Isolation and Characterization of Pigments from Protein-Carbonyl Browning Systems

Isolation, Purification, and Properties

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A method is described for the isolation and purification of protein-bound, brown pigments formed in protein-carbonyl browning reactions. The procedures include isoelectric washing of the protein, hydrolysis with proteolytic enzymes, and chromatographic purification by gel filtration and ion exchange chromatography. Some characteristics of the purified pigments are presented.

Colors and flavors result from nonenzymatic browning reactions which contribute to both the destruction and the improvement of many foods. Maillard reactions (the browning reaction of carbonyl compounds such as reducing sugars with proteins) reduce the nutritive value of foods (Henry and Kon, 1950; Lea and Hannan, 1950), but the brown pigments do not appear to be associated with any toxicological response (Adrian *et al.*, 1966; Jemmali and Petit, 1966).

The early steps of the Maillard reaction are well worked out (Hodge, 1953; Reynolds, 1963, 1965); they involve the formation of increasingly unsaturated multi-carbonyl compounds. In dry systems and in dilute solutions containing protein, the pigment causes cross-linking between protein chains (Hannan and Lea, 1952; Mohammed *et al.*, 1949) and thus reduced *in vivo* and *in vitro* enzymatic digestibility of the protein (Ford, 1965; Ford and Salter, 1966). Lea and coworkers have studied the effects of moisture content, pH, and temperature on the reactivity of the amine groups and on the formation of color in powdered casein-glucose and milk systems such as were used in our experiments (Lea and Hannan, 1949, 1950; Lea *et al.*, 1950).

Definitive research on the later steps of the browning reaction has been hindered by difficulties in purification of the pigments (Binkley, 1960a,b; Stinson and Willits, 1965) and by the fact that the color may result from more than one chromophore (Liggett and Deitz, 1954). The present paper introduces a new approach to solubilization, isolation, and purification of pigments and pigment-containing cross-links from a humidified protein-sugar browning reaction system. Several characteristics of the pigment will be presented.

EXPERIMENTAL

Materials. Extra grade, spray-dried, instant nonfat milk (Carnation Company, Los Angeles, Calif.) was purchased from a local supermarket. Hammersten Casein, an isoelectric precipitate containing 15% nitrogen, was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Glucose was obtained from Merck and Company, Inc., Rahway, N.J.

Preparation of Reaction System. The milk powder was used as supplied. Casein and glucose (2 to 1, w/w) were dissolved, with addition of 0.1*N* NaOH, in distilled water at pH 7.0 and freeze-dried. The samples were stored in open Erlenmeyer flasks at 36° or 55° C and 75% RH. The samples were coded for easy identification. For example, M-37d36 and CG-7d55 are respectively interpreted as nonfat, spray-dried milk stored 37 days at 36° C, and casein-glucose stored 7 days at 55° C.

Isolation. The procedure for isolation of the protein-bound brown pigment is shown in Figure 1. Removal of materials not bound to protein was accomplished by making a suspension in water (2.5 g per 100 ml), and by adjusting the pH to 6.5–6.8 (to partially dissolve and fully wet the material) and then to the isoelectric point. The precipitate which formed was filtered, rinsed, rewashed, and suspended in one-third the original volume at pH 8.0. A proteolytic enzyme complex from *S. griseus* (Pronase, Calbiochem, Los Angeles, Calif.), 0.25% based on initial sample weight, and isopropanol, 5% v/v, were added. The sample was maintained at pH 8.0 and room temperature for 48 hr to peptize the protein and bound pigment. Extent of browning was measured as the product of the absorbancy at 420 nm (A_{420}) and the sample volume. Absorbance of the isoelectric washings was also measured. The solution was concentrated seven- to eightfold on a rotary evaporator, refrigerated for 12 hr to complete precipitation, and filtered. The filtrate was made up to one-half the volume used during hydrolysis. Digestion, concentration, and filtration were repeated. Further amino acids could not be cleaved from the brown pigments by other proteolytic enzymes; this product is subsequently designated Limit-Peptide Pigment (LPP).

