Chemical Studies on Melanoidins

Part III. Effects of Heating on Chemical Properties of Melanoidin Prepared from Glycine-Xylose System

By Toshiharu GOMYO,* Hiromichi KATO, Kiyoshi UDAKA, Masayo HORIKOSHI* and Masao FUJIMAKI

Department of Agricultural Chemistry, The University of Tokyo, Tokyo
*Department of Nutrition, Kagawa Nutrition College, Tokyo

Received July 29, 1971

Nondiffusible melanoidin obtained from a glycine-xylose system was heated in aqueous media, and the resulting chemical changes, as affected by the presence of oxygen, pH value, temperature and the addition of transitional metals, were investigated.

Melanoidin, when heated at 90°C in an aqueous solution, caused remarkable discolorization accompanied by the development of fluorescence, oxygen consumption and a noticeable variation of reductone content. Heated melanoidin became polydispersive in molecular weight on gel filtration chromatograms. There appeared reductones, ninhydrin-positive substances, fluorescent substances, aromatic amines, aliphatic carbonyls, and aliphatic primary amines and/or methylene groups in diffusates of melanoidin heated in various media.

An increase in pH value favored oxidative discolorization, while an increase in the concentration of transitional metals except Mn²⁺ restrained the discolorization. In the absence of oxygen, heated melanoidin brought about a slight strengthening of color and the accumulation of reductones ca. fifteen times more than the initial level, while in the presence of oxygen the increase of reductone content at the early period was followed by a rapid decrease.

According to the results obtained, the ambivalent reactivity of melanoidin, i.e. polymerization (colorization) and depolymerization (discolorization), was discussed in relation to influencing factors. A mechanism for the production of reductones in heated melanoidin was also proposed.

Many works have already clarified a number of intermediates involved in the mechanism for the production of melanoidin during the amino-carbonyl or Maillard reaction.¹ However, little has been known about the chemical characteristics and/or chemical structure of melanoidin as a complicated macromolecule pigment.

In general, melanoidins are similar to humic substances²,³ and melanins⁴⁻⁶ in their chemical properties. These polymerized substances are random in structure, highly dispersive in molecular weight and gentle in absorption spectrum. Hence, there are great difficulties in investigating the structural feature of such substances in detail. As to melanins, the fundamental skeletons have been elucidated to a considerable extent along with the mechanism for polymerization.

Some reports are available in respect to the chemical structure of melanoidins prepared from model systems consisting of amino compounds and hexoses or pentoses.¹,⁷¹ However, there have been no more than descriptions of

¹ A part of this work was presented at the Symposium "Chemistry and Biochemistry of Reductones", in the Annual Meeting of the Agricultural Chemical Society of Japan, Fukuoka, April 2, 1970.
elementary compositions, some functional groups and general properties of melanoidins. In previous papers, the authors dealt with the measurement of reductone contents of nondiffusible fractions of melanoidin pigments from various model systems, and the characterization of fractions obtained from a browning reaction solution in relation to the degree of polymerization.

In the present paper, the chemical changes by heating, especially discolorization, of the nondiffusible fraction of melanoidin prepared from glycine and xylose are investigated in relation to influencing factors such as the presence of oxygen, pH value, temperature and transitional metals, and a mechanism for these changes is discussed in part.

MATERIALS AND METHODS

Preparation of melanoidin. An aqueous solution of a mixture of D-xylose 2M, glycine 2M and NaHCO₃ 0.05 M (pH 6.5) was heated at 90°C for 10 hr. The resulting blackish brown solution was dialyzed against flowing city water for 48 hr, distilled water for 24 hr and deionized water for 24 hr, one after another, in a cellophan tube (Visking Co.). The nondiffusible fraction was concentrated under reduced pressure at 40°C, and the concentrate was freeze-dried. A powder of nondiffusible melanoidin was obtained in a yield of 13.9 g from 30 g of xylose.

Heating melanoidin solution. The usual procedure for heating melanoidin solutions is as follows: melanoidin 1.25 mg/ml, NaHCO₃ 0.05 M, pH 8.5, heated at 90°C in sealed Pyrex tubes.

