Browning and Insolubilization of Ovalbumin by the Maillard Reaction with Some Aldohexoses

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The Maillard reaction of glucose, mannose, and galactose with ovalbumin was investigated by measuring the decrease in protein amino groups, brown colorization, and protein insolubilization. Ovalbumin was freeze-dried with each sugar in various mix ratios and stored for 0–10 days at 50 °C and 65% relative humidity. There were no large differences in the amino group decrease among the proteins stored with the three sugars. However, the protein mixed with glucose or mannose was almost completely soluble even after 10 days of storage, whereas no more than 40% of the protein stored with galactose was soluble. The brown coloring of the protein–galactose mixture stored for 4 days or more was 2–3 times as strong as that of the other sugar–protein mixtures. Thus, the reaction rate of the galactose system at the later stage of the Maillard reaction was faster than that of the other sugar systems.

INTRODUCTION

The protein–sugar complex produced in the early Maillard reaction was found to possess specific physicochemical properties such as high solubility and heat stability (Tybor et al., 1973; Morales et al., 1976; Kato et al., 1981, 1983). However, such protein properties fell to poor solubility, browning, and reduction of nutritional availability in the advanced stage of the reaction (Friedman, 1977; Ericksson, 1981).

The Maillard browning reaction may be desirable as in baked, fried, or roasted foods or undesirable as in concentrated and dried foods. In either case, it is important to study the factors affecting the intensity of Maillard browning. Many previous reports have shown the factors affecting the rate of Maillard reaction (Lea and Hannan, 1949, 1950; Hodge, 1953; Reynolds, 1965). Some of these reports have shown that the factors affecting the rate of Maillard reaction are the kinds or ratios of amino acids and reducing sugars under various conditions (Katchalsky and Sharon, 1953; Wolfrom et al., 1947; Ellis, 1959; Ashoor and Zent, 1984). However, these studies using amino acids could not satisfactorily explain the rate of protein–reducing sugar Maillard reaction, and few reports dealt with the rate of browning reaction between protein and reducing sugar.

The main objective of the present study is to compare the reaction rate of the amino–carbonyl reaction between ovalbumin and some aldohexoses having the same molecular formula and a different configuration, in order to elucidate the protein–reducing sugar Maillard reaction. The relationship between the sugar configuration and the reaction rate at the latter stage of Maillard reaction is discussed.

EXPERIMENTAL SECTION

Sample Preparation. Ovalbumin (OVA) was prepared from fresh egg white of White Leghorn hens by the ammonium sulfate method (Marshall and Neuberger, 1972). Glucose, galactose, and mannose were purchased from Wako Co., Ltd. (all reagents were super fine grade). Talose was obtained from Sigma Chemical Co.

The mixtures with ovalbumin and each sugar in various weight ratios (protein to sugar 1:0.01, 1:0.05, 1:0.1, 1:0.5, 1:1, and 1:3) were dissolved in distilled water (5% protein solution), and the solution was adjusted to pH 8.0 with diluted NaOH. These mixtures were freeze-dried and kept in desiccators for various periods (0–10 days) at 50 °C and 65% relative humidity maintained with saturated KI solution. As controls, ovalbumin and each sugar were stored individually for 10 days in a similar manner (see above).

Brown Color. Brown color was measured by absorbance at 420 nm of OVA–sugar mixtures (1 mg/mL of protein concentration). The insoluble protein–sugar mixture was dissolved by hydrolysis with Nagase (2 Penn Plaza, New York) for 2 h at 37 °C, and the brown color of the solution was measured.

Protein Solubility Measurement. Solubility of protein reacted with sugar was measured as follows. The ovalbumin and sugar mixture stored for various periods (0–10 days) was suspended at a level of 0.2% (w/v) in phosphate buffer (I = 0.1, pH 7.0), soaked for 1 h, and centrifuged at 3000 rpm for 15 min. Soluble protein in each supernatant fraction was determined by the method of Lowry et al. (1951). Solubility was expressed as the percentage of soluble protein to total protein.