Purification. Preliminary purification of the LPP was by gel filtration on a preparative 5- × 92-cm column of Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, Calif.) in 0.05*M* NH_4Ac buffer, pH 7.0. The fraction that contained material of about 1500 molecular weight (MW) was further purified on a 1.5- × 90-cm carboxymethyl cellulose (CMC) column (H. Reeve Angel & Co., Clifton, N. J., and Whatman, Clifton, N.J.) in 0.05*M* NH_4Ac with pH 6.0. The highest molecular weight pigments (5000 to 20,000 MW), which cannot be purified on CMC, were passed through a 1.5- × 90-cm Bio-Gel P-10 column to reduce their molecular weight dispersion.

On elution, the absorbance of each fraction was measured at 420 nm, and concentrations were measured by weighing the freeze-dried residue. The absorptivity was then calculated from the ratio of these measurements. An increased absorptivity was the criterion of increased purity.

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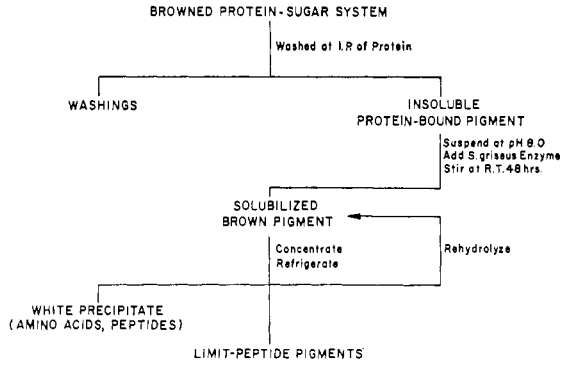

Figure 1. Isolation of brown pigment

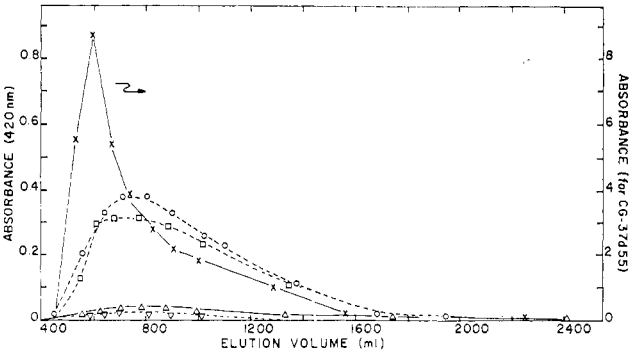

Figure 2. Chromatography of casein-glucose system on Bio-Gel P-4

▽ unstored casein
△ stored casein, 7 days, 55° C
□ casein-glucose, 37 days, 36° C
○ casein-glucose, 7 days, 55° C
× casein-glucose, 37 days, 55° C

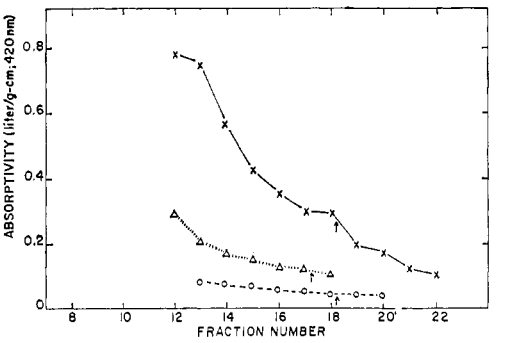

Figure 3. Chromatography of 1500 MW fraction of LPP's on carboxymethylcellulose at pH 6

↑ absorptivity before chromatography
○ casein-glucose, 37 days, 36° C
× casein-glucose, 37 days, 55° C
△ milk, 496 days, 36° C

