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Investigation of the influence of reaction conditions on the elementary composition of melanoidins

B. Cämmerer & L. W. Kroh

Technical University Berlin, Institute of Food Chemistry, Invalidenstr. 42, D-10115 Berlin, Germany

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The elementary composition of melanoidins produced in solvent-free reaction mixtures differs, markedly, depending on whether pentoses (ribose) or hexoses (glucose, fructose) are used. In the case of ribose, more than 4 mol sugar are incorporated into the melanoidin per mol amino acid, whereas, under the same reaction conditions, only about half the number of moles for hexoses are involved. The fundamental composition of model melanoidins under constant reaction conditions is only negligibly influenced by the molar ratio of the reactants. Reaction conditions have a significant influence on the composition of the polymers. Depending on the reaction temperature in a solvent-free milieu, at least 2 mol glucose per mol amino acid are incorporated into the polymer. In aqueous solution this ratio decreases to less than 1:1. In agreement with results of the elementary composition of melanoidins, a new fundamental structure of the polymer which is formed by reaction of dicarbonyl compounds with one another or with amino components is proposed. The structure is supported by IR, UV and CP-MAS NMR studies.

INTRODUCTION

Melanoidins are well-known brown to black coloured final products and represent a visible indication of the non-enzymatic browning reaction of carbohydrates and amino compounds. They can be detected in food and biological material, underlining their importance for food science as well as for physiological and medical studies. The formation of this complex group of substances is not the result of any single reaction but comprises a set of consecutive and parallel chemical reactions taking place during the Maillard reaction that are easily influenced by the reaction milieu and the choice and nature of reactants. As a result, little is known about structure and chemical properties of melanoidins.

In some reports, attempts have been made to describe some aspects of melanoidin structure and general properties using ^{13}C- and ^{15}N-CP-MAS NMR-spectrometry, where the formation was monitored with the help of labelled amino acids and sugars (Barbetti & Chiappini, 1976; Kato & Tsuchida, 1981; Hayase *et al.*, 1986; Benzing-Purdie & Ripmeester, 1987; Ledl & Schleicher, 1990). In model melanoidins the C_2 atom of amino acid is commonly incorporated into the polymer as an enamin-C (Hayase *et al.*, 1986). On the other hand, ^{15}N NMR spectroscopy also indicates pyrrole, indole and amide groups. Furthermore, the sugar-C_1 can exist in a broad range of oxidation grades in the polymer (e.g. –CHOH, > C = C, $-CH_3$) (Benzing-Purdie & Ripmeester, 1987; Huang & Feather, 1988). Melanoidins are generally known to show groups which are similar to these of Amadori compounds; even unchanged amino acids have been identified in melanoidins.

In addition to the instrumental–analytical methods described above further characterisation using microanalysis could also make a contribution to the structural elucidation of model melanoidins. With increasing reaction time and temperature, the total carbon content increases at the cost of the nitrogen ratio, thus promoting the aromatic character and the unsaturation of the melanoidins (Benzing-Purdie *et al.*, 1985).

MATERIALS AND METHODS

Materials

All the reagents were of analytical grade: D-ribose, D-glucose, D-fructose, sucrose, lactose and maltose were purchased from Merck (E. Merck, Darmstadt, Germany); D,L-glycine and D,L-phenylalanine were purchased from Fluka (Fluka Chemie AG, Buchs, Switzerland).

Preparation of model melanoidins

Solvent-free system

Carbohydrate and amino acid were mixed in the molar ratio 1:1 and heated in a flat sheet for 10–40 min at 170 or 180°C. The solid product was ground in a mortar and dialysed.

Aqueous system

(a) A 0·1 M solution of carbohydrate and amino acid was heated for 10 h under reflux. The pH-value (5 or 7·3) was monitored during heating by a sterilisable electrode for measuring the pH-values and corrected to constant values by adding 0·1 M NaOH. The amount depends on the initial pH value and its alteration (10–20 ml during the total heating time).

(b) A 0·1 M aqueous solution of sugar and amino acid was heated for 160 h at 60°C and the pH value was held constant at 5 with 0·1 M NaOH (see above).