Free Amino Group Determination. Free amino groups were measured by the fluorometric method using fluorescamine (Roche) according to Böhlen et al. (1973). The sample proteins were denatured by heating at 100 °C for 10 min and then mixed with fluorescamine. The fluorescence was determined by a Jasco spectrofluorometer FP-550A with the excitation at 390 nm and emission at 475 nm.

Electrophoresis. Polyacrylamide gel electrophoresis (7.5% acrylamide) was performed according to the method of Davis (1964). The gel sheets were stained with 0.2% Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Reactivity of Glucose, Mannose, and Galactose with Ovalbumin. The Maillard reaction of protein with reducing sugar was investigated by using typical monosaccharides, glucose, mannose, and galactose. Figure 1a–c shows the decrease in free amino group of ovalbumin stored with the sugars for various periods (0–10 days). The remaining amino groups were expressed as the relative value (%) of the free amino groups of native ovalbumin based on the fluorometric intensities. The fluorometric method was used for the free amino group determination in the present study, because the method is rapid and simple as compared with the traditional FDNB (1-fluoro-2,4-dinitrobenzene) method (Carpenter, 1960). The samples for the fluorometric assay were preheated at 100

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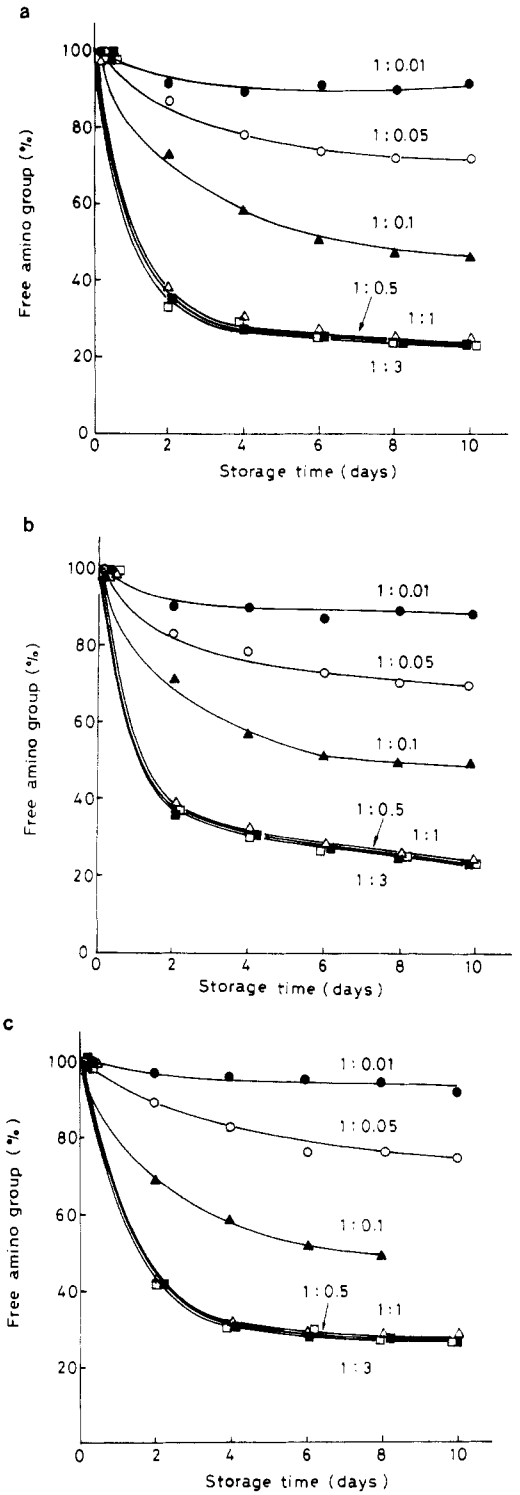

Figure 1. Decrease in free amino group of ovalbumin stored with glucose (a), mannose (b), and galactose (c) for 10 days. Ovalbumin was mixed with each sugar at the protein–sugar ratio of 1:0.01 (●), 1:0.05 (○), 1:0.1 (▲), 1:0.5 (△), 1:1 (■), and 1:3 (□) and stored at 50 °C and 65% relative humidity (RH).

