Antioxidative Effect of Maillard Reaction Products Formed from Honey at Different Reaction Times†

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Maillard reaction products (MRP) were synthesized from honey-lysine by refluxing with water for 4, 8, 12, 16, and 20 h. The MRP from each reaction time were added to and reacted with a linoleic acid buffered emulsion at 37 °C. The MRP from each of the five reaction times were added to the linoleic acid emulsion (LAE) at 1%, 5%, 10%, 15%, and 20% (v/v). The antioxidative effect of MRP on the LAE was determined spectrophotometrically at 234 nm. The MRP pH and brown pigment formation (450 nm) was measured. Absorbance at 450 nm increased from 0.6 to 1.6 between the 4 h and 20 h MRP treatments, respectively. The pH of the MRP decreased from 4 h to 20 h reaction solutions, ranging from 4.2 to 3.65. The antioxidative effect increased at each reaction time increment. Within each reaction time, the antioxidative effect was maximized between 10% and 15% addition levels.

Keywords: *Maillard reaction products; antioxidants; linoleic acid; honey*

INTRODUCTION

The Maillard reaction plays an important part in the formation of volatile flavorants from food precursors. In meat, amino acids and carbohydrates interact to form intermediates that are converted to meat flavor compounds by oxidation, decarboxylation, condensation, and cyclization. The O, N, and S heterocyclics, including furans, furanones, pyrazines, thiophenes, thiazoles, thiazoline, and cyclic polysulfides, contribute to the desirable flavor of meat (Bailey, 1983). Cooked meat aroma results from the total contribution of a large number of these heteroatomic compounds. During the first stages of cooking, thermally induced oxidation of lipids produces the desirable flavor of meat. Lipid-derived carbonyl products interact with amino groups, the amino group of phosphatidylethanolamine interacts with sugar-derived carbon, and free radicals from peroxidized lipids interact with Maillard reaction compounds to produce a range of desirable volatiles (Kanner, 1994). Different compounds have been isolated during different stages of the reaction. The specific aldehydes produced during the Maillard reaction are controlled mainly by the specific amino acids used in the reaction, whereas the amount of specific aldehyde produced is determined mostly by the type of sugar used in the reaction (Danehy, 1983).

The direct addition of honey to turkey breast meat prior to heating had an antioxidative effect on the meat, which was attributed to Maillard reaction products (MRP) formed during heating (Antony et al., 2000a). The addition of MRP formed by heating honey-lysine mixtures also had an antioxidative effect in a linoleic acid model system and in a turkey meat model system (Antony et al., 2000b). Reaction conditions including pH, reaction temperature and time, molar ratio of reactants and concentrations, water activity, and reaction medium greatly affect the induction of antioxidant activity (Namiki, 1988). Antioxidants are formed at several stages during the Maillard reaction, including degradation of Amadori compounds to amino reductones, or reductones, and the formation of polymers with antioxidant activity (Bailey, 1992). Fractionation procedures showed several different molecular weight fractions are necessary to have an antioxidative effect (Hofman, 1998). These studies indicated that several antioxidative products are formed by the Maillard reaction. These products differ in molecular size and chemical structure with a common single antioxidative functional group, though the presence of entirely different antioxidants with different modes of action cannot be excluded and has been reported (Lingnert et al., 1983). Liquid chromatography/mass spectrometric analysis has been used to study the antioxidative effect of separate fractions from the lactose/lysine Maillard reaction (Monti et al., 1999).

Since multiple antioxidant compounds are formed during different stages of the Maillard reaction, the objective of this study was to determine the antioxidative effect of honey/lysine MRP at different reaction times and added at different levels. The antioxidative effect was measured by use of the linoleic acid model system.

MATERIALS AND METHODS

Synthesis of Maillard Reaction Products. The MRP were synthesized by the method of Bedinghaus et al. (1995) by refluxing 0.2 M lysine monohydrochloride (Sigma Chemical Co., St Louis, MO) and 0.3 M dry honey (Groeb Farms, Onstead, MI) (expressed as percent reducing sugar [glucose]) in 100 mL of distilled water for 4, 8, 12, 16, and 20 h in a 250 mL Pyrex flask coupled to a reflux condenser unit. The percent reducing sugar was determined by refractometry, and the

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amount of reducing sugar was converted to moles of glucose. The MRP mixtures were stored overnight at 4 °C. After the reaction, the absorbance and pH of each heating time were measured at 450 nm with a Perkin-Elmer spectrophotometer (Perkin-Elmer, Norwalk, CT; model Lambda 3B UV/vis) and a pH meter (Orion, Boston, MA; model 420A), respectively.

