Maillard Reaction of Disaccharides with Protein: Suppressive Effect of Nonreducing End Pyranoside Groups on Browning and Protein Polymerization

Yasuko Kato,* Tsukasa Matsuda, Natsuki Kato, and Ryo Nakamura

The Maillard reaction of ovalbumin and several disaccharides (maltose, cellobiose, isomaltose, lactose, melibiose) having glucose at the reducing end was investigated by measuring several properties of the sugar–protein Maillard adducts after each sugar was kept with ovalbumin at 50 °C and 65% RH for 0–20 days. Isomaltose and melibiose mixed with the protein strongly induced brown colorization, production of fluorescent compounds, and protein polymerization, whereas maltose, cellobiose, and lactose did so very weakly. All five of the sugars decreased free amino groups of the protein to less than 20% within 1 week. The weaker production of advanced Maillard reaction products such as brown color and/or fluorescent compounds in the maltose, lactose, and cellobiose systems indicated that the terminal pyranoside groups bonded at the C-4 OH of glucose retarded further degradation to aldehyde components of their Amadori rearrangement products.

Earlier studies on the reaction between various reducing sugars and amino acids showed that the rates of the coupling reaction between amino and carbonyl groups and the browning reaction observed at advanced stages of the reaction were affected by the chemical structure of sugars and amino acids (e.g., Lewis and Lea, 1950; Pomeranz et al., 1962; Burton and McWeeney, 1963).

We have been investigating an amino–carbonyl reaction between proteins and reducing sugars (Kato et al., 1981a,b, 1986) and recently reported that slight differences in the chemical structure of the reducing sugars resulted in large differences in the formation of characteristic reaction products such as brown compounds and cross-linked proteins induced at the latter stage of the reaction (Kato et al., 1988). Galactose induced browning and protein cross-linking more strongly and quickly than glucose did, and a disaccharide lactose induced them much less strongly than glucose or galactose did (Kato et al., 1986, 1988). Those differences in the advanced amino–carbonyl reaction were explained by the chemical stability of Amadori compounds against further degradation into browning. The experiments on lactose- and 4-*O*-methyl-D-glucose–protein systems suggested that the C-4 hydroxy group of the reducing end glucose played an important role in the advanced stages of the amino–carbonyl reaction (Kato et al., 1988).

To get more information about the relationship between the sugar structure and the amino–carbonyl reaction, the reaction between ovalbumin and several disaccharides with a glucose reducing end having another glucose or galactose through $\alpha(1\rightarrow4)$, $\beta(1\rightarrow4)$, or $\alpha(1\rightarrow6)$ linkage was investigated by measuring browning, fluorescence, and protein polymerization of the protein–sugar reaction products.

MATERIALS AND METHODS

Sample Preparation. Ovalbumin (OVA) was prepared from the egg white of White Leghorn hen eggs by the ammonium sulfate method (Marshall and Neuberger, 1972). Maltose (mal), cellobiose (cel), isomaltose (iso), lactose (lac), and melibiose (mel) were purchased from Sigma Chemical Co., Ltd., of superfine-grade reagents.

Tokaigakuen Women's College, Tenpaku-ku, Nagoya 468, Japan (Y.K.), Department of Food Science and Technology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan (T.M., R.N.), and Chukyo Women's University, Ohbu 474, Japan (N.K.).

The mixture of OVA (8 mg) and each disaccharide (8 mg) was dissolved in 8 mL of distilled water adjusted to pH 8.0 with diluted NaOH solution. OVA has 20 amino groups per molecule. The sugar to amino group molar ratio of these mixtures is 1:6.6. Each solution was divided into eight tubes, and the sample solutions were freeze-dried. The dried mixtures were kept in a desiccator for various periods of time (0–20 days) at 50 °C, 65% relative humidity (RH) maintained with saturated KI solution. As control, OVA and each sugar were individually maintained for 20 days in a similar manner.

Measurements of Browning and Fluorescence Intensity. The OVA–sugar mixture was dissolved in phosphate-buffered saline (PBS) for measurement of the extent of browning and fluorescence. Browning was measured by spectrophotometry at 420 nm, and fluorescence was measured at 340 nm by a Jasco spectrofluorometer, Model FP-550A.

