Decolorization and Degradation Products of the Melanoidins by Hydrogen Peroxide

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Received May 7, 1984

Nondialyzable melanoidins prepared from a glucose–glycine system were incubated with glucose oxidase under optimal conditions and then the melanoidins were decolorized to 65%. The decolorization of melanoidins by glucose oxidase may be mainly caused by hydrogen peroxide produced from the enzyme-substrate reaction. Therefore, oxidative decomposition and decolorization of melanoidins were tried using hydrogen peroxide. Melanoidins were treated with hydrogen peroxide (final conc. 6.72%) under neutral (pH 7.0) and alkaline (pH 10.0) conditions at 37°C for 28 hr. Melanoidins were decolorized to 64% and 97%, respectively the under above optimum conditions. The mean molecular weight of melanoidins decreased from 5,300 to 3,500 after hydrogen peroxide treatment. The major components in the ether-soluble fraction obtained from melanoidins by oxidative degradation of alkaline hydrogen peroxide were identified as 2-methyl-2,4-pentanediol, N,N-dimethylacetamide, phenol, acetic acid, oxalic acid, methylpropanedioic acid, propanedioic acid, 2-furancarboxylic acid, butanedioic acid, 2-hydroxypropanoic acid, 2,5-furandicarboxylic acid and 5-(hydroxymethyl)-2-furancarboxylic acid. On the other hand, the major degradation product in aqueous fraction was identified as glycine, and glycine was produced in 1.73% yield per nondialyzable melanoidin.

The brown polymers, melanoidins, which are formed by the amino-carbonyl reaction, are analogous to humic substances of soils1) or melanins2) in regard to their chemical properties. In addition, they exist not only in various foods, but also in distillery and cane molasses wastes. Therefore, the degradation3,4) and decolorization5) by chemical and microbial treatments has been attempted on the melanoidins in order to characterize their chemical structures and from the environmental chemical aspects. However, the chemical structure of chromophores and the main skeleton of melanoidins have not been elucidated. The authors6,7) reported on the degradation products formed by pyrolysis of the melanoidins in order to clarify their partial chemical structure.

In this paper, we investigated the decolorization and decomposition products of melanoidins by use of hydrogen peroxide considering also environmental chemical aspects. Melanoidins prepared from a glucose-glycine reaction system were chosen for the present study.

MATERIALS AND METHODS

Preparation of nondialyzable melanoidins. Glucose (1 mol), glycine (1 mol) and sodium bicarbonate (0.2 mol) were dissolved in distilled and deionized water, made up to 500 ml (pH 6.8) and refuxed in an oil bath at 95°C for 7 hr. The resulting brown solution was dialyzed against distilled and deionized water for 2 weeks. The nondialyzable fraction was lyophilized and the dried powder was extracted with diethyl ether in order to remove low molecular substances. The residue was dried over phosphorus pentoxide in vacuo to obtain the nondialyzable melanoidins. The yield of purified nondialyzable melanoidins was 17.6 g from 180 g of glucose.

Incubation of melanoidins with glucose oxidase. Melanoidins were incubated with glucose oxidase under its optimal conditions as follows. Three milligrams of melanoidins, 3 mg (375 units) of glucose oxidase from Aspergillus niger (Type VII, Sigma) and 1 mmol of glucose
were dissolved in 0.1 M sodium acetate buffer, made up to 7 ml (pH 5.1) and incubated at 30°C for four days. The decolorization degree of melanoids was expressed as the decreasing ratio of the absorbance at 470 nm before and after incubation.

**Hydrogen peroxide treatment.** In order to determine the optimal decolorization conditions for melanoids by hydrogen peroxide, the influence of hydrogen peroxide concentration, effects of pH and temperature were investigated. Moreover, hydrogen peroxide treatment to investigate the degradation products was carried out by the method described below. Two grams of melanoids were dissolved in 200 ml of 33.6% hydrogen peroxide (Mitsubishi Gas Kagaku Co., Ltd.) and the solution was adjusted to pH 7.0 or 10.0 with HCl or NaOH, made up to 1 liter and incubated at 37°C for 28 hr. Residual hydrogen peroxide was decomposed by manganese dioxide. The amount of hydrogen peroxide was determined by iodo metric titration.