°C for 10 min, because only about half of the amino groups of native ovalbumin was reactive (Kato et al., 1983). The amino group content of ovalbumin determined by this method using *n*-pentylamine as standard was 21.1 (mol/mol of protein), which is relatively in good agreement with the theoretical value from the lysine content (20 mol/mol). Free amino groups of ovalbumin stored for 10 days with glucose in the 1:0.01, 1:0.05, and 1:0.1 ratios were decreased to 90, 73, and 48%, respectively. In the ovalbumin–glucose system, free amino group content decreased with an in-

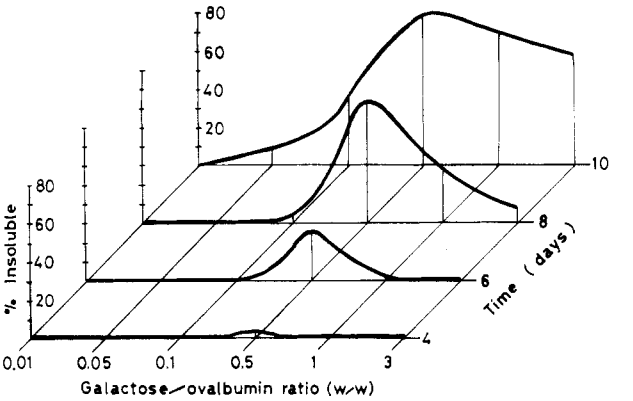

Figure 2. Insolubility of ovalbumin by storage with galactose for 4–10 days at 50 °C and 65% RH.

crease in the protein–sugar ratio to 1:0.5 (ovalbumin to glucose). The free amino group of ovalbumin with the protein–sugar ratio of 1:3 was decreased in a similar manner as that of the protein with the protein–sugar ratio of 1:0.5. In the system used in the present study, the glucose addition of more than half of the ovalbumin weight did not affect the rate of protein amino group blocking.

The ovalbumin stored with mannose and galactose also showed the profile of free amino group decrease similar to that of the protein stored with glucose. The amount of reducing sugar was reported to influence the rate of browning caused by Maillard reaction; on a glucose–lysine model system with a water activity of 0.52 stored at 45 °C, the rate of brown pigment formation increased linearly as the initial molar rate of glucose to available lysine increased from 0.5 to 3 (Warmbier et al., 1976). A study on a casein-glucose model system humidified to 0.70 water activity also indicated that lysine loss was maximum at an initial glucose-to-lysine (G/L) molar rate of approximately 3 (Lee and Hannan, 1950). In the present study, the maximum of lysine loss was attained by adding sugars at the protein–sugar weight ratio of 1:0.5, which corresponded to the amino group–sugar molar ratio of about 1:6. This result with the protein–sugar system did not seem to contradict the previous findings with amino acid–sugar systems.

The progress of the protein sugar reaction was monitored by measuring protein insolubilization during storage. The remarkable difference was observed in the protein solubility between the protein–galactose and the protein–other sugar systems; the protein stored with galactose gradually became insoluble with the storage time, while the protein mixed with glucose or mannose was almost completely soluble even after 10-day storage. The insolubilization profile of a OVA–galactose mixture stored for 4–10 days is shown in Figure 2. The protein–sugar mixture 1:0.5 was most insoluble among the samples with the six mix ratios throughout storage time (0–10 days). The sugar seemed to absorb moisture during the storage, resulting in increased water content of protein mixed with sugars, especially with a large amount of sugar. The dilution of protein or sugar by absorbed water might decrease the reaction rate of the samples with the protein–sugar ratios of 1:1 and 1:3.