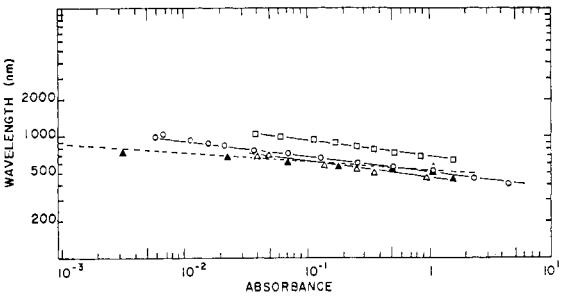

Figure 4. Logarithmic absorption spectra for various brown pigments

○ casein-glucose
□ furfural
△ kojic acid
▲ phenol

RESULTS AND DISCUSSION

Isolation. The pH of the aqueous suspension of the browning-reaction systems decreased as a function of storage time; M-496d36 dropped 1 pH unit and CG-124d55 dropped over 2 pH units. This effect, partially due to the reaction of the basic amino acids of the protein, was expected.

Most of the color in these reaction systems is bound to protein. Isoelectric washing removed less than 5% of the total color in the casein-glucose system and only 10 to 25% of the color in the (less homogeneous) milk system.

Preparative Chromatography. Figure 2 shows typical elution chromatograms from the Bio-Gel P-4 column for the pigments isolated from a series of casein-glucose samples stored under increasingly severe conditions. Increased severity of storage increased both the proportion and the absorptivity of the high molecular weight LPPs. This increase in the molecular weight of the LPP has indirectly been shown by Ford (1965) and is related to reduction of *in vivo* digestibility. In all cases, the materials with highest molecular weights are darkest.

Analytical Chromatography. The elution curve from the CMC column, showing absorptivity at 420 nm for several 1500 MW fractions, is presented in Figure 3. Purification was due not to a sharp separation of pigmented and nonpigmented materials, but rather to a slightly greater retention of nonpigmented materials on the column. Purification was greatest with the darkest 1500 MW pigments. Those pigments with high molecular weights could not be further purified on CMC.

The molecular weight distribution of the pigments with high molecular weights was examined by chromatography on Bio-Gel P-10. Individual fractions had less molecular weight dispersion, but no increase in purity. Increased severity of storage resulted in shifts of the molecular weight distribution to higher molecular weights. The largest LPP's isolated were about 18,000 MW. No effort was made to relate this shift directly to the extent of color formation or severity of storage conditions.

Characterization of Purified LPP's. Visible spectroscopy of the purified pigments gave typical, featureless end absorption, becoming more intense as wavelength decreased. Plotting the log absorbance as a function of the log wavelength from 450 to 1000 nm gives a straight line (slope −8.0) (Figure 4). Kojic acid and furfural, which had turned brown during storage, yielded the same relationship, whereas a reddish-brown sample of phenol was different. The slope is in agreement with Liggett and Deitz (1954), who reported values of −2.5 to −9.0 for browned sugar solutions, and in contrast to Grover (1968), who felt that log absorbance *vs.* wavelength yielded more linear results for several other brown pigments.

The pigment could be bleached completely with hot H_2O_2. Only partial bleaching was possible at room temperature with $NaBH_4$ (45% color removal), NaOCl followed by $NaBH_4$ (90% color removal), or $NaBH_4$ followed by NaOCl (95% color removal). The effect of these bleaching agents on the molecular weight of a high molecular weight pigment derived from CG-37d55 was examined. The pigment was bleached,

and elution patterns from the 1.5- × 90-cm Bio-Gel P-10 column were monitored at 280 nm (Figure 5). Visual examination of the freeze-dried residues of each fraction, which were too small to weigh accurately, indicated that the absorption at 280 nm was a valid representation of the distribution of the material. $NaBH_4$ caused only slight changes in the elution pattern (although intensity decreased). NaOCl decreased molecular weight [possibly by reverse aldol condensation of the hypochlorite-oxidized product (Stewart, 1964)] and greatly increased molecular weight dispersion. H_2O_2 cleaved the pigment to very low molecular weight materials, some of which were adsorbed slightly to the column (Figure 5).