**Fractionation, characterization and determination of degradation products.** The reaction mixtures of melanoids treated with hydrogen peroxide were fractionated into an aqueous fraction and an ether-soluble fraction. The ether-soluble fraction was fractionated into neutral, basic and acidic fractions according to the method described in the previous paper. Acids in the acidic fraction were methylated with diazomethane. Fifty micrograms and 100 μg of H-tridecane as internal standard was added to the neutral and acidic fractions, respectively. Low molecular substances in the ether-soluble fraction were identified and determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

One part of the aqueous fraction was applied to a Sephadex G-50 column (2.2 x 48 cm) and developed with 0.1 M NaCl containing 1 mM sodium azide at a flow rate of 4 ml/hr. Molecular weight was estimated by calibration of the elution volume corresponding to a maximum peak of sample against that of the molecular markers for pullulans (Shodex, P-5, MW = 5300; P-10, MW = 12000) and stachyose. The other part of the aqueous fraction was dialyzed against distilled and deionized water for three days, and the nondialyzable fraction was applied on SDS-polyacrylamide gel electrophoresis. Ninhydrin positive compounds in the dialyzable fraction were identified and determined using a TLC (Avicel SF, Funacosi Co., Ltd.), 13CNMR spectrometer (Jeol FX-100) and Amino acid Analyzer (Hitachi 835 type Amino acid Analyzer). TLC was developed by the solvent system, butanol-acetic acid-water, 4:1:2.

**Gas chromatography (GC).** Each ether-soluble fraction obtained by hydrogen peroxide treatment of melanoids was analyzed with a Shimadzu Model 7A Gas Chromatograph equipped with a flame thermionic detector (FTD). A fused silica WCOT capillary column (50 m x 0.25 mm i.d.) coated with PEG-20 M was used. The column oven temperature was programmed from 60°C to 190°C at a rate of 4°C/min. The injection port and detector temperature were kept at 200°C. Nitrogen was used as the carrier gas at a flow rate of 1.5 ml/min with a split ratio of 1:25. Measurement of the gas chromatographic peak area was determined by a Shimadzu Model Chromatopac E1-B integrator connected with the gas chromatograph.

**Gas chromatography-mass spectrometry (GC-MS).** GC-MS spectra were analyzed with a JEOL JMS-DX-300 Mass Spectrometer. Ionization voltage was 70 eV and ion source temperature was kept at 220°C. A fused silica WCOT capillary column (50 m x 0.25 mm i.d.) coated with PEG-20 M was used and the column oven temperature was programmed from 60°C to 170°C at a rate of 2°C/min and injection port temperature was 170°C. Helium was used as carrier gas at a flow rate of 1.0 ml/min with a split ratio of 1:50.

**SDS-polyacrylamide gel electrophoresis.** Disc SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn. The electrophoresis was performed at pH 7.2 using 0.1 M sodium phosphate buffer in the presence of 0.1% SDS at 8 mA per gel (0.5 x 8 cm) for 3 hr, 10% acrylamide gel being used. After electrophoresis, each gel was removed from the tubes and the relative intensity of bands on polyacrylamide gel was determined by scanning at 450 nm using a Toyo DMU-33C densitometer.

**RESULTS**

**Decolorization by glucose oxidase**

Melanoids were incubated with glucose oxidase under optimal conditions. Table I shows that the enzymatic treatment has caused the decolorization of melanoidin pigment. The melanoids, however, were not decolorized in

**Table I. Decolorization of Melanoids by Glucose Oxidase**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Decolorization degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidase</td>
<td>Glucose</td>
<td>65.0</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

Glucose oxidase, melanoids and glucose were allowed to incubate in 0.1 M sodium acetate buffer (pH 5.1) at 30°C for four days.