After the heat treatment the solutions were lyophilysed and the dry residue dialysed.

Dialysis

The dialysis tubing (Spectrum Medical Industries, Houston, USA) was made from cellulose with MWCO 12000–14000 Da and pore size 1·5–3·0 nm. Batch dialysis was performed using 5 g melanoidin in a 10-cm dialysis tubing in 1 litre of distilled water, with a change of water every 8–10 h. Total dialysis time was 136 h. After dialysis, the samples were freeze-dried.

HPLC

A Kontron (Kontron Instruments, Neufahrn, Germany) 325-gradient pump and a programmable photodiode array detector (DAD), model 440 with the data management system 450-DAD, were used. Water (distilled and filtered) was degassed and used as eluent at a flow rate of 2·0 ml/min and the pressure was maintained at 1200PSI. Separations were performed on a Nucleogel column (Nucleogel aqua-OH 40, 7·7 mm × 300 mm, Macherey-Nagel, Düren, Germany). Identification was conducted using a dioden array detector in the range 190–450 nm.

Microanalysis

The microanalysis was performed using a Analyzer CHNS 932 (Fa. Leco); the weight was about 1·5 mg.

IR spectroscopy

The IR spectra of the melanoidins were done on a Specord 71 IR (Fa. ZEISS) in pallets of KBr at similar weight concentrations.

RESULTS AND DISCUSSION

With the help of microanalysis data, it is possible to draw conclusions about the influence of the nature of reactants and milieu conditions on the chemical composition of the prepared melanoidins. Conclusions are

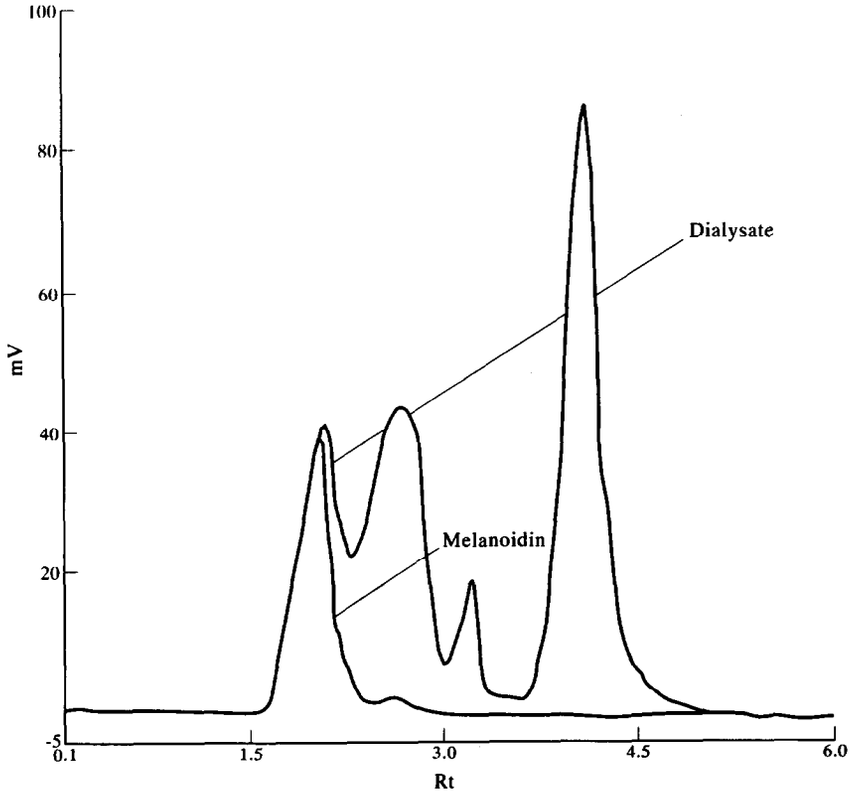

Fig. 1. HPLC-chromatograms of the dialysate (8 h) and the non-dialysable melanoidin obtained after dialysis (112 h). UV-detection: λ_{max}; i.e. generated from the highest absorbances in the selected spectral range. Melanoidin glucose/glycine (1:1); 10 h/100°C, pH 5.

