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Stability of Antioxidants Formed from Histidine and Glucose by the Maillard Reaction

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Maillard reaction products (MRP) have been reported by several authors to inhibit lipid oxidation in model systems as well as in food products. The antioxidative compounds formed have so far not been identified, nor has the mechanism of their antioxidative effect been elucidated. In the course of trying to isolate antioxidative components formed by the Maillard reaction between histidine and glucose, it was found that part of the antioxidative effect was lost during the isolation processing. The stability of the antioxidants was therefore studied in more detail. The antioxidants were found to be unstable in solution when exposed to air. When they were stored in nitrogen atmosphere, no loss of antioxidative effect was noticed. The oxygen sensitivity was found to be pH dependent. Within the studied pH range 2–10, the stability of the antioxidants decreased with increasing pH. An oxygen electrode could be used to measure the consumption of oxygen by the MRP. In the dry state and in concentrated solutions at low temperatures, the MRP were found to be fairly stable. The significance of this instability in a food system and in laboratory work with the antioxidants is discussed.

The Maillard reaction between amino and carbonyl compounds has gained increased attention in recent years. The reaction is very common in many foods, and the reaction products are of great importance for the color and the flavor of the foods. Maillard reaction products (MRP) from amino acids and sugars have also been shown to inhibit lipid oxidation in model systems (Kirigaya et al., 1969; Lingnert and Eriksson, 1980; Eichner, 1980) as well as in storage experiments with food products (Tomita, 1972; Lingnert, 1980; Lingnert and Lundgren, 1980). In spite of the large amount of research that has been done on the Maillard reaction, many parts of this complex reaction are still obscure. For example, the mechanism for the formation of the brown pigments is not yet fully understood. No antioxidative compounds formed by the reaction have, so far, been identified, nor has the mechanism of their antioxidative effect been elucidated.

In the course of trying to isolate and identify antioxidative MRP it was found that part of the antioxidative effect was lost during the isolation processes. It was therefore decided to investigate more carefully the stability of these antioxidants. Knowledge of the stability is of importance when trying to utilize MRP as antioxidants in food, as well as when designing separation methods for the further work on identification of the antioxidants. The results might also give information about the chemical nature of the antioxidative MRP. The reaction products from histidine and glucose were chosen for this study. This combination was previously found to be one of the most effective in forming antioxidative MRP (Lingnert and Eriksson, 1980).

MATERIALS AND METHODS

Synthesis of MRP. MRP were obtained by refluxing 100 mL of distilled water containing 0.1 mol of L-histidine monohydrochloride monohydrate (Sigma Chemical Co., St. Louis, MO) and 0.05 mol of D-glucose (Sigma Chemical Co.) for 20 h. The pH of the reaction mixture was adjusted to 7.0 with potassium hydroxide before starting the reaction.

Dialysis of MRP. The Maillard reaction mixture was diluted with distilled water to double its volume and dialyzed against distilled water (250 times the volume of the MRP solution) for 2 × 12 h. Spectrapor 6 dialysis tubing with a molecular weight cutoff of 1000 was used (Spectrum Medical Industries, Inc., Los Angeles, CA).

Measurement of Antioxidative Effect. The antioxidative effect of the MRP was evaluated by the polarographic method described previously (Lingnert et al., 1979) with the following modifications: The volume of the reaction vessel was 3.2 mL. The vessel was equipped with an oxygen electrode connected to an YSI 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH).

Measurement of Oxygen Consumption by MRP. The equipment described above was also used for measuring the rate of oxygen consumption by the MRP. The reaction vessel was filled with air-saturated distilled water. The MRP solution (0.2 mL) was injected, and the oxygen consumption and time elapsed were recorded.

Incubation of MRP for Stability Studies. Where not otherwise stated, the Maillard reaction mixture was diluted 40-fold with distilled water, and 2-mL portions were transferred to 15-mL test tubes with screw caps containing Teflon seals. In some cases nitrogen was bubbled through the solution for 3 min before closing the test tubes to study the influence of inert atmosphere on the stability. (The distilled water used for the dilution of the Maillard reaction mixture was, in these cases, first degassed with vacuum and then bubbled with nitrogen.) The tubes were incubated in a shaker (G-24 Environmental Incubator Shaker, New Brunswick Scientific, NJ) at 25 °C and 200 rpm. At various times tubes were withdrawn for measurement of the antioxidative effect. The tube was then bubbled with nitrogen, frozen in a mixture of solid carbon dioxide and methanol, and kept in a freezer (−18 °C) until the antioxidative effect was measured.

