Studies on the Relationship between Molecular Weight and the Color Potency of Fractions Obtained by Thermal Treatment of Glucose/Amino Acid and Glucose/Protein Solutions by Using Ultracentrifugation and Color Dilution Techniques

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In heated aqueous solutions of glucose and alanine or glycine, respectively, the majority of colored compounds formed were shown to have molecular weights <1000 Da. Compounds with molecular weights >3000 Da were found in only trace amounts, whereas high molecular colorants could not be observed under the conditions applied, which are typical for cooking processes of foods. In contrast, the reaction between casein and glucose leads to a drastic increase in the molecular weights by a carbohydrate-induced oligomerization of the protein. More than 43% of the reaction mixture was shown to be pentamers or even higher oligomers of casein exhibiting molecular weights of >100000 Da. This cross-linking of casein was found to run in parallel with the color intensity of the products formed, indicating that chromophoric substructures, derived from carbohydrates, are incorporated into these oligomers. The data indicate that the formation of melanoidins during cooking of foods by polymerization of reactive low molecular weight compounds should be very unlikely in the Maillard reaction between carbohydrates and amino acids.

Keywords: *Melanoidins; Maillard reaction; protein oligomerization; nonenzymatic browning; protein cross-linking; color dilution factor*

INTRODUCTION

The Maillard reaction between reducing carbohydrates and compounds bearing an amino group is chiefly responsible for the development of the brown color that occurs during the thermal processing of foods. Depending on their molecular weight, the colored components affecting this nonenzymatic browning may be divided into two classes, namely, the low molecular weight colored compounds, consisting of up to four linked rings with molecular weights <1000 Da (Severin and Krönig, 1972; Nursten and O'Reilly, 1986; Arnoldi et al., 1997; Hofmann, 1997a,b, 1998a), and the melanoidins, which are assumed to be water-soluble, high molecular weight colored compounds with masses up to 100000 Da.

A number of chromatographical and electrophoretical attempts have been undertaken to isolate and to purify melanoidins from foods, for example, from coffee (Maier et al., 1968; Maier and Buttle, 1973; Steinhart et al., 1989), soy sauce (Hashiba, 1973), malt (Obretenov et al., 1991), and dark beer (Kuntcheva and Obretenov, 1996). Despite the high amount of melanoidins in processed foods, for example, 28% in coffee malt (Obretenov et al., 1991), it has as yet not been possible to isolate and characterize a pure melanoidin.

Extensive studies on melanoidins prepared in model reactions have been, therefore, performed to clarify the chemical species responsible for the typical brown color; however, the findings are very heterogeneous. The most common hypothesis discussed, is that high molecular weight colored structures were formed by polymerization of low molecular weight intermediates from the Maillard reaction between carbohydrates and amino acids. Most model reactions were, therefore, performed with carbohydrates and amines or amino acids. The browing reaction between glucose and glycine was especially extensively investigated, for example, by Maillard (1912, 1916), McWeeny et al. (1969), Gomyo et al. (1972), Motai (1974), Motai and Inoue (1974), Hayase and Kato (1981), Homma et al. (1982), Hayase et al. (1984), Kim et al. (1985), Kim and Park (1986), Kato et al. (1968, 1969, 1986, 1987), Obretenov et al. (1986), Wedzicha and Vakalis (1988), Taguchi and Sampei (1986), Feather and Nelson (1984), Benzing-Purdie et al. (1983), Nam and Kim (1984), Nursten and O'Reilly (1986), Wedzicha and Kaputo (1987, 1992), and recently by Cämmerer and Kroh (1995).

Despite extensive studies, the data reported on the structural characteristics of melanoidins are very contradictory; for example, furanoyl structures (Heyns and Hauber, 1970), Schiff bases of 3-deoxyosones (Kato and Tsuchida, 1981), and enamines of 1-amino-1-deoxyosuloses (Cämmerer and Kroh, 1995) were assumed as the repeating unit in melanoidins.