The protein polymerization induced by the storage with sugars was analyzed by polyacrylamide gel electrophoresis. Figure 3 shows the electrophoretogram of samples stored for 4 days. The high molecular weight polymers were observed especially for the protein–galactose mixture with the mix ratio of 1:0.5 or more. The protein stored with sugar, especially at the ratio of 1:0.5 or more, showed an electrophoretic mobility greater than that of native protein, indicating that the positive charge of protein was decreased

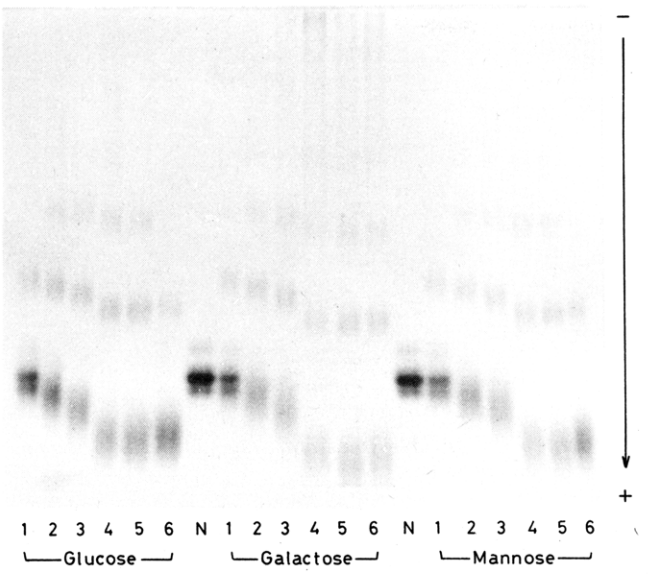

Figure 3. Polyacrylamide gel electrophoretogram of ovalbumin–glucose, –galactose, and –mannose mixtures stored for 4 days at 50 °C and 65% RH. The protein–sugar ratios are 1:0.01 (1), 1:0.05 (2), 1:0.1 (3), 1:0.5 (4), 1:1 (5), and 1:3 (6). Native ovalbumin (N) also was applied to the gel for comparison.

by the blocking of amino groups with sugars. The protein–galactose mixture with the mix ratio of 1:0.5 or more showed a monomer protein band rather weaker than those of other protein–sugar mixtures, and protein polymers that could not migrate into the lower gel were observed for the protein–galactose mixture with the mix ratio of 1:0.5 or more.

The color development profiles of the protein–sugars are depicted in Figure 4a–c. The brown color is plotted against the storage day and the protein–sugar ratio. The color intensity of the three protein–sugar mixtures increased with the storage time and also with increase in the sugar ratio; the sample with the protein-to-sugar ratio of 1:3 showed the strongest brown color of the six samples with various mix ratios. The brown coloring was observed also for the protein–glucose and –mannose mixtures that were soluble even after 10-day storage, and the relationship between the color development and the protein-to-sugar ratio for the galactose–protein did not correspond with the relationship between protein solubility and protein–sugar ratio (Figures 2 and 4). These results suggested that the protein polymerization and browning reaction were not necessarily connected and proceeded independently through the Maillard reaction. The color intensity of OVA–galactose mixture stored for 4 days or more was remarkably stronger than that of the other protein–sugar mixtures. The galactose seemed to accelerate not only protein polymerization but also brown colorization more strongly than the other sugars.

The Maillard reaction of protein with reducing sugar could be classified into two steps. The initial step is the combination of reducing sugars to protein amino groups, followed by the formation of Schiff base and Amadori compound, and the advanced step contains several complex reactions that cause brown colorization and protein unsolubilization. The decrease in protein free amino groups by the reaction with the three sugars (Figure 1) suggested that the rate of amino group decrease did not strongly depend on the aldohexose configurations. On the other hand, the protein insolubilization and brown colorization of protein stored with the sugars indicated that the reaction rate of the advanced step of Maillard reaction between protein and galactose was faster than that of the