applicable only to the soluble high molecular weight fraction of melanoidins. During the dialysis of all melanoidin preparations investigated, not only the low molecular weight fraction (as characterised by the retention time of HPLC) containing the unreacted sugar and amino acid, HMF and Amadori compound but also a part of the higher and also the highest molecular weight fraction were washed out. The amount depended on the length of dialysis time (Fig. 1.). As this is typical for the dialysis of compounds and unknown molecular distribution, it was not possible to apply this method to isolate and characterise the low molecular weight fraction of model melanoidins. Depending on the reactivity and the water/solubility of the reactants used, optimal dialysis conditions were characterised with the help of HPLC to prepare high-molecular-weight melanoidins for further investigations. The latter were practically free of starting materials and interfering melanoidin precursors.

For the evaluation of the microanalysis data of model melanoidins, a correlation method reported by Wedzicha and Kaputo (1992) was used, which allowed the calculation of the amount of carbonyl compound incorporated into the polymer per mol amino acid. Considering that the overall reaction for the formation of the melanoidin is a combination of a carbonyl molecules consisting of l, m and n atoms of C, H, and O, respectively, and b molecules of amino acid comprising p, q and r atoms of C, H and O, respectively, and one atom for N, then the formula of the melanoidin can be represented with $C_{la+pb-x}H_{ma+qb-2y}O_{na+rb-2s-y}H_b$, where x and y are the amounts of CO_2 or H_2O liberated. As a result of putting the respective numbers into and solving the equation, the amount of carbonyl compound incorporated per mol amino acid can be calculated, making a comparison of different melanoidins on the basis of one atom N possible.

At the beginning the amino acid (glycine) and the reaction conditions were maintained constant so that the influence of the nature of the carbonyl component on the chemical composition of melanoidins could be investigated (Table 1). The microanalysis data of melanoidins derived for pentoses (ribose) and for hexoses (glucose, fructose) differ considerably. In the case of ribose, more than 4 mol of sugar were incorporated into the melanoidin per mole of amino acid, whereas under the same reaction conditions 2 mol of hexoses were needed. There is also a difference between the ratio of nitrogen to carbon in the melanoidins of the two hexoses investigated. In the literature (Bobbio *et al.*, 1981), a decrease of the C:N ratio was reported for the formation of melanoidins in citric buffer, when the carbonyl compound used was changed from glucose to fructose. This can probably be explained by the different thermal fragment abilities of these two sugars which cause differences in the contribution of the two alternative mechanisms discussed in the literature as radicalic and ionic to the early stage of the Maillard reaction (Cämmerer & Kroh, 1995). The investigated disaccharides can be divided into two groups. Lactose and sucrose can be used to prepare melanoidins with a high nitrogen content. On the other hand, the behaviour of maltose is similar to that of glucose under the same reaction conditions. Two mol of sugars are incorporated here into the polymer per mol amino acid. Possibly the explanation for these differences in the molecular structure of melanoidins is to be found in the different reactivity of the various sugars towards the amino acids. It seems that maltose prefers a reaction involving the reducing hydroxyl-group, whereas sucrose and lactose have to be degraded to some extent into lower sugar and/or dicarbonyl units to produce melanoidins.

Of further interest is a comparison of microanalysis data of glucose/glycine model melanoidins produced with various molar ratios of reactants, as shown in Table 2. Even a significant excess of glycine (2:8) in the reaction system can hardly affect the molecular composition of the polymer. So it may be concluded that under the same reaction conditions the basic structure of melanoidins is only negligibly influenced by molar ratios of reactants (see also Wedzicha & Kaputo, 1992). In the presence of excess sugar, it is very likely that low-molecular-weight caramel products are produced, which are then removed by the following dialysis. Even the reaction products of amino acids (expected to bring a high nitrogen moiety into the polymer) remain without influence.

