO (Ferrar & Walker, 1996; Janovitz-Klapp, Richard, Goupy, & Nicolas, 1990). This was important to the soy sauce and soybean paste fermentation since the moromi fermentation is conducted in brine solution. This finding suggested that high salt concentration would lead to less browning during the process. NaCl concentration of the brine used is a critical factor in browning development during the fermentation.

Although tyrosinase is widespread among the *Aspergilli*, Yong and Wood (1977) reported that tyrosinase was not detected in their soybean koji fermentation. Tyrosinase catalyses the hydroxylation of monophenols to *o*-diphenols, as well as the oxidation of *o*-diphenols to *o*-quinones (Jimenez, Chazarra, Escribano, Cabanes, & Garcia-Carmona, 2001). On the other hand, tyrosinase in rice koji, for rice wine fermentation, has been reported (Yong & Wood, 1977). This might be due to a different strain of the mould used or an inappropriate approach to enzyme activity assay. Yong and Wood (1977) conducted tyrosinase assay by incubating the koji with 3,4-dihydroxy-phenylalanine as a substrate in the assay, for 5 min. Obviously, induction time for browning was much shorter than in our experiment (e.g. 2 h).

Generally, soy sauce and soybean paste, are produced by similar processes (Mongkolwai et al., 1997). First, the koji mould (*A. oryzae* or *A. sojae*) is grown on soybean coated with wheat flour. The koji is then

Table 1
Enzymatic browning activity of soybean paste moromi by different substrate

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
H_2O_2	nd[a]
Hydroquinone/H_2O_2	0.39±0.04

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

[a] Not detectable.

Table 2
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.

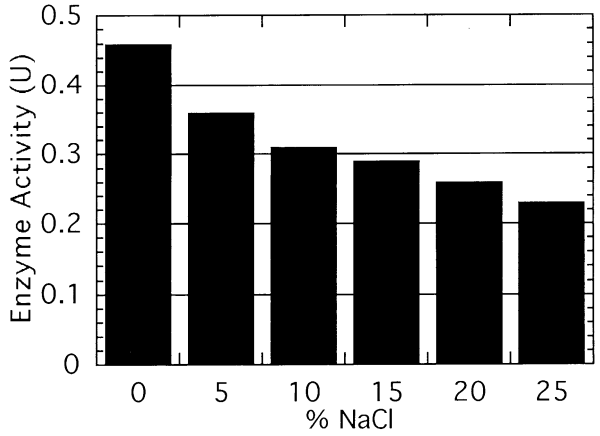

Fig. 1. Effect of NaCl concentration on enzymatic browning activity in soybean paste moromi. Filtrate of the moromi induced browning by hydroquinone as substrate. Moromi pH was 6.5.

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 of the brine solution is used, since the solid soybean in the moromi is consumed. Unlike the soy sauce, the moromi of soybean paste is heated and bottled 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 than in soy sauce. This might facilitate our assay of the activity of the DPO, which is a minor enzyme in the mash fermentation. The results in this study strongly implied that the enzymatic browning also occurred during soy sauce fermentation.

From our previous study, we found that browning (OD 420 nm) during the mash fermentation of Thai soy sauce can be expressed as a powered regression with fermentation time-course (Lertsiri et al., 2001). The rate was high over the first 3 days and declined at the later stages. Considering the contradiction between the rates of the Maillard reaction and browning, the Maillard reaction rate is constant from the beginning to the end, throughout the process, while the browning rate alters from time to time, decreasing during the fermentation. Thus, in conclusion, the Maillard reaction partially explains this browning. We assume that the enzymatic browning and the Maillard reaction occur simultaneously. The enzyme might play an important role in enhancement of the browning, particularly during the first 3 days of the mash fermentation.

To verify that such enzymatic browning originated from the koji mould, a culture of the mould used in a starter culture, *A. oryzae* MUTK, was assayed for enzymatic browning activity. Since such enzyme activity might relate to polyphenolic compounds in soybean, soybean extract (SE) was added to YM broth to promote the induction of enzyme activity. As a result, the enzymatic browning activity was detected on the second day of fermentation in the culture supplemented with SE (Fig. 2). On the other hand, such activity was found on the fourth day in the culture without SE. Enzyme activity, in both cases, reached maxima on the seventh day of the cultivation. Laccase-like activity, expressed in cultured broth of *A. oryzae* MUTK, also showed similar substrate specificity, as seen in the moromi. Hydroquinone yielded the highest activity while resorcinol, guaiacol, and catechol gave 47%, and tyrosine gave 31% of activity due to hydroquinone. Hydrogen peroxide did not enhance the browning in the assay system.