Adjustment of the LPP over the range from pH 1 to pH 12 resulted in up to 20% decrease or increase, respectively, of the absorbance at any visible wavelength compared to that at pH 8.0. Reduction of color intensity by bleaching or pH adjustment caused no change in the slope of the log absorbance *vs.* the log wavelength spectrum.

The ultraviolet absorption spectra of these purified pigments were relatively featureless, rising slowly to a weak shoulder around 270 nm (more rapidly below 240 nm). Addition of acid or base slightly increased absorbance and shifted the shoulder to slightly higher wavelengths. Treatment with $NaBH_4$ decreased the intensity but had no effect on the shape. Treatment with NaOCl removed all features except for the end absorption.

The infrared spectra of purified pigments gave only broad bands characteristic of proteins and sugars. Except for slight increases in carbonyl absorption intensities, no clues to pigment structure were obtained.

Uncorrected fluorescence spectra were broad and changed shape with different excitation wavelengths. According to Berlman (1965), such changes are a consequence of the presence of more than one absorbing chromophore. Furthermore, the excitation spectrum is different from the ultraviolet absorption spectrum, indicating that the pigment is not a pure compound (Udenfriend, 1962).

Since Mitsuda *et al.* (1965) have reported free radicals in browning reactions, we decided to examine the samples by electron paramagnetic resonance spectroscopy. No free radicals were detected. This indicates that although free radicals may be involved in the reaction mechanism, stable free radicals do not remain and do not contribute to the visible absorption of the pigments.

LPP's were very soluble in water (at least 10 to 50 g per 100 ml); no attempt was made to determine maximum solubility. They showed negligible or slight solubility in a variety of organic solvents.

Qualitative spray reagents were used for functional group analyses of samples of purified pigments spotted on cellulose or silica gel tlc plates. The darkness of the pigment interfered with many of the tests, and the reactivity of control materials from sugar free systems further confused the results. Because of the free terminal amine group liberated by enzymatic hydrolysis, all pigments reacted with ninhydrin. The alkaline potassium permanganate test for reducing groups (including double bonds) was positive.

Trace metals, determined by emission spectrography (Schwarzkopf Microanalytical Laboratories, Woodside, N.Y.), apparently are not important in the pigment structure. Heating of a 1500 MW pigment (CG-37d36) to 100° C with an EDTA solution gave a 16% increase in A_{420}. The metal content of a darker 1500 MW pigment (CG-37d55) indicated a stoichiometry of about 1 phosphorus and 0.1 each of calcium and silicon per molecule. Heavier metals, such as Mg, Al, Cu, and Fe, were present only to the extent of about 0.01 or less per molecule.

Table I. Amino Acid Composition of Some Purified Limit-Peptide Pigments

	Recovered Amino Acids (%)		
Amino Acid	Casein Control	Casein-glucose (37 days at 55° C)	Milk (496 days at 36° C)
Aspartic acid	7.17	9.23	10.90
Threonine	4.28	5.35	5.93
Serine	6.29	7.53	7.23
Glutamic acid	22.07	33.81	30.20
Glycine	1.73	1.98	2.80
Alanine	2.92	3.33	3.27
Valine	6.40	5.19	4.49
Cysteine	0.46	0.95	2.72
Methionine	2.79	4.37	4.19
Isoleucine	5.05	7.04	6.22
Leucine	10.01	5.96	5.16
Tyrosine	6.35	3.28	1.42
Phenylalanine	5.81	2.65	1.88
Lysine	8.63	5.04	4.92
Histidine	3.24	3.60	3.31
Arginine	4.38	0.70	1.80