Estimation of oxygen consumption. Oxygen partial pressure was estimated on the head space of a sealed tube by means of gas chromatography. The condition for gas chromatography: Linde Molecular Sieve 5A, 3 m; the flow rate of helium gas 15~20 ml/min; room temperature.

Estimation of reductone content. Reductone content was estimated by a colorimetric method using 2,6-dichlorophenol indophenol.

Estimation of fluorescence. An anthranilic acid solution was used as a standard. The equipment employed for analysis was a Shimadzu-Kotaki-Dr. Yagi’s Ultra Micro Fluoro-photometer Model SKM 4L; the filters applied were as follows: light source filter, UV-V; fluorescence-selection filter, FL-B₂ (77); ultraviolet cut-off filter, UV-O₂.

Gel filtration on Sephadex G-25 column. A melanoidin solution after heat treatment was applied to a Sephadex G-25 column (132×1.1 cm) and developed with 0.1 M acetic acid at the flow rate of 20 ml/hr. Each fraction was measured for $E_{470}$, fluorescence and potassium permanganate consumption.

RESULTS

Discolorization of melanoidin by heating

Figure 1 shows that heat treatment (90°C) has caused discolorization of melanoidin pigment. Under this condition, $E_{470}$, an indicator of the color intensity of the pigment, decreased to about 70% within 10 hr and went down below 50% within 48 hr. Longer-time heating
resulted in fading away of the color of melanoidin pigment, from blackish brown to pale yellow. The spectral change of melanoidin by heating is shown in Fig. 2. Seventy-two hours heating caused the disappearance of the broad peak at about 320 mµ. An increase in absorbance in the shorter range and a decrease in absorbance in the longer range of wave length were observed. The decrease of $E_{470}$ is closely related to oxygen consumption and the development of fluorescence as seen in Fig. 1. On the other hand, the amount of reductones changed in a queer manner as seen in this Figure. In the earlier stage of heating, the amount of reductones increased and then decreased rapidly below the initial level.

Changes in gel filtration patterns of melanoidin by heating

In order to make the changes in the patterns clearer, the nondiffusible melanoidin was further purified on a Sephadex G-25 column to give a narrower peak in the chromatogram and then used as a sample for heating experiments. The results are shown in Fig. 3. After being heated at 90°C for 72 hr, the major peak migrated to the slower side or the lower region of molecular weight. The shoulder of the major peak of the heated melanoidin corresponds to the peak of non-heated melanoidin.
TABLE I. CHEMICAL ANALYSIS OF DIFFUSABLE FRACTION FROM HEATED MELANOIDIN*1

<table>
<thead>
<tr>
<th>Heating media</th>
<th>Control*1</th>
<th>HCl</th>
<th>NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.81</td>
<td>14.6</td>
<td>25.2</td>
</tr>
<tr>
<td>KMnO₄ consumed&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color intensity</td>
<td>+</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Flourescence</td>
<td>+</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indophenol</td>
<td>#</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Sugars&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ninhydrin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>Nessler</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Dimethylaminobenzaldehyde</td>
<td></td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td></td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>Sodium 1,2-naphthoquinone-4-sulfonate</td>
<td>#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric chloride</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>*1</sup> After being heated at 90°C for 24 hr, 4 ml of melanoidin solution (6.25 mg/ml) was dialyzed against 100 ml of distilled water for 24 hr.

<sup>*2</sup> Heated under the same condition without HCl or NaOH; the initial pH value was 8.5.

<sup>*3</sup> The percentage of the diffusate to the whole in KMnO₄ consumption.

<sup>*4</sup> Tested by anthrone-H₂SO₄, aniline hydrogen phthalate and p-anisidine-HCl.

<sup>*5</sup> The ninhydrin-positive matters mostly consisted of glycine on the basis of Rf value on the paper chromatogram.

