Separation of Model Melanoidin into Components with Copper Chelating Sepharose 6B Column Chromatography and Comparison of Chelating Activity

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Nondialyzable model melanoidin prepared from glucose-glycine was separated into 6 components by pH gradient elution on a copper chelated Sepharose 6B column. The separated components were measured for copper chelating activity, UV-VIS spectrophotometry, and electrofocusing. The UV-VIS spectra of these components were found to be almost the same, but the melanoidin components with stronger affinity to the Cu column were found to be smaller in *K_d*.

Their electrofocusing profiles were similar, and the electrofocused bands were categorized into four major groups with pI 2.5-4.0. Major chelating activity of the melanoidin components was due to a band of pI 2.7 on the electrofocusing.

Melanoidin is a colored compound generally found in food in a minute quantity, and it is formed during food processing and preservation through amino-carbonyl reactions. The metabolic fate of these food melanoidins in animals is not known. In general separation of food melanoidin from other food constituents is so difficult that chemical and biological studies on melanoidin have been done on model melanoidin.

Nondialyzable model melanoidin prepared in a glucose-¹⁵N-glycine model system, was found to consist of more than 14 components on electrofocusing gel with pIs from 2.8 to 3.4.¹⁾ When this ¹⁵N-melanoidin was fed to rats, 78% of the ¹⁵N was excreted in feces and 2% was in urine,²⁾ and a part of ¹⁵N-melanoidin was metabolized into urine.³⁾ Compared with the original melanoidin, the melanoidin excreted in feces consisted of less pI and lower molecular weight components according to electrofocusing and gel permeation chromatography, respectively.¹⁻³⁾

Melanoidin in foods has been characterized by molecular weight distribution, reducing activity, spectrophotometric property, and electrofocusing profile.⁴⁻⁶⁾ It has been ex-

pected that another method to characterize melanoidin will be developed. It has been reported that model melanoidin and food melanoidin were found to have metal chelating activity,⁷⁻⁹⁾ which might retard the availability of trace elements in animals and autoxidation of lipids in food systems. We have tried to make use of chelation of melanoidin in the separation and characterization of melanoidin.

The purposes of this study are: (1) to separate nondialyzable model melanoidin into components by copper(II)-chelating Sepharose 6B column chromatography; and (2) to compare chelating activity of separated melanoidin components.

Materials and Methods

Preparation of nondialyzable melanoidin. An aqueous solution of 2 M D-glucose, 2 M glycine, and 0.2 M NaHCO₃ was refluxed on heating for 2 hr, with stirring on a hot plate. The resulting brown solution was rapidly cooled in an ice bath followed by dialysis using Amicon membrane filtration system and lyophilization. The membrane filtration was done in a 4°C cold room with a 350-ml cell using a P.M. 10 membrane of *M_w* 10,000 cut off. The total amount of dialyzing water was 3 l. The yield against the weight of the starting material (D-glucose plus glycine) was

3.74%. The nitrogen content of the melanoidin was 6.81%.

Fractionation of nondialyzable melanoidin by Cu-chelating Sepharose 6B column chromatography. A chelating Sepharose 6B column (3.5 × 10 cm) (Pharmacia Fine Chemicals), was filled with about 50 ml of 0.1 M CuSO₄ solution and then eluted with 250 ml of water. The resulting copper(II) chelating Sepharose 6B column was equilibrated with 300 ml of pH 7.65 phosphate buffer (0.05 M) containing 0.5 M NaCl. Nondialyzable model melanoidin (280 mg) was dissolved in 2 ml of pH 7.65 phosphate buffer (0.05 M), and 1 ml of the sample was applied on the Cu(II)-chelating Sepharose 6B column, which was eluted with 280 ml of the pH 7.65 phosphate buffer. One milliliter of the sample was put again on the column, and 280 ml of the pH 7.65 phosphate buffer was eluted. Since the application of excess sample volume disturbed the formation of a sharp brown band adsorbed on the top of the column, the melanoidin solution was repeatedly put on the column by small portions. The elution was done at a flow rate of 3.20 ml/min by gradient pH change from 7.65 to 5.5. All the eluant contained 0.5 M NaCl. All the chromatographic performances such as sample charge, elution, gradient pH change and detection were done with a Pharmacia LCC-500 Fast Performance Liquid Chromatograph. Elution was monitored by absorbance at 280 nm and pH, and the eluate was collected into 16-ml fractions. The eluants for gradient pH change were prepared by a gradient mixture of the following two buffers:

For pH 7.65–6.5, 400 ml of a gradient formed by mixture of pH 7.65 phosphate buffer (0.05 M KH₂PO₄–Na₂HPO₄/0.5 M NaCl) and pH 5.5 acetate buffer (0.05 M CH₃COONa–CH₃COOH/0.5 M NaCl); for pH 6.5–5.5, 718 ml of a gradient formed by mixture of pH 5.5 acetate buffer (0.05 M CH₃COONa–CH₃COOH/0.5 M NaCl) and 0.05 M acetic acid/0.5 M NaCl; finally 100 ml of 0.05 M EDTA/0.5 M NaCl.

The fractions of 6 peaks detected by absorbance at 280 nm were collected and dialyzed by the Amicon membrane filtration system, and then lyophilized.

Absorption spectra of melanoidin. The fractionated melanoidin components were dissolved in pH 7.65 phosphate buffer (0.05 M) and their ultraviolet and visible range absorption spectra were measured with a Hitachi 200-20 spectrophotometer.

Gel filtration chromatography of melanoidin with copper ion. Twenty milligrams of melanoidin fractionated on a Cu(II)-chelating Sepharose 6B column was dissolved in 1 ml of pH 4.0 acetate buffer (0.01 M) containing 0.05 mM CuSO₄. Gel filtration chromatography was done with a Hitachi 635 high performance liquid chromatograph (HPLC) using a TSK G-3000PWXL column (7.5 × 300 mm, Tosoh, Japan) connected to a precolumn of TSK PWX (7.5 × 75 mm, Tosoh, Japan). The elution was done

at a flow rate of 1.0 ml/min with pH 4.0 acetate buffer (0.01 M) containing 0.05 mM CuSO₄ and was monitored by absorbance at 500 nm. The eluate was collected into 5-ml fractions, the copper concentrations of which were measured by atomic absorption spectrophotometry (Shimadzu AA-670) without ashing.

Measurement of binding activity of copper. The measurement of binding activity for copper ion was done with some modifications by the TMM (tetramethylmurexide; see Fig. 4) method of T. Asakura *et al.*⁹⁾ CuSO₄ (0–400 μM), samples (100 μg/ml) and TMM (1 mM or 5 mM) were respectively dissolved in pH 5.0, 10 mM hexamine–HCl buffer containing 10 mM KCl. To 2 ml of a solution consisting of 1.0 ml CuSO₄ and 1.0 ml of sample was added 0.1 ml of TMM, and the absorbance at 460 nm and 530 nm of the reaction mixtures (2.1 ml) were measured. The total copper content in fractionated melanoidin samples was measured by atomic absorption spectrophotometry and the percentage of bound copper was calculated.

Measurement of molecular weight of melanoidin. The molecular weight of the intact and the fractionated melanoidins were measured by HPLC using a TSK G-3000PWXL column (7.5 × 300 mm, Tosoh, Japan) connected to a precolumn of TSK PWX (7.5 × 75 mm, Tosoh, Japan). The eluant was pH 4.0 acetate buffer (0.01 M CH₃COONa–CH₃COOH) to contain 0.02 mM CuSO₄ and the flow rate was 1.0 ml/min. The eluate was detected by absorbance at 500 nm and RI (Shodex RI SE-11) using polyethylenoxide (TSK standard) as the standard for molecular weight.

Preparative flat-bed electrofocusing in a granulated gel. A granulated gel of Sephadex G-75 was washed with water, dried, and used as the stabilizing medium in flat-bed electrofocusing. The experimental procedure followed our previous report¹⁾ and the manual of the LKB 2117.¹⁰⁾ The gel slurry consisted of 5 g of Sephadex G-75, 5 ml of pH 2.5–4 carrier ampholine (LKB 1809-106, 20% w/v), and 95 ml of water. The size of the gel bed for electrofocusing was 10 × 20 cm. Twenty milligrams of the fractionated melanoidin component was dissolved in 1 ml of the 1% carrier ampholine solution, and 100-μl samples of the melanoidin solution were put on an area 3–5 cm from the cathode. The electrophoresis was done at a constant power of 5.0 watt for about 15 hr, and the initial and final voltages were 210 and 360 V, respectively. Finally, the voltage was raised to 750 V, which was maintained for 3 hr. The gel surface of each band was measured for pH by a combined pH electrode (SE-1600GC, Fuji Kagaku Keisoku, Japan), and the gel of each band was scraped off and transferred into a small column. Pigment in the scraped gel was eluted with pH 7.65 phosphate buffer (0.05 M). Each eluate was measured for copper concentration by atomic absorption spectrophotometry and for absorption spectrum in

ultraviolet and visible ranges.