C, H, N, and O Analysis. Analysis for carbon, hydrogen, nitrogen, and oxygen in the samples were performed by Galbraith Laboratories, Inc., Knoxville, TN.

RESULTS

The effect of the incubation time on the antioxidative effect of the histidine–glucose reaction products is shown in Figure 1. A considerable loss of antioxidative effect was noticed for the samples incubated in an air atmosphere. Most of the antioxidative effect was lost during the first 10 h of incubation. In Figure 1 it can also be seen that no antioxidative effect was lost when the MRP were incubated in nitrogen for 96 h. The antioxidative products in the reaction mixture are, obviously, sensitive to oxygen.

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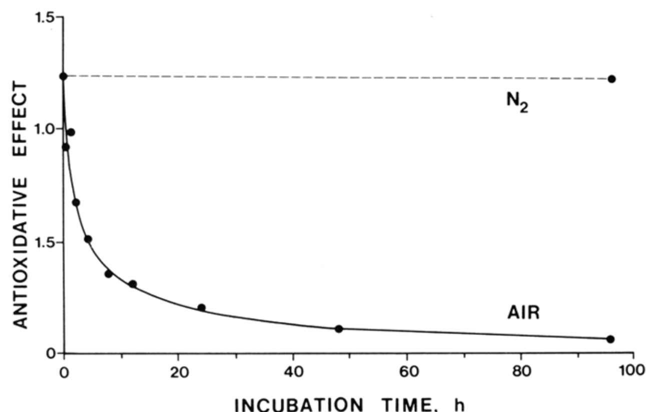

Figure 1. Loss of antioxidative effect of MRP from histidine and glucose incubated at 25 °C in an atmosphere of air or nitrogen.

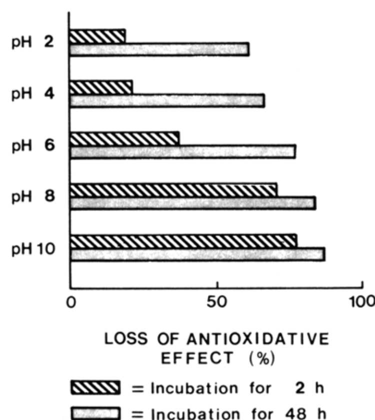

Figure 2. Influence of pH on the loss of antioxidative effect of MRP from histidine and glucose when incubated at 25 °C in an air atmosphere.

The influence of the pH on the stability of the antioxidants in air was also studied. Hydrochloric acid and potassium hydroxide were used to adjust the pH to 2.0, 4.0, 6.0, 8.0, and 10.0, respectively, before starting the incubation; samples were withdrawn after 2 and 48 h for measurement of the antioxidative effect. The results are given in Figure 2, where the loss of antioxidative effect is shown. It can be seen that such loss is considerably slower at low pH than at high pH. In particular, this is obvious when the losses of antioxidative effect after 2 h at the various pH values are compared. The differences are not as large after 48 h, since after this time all samples probably have reached the phase of slow decrease in antioxidative effect at a low level as seen in Figure 1.

Since the MRP were found sensitive to oxygen, experiments were conducted to determine whether any reaction with oxygen could be detected. The results are shown in Figure 3. The Maillard reaction mixture was diluted 3 times with degassed, nitrogen-bubbled, distilled water, and the pH was adjusted with hydrochloride acid or potassium hydroxide to 2.0, 4.0, 6.0, 8.0, and 10.0, respectively. To the reaction vessel filled with air-saturated, distilled water was added 0.2 mL of one of these MRP solutions. Owing to dilution with the water in the reaction vessel the pH changed to the final values of 2.6, 4.2, 5.9, 8.0, and 9.8, respectively. Figure 3 shows that a substantial oxygen consumption by the MRP could be measured with the oxygen electrode. In accordance with the results from the stability studies at various pH values, the influence of pH was considerable, increased pH resulting in increased oxygen consumption. The initial rapid drop in oxygen content that is seen especially at pH 2 and pH 4 is due to the