Due to the complexity of the melanoidins, another, more synthetic approach to clarify the mechanisms of melanoidin formation is to study the polymerization of certain low molecular weight Maillard intermediates under controlled conditions. In a very recent work, Tressl et al. (1998) investigated the reaction of 2-deoxy-D-ribose with methylamine and identified *N*-methyl-2-(hydroxymethyl)pyrrole, the corresponding dimeric bis(*N*-methyl-2-pyrrolyl)methane, as well as the trimeric *N*-methyl-2,5-bis(*N*-methyl-2-pyrrolylmethyl)pyrrole as minor reaction products. Synthetic studies in combination with MALDI-TOF-MS analysis revealed an oligo-

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merization of the *N*-methyl-2-(hydroxymethyl)pyrrole monomer up to six units.

In addition to the nonenzymatic browning of carbohydrates and amino acids, the reaction between carbohydrates and proteins is also known to result in color formation (Hannan and Lea, 1952; Clark and Tannenbaum, 1970, 1974). Further investigations on heated protein/glucose mixtures revealed that the browning is accompanied by polymerization of the protein (Okitani et al., 1984; Cho et al., 1984; Kato et al., 1986).

Hydrolysis of food melanoidins afforded amino acids and peptides (Aurich et al., 1967; Maier et al., 1968; Maier and Buttle, 1973) as well as carbohydrates (Maier und Buttle, 1973) as the consituents. Furthermore, very recently we showed that food melanoidins are generated by a cross-linking reaction between low molecular weight colored Maillard reaction products and high molecular weight noncolored proteins (Hofmann, 1997b, 1998b). The characterization of a brown-orange melanoprotein bearing a lysine-bound 3(2*H*)-pyrrolinone chromophore (Hofmann, 1997b, 1998b) recently demonstrated for the first time that low molecular weight chromophores can be bound covalently to a noncolored polymer.

It is, however, as yet not clear whether food melanoidins are produced by polymerization of low molecular weight reaction products or by cross-linking and color-generating reactions between biopolymers, such as proteins, and reactive carbohydrate degradation products. Because in foods amino acids as well as proteins occur in addition to carbohydrates, it should, therefore, be a helpful approach to correlate the degree of polymerization with the browning ability of amino acids and proteins in the presence of glucose.

In the present investigation, we therefore studied the relationship between the molecular weight and the browning intensity of fractions, obtained by ultracentrifugation of the reaction products formed upon thermal treatment of aqueous solutions of glycine and L-alanine or β-casein, respectively, in the presence of glucose.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: D-glucose, L-alanine, and glycine were from Aldrich (Steinheim, Germany); β-casein was from Sigma (Deisenhofen, Germany).

Model Reactions. Solutions of glucose (8 mmol) and the amino acid (1.6 mmol) or β-casein (2.02 g), respectively, in phosphate buffer (40 mL, pH 7.0, 0.1 mol/L) were heated for 4 h at 95 °C. The amount of casein used in the model experiment corresponds to ~1.6 mmol as the sum of the reactive side chains of L-lysine (8.2% in casein) and L-arginine (4.1% in casein).

Fractionation of the Reaction Mixtures by Ultracentrifugation. An aliquot (20 mL) of the reaction mixtures was diluted with water (20 mL) and then fractionated by ultracentrifugation starting with a cutoff of 100000 Da. Two aliquots of the solution were successively added to the sample reservoir and were then centrifuged in a centrifuge with a swinging-bucket rotor adapter at 3000*g* until filtration was complete. Water (2 mL) was added into the reservoir and centrifuged again. The retentate was dissolved in water (5 mL) and stored at −20 °C, whereas the filtrate was further fractionated by ultracentrifugation with a cutoff of 50000 Da. This procedure was performed step by step using filters with cutoffs of 30000, 10000, 3000, and 1000 Da. Filters (Centricon and Centriplus) were supplied by Amicon (Witten, Germany).

Table 1. Yield and Browning Intensity of Molecular Weight Fractions of a Heated Glucose/Glycine Solution

fraction	mol wt (Da)	amount		CD factor[a]
		mg	%	
1	>100000	<0.5	<0.1	<1
2	100000−50000	<0.5	<0.1	<1
3	50000−30000	<0.5	<0.1	<1
4	30000−10000	0.6	0.1	<1
5	10000−3000	5.4	0.8	8
6	3000−1000	154.9	19.8	128
7	<1000	632.0	78.4	1024
	Σ (1−7)	792.9	96.7	nd[b]
	complete mixture	820.0	100.0	2048

[a] The color dilution (CD) factor is defined as the dilution of the colored sample in water, at which the color is just detectable visually in a triangle test using water as the blank. The data are the means of triplicates. [b] nd, not determined.