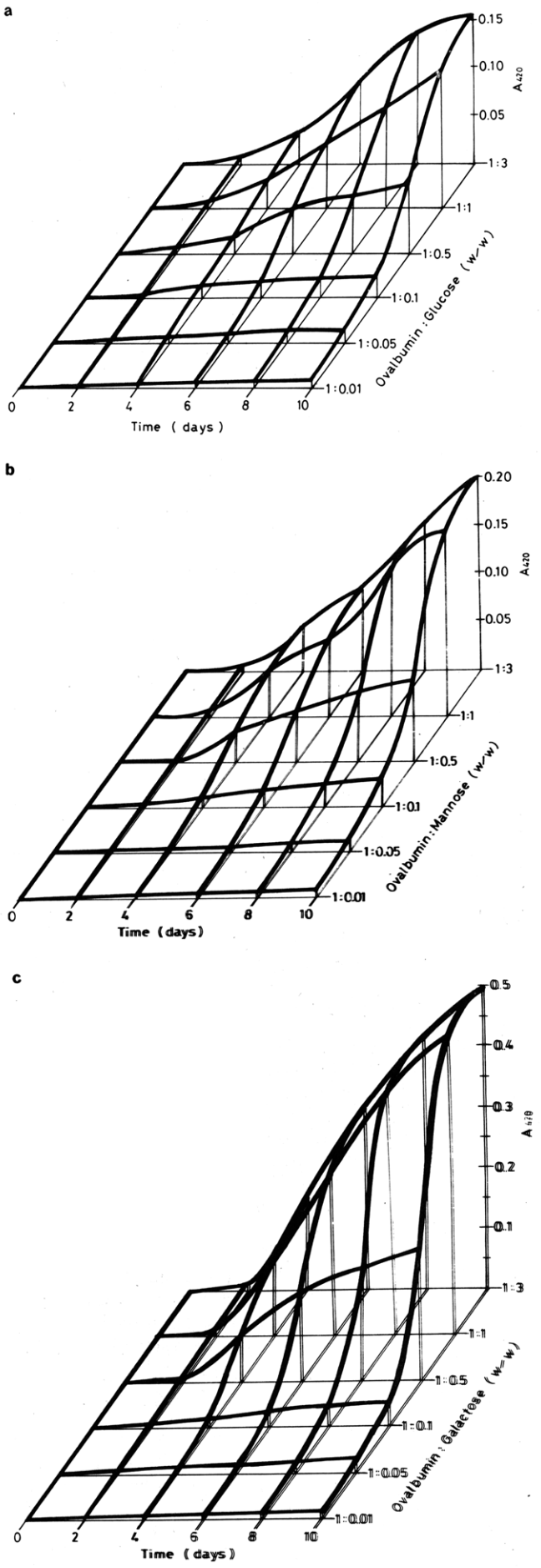

Figure 4. Brown color development of ovalbumin stored with glucose (a), mannose (b), and galactose (c) for 10 days.

Table I. Brown Color and Free Amino Groups of Ovalbumin Stored with Galactose, Talose, and Glucose

	storage time, days			
	4	6	8	10
free amino groups, %				
galactose	39	32	30	25
talose	32	31	28	22
glucose	37	35	31	24
brown color (420 nm)				
galactose	0.14	0.23	0.35	0.50
talose	0.24	0.35	0.50	0.64
glucose	0.03	0.05	0.11	0.16

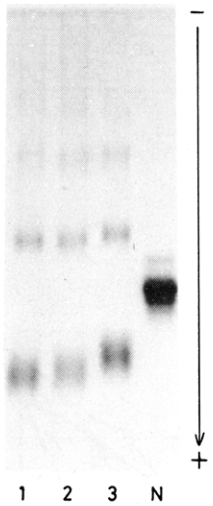

Figure 5. Polyacrylamide gel electrophoretogram of ovalbumin–talose (1), –galactose (2), and –glucose (3) mixtures stored for 4 days at 50 °C and 65% RH and native ovalbumin (N).

reaction between protein and the other sugars (glucose, mannose). Such a remarkable difference in the reaction rate among sugars might be explained by the stereochemistries of each sugars; the C-3 and C-4 hydroxy groups seemed to be important because galactose has the reverse configuration of C-4 OH to that of glucose or mannose.