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

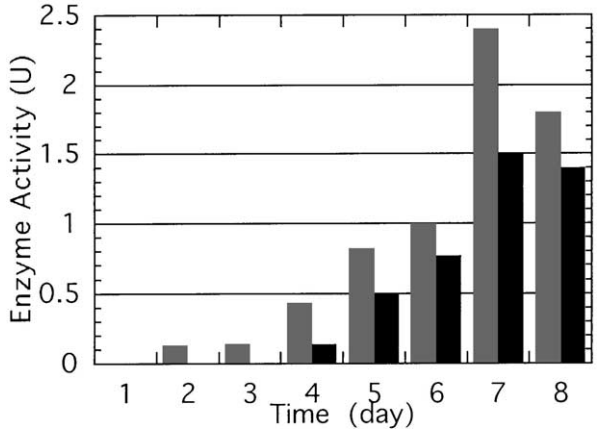

Fig. 2. Effect of soybean extract on laccase-like enzyme activity in cultured broth of *Aspergillus oryzae* MUTK. The mould was inoculated into YM broth with/without soybean extract supplementation. The cultured broth was withdrawn, filtered, and laccase-like enzyme activity measured. The gray bar and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

Generally, to measure the enzyme activity of DPO, the procedures of spectrophotometry (Das, Bhat, & Gowda, 1997; Duangmal & Apenten, 1999; Ferrar & Walker, 1996) and polarography (Ferrar & Walker, 1996; Weemaes, Rubens, De Cordt, Ludikhuyze, Van der Brock, Hendrickx et al., 1997) have been employed. In this study, the aim was to elucidate the role of DPO in browning which occurs in soybean paste production. Hence, the enzyme activity assay based on browning (OD 420nm) measurement was preferable. To avoid possible self-polymerization of the substrate itself, including polymerization of autoxidation products (quinone) corresponding to the substrate, a low concentration of the

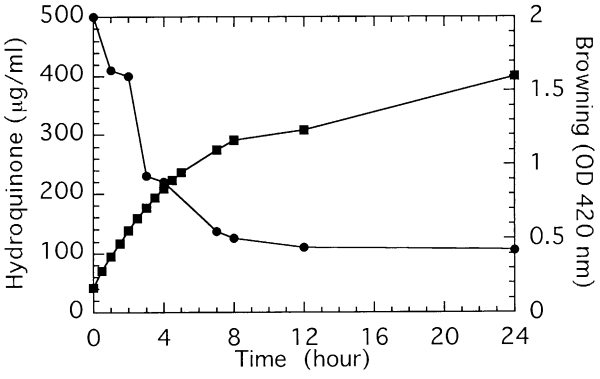

Fig. 3. Time-course for browning development (OD 420 nm; ■—■) and the change of phenolic compound (μg/ml hydroquinone equivalents; ●—●) during the incubation of *Aspergillus oryzae* MUTK cultured broth with hydroquinone. Aliquots of the broth were withdrawn at specific time intervals for analyses.

substrate (0.33 mM) was employed in this study. In the enzyme activity assay, the accumulation of the pigment formed should be sufficient for accurate measurement at 420 nm; consequently the incubation time of 2-h duration was necessary. The effect of temperature on the browning due to DPO was preliminarily studied and it was found that 37 °C gave the highest OD at 420 nm among other temperatures tested, e.g. 4 °C, 30 °C, and 45 °C. Moreover, shaking of the assay system enhanced browning up to three folds more than the system without shaking.

Within a 3-h period, the browning due to *p*-DPO increased in linear manner as seen in Figs. 3 and 4. Therefore, enzyme activity during a 2-h reaction was calculated from the slope of initial linear portion of the increasing absorbance. With the conditions employed, the browning rate depended on substrate concentration (Fig. 4). During the assay, the incubation at 37 °C for 2 h, without the substrate, did not cause significant changes in apparent colour or increase in OD at 420 nm of the reaction mixture.