From the microanalysis data of melanoidins obtained under various milieu conditions it can be seen that the elementary composition of the polymers is significantly influenced by temperature and water activity (Table 3). Some general remarks have been reported on

Table 1. Microanalysis data (%) of non-dialysable melanoidins from D-carbohydrates/glycine (1:1) model systems[a]

Sugar	C	H	N	O	a[b]	y/a[c]
Ribose	50·28	6·28	2·41	41·03	4·74	1·69
Glucose	53·42	5·38	4·26	37·61	2·19	3·05
Fructose	47·39	6·42	5·00	41·19	1·53	3·54
Maltose	42·34	6·09	1·61	49·96	2·34	0·76
Lactose	42·03	5·41	4·37	48·19	0·71	2·31
Sucrose	42·90	5·53	5·80	45·77	0·52	2·98

[a]Reaction conditions: solvent free, 10 min, 180°C.
[b]Mol sugar (C_6- or $2C_3$-fragments) which is incorporated into the polymer per mol amino acid.
[c]Mol water which is liberated per mol sugar.

Table 2. Microanalysis data (%) of non-dialysable melanoidins from glucose/glycine model systems[a]

Molar ratio	C	H	N	O	a[b]	y/a[c]
8:2	50.32	6.19	4.15	39.34	2.16	2.31
1:1	53.42	5.39	4.26	37.61	2.19	3.05
2:8	47.81	6.21	3.50	42.48	2.43	1.92

[a]Reaction conditions: solvent free, 10 min, 180°C.
[b]Mol sugar (C_6- or $2C_3$-fragments) which is incorporated into the polymer per mol amino acid.
[c]Mol water which is liberated per mol sugar.

Table 3. Microanalysis data (%) of non-dialysable glucose/glycine (1:1) melanoidins produced under various reaction conditions

Conditions	C	H	N	O	a[a]	y/a[b]
170°C/20 min	53·42	4·38	4·26	37·61	2·19	3·05
100°C/10 h/pH 5	55·58	5·38	6·97	32·07	1·28	3·75
100°C/10 h/pH 7	49·05	5·25	6·12	39·57	1·20	3·10
90°C/22 h/pH 5[c]					1·06	2·96
60°C/160 h/pH 5	43·02	4·78	6·94	45·25	0·75	2·89

[a]Mol sugar (C_6- or 2 C_3-fragments) which is incorporated into the polymer per mol amino acid.
[b]Mol water which is liberated per mol sugar.
[c]Wedzicha & Kaputo (1992).

the influence of raising temperature on the aromatic properties of melanoidins (Benzing-Purdie & Ripmeester, 1985). The current investigations now allow some fundamental conclusions. In solvent-free milieu, depending on the reaction temperature, at least two mol glucose or the corresponding decomposition product are incorporated into the polymer per mol amino acid. By variation of reaction conditions, the nitrogen/carbon ratio of the melanoidins can be changed. Independent of the pH value, in boiling reaction mixtures (100°C) the ratio of amino to carbonyl compound falls to around 1:1. Under more gentle conditions (60°C), a slightly increased amount of amino acid will be incorporated. In all experiments carried out, three mol water were eliminated per mol carbonyl compound.

The results show that the structure of the brown polymers formed during the Maillard reaction can be influenced easily by changing the reaction conditions. This mean that it is difficult to determine the fundamental structure of the polymers and it cannot be assumed that the melanoidins have a regular composition with repeating units. Therefore, it is unlikely that the fundamental melanoidin structure suggested by Kato and Tsuchida (1981) has a general validity. Up to now, all structural proposals assume that the sugar and amino compound form the polymer in a 1:1 ratio on the basis of Amadori products. This appears not be in agreement with the results described above, especially for those investigations in solvent-free milieu.

The hypothetical structure proposed in Fig. 2 is based on the reactions of dicarbonyl compounds (in this proposal especially 3-desoxyhexosulose) which are proven as reactive intermediates of the Maillard reaction and caramelisation (Ledl & Schleicher, 1990; Kroh, 1994). A reaction among themselves (aldol reaction or nucleophilic addition) as well as a substitution reactions with amino compounds are possible. It is possible to explain a variation of the C/N ratio of model melanoidins caused by different reaction conditions without changes in this basic structure. Enamines of 1-amino-1-deoxyosuloses or decomposition products of diaminoketosyl- and diketosylamino-compounds (Kroh & Westphal, 1983) are easily inserted into this structure and, in the same way, it is also likely that the products of caramelisation are constituted in this manner. Since many reactive centres are present in the main chain and also in the side chain, the formation of furan bodies or *N*-heterocyclics such as pyrrolaldehyde (Kato *et al.*, 1981) can be explained by intramolecular cyclisation. Also decarboxylations and the elimination of water are possible, and the structure of the real melanoidins could be a result of many reactions in the basic framework.