Decolorization degree was indicated by the measurement of the absorbance at 470 nm.
Decolorization and Degradation of Melanoidin by Hydrogen Peroxide

6mg of melanoidins and 7ml of each concentration of hydrogen peroxide were allowed to incubate at 25°C for one week.

the absence of glucose which is the substrate of glucose oxidase.

Determination of the optimal decolorization conditions for melanoidins by hydrogen peroxide

(1) Influence of hydrogen peroxide concentration. Figure 1 shows the decolorization degree of melanoidins on each concentration of hydrogen peroxide. Decolorization degree of melanoidins reached about 75 and 90% in the presence of 0.45 and 6.72% hydrogen peroxide, respectively. The decolorization degree increased only slightly by the addition of hydrogen peroxide over 6.72%. Accordingly, the optimal concentration of hydrogen peroxide was determined as 6.72%.

(2) Effect of pH. Figure 2 shows the decolorization degree of melanoidins using 6.72% hydrogen peroxide in various pH ranges from pH 3 to pH 13. Decolorization of melanoidins on the alkaline side proceeded more rapidly than that on the acidic side and neutral, and it reached 94% at pH 10. The decolorization degree was found to be dependent on pH.

(3) Effect of temperature. Figure 3 shows the decolorization degree of melanoidins by 6.72% hydrogen peroxide treatment under neutral (pH 7.0) and alkaline (pH 10.0) conditions at 37°C or 90°C, respectively. Decolorization degree was predominantly influenced by the difference of temperature under neutral condition, but the differences were small under alkaline conditions in comparison with neutral conditions. A high decolorization degree was obtained by more mild reaction using hydrogen peroxide under alkaline rather than neutral conditions.
(4) The relationship between decolorization of melanoids and consumption of hydrogen peroxide. Figures 4 and 5 show the relationship between the decolorization degree of melanoids and the consumption of hydrogen peroxide under neutral and alkaline conditions, respectively. Melanoids were decolorized to 50% after 16 hr under neutral condition and 43 mg of hydrogen peroxide were consumed. Under alkaline condition, the decolorization degree of melanoids reached 87% after 1.7 hr and the consumption of hydrogen peroxide at that time was 620 mg.

Degradation products of melanoids treated with hydrogen peroxide
(1) The changes in molecular weight and the yield of each fraction. Nondialyzable melanoids were decolorized and decomposed with 6.72% alkaline hydrogen peroxide at 37°C for 28 hr and then the reaction mixtures were fractionated into the ether-soluble and aqueous fractions using ethyl ether. The aqueous fraction was separated into nondialyzable and dialyzable fractions. The weight percent of the nondialyzable and dialyzable fractions per melanoids was 47.8 and 51.0%, respectively. In order to investigate the change of molecular weight of melanoids with hydrogen peroxide treatment, gel filtration by Sephadex G-50 and SDS-PAG electrophoresis were performed. It became clear that the mean molecular weight of melanoids decreased with hydrogen peroxide treatment as shown in Figs. 6 and 7. From the results of gel filtration, it was estimated that the mean molecular weight of melanoids decreased from 5300 to 3500 by hydrogen peroxide treatment.
Decolorization and Degradation of Melanoidin by Hydrogen Peroxide

**TABLE II. THE MAIN COMPOUNDS OF THE NEUTRAL FRACTION OBTAINED FROM ETHER EXTRACTS OF MELANOIDINS TREATED WITH ALKALINE HYDROGEN PEROXIDE**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Peak No.</th>
<th>Ratio to IS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methyl-5-formylfuran</td>
<td>7</td>
<td>0.63</td>
</tr>
<tr>
<td>2-Methyl-2,4-pentanediol</td>
<td>8</td>
<td>69.30</td>
</tr>
<tr>
<td>N,N-Dimethylacetamide</td>
<td>9</td>
<td>5.96</td>
</tr>
<tr>
<td>Phenol</td>
<td>13</td>
<td>4.47</td>
</tr>
<tr>
<td>Unknown</td>
<td>18</td>
<td>2.67</td>
</tr>
</tbody>
</table>

* IS: Internal Standard.