Linoleic Acid Model System. The model system was prepared [modified from Decker et al., (1986) and Wills (1965)] by emulsifying linoleic acid with Tween 20 in 0.1 M potassium phosphate buffer, pH 6.2. The buffer was made from a 0.2 M stock solution of 81.5% monobasic sodium phosphate and 18.5% dibasic sodium phosphate, diluted to 200 mL. Linoleic acid (Sigma, 99% pure) was dissolved in 95% ethanol (70.1 mg of linoleate/ml of ethanol) and stored in 2 mL glass vials at −20 °C prior to use. Two milliliters of the linoleic acid was diluted to 5 mL with additional 95% ethanol. This was added to 45 mL of 0.1 M potassium phosphate buffer, pH 6.2, to form a 10 mM linoleate emulsion. Tween 20 (J. T. Baker, Phillipsburg, NJ) at 1% was added to obtain a stable emulsion.

Measurement of Antioxidative Effect. Synthesized MRP from each reaction time were added at 1%, 5%, 10%, 15%, and 20% (v/v) to 2 mL of the linoleic acid emulsion in test tubes by a modification of the general procedure of Lingnert et al. (1979). The tubes were placed in darkness to exclude light effects and at 37 °C to accelerate oxidation. Then 0.2 mL of the substrate−antioxidant mixture was withdrawn, diluted to 5 mL with 95% ethanol and 0.1 M phosphate buffer mixture, and centrifuged for 3 min, and the spectrophotometric absorption of the supernate conjugated diene at 234 nm was measured. Absorption was measured prior to incubation and after incubation for 15 h. The antioxidative effect (AE) was calculated according to

$$\mathrm{AE} = \frac{\Delta A_{234}(\mathrm{C}) - \Delta A_{234}}{\Delta A_{234}(\mathrm{C})}$$

where ΔA_{234} = increase of absorption at 234 nm during the incubation time (15 h) and ΔA_{234} (C) = corresponding increase in control (no MRP added). The absorbance was measured to 20 h to verify that the concentration of the dienes had not passed through the maximum since the hydroperoxides are intermediates in the lipid oxidation sequence.

Headspace GC−Mass Spectrometry. The volatiles from the MRP prepared at different reaction times were identified by use of a Hewlett-Packard (HP; Wilmington, DE) Model 6890 series GC system with HP 5973 mass selective detector. Relative EM voltage was 106 and a mass range of 40−300 was used. Three grams of the reaction mixture was heated in an HP 7694 headspace sampler at 90 °C for 20 min in a 10 mL glass vial, after which a sample of the headspace volatiles was automatically injected onto the head of a 30 m HP-5 MS capillary column (30.0 m × 250 μm × 0.25 μm) in GC. The GC was programmed from −20 to 250 °C at the rate of 10.0 °C/min. Peak identification was performed by the HP Chemstation system by use of the NBS75K database.

Statistical Analysis. The study was designed as a randomized complete block design. The experiment was replicated three times with each replication used as a blocking variable. The data were analyzed as a two-factor factorial arrangement. Time of heating was one factor and percent levels of MRP for each heating time was the second treatment factor. The data were analyzed by the Proc Mixed procedure (SAS Institute Inc., Cary, NC) for treatment effects and interaction between treatments at $p \leq 0.05$. For pH and absorbance, the best ($p \leq 0.05$) polynomial model was fit with orthogonal contrasts for treatment effects, after analyzing for all interaction effects between treatments.

The equation best describing the relationship between treatment time and percent factors to the response variable was fit by trend analysis by following the hierarchy principle approach (Maxwell & Delaney, 1989). Terms of increasing order for the individual components were added in successive steps. The lack of fit was tested for each term by type I sum of squares. The highest order term for each component that had a significant effect ($p \leq 0.05$) on the response variable (antioxidative effect) was determined. To obtain the best possible model, the highest order term showing significance and all lower order terms for that component were included in the model. Bonferroni's multiple comparison procedure was used for comparisons between means.