Results and Discussion

Separation of melanoidin by copper chelating Sepharose 6B column chromatography

The fractionation of melanoidin by metal chelating Sepharose 6B column chromatography were tested by preliminary study, in which Cu(II), Fe(II), and Zn(II) ions were chelated to the column. The number of the fractionated melanoidin components detected at 405 nm was the largest in Cu(II) chelating Sepharose 6B; the zinc column was too weak to adsorb melanoidin, and the iron(II) column

gave two large peaks.

Nondialyzable melanoidin prepared from glucose and glycine was put on the Cu(II)-chelating Sepharose 6B column. As shown by the A_{280} elution profile in Fig. 1, the melanoidin was separated into 6 components. Recovery of the total melanoidin from the Cu(II)-chelating column was 52% after ultrafiltration and lyophilization.

The first component is considered to consist of melanoidin components that had no affinity for the Cu(II)-chelating column in pH 7.65 and/or stronger chelating activity to copper ion than the imino acetic acid group of the column. Components from 2 to 5 appeared

Fig. 1. Elution Profile of Melanoidin on Cu-chelating Sepharose 6B Column.

—, absorbance at 280 nm; ----, pH; ·····, concentration of Cu(II).

Table I. CHELATING ACTIVITY OF MELANOIDIN COMPONENTS FRACTIONATED BY Cu(II)-CHELATING SEPHAROSE 6B COLUMN CHROMATOGRAPHY

Yield (%)	$E_{500\text{nm}}^{1\%}$	Content of Cu ($\mu\text{g Cu/mg melanoidin}$)	Chelating activity		TMM method		M_w	Molecule/Cu	
			Bound Cu ($\mu\text{g Cu/mg melanoidin}$)						
			Positive peak	Negative peak	Kd (μM)	n			
Melanoidin	100	30.0	0.5	12.1	12.8	4.0	7	12,000	1700
Component 1	34.6	28.8	17.8	21.5	12.1	5.6	12	15,000	1300
Component 2	8.4	29.2	6.9	13.5	15.1	3.4	15	22,000	1500
Component 3	2.7	31.0	5.9	13.8	15.8	3.2	21	32,000	1500
Component 4	2.6	31.6	5.2	14.5	15.1	2.7	19	30,000	1600
Component 5	3.4	35.0	6.0	12.8	14.3	1.2	18	31,000	1700
Component 6	0.6	32.6	1.9	13.0	15.4	—	—	—	—
Total	52.3								

during the gradient pH fall, and a final component appeared accompanying Cu(II) ion with EDTA (Fig. 1). The chromatogram shows that each melanoidin component was eluted from the column accompanying with copper ion and that these melanoidin components had stronger chelating activity for Cu(II) ion than the imino acetic acid group on Sepharose 6B under eluting pH conditions.

After dialysis and lyophilization of these melanoidin components, the copper bound to these components was measured, and is shown in Table I. The smallest level of copper bound to component 6 was thought to be due to the ultrafiltration procedure to liberate Cu(II) ion with EDTA from Cu(II)-melanoidin complex in the final eluant.

Spectra of melanoidin components

Figure 2 shows UV-VIS absorption spectra of melanoidin components 1-6. The spectrum of each component had a shoulder around 320nm, and showed similar patterns of a general absorption to melanoidin. $E_{500nm}^{1\%}$ of each component is shown in Table I. The pigment intensity of these components was larger as they were eluted with lower pH eluants from the column.

Amount of chelated copper with melanoidin components

Chelating activity of the melanoidin compo-

Fig. 2. UV-VIS Absorption Spectra of the Melanoidin Components Fractionated by Cu-chelating Sepharose 6B Column Chromatography.

Sample concentration: 0.05 mg/ml pH 7.65 phosphate buffer (0.05 M).

Fig. 3. Chromatographic Pattern of Melanoidin on HPLC.