**TABLE III. THE MAIN COMPOUNDS OF THE ACIDIC FRACTION OBTAINED FROM ETHER EXTRACTS OF MELANOIDINS TREATED WITH ALKALINE HYDROGEN PEROXIDE**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Peak No.</th>
<th>Ratio to IS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td></td>
<td>(239.84)</td>
</tr>
<tr>
<td>2,3-Epoxybutanoic acid</td>
<td>6</td>
<td>1.28</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>7</td>
<td>2.02</td>
</tr>
<tr>
<td>Methylpropanedioic acid</td>
<td>8</td>
<td>1.54</td>
</tr>
<tr>
<td>Propanedioic acid</td>
<td>9</td>
<td>3.97</td>
</tr>
<tr>
<td>2-Furancarboxylic acid</td>
<td>11</td>
<td>2.79</td>
</tr>
<tr>
<td>Butanedioic acid</td>
<td>13</td>
<td>7.64</td>
</tr>
<tr>
<td>2-Hydroxypropanoic acid</td>
<td>14</td>
<td>5.66</td>
</tr>
<tr>
<td>2,5-Furandicarboxylic acid</td>
<td>21</td>
<td>2.25</td>
</tr>
<tr>
<td>5-(Hydroxymethyl)-2-furancarboxylic acid</td>
<td>22</td>
<td>2.54</td>
</tr>
</tbody>
</table>

* IS: Internal Standard.

The value in parenthesis indicates “not methylated.”

On the other hand, the ether soluble fraction was fractionated into neutral, basic and acidic fractions. The yield of neutral, basic and acidic fractions was 0.1%, trace and 1.1% per melanoidins, respectively.

(2) Ether soluble fraction. Figures 8 and 9 show the gas chromatograms of the neutral fraction and acidic fraction methylated with diazomethane and the major compounds identified and their relative amounts are shown in Tables II and III. Butylated hydroxy toluene (BHT) is a contaminant originated from ether used as the solvent. N,N-Dimethylacetamide was also confirmed by FTD. In the neutral fraction, 2-methyl-2,4-pentanediol was the most abundant component, while in the acidic fraction acetic acid was the main component as shown in Table III. The other major peaks on the gas chromatograms of the acidic fraction were identified as propanedioic acid (peak 9), butanedioic acid (peak 13), 2-hydroxypropanoic acid (peak 14) and so on.

(3) Aqueous fraction. The dialyzable fraction was concentrated and analyzed by TLC. In TLC, the main spot (Rf/0.24) gave a positive reaction with ninhydrin reagent. The main spot was collected by preparative TLC and its $^{13}$CNMR spectrum ($\delta_{\text{TMS}}$ ppm: 1.45, 2.15) was measured. From these results, the main spot was identified as glycine. The quantity of glycine was calculated by amino acid analyzer. Glycine was produced in 1.73% yield per nondialyzable melanoidin by hydrogen peroxide treatment of melanoidins.

**DISCUSSION**

Melanoidins were reported to be decolorized by sorbose oxidase purified from *Coriolus* sp. No.20. In the present experiment, melanoidins were predominantly decolorized by glucose oxidase (Table I), but when glucose as substrate of glucose oxidase was not used, melanoidins were not decolorized. From these findings, melanoidins are suggested to be decolorized by the active oxygens such as hydrogen peroxide which is secondarily produced by the enzymatic oxidation reaction from glucose, used as substrate, to gluconic acid. Accordingly, we investigated the optimal conditions for the decolorization and degradation of melanoidins using hydrogen peroxide. The decolorization and degradation of melanoidins were considerably affected by pH of the reaction mixture and took place markedly in the alkaline pH region rather than in the acidic and neutral pH regions (Figs. 2 ~ 5).