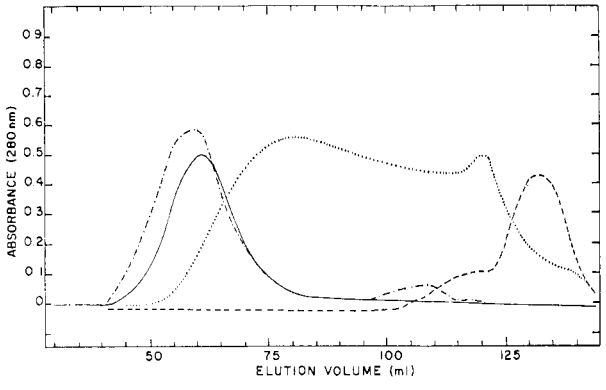

Figure 5. Effect of chemical bleaching on the chromatographic behavior of LPP's on Bio-Gel P-10

—— untreated
– – – hydrogen peroxide
-·- sodium borohydride
··· sodium hypochlorite

Elemental analysis of an LPP derived from a milk system and purified by both gel filtration and ion exchange chromatography gave C = 43.70%, H = 6.04%, and N = 11.27%. The amino acid composition of this pigment, given in Table I, is compared to a casein-glucose pigment and a stored casein control. The expected elemental analysis of the peptide portion of the milk LPP (derived from Table I) is C = 21.8%, H = 2.96%, N = 7.23%, O and S = 12.62%. A differential elemental analysis of the pigment portion would then be C = 22.52%, H = 3.08%, N = 4.04%, and O plus other = 26.47%. This would be consistent with an elemental formula $C_{19}H_{30}N_3O_{16}$, which indicates a fairly complex structure.

For example, this analysis could suggest 1.5 μm of C_6 sugar residue-lysine complex per mg LPP or 0.75 μm of C_{18} sugar residue-arginine complex per mg or 1 μm sugar residue-histidine per mg, or any combination of these along with smaller amounts of sugar which could have reacted with *N*-terminal amino acids. If all of the product were a sugar-lysine complex, there would be nine sugar-lysine complexes per mole (about 6000 MW by gel filtration).

CONCLUSIONS

Brown pigments formed by reaction of sugars and proteins were purified by enzyme hydrolysis and column chromatography. The most highly purified fractions still retained a peptide fragment, presumably because of steric factors; the resulting material is therefore an LPP. An increase in duration or temperature of storage of a protein-sugar system leads to increased molecular weight of the LPP. The UV-visible spectrum of an LPP is characterized by a linear relationship between log absorbance and log wavelength which is not affected by bleaching with NaOCl, $NaBH_4$, H_2O_2, or acidification. Spot tests were positive for reducing compounds, but other spectroscopic analyses were inconclusive or negative. Results of elemental and amino acid analyses were also too complex for unequivocal interpretation. It would appear that LPP's are not homogeneous, but represent a distribution of highly degraded carbohydrate structures bonded to a variety of sites on the reacting protein. The enrichment of dicarboxylic amino acids in the adjacent peptide structures relative to the enrichment of the starting material may be linked to the participation of carboxylates as catalysts of pigment formation (Kato *et al.*, 1969).

Further interpretation of the composition of the remaining peptide is not feasible with a protein as complex as casein and with an approach relying solely on chemical analysis. Supplementary research efforts are in progress using radioactive carbon- and hydrogen-labeled sugars with casein and with insulin. Because these pigments do not appear to be single chemical entities, elucidation of their structure is expected to be a long range research objective. Even a partial understanding of their structure, however, may greatly further the understanding of biological problems such as those related to losses in the nutritive value of protein foods.

ACKNOWLEDGMENT

The authors would like to thank John E. Kimura for performing the EPR spectroscopy at the Woods Hole Biological Research Laboratories, Woods Hole, Mass.

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Received for review March 12, 1970. Accepted May 28, 1970. This work was supported by Public Health Service Research Grants UI1033 and 5POIES00183 from the National Center for Urban and Industrial Health. Contribution No. 1607 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.