—, absorbance; —○—, concentration of Cu(II). Eluant was pH 4.0, 10 mM acetate buffer with 0.05 mM CuSO_4 .

nents towards Cu(II) was measured by HPLC method.¹¹⁾ Original melanoidin and components 1–5 had similar chromatograms. The typical HPLC pattern of the melanoidin is shown in Fig. 3. This HPLC method involves the equilibration of a gel filtration column with a pH 4 acetate buffer containing Cu(II). The chromatogram shows a melanoidin–Cu(II) complex peak at the retention volume of melanoidin followed by a negative peak. The amount of bound Cu(II) was estimated by the negative peak area of HPLC and found to be almost the same level for the melanoidin components (Table I) except for the component 1. The amounts of chelated Cu(II) ion calculated by positive and negative peaks were not always similar. When the melanoidin sample was dissolved in the elution buffer containing 0.05 mM copper ion, an insoluble compound was not formed, and the samples were directly injected into the column. But when the positive peak area was smaller than the negative peak area, insoluble Cu(II)–melanoidin complex might be formed or Cu–melanoidin complex might be adsorbed to the column during the passage through the column equilibrated with the Cu(II)-containing buffer.

Measurement of the binding activity of copper by TMM method and Scatchard plot of the intact melanoidin and the fractionated melanoidin

As shown in Table I, the molecular weight of the fractionated melanoidin was larger than that of the intact melanoidin. It is speculated that the melanoidin was changed into larger

molecules through chelation of copper during the fractionation process. Since the recovery of melanoidin from the chelating Sepharose 6B column was 52.3% (Table I), the melanoidin components of molecular weight less than 15,000 might be adsorbed to the column.

We suggest either of the following two possibilities for the amount of the melanoidin not accounted for in the eluates: (a) the melanoidin components of molecular weight less than 15,000 were adsorbed to the column, and they were gradually eluted in small amounts without forming distinct peaks as shown by the slightly raised base lines of the chromatograms; (b) they were eluted with water after the elution of EDTA. The adsorbed melanoidin components hardly disturb the chromatography because the chromatographic patterns had been reproducible regardless of the repeated performances.

The TMM method (Fig. 4) was used for the measurement of Scatchard plot (Fig. 5) of the melanoidin component for copper(II). The melanoidin components with strong affinity to the Cu-column were found to be smaller in *K_d* and larger in molecular weight. The number of bound copper molecules per mole of melanoidin component was found to be a maximum of 21 for component 3 and to be reduced as the *K_d* of a component became larger or smaller. These results were given by the measurement of copper ion by the TMM method at pH 5.0, nevertheless, the results given by the HPLC method at pH 4.0 also show the maximum value of 8.0 moles of bound copper per molecule for component 3. The smaller

Fig. 4. Measurement of Bound Cu(II) by the Tetramethylmurexide.

TMM (1 or 5 mM), 0.1 ml was added to the mixture of 1 ml of CuSO₄ (0–400 μM) and 1 ml sample (100 μg/ml). The reaction medium was pH 5.0, 10 mM hexamine–HCl buffer containing 10 mM KCl.

value of bound copper molecule measured by the HPLC method than the TMM method seems to be mainly due to the lower pH and acetate concentration, which were a disadvantage for complex formation compared to the pH 5.0 in the TMM method.

Component 1 had an extremely large value of bound copper molecules in the positive peak compared to the negative peak. The value for the difference is mainly due to the copper derived from the Cu column and already connected with strong chelating sites in the molecule. The copper content in component 1, 17.8 $\mu\text{g}/\text{mg}$ melanoidin, supports this speculation.

The ratio of molecular weight of each melanoidin component to the number of bound copper molecules measured by the TMM method is also shown in Table I. It means the size of melanoidin residues to bind one copper molecule. The ratio increases as the K_d value decreases. These data imply that strong chelation of melanoidin for copper ion is

Fig. 5. Scatchard Plot of Binding of Cu(II) by Melanoidin.

r , μmoles bound Cu(II)/ μmole melanoidin; n , number of binding sites; K_d , dissociation constant.

Fig. 6. Electrofocusing of the Melanoidin Components Separated by Cu-chelating Sepharose 6B Column Chromatography.

0, original melanoidin; 1-6, component 1-6.