Reactivity of Talose with Ovalbumin. The talose configuration is the same as that of galactose at the C-3 and C-4 hydroxy groups, and different from glucose and mannose at the C-4 hydroxy group. Thus, the authors chose talose as a model sugar to confirm that the configuration of hydroxy groups affected the advanced step of Maillard reaction between the protein and sugars. The Maillard reaction of the ovalbumin–talose system was compared with that of ovalbumin–galactose and of ovalbumin–glucose systems. The protein was mixed with each sugar (protein-to-sugar ratio of 1:1), freeze-dried, and stored as above.

Table I shows the amino group decrease and the brown colorization of the three protein–sugar systems. There were no significant differences in the free amino group decrease among the three systems. However, the protein–talose mixture showed a brown color stronger than that of the protein–glucose system, and the browning profile of the protein–talose system was similar to that of the protein–galactose system.

Figure 5 shows the electrophoretic patterns of the three protein–sugar systems. The formation of protein polymers that could not migrate into the gel was observed for the protein–talose and the protein–galactose systems, but not for the protein–glucose system. These characteristics of the protein–talose Maillard reaction suggested that talose was classified as a galactose type aldohexose rather than the glucose type one in terms of reactivity in the protein–sugar Maillard reaction.

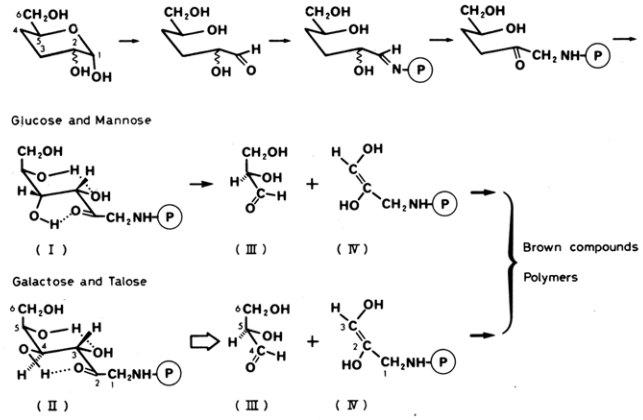

Figure 6. Conceivable transition states of sugar–ovalbumin Amadori compounds.

The conceivable reaction mechanisms for the galactose type and glucose type of protein–sugar Maillard reaction are shown in Figure 6. After the chirality at C-2 is lost at Amadori rearrangement, the configurations of C-3 and C-4 of the reaction intermediate (Amadori compound) play an important role in the reaction rate. OVA–glucose and –mannose complexes convert into intermediates that constitute the unstable cis-chair,chair conformation (Figure 6, I). However, the reaction intermediates from the protein–galactose and –talose form the energetically favorable trans-chair,chair conformation (Figure 6, II) by two hydrogen bonds between the C-2 carbonyl and C-4 hydroxy groups and the C-3 and C-5 hydroxy groups. In the next reaction step, the intermediates convert into glyceraldehyde compounds (Figure 6, III and IV) that become brown polymers. The aldehyde compounds are produced smoothly and rapidly from the stable intermediates via a concerted cleavage reaction.

The study of the reaction rate of various monosaccharides with hemoglobin showed that the rate of Schiff base formation depended on the carbonyl form proportion of each sugar in an aqueous solution (Bunn and Higgins, 1981). It has been reported that the carbonyl form proportion of galactose and talose were higher than those of mannose and glucose (Hayward and Angyal, 1977). Hence, the binding reaction of galactose and talose was expected to be faster than those of glucose and mannose. In fact, however, no large difference in the amino group decrease was detected among the proteins stored with each sugar (Figure 1; Table I). Hence, the sugar-dependent differences in reactivity at an advanced stage of Maillard reaction could not be explained simply by the ratio of the sugar carbonyl form to the hemiacetal form in an aqueous solution. The reaction rate of sugar to protein amino group in a dry system is much faster than that in an aqueous system, and the browning reaction and the protein polymerization would depend on the formation of degradation compounds rather than upon the sugar binding reaction to protein amino groups.