Determination of the Molecular Weight Distribution. The retentate of each fractionation step and an aliquot (20 mL) of the complete reaction mixtures of the single fractions were freeze-dried, and the residues were weighed. The weight content of each fraction from the complete mixture was then calculated in percent.

Determination of the Browning Intensity. For the determination of the browning intensity, the freeze-dried retentates as well as the freeze-dried aliquot (20 mL) of the complete reaction mixture were dissolved in water (2 mL) and then diluted step by step (1+1; by vol) until a color difference between the sample (2 mL) and two blanks (tap water; 2 mL) in a glass vial (1 cm i.d.) could just be visually detected using a triangle test. Using this procedure, a color dilution (CD) factor could be defined for each fraction.

UV−Vis Spectrocopy. UV−vis spectra were obtained by means of a U-2000 spectrometer (Colora Messtechnik GmbH, Lorch, Germany).

RESULTS AND DISCUSSION

Thermal treatment of aqueous solutions of glucose in the presence of alanine, glycine, or β-casein led to an intense browning of the reaction mixtures. To gain insight into the molecular weight of the products formed, the heated mixtures were separated into seven fractions by using ultracentrifugation with cutoffs of 100000, 50000, 30000, 10000, 3000, and 1000 Da, respectively. This fractionation technique was favored in comparison to gel chromatography because some of the browning products formed were found to be irreversibely bound to the gel material, making quantitative measurements unreliable.

To correlate the molecular weight ranges to their relative browning intensities, each fraction was diluted step by step with tap water and the color intensity of each dilution was compared with two blanks of tap water using the triangle test. The dilution, at which a color difference between the sample and the blanks could just be visually detected, was defined as the color dilution (CD) factor. Because the CD factor, by definition, corresponds to the visual threshold of the colored fraction in water, a colorless solution has a CD factor of <1. The CD factor, therefore, ranks the single fractions on the basis of their relative color contributions.

Glucose/Amino Acid. After fractionation of the intense brown glucose/glycine (Table 1) and glucose/alanine mixtures (Table 2), respectively, nearly 97% of the total weight could be recovered after ultracentrifugation. This result demonstrates that this fractionation

Table 2. Yield and Browning Intensity of Molecular Weight Fractions of a Heated Glucose/Alanine Solution

fraction	mol wt (Da)	amount		CD factor[a]
		mg	%	
1	>100 000	<0.5	<0.1	<1
2	100000−50000	<0.5	<0.1	<1
3	50000−30000	<0.5	<0.1	<1
4	30000−10000	<0.5	<0.1	<1
5	10000−3000	3.5	0.4	2
6	3000−1000	140.4	16.9	32
7	<1000	661.8	79.8	1024
	Σ (1−7)	805.7	97.1	nd[b]
	complete mixture	829.0	100.0	2048

[a] The color dilution (CD) factor is defined as the dilution of the colored sample in water, at which the color is just detectable visually in a triangle test using water as the blank. The data are the means of triplicates. [b] nd, not determined.

Table 3. Yield and Browning Intensity of Molecular Weight Fractions of a Heated Glucose/Casein Solution

fraction	mol wt (Da)	amount		CD factor[a]
		mg	%	
1	>100000	895.3	43.4	2048
2	100000−50000	373.8	18.1	512
3	50000−30000	93.1	4.5	8
4	30000−10000	76.5	3.7	<1
5	10000−3000	9.1	0.4	<1
6	3000−1000	36.0	1.7	<1
7	<1000	488.5	23.7	2
	Σ (1−7)	1972.3	95.5	n.d.
	complete mixture	2061.0	100.0	4096

[a] The color dilution (CD) factor is defined as the dilution of the colored sample in water, at which the color is just detectable visually in a triangle test using water as the blank. The data are the means of triplicates. [b] nd, not determined.

technique is very suitable for quantitative measurements.