Table II. AMOUNT OF COPPER CHELATED IN MELANOIDIN COMPONENTS FRACTIONATED BY ELECTROFOCUSING OF pH 2.5–4 ($\mu\text{g Cu}/\text{OD}_{500}$)

Melanoidin band	Intact melanoidin	Component					
		1	2	3	4	5	6
A	0.536	0.241	0.409	—	0.200	1.25	23.6
B	—	0.292	0.473	0.0893	—	—	—
C	1.67	18.8	7.20	—	—	0.517	23.0
D	0.207	126.2	68.3	17.9	16.3	21.2	126.0
E	0.400	0.643	0.914	0.647	0.964	0.547	0.534

supported by a larger size of melanoidin residue. These results suggest that the repeating unit of melanoidin differs in each melanoidin component. We speculated that polymerization process involves some modifications of active groups in monomers such as reductone and that the number and affinity to copper ion of the modified active groups in high polymers are larger than in the low polymers.

The results suggest that the size of repeating unit in melanoidin molecule seems to be from 1300 to 1700.

Electrofocusing profile of melanoidin components

Fractionated melanoidins were analyzed by flat-bed electrofocusing of the pH range 2.5–4 and their electrofocusing profiles are shown in Fig. 6. All the melanoidins put on were electrophoresed from the starting position towards the anode, resulting in the formation of four major groups of bands. The number of electrofocused melanoidin bands were larger in the original melanoidin than in the fractionated melanoidin.

The electrofocusing profiles of the fractionated melanoidins was found to be similar, except that component 1 was found to contain more pigment of pI 2.3. The ratio of the unelectrophoresed pigment, which was retained in the charged area, increased as the melanoidin components were eluted later from the copper chelating Sepharose 6B. This result shows that the melanoidin components eluted later from the Cu(II)-chelating column seem to give many spots of pI around 3.5, therefore,

these samples were not electrophoresed from the charged area. Expanding pH range of the used carrier ampholine to pH 2.5–6.0, the residual melanoidin was electrophoresed from the charged area to the cathode.

The melanoidin bands on the electrofocusing gel were categorized into five groups and named A (pI 2.3), B (pI 2.5), C (pI 2.6), D (pI 2.7), and E (pI 3.4–3.6). Copper in each melanoidin group was measured, and the amount of copper chelated was shown by μg per pigment quantity, that is the absorbance units at 500 nm of extracted solution (Table II). Band D showed the largest quantity of copper and band B the smallest for every melanoidin component. Although, band D of the original melanoidin, band A of component 1, and band E of component 6 were apparently intense in color compared to other bands (Fig. 6), amounts of chelated Cu(II) of these bands were small. It follows that most parts of copper derived from the Cu-chelating Sepharose 6B column were bound to the melanoidin components electrofocused to the D group of which pI was 2.7.

Nondialyzable model melanoidin was separated into 6 components by a copper-chelating Sepharose 6B column chromatography. The order of their elution was correlated with $E_{500\text{nm}}^{1\%}$ except for component 6, and was reversely correlated with the K_d of the melanoidin components.

Electrofocusing profile and UV-VIS spectra of the intact and the fractionated melanoidins were found to be almost the same. Major chelating activity of the melanoidin compo-

nents is due to band D of which the pI is about 2.7. It is expected that we will investigate active groups involved in the chelation and raise the recovery of melanoidin from the copper column. These improvements would lead to the separation and characterization of melanoidin in food systems.

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References

- 1) S. Homma, T. Tomura and M. Fujimaki, *Agric. Biol. Chem.*, **46**, 1791 (1982).
- 2) S. Homma, Y. Endo and M. Fujimaki, *Agric. Biol. Chem.*, **53**, 1861 (1989).
- 3) M. Fujimaki, S. Homma, N. Arakawa and C. Inagaki, *Agric. Biol. Chem.*, **43**, 497 (1979).
- 4) T. Gomyo and M. Miura, *Nippon Eiyo Shokuryo Gakkaishi*, **36**, 331 (1983).
- 5) H. Kato, *Nippon Nōgeikagaku Kaishi*, **61**, 967 (1987).
- 6) Y. S. Lee, S. Homma and K. Aida, *Nippon Shokuhin Kogyo Gakkaishi*, **34**, 313 (1987).
- 7) T. Gomyo and M. Horikoshi, *Agric. Biol. Chem.*, **40**, 33 (1976).
- 8) J. A. Rendleman, Jr., *J. Food Sci.*, **52**, 1699 (1987).
- 9) T. Asakura, Y. Nakamura, N. Inoue, M. Murata and S. Homma, *Agric. Biol. Chem.*, **54**, 855 (1990).
- 10) A. Winter, H. Perlmutter and H. Davis, LKB Application Note, No. 198, Sept. 11, 1975.
- 11) B. Seville and C. N. Thuavd, *J. Chromatogr.*, **167**, 159 (1978).