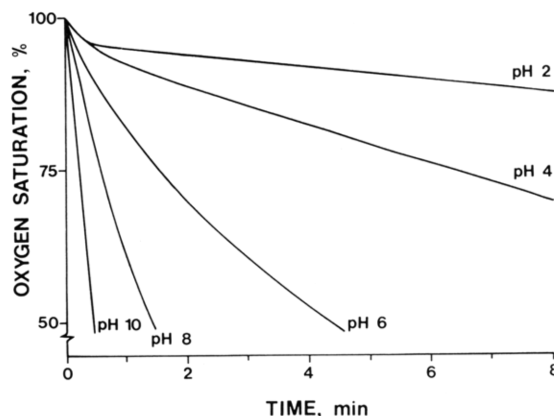

Figure 3. Influence of pH on the oxygen consumption of MRP from histidine and glucose at 25 °C.

Table I. Oxygen Consumption of MRP from Histidine and Glucose at 25 °C at Various pH Values

pH	oxygen consumption, nmol of O_2 (mg of MRP)$^{-1}$ s^{-1}
2	0.01
4	0.04
6	0.26
8	0.62
10	1.68

Table II. Antioxidative Effect, Oxygen Consumption, and Elementary Composition of Fresh MRP from Histidine and Glucose and of MRP Incubated at 25 °C for 6 Days

	antioxidative effect	oxygen consumption[a]	composition, % C	H	N	O
fresh MRP	12.0	0.126	50.7	5.4	16.9	26.9
incubated MRP	0.5	0.005	47.6	5.4	16.3	30.5

[a] Nanomoles of O_2 per milligram of MRP per second.

addition of the MRP solution that contained no oxygen. Data in Table I show the initial rate of oxygen consumption per weight unit of MRP as calculated by using the value 258 μmol of O_2/L air-saturated water at 25 °C (Estabrook, 1967) and excluding the initial changes in the oxygen concentration that resulted from the mixing with the nitrogen-saturated MRP solutions.

When the antioxidative effect of the MRP had been reduced to a low level by shaking in air for a long time, the oxygen consumption that could be measured by the oxygen electrode had decreased as well. This was shown in an experiment where 1.0 g of MRP was shaken in 50 mL of water for 6 days. The antioxidative effect and the oxygen consumption of the fresh and the oxidized MRP are shown in Table II. The table also shows the C, H, N, and O content of the two samples. The oxidation involved not only an increase in the oxygen content but also a disproportionate decrease in the carbon content, indicating that some volatile carbon compound was formed and lost during the oxidation. (Oxygen uptake alone, without loss of any constituents, would result in an increase in the oxygen content and proportional decreases in the carbon, hydrogen, and nitrogen contents.) The oxidation of the MRP did not involve any noticeable changes in either the UV spectrum or the IR spectrum.

Since MRP previously have been stored in this laboratory for months in the refrigerator without any noticeable loss of antioxidative effect, storage experiments were also conducted with undiluted Maillard reaction mixtures that

Table III. Changes of the Antioxidative Effect of MRP from Histidine and Glucose When Stored in the Dry State at Ambient Temperature for 55 Days

	antioxidative effect	
storage conditions	crude reaction mixture[b]	retentate from dialysis of MRP[b]
control[a]	1.5	0.4
in room atmosphere	1.7	0.4
in desiccator	1.4	0.4

[a] Sample dissolved in distilled water under nitrogen and stored at -18 °C was used as the control. [b] The concentration of the crude reaction mixture was 1.0 mg/mL of linoleic acid emulsion, while the concentration of the retentate was 0.1 mg/mL of linoleic acid emulsion.

were not subjected to shaking. Over a 70-day storage period no significant difference could be observed between samples stored in the refrigerator (+7 °C) and samples stored in the freezer (−18 °C) under nitrogen. However, some loss of antioxidative effect was noticed for samples stored for the same time at room temperature in the dark.