Generally, hydrogen peroxide reacts with the hydroxyl anion to give mainly perhydroxy anion (HOO$^-$) which has a strong nucleophilic activity. The perhydroxy anion is considered to attack nucleophilically the carbonyl groups in melanoidins. The findings that both decolorization and degradation of melanoidins occur markedly in the alkaline medium is due to the abundant presence of the perhydroxyl...
anion. The presence of metals (Fe, Cu etc.) can induce an autodegradation of hydrogen peroxide to form the free radicals such as $\cdot OH$, $\cdot OOH$ and $\cdot OO^-$, which may positively react with melanoidins. It is, however, well known that melanoidin itself is capable of producing reductones and forming chelates with metalic ions. Consequently, it is suggested that very small amounts of metallic ions existing in the present reaction system interact with melanoidins and radical reactions due to hydrogen peroxide may be suppressed by the formation of the melanoidin–metal complex.

In the ether-soluble fraction obtained from melanoidins which were treated with alkaline hydrogen peroxide, the yield of acidic compounds was ten-fold that of neutral compounds. A part of the chemical structure of melanoidin is postulated below from compounds identified in the present investigation. C-terminal structures originated from glucose existing in melanoidins are suggested as follows: $\text{CH}_3\text{CO-}R$ (I), $\text{CH}_3\text{C(H or OH)}=\text{C(H or OH)-CO-}R'$ (II), $\text{R-CO-}CO-\text{R'}$ (III), $\text{R-CO-CH(CH}_3\text{)-CO-}R'$ (IV), $\text{R-CO-CH}_2\text{CO-}R'$ (V), $\text{R-CO-CH}_2\text{CH}_2\text{-CO-}R'$ (VI), $\text{CH}_3\text{-CH(OH)-CO-}R$ (VII) and so on. From the results in which acetic acid was a major product in acidic fraction, compound I is estimated to exist for the most part in melanoidins. Nyhammar et al. investigated the formation mechanisms of methylpyrrol-2-yl-ketone and 2-methyl-3-pyrinol obtained from a (1-13C)-D-glucose-glycine reaction, and confirmed that the methyl group of the above Strecker degradation compounds is derived from the C-6 position of glucose. Acetic acid has been identified as the major volatile component in the pyrolyzate of melanoidins prepared from the glycine–glucose system, Amadori compounds and glucose.

In addition, it appears that branched chains such as compound IV and compounds containing a furan ring also exist. The presence of carbon branched chains is supported by the formation of 2-methyl-2,4-pentanediol identified in the neutral fraction. The formation of $N,N$-dimethylacetamide in the neutral fraction suggests that a part of the linkage of the nitrogen atoms in the melanoidin skeleton is a tertiary amine type.

On the other hand, glycine identified in the aqueous fraction is considered to have a configuration susceptible to liberation by hydrogen peroxide treatment. A recent study using N-15 labeled melanoidins, revealed that the nitrogen in the melanoidins was mainly in the secondary amide type. In the present investigation, glycine seems to be formed by the cleavage of amide type linkages in melanoidins by hydrogen peroxide.

Furthermore, the decrease of the mean molecular weight of melanoidins was shown by the gel filtration of aqueous fraction (Fig. 6). The decrease of mean molecular weight is supposed to result from the cleavage of the C–CO bond, NH–CO bond etc. in melanoidins. Other approaches, however, are also needed for further characterization of the melanoidin structure. We are now studying the decolorization and degradation of the melanoidins by ozone.

REFERENCES

1) G. T. Felbeck, Jr., _Adv. in Agronomy_, 17, 328 (1965).