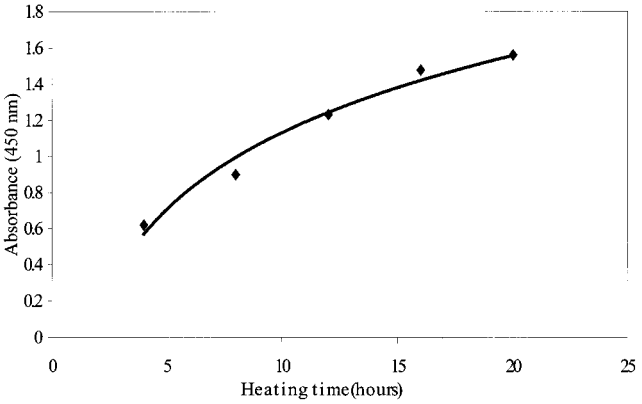

Figure 1. Change in absorbance (450 nm) with heating time for honey-lysine MRP ($n = 6$) (1:5 dilution).

RESULTS AND DISCUSSION

The antioxidative activity of the Maillard reaction has been related to the pigments formed during the course of the reaction. However, correlation between antioxidant capacity and degree of browning has not been strongly established. Absorption at 450 nm was used to measure color of the reaction mixture with time. The absorbance of the honey-lysine mixture increased with increasing reaction time up to 20 h (Figure 1). Namiki (1988) reported that antioxidative activity increased in proportion to the color intensity of reaction mixture. Kim et al. (1989) reported that antioxidative effect of Maillard reaction mixture did not increase in proportion to length of reaction time. In a glucose−histidine system (Lingnert et al., 1980), antioxidative activity was found to increase with browning in the first stage but decreased with further heating after reaching a maximum. A decrease in color and antioxidative effect was observed after 20 h of reaction. This was attributed to precipitation of high molecular weight material, since measurements are made on the soluble part of the mixture. In our study, the formation of a brown-black precipitate around the flask was observed after 16 h of reaction. No decrease in absorbance was observed. The Maillard reaction is usually accompanied by a decline in pH. In the reaction of histidine and glucose at 100 °C in 0.1 M phosphate buffer, a decrease in initial pH 7.0 to pH 5.0 was observed within 5 h of reaction (Lingnert, 1990). In the synthesis of antioxidative products from arginine and xylose (Waller et al., 1983), the pH of the crude reaction mixture reached a constant minimum pH value after 2−5 h. The reaction time required for antioxidative activity varied between 10 and 20 h. In both buffered and nonbuffered systems, no significant difference in antioxidative effect was observed. In nonbuffered systems having different initial pH values, a minimum and constant value was attained within 5 h. The reaction mixtures characterized by initial pH values of 5.0 and 7.0 gave maximum antioxidative effect and attained a final pH of 3.5 and 4.0, respectively. For the honey-lysine mixture, the pH decreased from an initial pH of 5.8 to 4.23 in 4 h (Figure 2). The pH continued to decrease and the rate of pH decline was lower after 12

Table 1. Volatiles from Honey-Lysine Maillard Reaction Products Prepared At Different Reaction Times

volatiles	reaction time (h) 4	8	12	16	20
pyrazine	− − − − −	+ + + − +	+ + + + +	+ + + + +	+ + + + +
1,5-dihydro-1-methyl-2*H*-pyrrol-2-one or 3,4,4-trimethylcyclohexene[b]	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +
1-(2-furanyl)ethanone	− − − + −	− − + − −	+ + + + −	+ + + + +	+ + + + +
2-furancarboxaldehyde	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +
5-methyl-2-furancarboxaldehyde	− − − − −	− − − − −	− − − − −	− + + + +	+ + + + +
2,4-dimethyl-1*H*-imidazole or 1,4-dimethyl-1*H*-pyrazole or 2,5-dimethylfuran[b]	+ + − − +	+ + + − −	+ + + − −	+ + + − −	+ + + − −
1-methyl-1*H*-pyrrole[c]	− − − − −	− − − − −	− + + − −	− − − − −	− − − − −
4-ethyl-1*H*-pyrazole-1-carboxaldehyde, or 5,6-dihydro-6-(2-P)-2*H*-pyran-2-one[b]	− − + − −	− − + − −	+ − − − −	+ − − − −	+ + + − −
butylated hydroxytoluene or 6,7-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran[b]	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +

[a] (+) Detected; (−) not detected. Five separate replications were analyzed for each reaction time. Compound identification was from the NBS75K database. [b] Identified as either of these compounds. [c] Formed in some trials.