It was also of interest to study the stability of MRP in the dry state. Lyophilized, crude histidine–glucose reaction mixture as well as lyophilized retentate from dialysis of the reaction mixture was stored for 55 days at ambient temperature in a desiccator as well as in the room atmosphere. (The dialysis through a membrane with a nominal molecule weight cutoff of 1000 involved a concentration of antioxidative products in the retentate.) The control consisted of samples that were dissolved in degassed, nitrogen-bubbled, distilled water and stored at −18 °C under nitrogen. The results are shown in Table III. No changes were observed with the dialyzed material. The crude reaction mixture was very hygroscopic and could be observed to take up water when stored at room atmosphere. (No hygroscopic properties could be observed for the dialyzed material, since the unreacted sugars and other low molecular weight compounds had been removed.) In spite of the water uptake the results indicate a small increase of the antioxidative effect. A speculative explanation for this is that more antioxidative MRP were formed during the storage since the sample probably contained the precursors needed.

Although the products from the reaction between histidine and glucose were used in the present study, the instability is not limited to MRP from this combination. Preliminary experiments have shown that similar results are obtained with the combination arginine–xylose.

DISCUSSION

The instability of the antioxidative MRP was rather surprising since previously no loss of antioxidative effect had been noticed after storing the Maillard reaction mixtures for long time. The MRP were then kept in concentrated solutions at low temperatures, and this study confirmed that no significant loss of activity was obtained during these conditions. The possible explanation for this is that the reaction is slow at the low temperature and that the oxygen availability is very limited because of very slow oxygen diffusion through the viscous, concentrated solution.

Since the Maillard reaction mixture is a dynamic system containing everything from the original reactants, through several intermediate products, to more or less stable final products, the formation of more of the antioxidative compounds during storage cannot be excluded. The apparent loss (or gain) of antioxidative effect would then represent the net result of the opposing effects of degradation and formation of new antioxidants. Each of these processes might be separately influenced by factors such as temperature, pH, oxygen availability, and water activity. This may explain increased antioxidative effect in some cases, as indicated with the storage of dry samples of MRP.

The main question is how this oxygen sensitivity affects the possibilities of utilization of MRP as antioxidants in food, regardless of whether these are added as preformed antioxidative MRP to the food or formed in the food during processing. The aspects discussed above about the balance between degradation and new formation of antioxidants should be valid also in this case. The type of food (its water content, pH, etc.) as well as the temperature and oxygen availability during processing and storage is probably of importance for success in using MRP to protect against lipid oxidation. In a previous study on application of antioxidative MRP in cookies, the addition of preformed histidine–glucose products to the dough failed to inhibit lipid oxidation in the cookies, while addition of free histidine and glucose to the dough before baking was effective (Lingnert, 1980). It was then suggested that the concentration of performed MRP used was too low. In the light of the results from the present investigation another explanation could be that the antioxidants were oxidized during the baking.

The results presented in this study may also give some information about the chemical structure of the antioxidative MRP. They indicate that the antioxidative compounds contain at least one functional group that is readily oxidized by direct attack by oxygen. Furthermore, this oxygenation is obviously pH dependent. The reaction of MRP with oxygen also seems to involve the formation of some volatile carbon compound, possibly carbon dioxide or a similar product.

Since the MRP react with oxygen it could be asked if the mechanism for the antioxidative effect is simply that the MRP are more readily oxidized than the lipids, thereby making the oxygen unavailable for the lipids. This is, however, unlikely to be a main mechanism, since the antioxidative effect in this study was evaluated by measuring the oxygen consumption and the MRP in fact retarded the oxygen consumption in the linoleic acid emulsion. The antioxidative effect is possibly underestimated by this method, since the MRP per se can be responsible for part of the oxygen consumption in the test. However, approximately 10 times higher concentrations of MRP would be needed in a water system in order to reach an oxygen consumption of the same magnitude as that obtained in the linoleic acid system.

Finally, it may be concluded that caution must be used when studying the chemistry of the Maillard reaction, in particular with research on the antioxidative reaction products. It is especially important to avoid all exposure to oxygen in order not to change the MRP originally formed and not to lose antioxidative effect during, for example, isolation of the antioxidants.