Fractionation of the compounds generated by heating glucose in the presence of glycine and alanine, respectively, yielded 78.4 and 79.8% of the reaction mixture in fraction 7 containing the products having molecular weights <1000 Da (Tables 1 and 2). These data clearly indicate that the predominant part of the glucose/amino acid mixtures is still of low molecular weight. Nearly 20 or 17% of the compounds formed in the glucose/glycine or the glucose/alanine mixture, respectively, exhibited molecular weights between 1000 and 3000 Da, indicating that an oligomerization of low molecular weight Maillard reaction products had occurred. Neither amino acid, however, yielded significant amounts of high molecular weight compounds (Tables 1 and 2).

To correlate the molecular weight distribution of the reaction products with their browning intensities, the CD factor was determined for each fraction. The results, also given in Tables 1 and 2, show that, independent from the amino acid moiety, the low molecular weight fraction (7 in Tables 1 and 2) showed with a CD factor of 1024 by far the highest browning intensity. In fraction 6, containing compounds with molecular weights between 1000 and 3000 Da, a CD factor of 128 or 32 was approximated for the glucose/glycine (Table 1) or the glucose/alanine mixture (Table 2), respectively. These data show that the browning intensity of Maillard products with molecular weights between 1000 and 3000 Da is much lower than that of the low molecular weight fraction 7. These data demonstrate that only a minor part of the colored compounds formed in the reaction mixture exhibits molecular weights >1000 Da. A comparison of the results for the glucose/glycine mixture (Table 1) with those for the glucose/alanine mixture (Table 2) showed no significant difference in the molecular weight distributions or in the color intensity, implying that the formation of colored compounds from glucose should not be strongly influenced by the amino acid moiety.

Glucose/β-Casein. To compare the results of the glucose/L-alanine experiment with those of the reaction of proteins, the amino acid was substituted with casein. To guarantee similar concentrations of reactive amino groups, casein was heated in such an amount that the content of reactive side chains of L-lysine (8.2% in casein) and L-arginine (4.1% in casein) corresponded to the amount of L-alanine in the L-alanine/glucose solution. Heating the aqueous casein solution in the presence of glucose led to a rapid brown colorization of the reaction mixture, being reflected by the high CD factor of 4096.

Fractionation of the reacted mixture and determination of the weight distribution revealed that 62% (by wt) of the products formed showed molecular weights >50000 Da, among which 44% showing molecular weights >100000 Da. These data clearly demonstrate that the predominant part of the reaction products formed are high molecular weight. On the basis of the molecular weight of β-casein of 24000 Da, these data allow the conclusion that at least 18% of the β-casein is cross-linked to trimeric or tetrameric aggregates and, in addition, that 44% of β-casein is aggregated to pentamers or even higher oligomers. In contrast, nearly 24% of the reacted mixture was found in fraction 7, which contained compounds with molecular weights <1000 Da. These most likely correspond to unreacted glucose and low molecular weight Maillard reaction products. The lowest amounts of compounds were evaluated in fractions between 1000 and 30000 Da, indicating that only a small amount of β-casein did not react with glucose or its degradation products during thermal treatment.

The evaluation of the browning intensities in the seven fractions revealed that the colored products formed showed exclusively molecular weights above 50000 Da (Table 3). The highest CD factor, evaluated as 2048, was determined for the high molecular weight fraction 1 containing compounds with molecular weight >100000 Da, followed by the compounds with molecular weights between 50000 and 100000 Da, for which a 4-fold lower CD factor was measured. The low molecular weight fraction 7, however, was colorless, although 23.7% of the reaction products exhibited molecular weights <1000 Da.

DISCUSSION

The data presented indicate that in glucose/amino acid mixtures heated under conditions typical for cooking processes, the majority of colored compounds formed showed molecular weights <1000 Da. In contrast to data reported in the literature (Cämmerer and Kroh, 1995; Wedzicha and Kaputo, 1987), only trace amounts of compounds with molecular weights >3000 Da were found.