By heating, a few minor peaks appeared before and after the major peak. These fractions emitted rather strong fluorescence. The paper chromatogram in Fig. 3 also shows the production of fluorescent matters during heating. Melanoidin solutions varying in the concentration of H⁺ were heated at 90°C for 24 hr. The resulting solutions were dialyzed to obtain diffusible fractions, which were examined in respect to some chemical properties as shown in Table I. It is obvious that heat treatment has brought about fragmentation of melanoidin molecules, depending on acidity or alkalinity. The fragmentation may be based on a certain hydrolysis of linkages in melanoidin. On account of unstability in drastic media, the amount of reductones is greater in a mild condition such as in control, which is reverse to potassium permanganate consumption. The ninhydrin-positive matters mostly consisted of glycine on the basis of Rf value on the paper chromatogram. Aromatic amines, aliphatic carbonyls and methylene groups and/or aliphatic primary amines appeared in the diffusates of melanoidin heated in various media; only a trace of ammonia was detected; neither sugars nor ferric chloride-positive substances appeared.

**Effects of oxygen on discolorization of melanoidin**

Figure 4 shows that in the absence of oxygen the discolorization of melanoidin and the development of fluorescence do not occur. In this condition, heat treatment caused a slight increase in color intensity, followed by precipitation, indicating that polymerization of melanoidin occurred to some extent. The reductone content, however, increased to reach a constant level which was ca. fifteen times higher than the initial level. The shape of the curve of reductone content shown in Fig. 1 can be explained on the basis of the difference between the accumulation of reductones and their oxidation.

Peroxides were produced during heating in the presence of oxygen as shown in Table II, but their amount was too small to compensate the difference of reductone contents between the presence and absence of oxygen. Further
Chemical Studies on Melanoidins. Part III


Reaction condition: melanoidin 1.25 mg/ml, NaHCO₃ 0.05 M, heated at 90°C in a sealed Pyrex glass tube after degassing.

TABLE II. Peroxide Value and Reductone Content of Melanoidin

<table>
<thead>
<tr>
<th></th>
<th>Non-heated</th>
<th>Heated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>+O₂</th>
<th>−O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxides&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reductones&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75</td>
<td>38</td>
<td>884</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Heated at 90°C for 20 hr under the same condition as shown in Fig. 1.

<sup>b</sup> meq of Na₂S₂O₃ per 1 kg of melanoidin.

<sup>c</sup> meq of ascorbic acid per 1 kg of melanoidin.

Effects of pH on discolorization of melanoidin

The pH dependence of discolorization of melanoidin is significant as seen in Fig. 5. Generally, the discolorization and the development of fluorescence increased with the rise of pH value. In the lower region of pH, lower than about pH 5.0, E₄₇₀ increased in spite of the development of fluorescence.

The change of reductone contents in the presence or the absence of oxygen also corresponds to the above tendency as seen in Fig. 6.

FIG. 5. pH Dependency of Heating Discolorization of Melanoidin.

Reaction condition: melanoidin 0.625 mg/ml, NaHCO₃ 0.05 M, pH was adjusted with HCl or NaOH, heated for 5 hr at 90°C.

FIG. 6. pH Dependency of Reductone Content of Melanoidin on Heat Treatment with or without Oxygen.

Reaction condition: melanoidin 0.625 mg/ml, NaHCO₃ 0.05 M, pH was adjusted with acetate, phosphate or borate buffer, respectively; heated for 4 hr at 90°C.
In this case, the change was remarkable in the neutral or slightly alkaline region of pH, from 6 to 9.

Browning by Maillard reaction is usually promoted in the higher region of pH, but melanoidin itself discolorizes remarkably in this region.

Effects of temperature on discolorization of melanoidin

Figure 7 shows the temperature dependence of the discolorization and the development of fluorescence of melanoidin. The development of fluorescence is more significantly dependent on temperature than the discolorization. The development of fluorescence begins to increase abruptly at around 50°C.

Effects of transitional metals on discolorization of melanoidin

Such kinds of transitional metals as Fe, Cu, Co and Mn were examined on their effects on the discolorization of melanoidin. The results are shown in Fig. 8. Transitional metals showed common effects on the discolorization and the development of fluorescence of melanoidin with an exception of Mn. With the increase in the concentration of a metallic ion, both changes of melanoidin were restrained. In case of Mn, however, there appears to be a maximum between 10^{-5} M and 10^{-4} M. The inhibitory effect of Cu^{2+} was the strongest among the metallic ions examined. The presence of Cu^{2+} at 10^{-2} M intensified the color of melanoidin on heating, while fluorescence increase over the initial level to a small extent.