In principle, the suggested structure could be confirmed by the interpretation of IR absorptions (Table 4). The spectra are typical of polymeric compounds with spreading bands. Nevertheless some characteristic absorptions are observed which allow an assignment to group frequencies. The position and the type of characteristic IR absorptions found for model melanoidins are not affected by the different reaction conditions used for their formation, although the microanalysis data differ to a large extent. Absorptions are in agreement with the general hypothetical melanoidin structure presented in this study.

The chemical structure of investigated melanoidins described above (Fig. 2) is also supported by the

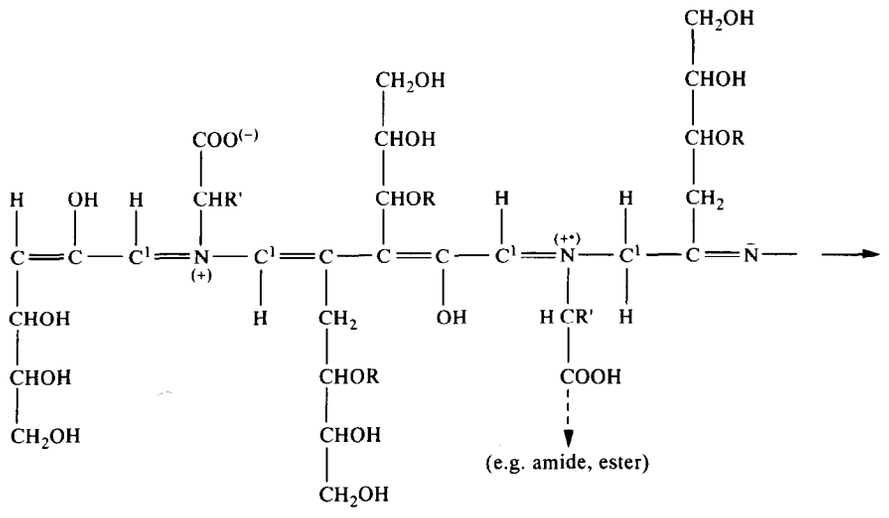

Fig. 2. Proposal for the general structure of the melanoidin polymer (from 3-desoxyhexosuloses and amino acids). R:H or saccharides. R': side chain of amino acid.

Table 4. Infrared absorptions of melanoidins from glucose and glycine under various reaction conditions and their assignments

Infrared absorptions, ν (cm^{-1})			
Aqueous (100°C, 10 h)	Solvent/free (170°C, 20 min)	Intensities	Assignment
3340	3420	s	–O–H; –N–H stretching
2920	1930	m	–C–H (aliphatic) stretching
1700	1710	m (sh)	>C=O stretching
1615	1630	s	>C=N–; >C=C< stretching
1420	1420	m (sh)	–C-H deformation
1375	1380	m	–O–H deformation
1215	1200	m	–C–O; –C–N– stretching
1050	1020	m (broad)	–C–O; –C–N–; –C–C– stretching

observed signals in the ^{13}C-CP-MAS NMR spectra (Engelke *et al.*, 1994). In ^{15}N-CP-MAS NMR investigations the –N=C– or $>N^{+}$=C– group (100–130 ppm) is postulated to be the main structural element which may be predominant for the incorporation of nitrogen into the melanoidin polymer. On the other hand, there are some signals found with lower intensities of secondary amides and also of secondary and tertiary amines (30–90 ppm) corresponding with the literature (Hayase *et al.*, 1986). Further investigations include those by ^{1}H and ^{13}C NMR (600MHz) spectroscopy to characterize the composition of high molecular weight melanoidins obtained by controlled dialysis.

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