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Effect of Oat Constituents on Aggregation Behavior of *Oryzaephilus surinamensis* (L.)

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Components isolated from pentane extracts of rolled oats that stimulate aggregation of *Oryzaephilus surinamensis* (L.) (saw-toothed grain beetle) adults have been identified. These materials include mixtures of fatty acids consisting mainly of palmitic, oleic, and linoleic acids and triglycerides with acyl substituents made up of similar mixtures of these same fatty acids. Food-grade coconut, corn, and sunflower oils also induce aggregation when administered in the bioassay. Triglycerides from rolled oat extract containing 9,10-epoxyoctadecanoic and 9,10-epoxy-*cis*-12-octadecenoic acids showed no activity. Appreciable amounts of C_{18} hydroxydiene acids were identified in triglyceride fractions that induced a distinct avoidance response in the insects.

The sawtoothed grain beetle [*Oryzaephilus surinamensis* (L.)] is an economically important stored-product pest of worldwide distribution. Although numerous investigations have dealt with food attractants for *Tribolium*, *Trogoderma*, and *Sitophilus* species [see Nara et al. (1981) for a summary], only recently have two reports appeared concerning oat constituents that elicit chemosensory responses in *O. surinamensis* adults (Pierce et al., 1981; Freedman et al., 1982). Freedman et al. (1982) presented results indicating that crude mixtures of essentially nonvolatile triglyceride materials from pentane extracts of rolled oats stimulate aggregation of the saw-toothed grain beetle; however, both groups of workers also suggested that volatile chemical stimuli are also present in oats and oat extracts and are responsible for the actual olfactory attraction that was observed in pitfall and olfactometer bioassays.

Since none of the oat constituents had been identified, we conducted this investigation to extend the work of Freedman et al. (1982) by isolating and identifying those constituents responsible for the observed insect behavior.

MATERIALS AND METHODS

Insect Rearing and Bioassay. *O. surinamensis* rearing, Petri dish bioassays, and statistical treatment of data were all done as previously described (Freedman et al., 1982). Test beetles were reared on a diet of rolled oats containing 5% of brewers yeast, were 3–5 weeks old, and had been starved from 5 to 7 days prior to being used in a bioassay. Petri dishes with two filter paper disks were used as test arenas. Percent response data shown in Tables I and II are calculated by the formula $100(T - C)/N$, where T and C are the number of beetles aggregating at the treated and control disks, respectively, and N is the total number of beetles used. Reference bioassays using pentane extract of rolled oats (Quaker Old Fashioned) at 5×10^4 and 1×10^4 μg/test were conducted with each set of four replicates. All solvents were redistilled before use.

Table I. Aggregation Response of *O. surinamensis* to Oat Extract Fractions

sample designation: new	sample designation: previous[a]	subfractions	type of material	recovery from extract, %	% response[b,c]
A	2		triglyceride	76.4	67**[a]
B	3-3		triglyceride	0.57	42**[a]
C	4-3	1	triglyceride	0.28	11
		2	triglyceride	0.44	6
		3	triglyceride	0.59	−9
D	5-1, 5-2, 5-3		free fatty acids	0.79	61**
E	5-5	1	triglyceride	0.14	−56**
		2	triglyceride	0.27	−30**
		3	free fatty acids	0.04	20*
F	5-6	1	mixture	0.48	3
		2a	triglyceride	0.04	*d*
		2b	triglyceride	0.03	*d*
		2c	di- + monoglyceride	0.16	20*[e]
		2d	di- + monoglyceride	0.91	23*[e]
		3	mixture	0.93	3
		4	free fatty acids	0.04	52**
G	6-2, 6-3, 6-4		free fatty acids	0.55	24**[f]

[a] From Freedman et al. (1982), Table 6. [b] Based on 4 replicates, 25 beetles per replicate, and 2500 μg of material/test. [c] (*) and (**) are significant at $P < 0.05$ and $P < 0.01$, respectively, based on χ^2 analysis. [d] Insufficient quantity for reliable bioassay. [e] 0.025 μg/test; eight replicates. [f] 2.5 μg/test.

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