In general, the reaction conditions chosen in the literature for most of the experiments are not suitable to simulate cooking processes of foods. Results obtained

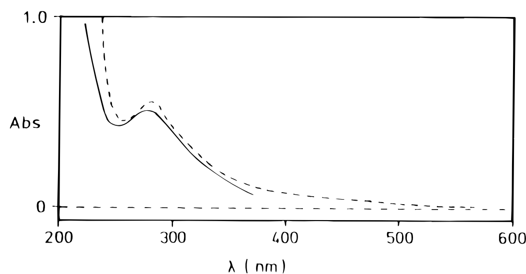

Figure 1. UV−vis spectrum of (- - -) melanoidins (MG ≥ 100000 Da) formed from glucose/casein and (—) of melanoidins isolated from dark beer (Kuntcheva and Obretenov, 1996).

from carbohydrate/amino acid mixtures dry-heated at 170 °C for up to 40 min (Cämmerer and Kroh, 1995) or heated in solution at 90 °C for 22 h (Wedzicha and Kaputo, 1987) can be regarded as unsuitable to match color formation in foods and to gain insights into melanoidin formation pathways.

The result that high molecular weight colorants with molecular weights up to several thousand daltons could not be observed indicates that the formation of melanoidins by polymerization of reactive low molecular weight compounds is very unlikely in the Maillard reaction performed under more food-related conditions.

The reaction between casein and glucose, however, led to a drastic increase in the molecular weights by carbohydrate-induced oligomerization of the protein backbone. The data presented show, furthermore, that protein oligomerization runs in parallel with an intense browning. These results confirm the earlier findings of Clark and Tannenbaum (1974) that the reaction between proteins and carbohydrates leads to browning and polymerization of the reaction mixture. The results of the present investigation, however, indicate that the colored structures generated when protein and glucose are heated are incorporated into protein oligomers and that no low molecular weight colored compounds are formed. Such protein-linked chromophores were very recently identified (Hofmann, 1997b, 1998a,b).

Due to the high protein content and the comparatively much lower content of free amino acids in foods, it is, therefore, very likely that food melanoidins are formed by carbohydrate-induced oligomerization and browning of proteins. This is well in line with the findings of Aurich et al. (1967), Maier et al. (1968), and Maier and Buttle (1973), who identified amino acids and peptides in melanoidins isolated from roasted coffee. Comparison of the UV−vis spectra of the glucose/casein melanoidin with a molecular weight >100000 Da (fraction 1 in Table 3) and melanoidins isolated recently from dark beer (Kuntcheva and Obretenov, 1996) demonstrated the similarity between this model melanoidin and the real food melanoidin (Figure 1). UV−vis spectra exhibiting both featureless end absorption and increased intense as the wavelength decreases are typical of melanoidins (Clark and Tannenbaum, 1970).

The data presented clearly demonstrate that in the presence of reducing carbohydrates proteins are effective precursors of food melanoidins. The latter protein might act as a noncolored skeleton of melanoidins, to which a variety of chromophoric substructures derived from carbohydrate degradation can be covalently bound via reactive side chains of, for example, lysine, arginine, or cysteine. As indicated in Figure 2, the featureless total absorption of a melanoidin is well in line with the proposed presence of several discrete chromophores

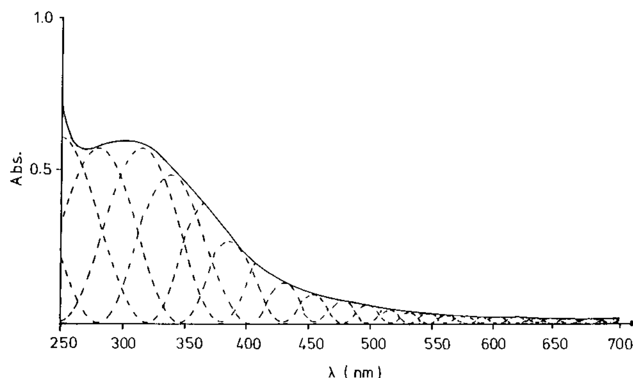

Figure 2. UV−vis spectrum of (—) a typical melanoidin and (- - -) of individual chromophoric substructures [based on Clark and Tannenbaum (1974)].

varying in their absorption maxima. The formation of chromophoric cross-linking structures between low molecular weight Maillard reaction products and reactive side chains of several proteins then gives rise to the formation of colored high molecular weight oligomers, the melanoidins.

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