The inhibitory effects by transitional metals are interesting, when we consider that the discolorization of melanoidin is ascribed to the oxidative processes which are generally catalyzed by heavy metallic ions.

DISCUSSION

Melanoidin pigment as a nondiffusible fraction from a Maillard reaction solution is a highly reactive substance, especially unstable to heating in aqueous media. The reaction condition for heating of melanoidin applied in this experiment is the same to that for the preparation of melanoidin. Melanoidin is discolorized on heat treatment in the presence of oxygen. Therefore, it is reasonable to conclude.
that both colorization and discolorization are occurring simultaneously during the browning reaction or the preparation of melanoidin. The browning rate, an increase of general absorption, may be influenced by the difference in the reaction velocities between colorization and discolorization.

As described in a previous paper, the color intensity of melanoidin becomes higher with the degree of polymerization. On the other hand, it is considered that discolorization is closely related to the depolymerization of melanoidin molecules. It is evident from the present work that melanoidin has an ambivalent reactivity, depolymerization and polymerization, on heat treatment. The factors influencing such changes of melanoidin are summarized in Fig. 9. Heat treatment of melanoidin results in higher dispersiveness in molecular weight or molecular size as shown in Fig. 3.

It should be noted that melanoidin itself is capable of producing reductones. Reductones are well known as important intermediates in the process of browning. However, it is considered from the result in Fig. 4 that most of reductones appearing on heat treatment of melanoidin are bound to the nondiffusible fraction. Hence, one of the effective sources of reductones which are formed in Maillard reaction may be accounted for by the intramolecular change of melanoidin as macromolecules. The constant level of reductones attained in the anaerobic environment seems to be independent of heating time and dependent on the amount of melanoidin present (Fig. 6). This suggests that the precursors producing reductones are specific functional groups in melanoidin molecule. It is assumed that there occur Schiff bases in melanoidin molecules during the browning reaction. The occurrence of Schiff bases in melanoidin molecules is presumed partly on the basis of remarkable liberation of glycine during heating in an acidic or an alkaline medium (Table I). If these Schiff bases have hydroxyl groups on the adjacent carbons, amino reductones such as enaminoles are easily produced by way of immonium ions. The following reaction scheme has been proposed for the Amadori rearrangement.

$$\text{RN-CH} \quad \begin{array}{c} \text{H}^+ \\ \text{H-C-OH} \end{array} \xrightarrow{\text{H}} \text{RN-CH} \quad \begin{array}{c} \text{C-OH} \\ \text{C} \end{array} \xrightarrow{\text{H}^+} \text{RNH-CH} \quad \text{RNH-CH}_2$$

The pH dependency of reductone formation can be explained on the basis of tautomerism between enaminoles and the keto-form, which inclines towards enaminoles with the increase of pH value. In addition, the disconnection of H from the a carbon of immonium ion is the rate regulating process where the increasing pH value accelerates formation of enaminoles. Consequently, higher values of pH favor the production of reductones according to the above reaction scheme.

Rapid decomposition of reductones in the presence of oxygen was expected to produce a significant amount of peroxides, but as shown in Table II, the peroxide value of heated melanoidin was quite low. At present, the authors assume that hydrogen peroxide or other
peroxides which may be formed by oxidation of reductones are consumed for the oxidative discolorization of melanoidin. The peroxides may be too unstable to be estimated under these experimental conditions. Further extensive work should be carried out to elucidate the mechanism of the discolorization of melanoidin in relation to the production and the extinguishment of reductones.

The inhibitory effects of transitional metals on the discolorization as well as the development of fluorescence of melanoidin on heat treatment were contrary to authors' expectation that these oxidative changes of melanoidin would be catalyzed by heavy metallic ions. In the autoxidation of lipids, however, transitional metals are known to be inhibitory in the propagation stage and to promote termination, which are thought to be based on the stabilization or inactivation of free radicals involved in the reaction. According to this viewpoint, it may be reasonable to assume that transitional metals cause inactivation of free radicals involved in the oxidation process of melanoidin. The detailed mechanism for these phenomena is a subject for future investigation.

REFERENCES