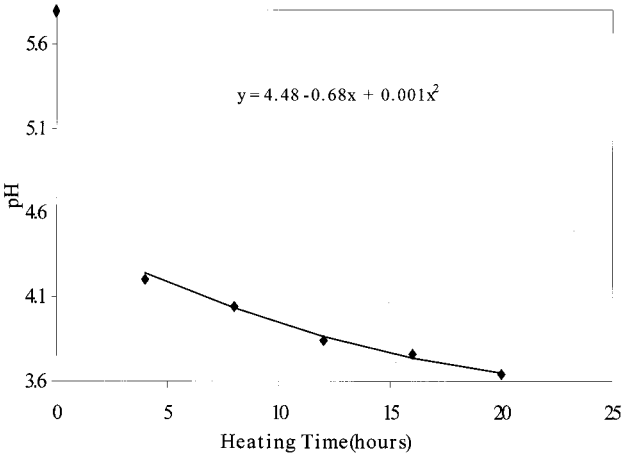

Figure 2. Change in pH with heating time for honey-lysine MRP ($n = 6$).

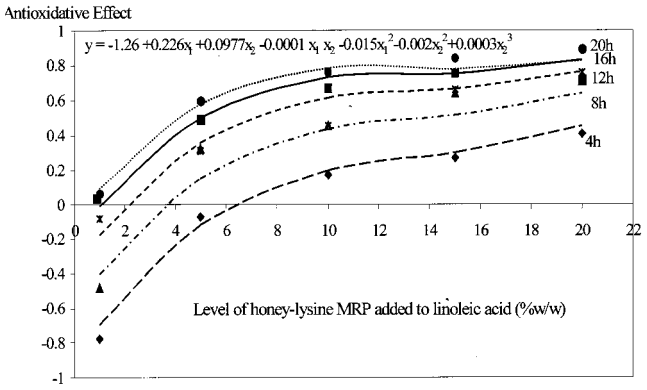

Figure 3. Antioxidative effect of honey-lysine MRP formed at different reaction times tested in a linoleic acid model system ($n = 6$). AE = $[\Delta A_{234}(C) - \Delta A_{234}]/\Delta A_{234}(C)$, where ΔA_{234} = increase of absorption at 234 nm during the incubation time (15 h) and ΔA_{234} (C) = the corresponding increase in control (no MRP added). (···) 20 h; (—) 16 h; (- - -) 12 h; (- · -) 8 h; (— — —) 4 h.

h of reaction. The maximum pH decline was during the first 4 h of reaction. The pH of the 20 h heated honey-lysine mixture and glucose-lysine mixtures was 3.66 and 3.77, respectively (Antony et al., 2000b). Antioxidative effect of MRP has been shown to strongly depend on the reactants. However, there is no evidence that the most effective amino acid when reacted with one sugar is most effective when reacted with other sugars. Honey contains reducing sugars, with glucose and fructose making up a major portion, and this could affect the color and pH as well as the antioxidative effect provided by the final MRP mixture. The pH of the reaction mixture did not differ significantly from that of the glucose-lysine mixture for the 20 h reaction time under the synthesis conditions.

The antioxidative effect increased with heating time and percent MRP added. There was no interaction ($p \geq 0.05$) between percent MRP added and hours of heating on the antioxidative effect. The antioxidative effect indicated a quadratic response with time and a cubic behavior with respect to levels of MRP at each heating time (Figure 3). No antioxidative effect was observed at the 1% level for the 4, 8, or 12 h reaction mixture under our study conditions. In fact, a pro-oxidative effect was observed at the 4 and 8 h reaction times for the 1% level. Pro-oxidative effects for lysine have been observed (Porkorny, 1987) when trace amounts of metal ions are present. This does not fully explain why greater levels of the 1% reaction mixture were not pro-oxidative unless the longer reaction times produced more antioxidative compounds. These compounds might compensate and overcome the pro-oxidative effect of the trace metal ions in the lysine mixture. There was no attempt to verify the presence of ions in the lysine mixture. At the 10% level, a 17% increase in antioxidative effect was observed as compared to the control for the 4 h heated MRP reaction mixture. When 20% MRP was added, a 30% greater antioxidative effect was observed between MRP prepared at 8 h reaction time as compared to 4 h reaction time. At levels of 10% and higher there was no significant difference ($p \geq 0.05$) in the antioxidative effect after 8 h reaction time. An increase of 46% and 53% was observed between the 1% and 5% MRP levels, respectively, for the 16 and 20 h heated reaction mixture. At 4 and 8 h of heating time, not enough antioxidative compounds were formed, resulting in a low antioxidative effect. To summarize, the higher the reaction time, the greater the antioxidative effect. For the 16 and 20 h reaction mixture, the antioxidative effect leveled off at MRP concentrations greater than 10%. The equation in Figure 3 includes both the heating time (x_1) and the level of added honey (x_2), allowing the effect of both factors on the antioxidative effect to be estimated throughout the ranges used in the experiment.