Registry No. Glucose, 50-99-7; mannose, 3458-28-4; galactose, 59-23-4; talose, 30077-17-9.

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Effect of Maillard Reaction Products on Protein Digestion. In Vitro Studies

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The low molecular weight fraction of a glucose–lysine reaction mixture, previously shown to affect the in vivo uptake of proteins in the rat, was tested for its effect on a number of gastrointestinal proteases and peptidases in vitro. Of these, carboxypeptidase A was significantly inhibited by 0.5 mg/mL of the Maillard products, and aminopeptidase N (a key enzyme in the brush border hydrolysis of peptides) was strongly inhibited by 0.25 mg/mL. The inhibition of aminopeptidase N was of the mixed type while the inhibition of carboxypeptidase A could not be described by means of classical enzyme kinetics. Gel filtration chromatography indicated that a number of different compounds in the Maillard reaction mixture may inhibit the two enzymes.

INTRODUCTION

Because the ϵ-amino group of lysine reacts in the Maillard reaction, heating proteins with carbohydrates may lead to a reduced nutritional value of the protein. The degree of reduction is dependent on a number of factors, including water activity, type and amount of reducing sugar, and type of protein as well as temperature and time of the heat treatment (Dworschak, 1980; Mauron, 1982). The loss of protein quality may include both a reduced biological value and digestibility. When the heat treatment is mild, a loss in the BV often corresponds to the loss of biologically available lysine caused by the Maillard reaction, provided lysine is the limiting amino acid in the protein (Burvall et al., 1978; Mauron, 1982; Björck et al., 1983). When the heating is more intense, the reduction of protein quality may be greater than the apparent loss of available lysine (measured in vitro) and there may also be a reduction in digestibility (Boctor and Harper, 1968; Adrian, 1982; Björck et al., 1983; Öste and Sjödin, 1984).

There are some reports on possible mechanisms behind these effects of severe heat treatment based on studies of rats. Adrian (1982) has shown that water-soluble premelanoidines from a glucose–glycine reaction mixture reduce the protein digestibility and also affect the utilization of absorbed amino acids. Valle-Riestra and Barnes (1970) suggest that the reduced uptake of a severely heated glucose–egg albumin mixture is the effect of a decreased pancreatic enzyme secretion. Percival and Schneeman (1979) suggest that the enhanced fecal excretion of nitrogen following intake of heated casein is partly due to undigested endogenous protein. The observed decrease in biological value (i.e., enhanced urinary nitrogen excretion) may also be explained by the uptake of nonmetabolizable nitrogen-containing compounds from the gut (Valle-Riestra and Barnes, 1970; Ford and Shorrock, 1971; Pronczuk et al., 1973).

A previous study on the effects of Maillard reaction compounds on the digestion of dietary protein has indicated that a low molecular weight (LMW) fraction of a glucose–lysine reaction mixture affects the utilization of dietary protein in the rat (Öste and Sjödin, 1984). The present paper reports effects of this fraction on the in vitro activities of gastrointestinal proteolytic enzymes.

EXPERIMENTAL SECTION

Glucose–Lysine Reaction Mixture. A low molecular weight (LMW) fraction from a reflux-boiled mixture of equimolar amounts of D-glucose and L-lysine was prepared as described by Öste and Sjödin (1984).

Gastric and Pancreatic Enzymes. Pepsin (EC 3.4.23.2; 2× crystallized, from porcine stomach mucosa), trypsin (EC 3.4.21.4; 2× crystallized, from bovine pancreas), chymotrypsin (EC 3.4.21.1; 3× crystallized, from bovine pancreas), carboxypeptidase A (EC 3.4.17.1; 2× crystallized, from bovine pancreas), and carboxypeptidase B (EC 3.4.17.2; chromatographically pure, from porcine

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