On the other hand, peroxidase (EC 1.11.1.7) may also contribute to enzymatic browning. The enzyme primarily oxidizes hydrogen donors, including polyphenol, at the expense of peroxide. This enzyme is highly specific for hydrogen peroxide (Forget & Gauillard, 1997). From the results (Table 1), when hydrogen peroxide was added to the assay system concomitantly with hydroquinone, or hydroquinone omitted, there was no further promotion of the browning in the former, or such browning occurred in the latter case. If the assay system could detect the browning activity due to peroxidase associated with DPO, then the browning should be enhanced by hydrogen peroxide addition (Forget & Gauillard, 1997). Consequently, the possibility of peroxidase presence in the culture of *A. oryzae* was low in this experiment. Furthermore, there is no report on genes coding for peroxidase originating from *A. oryzae* (Gomi, 2000).

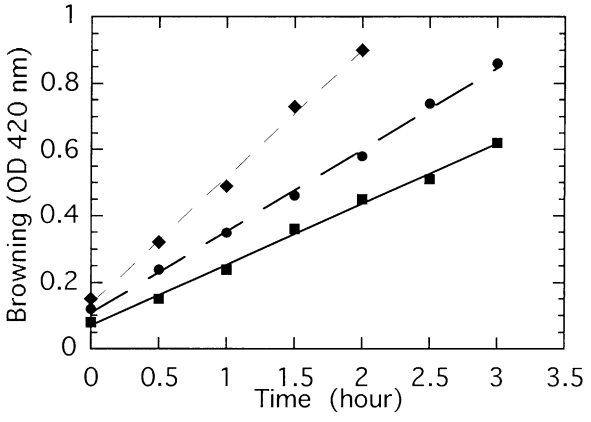

Fig. 4. Time-course for browning development (OD 420 nm) during the incubation of *Aspergillus oryzae* MUTK cultured broth with various concentrations of hydroquinone (0.33 mM, ■—■; 0.66 mM, ●—● ; 0.99 mM, ▲—▲). Aliquots of the broth were withdrawn at specific time intervals for OD measurement.

In conclusion, enzyme activity (DPO) is involved in browning of Thai soybean paste. Enzyme substrate specificity indicated that DPO found should be classified as a 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 occurring was correlated to residual hydroquinone in the system. Since NaCl inhibited this enzymatic browning, NaCl concentration in the fermenting mixture was also a critical controlling factor.

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References

AOAC. In W. Sydney (Ed.), *Official methods of analysis of the Association of Official Analytical Chemists* (14th ed.). Washington DC: Association of Official Analytical Chemists.

Das, J. R., Bhat, S. G., & Gowda, L. R. (1997). Purification and characterization of a polyphenol oxidase from the kew cultivar of Indian pineapple fruit. *Journal of Agricultural and Food Chemistry*, *45*, 2031–2035.

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.

Duangmal, K., & Apenten, R. K. O. (1999). A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Salanum tuberosum* var. Romano). *Food Chemistry*, *64*, 351–359.

Ferrar, P. H., & Walker, J. R. L. (1996). Inhibition of diphenol oxidases: a comparative study. *Journal of Food Biochemistry*, *20*, 15–30.

Forget, F. C. R., & Gauillard, A. (1997). Oxidation of Chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of Pear (*Pyrus communis* v. Williams) polyphenol oxidase and peroxidase: A possible invlovement of peroxidase in enzymatic browning. *Journal of Agricultural and Food Chemistry*, *45*, 2472–2476.

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.

Gomi, K. (2000). *Aspergillus oryzae*. In J. Loderberg (Ed.), *Encyclopedia of microbiology* (2nd ed.) (pp. 66–72). San Diego: Academic Press.

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*, 931–962.

Jimenez, M., Chazarra, S., Escribano, J., Cabanes, J., & 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.

Weemaes, C., Rubens, P., de Cordt, S., Ludikhuyze, L., Van der Broeck, I., Hendrickx, M., Hermans, K., & Tabback, P. (1997). Temperature sensitivity and pressure resistance of mushroom polyphenoloxidase. *Journal of Food Science*, *62*(2), 261–266.

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.