Compounds such as maltol (3-hydroxy-2-methyl-4H-pyran-4-one), isomaltol [1-(3-hydroxy-2-furanyl)ethanone], 5-hydroxy, 5,6-dihydromaltol, 4-hydroxy-5-methyl-3-(2*H*)-furanone, and similar compounds arise from the 1-deoxysone pathway by dehydration. These compounds are potential precursors for antioxidant and flavor compounds, particularly when heated with amines.

Pyruvaldehyde, diacetyl, acetaldehyde, and reductones are formed by the degradation of the enol form of deoxysone (1-deoxy reductone). Reductic acid (2,3-dihydroxy-2-cyclopenten-1-one) and maltol, when added to ground beef, reduced the level of WOF (Bailey, 1987). Kim et al. (1992) identified imidazole, lactic acid, furan, furfuryl alcohol, and furfural as important intermediates of the Maillard reaction that showed antioxidative activity in soybean oil. Paik et al. (1978) reported an antioxidant activity for a methylene chloride extract from a glucose−ammonia mixture that was subjected to optimum conditions for pyrazine formation. Kim et al. (1992) investigated the antioxidant activity of pyrazine, its derivatives, and pyrrole, intermediate products of the Maillard reaction. Their results indicated that pyrrole possessed considerable antioxidant activity, explained by resonance stabilization of pyrrole radical that can be formed by interaction of pyrrole with free radicals including the peroxy radical. Although pyrazines did not show any activity, their alkylated derivatives 2,3,5-trimethylpyrazine and 2,3-diethyl-5-methylpyrazine showed weak antioxidative activity. These compounds, being weakly basic, partially neutralized the free fatty acids formed from the substrate, thereby lowering their acid values.

Analysis of the volatiles from MRP produced by different heating times showed the formation of several different compounds. The formation of furanone, pyranone, pyrazine, imidazole, pyrazole, ethanone, and furan derivatives and pyrrole compounds was identified (Table 1). Eiserich et al. (1992) showed that alkylthiophenes, 2-thiophenethiol, 2-methyl-3-furanthiol, and furfuryl mercaptan have antioxidant properties. The antioxidative capacity of these compounds depended on the degree of unsaturation in the heterocyclic ring, as well as the substituent type. Formation of furfural, pyrazines, and furanylethanone in the present study were observed only at longer heating times and could be contributing to the antioxidative effect observed. This could explain the higher antioxidative effect observed with increased heating time.

A compound tentatively identified as BHT or 6,7-dimethoxy-2,2-dimethyl-2*H*-1- benzopyran is believed to be a polymerization product from the heating of lysine. The comparison with glucose-lysine MRP in our previous study and the lower antioxidative effect observed up to 10% levels does indicate that there is no antioxidative compound like BHT present in lysine. Amino acids on heating can form dipeptides or form cyclic compounds; therefore this compound could be some dimeric form of the amino acid, since it was found at small levels in all treatments with no trend. Since the identification of the volatiles from GC−mass spectrometry analysis was initiated through a volatile headspace injection, other nonvolatile antioxidative reaction products that are in solution may be present. Further isolation and identification by LC−mass spectrometry or other techniques would be desirable before more definite conclusions are drawn on antioxidant compounds formed during the honey-lysine Maillard reaction.

CONCLUSIONS

Control of the browning reaction to produce desirable flavors and odors is an intriguing possibility considering the complexity and number of variables that influence this reaction. Soup mixes and gravy mixes are practical examples of Maillard technology. A number of patents have been directed to the production of meat flavors based on the Maillard reaction. However, to get reproducible results, precise control should be exercised at every stage of the process. On the basis of these and the results of others, antioxidative compounds could be prepared by combining honey with meat proteins and adding them to the products prior to thermal processing.

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