Measurement of Free Amino Group Content. The free amino group content of the Maillard-reacted protein was measured by the fluorometric method using fluorescamin (Roche Co., Ltd) according to Böhlen et al. (1973). The emission fluorescence at 475 nm was determined as described previously (Kato et al., 1986).

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8% acrylamide) (SDS-PAGE) was performed by the method of Laemmli (1970). Samples were heated at 90 °C for 3 min in the presence of 1% SDS and 5% 2-mercaptoethanol (2-ME).

Gel Permeation HPLC. The formation of protein polymer was analyzed on a Jasco HPLC system by a gel filtration column (TSK-Gel G-3000) equilibrated with PBS. Elution profiles were monitored by spectrophotometry at 280 nm, and the peak area was determined by an integrator of Shimazu Type C-R3A.

RESULTS AND DISCUSSION

Ovalbumin glycosylation was examined for several disaccharides: maltose, cellobiose, isomaltose, lactose, and melibiose. The decrease in free amino groups of OVA during storage for 20 days due to Maillard reaction is shown in Figure 1. The free amino groups of OVA stored with isomaltose and melibiose decreased slightly faster than those of the others. However, all five of the sugars decreased the free amino groups of OVA to less than 20% within 1 week. The decrease of free amino groups in OVA did not obey first-order kinetics, since the 20 amino groups of OVA might not react simultaneously with reducing sugars because of its conformation and slight conformational change through the reaction.

Figure 2 shows the increase in brown color of protein–sugar mixtures during the 20-day storage as measured by A_{420}. OVA–iso and OVA–mel systems strongly induced brown colorization during the storage, whereas the other

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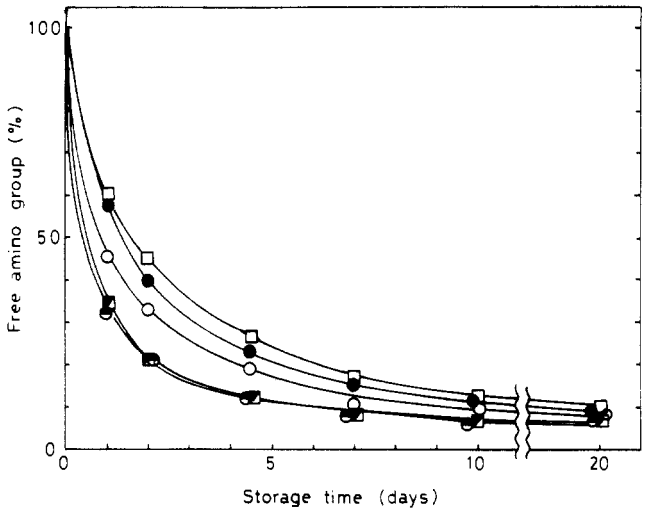

Figure 1. Decrease in free amino groups of ovalbumin during storage with maltose (●), cellobiose (○), isomaltose (◓), lactose (□), and melibiose (◩) for 20 days.

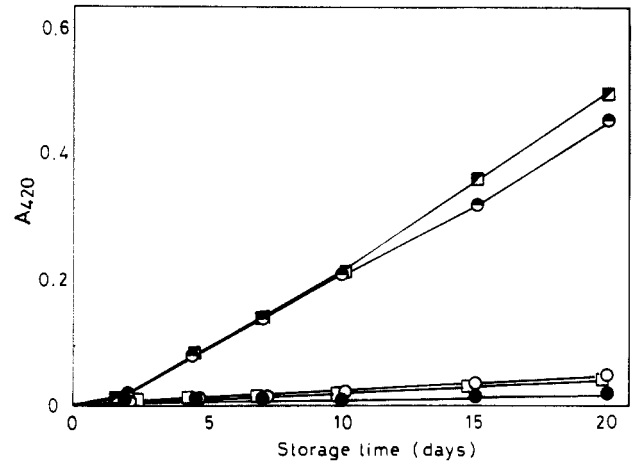

Figure 2. Brown color development of ovalbumin stored with maltose (●), cellobiose (○), isomaltose (◓), lactose (□), and melibiose (◩) for 20 days.

systems (OVA–mal, OVA–cel, OVA–lac) gave almost no browning. It seems interesting that almost no browning was induced even after more than 80% of the protein amino groups had been glycosylated with maltose, cellobiose, or lactose during 10- or 20-day storage (figure 1).

It has been reported that a brown cross-linking product in the advanced Maillard reaction showed characteristic fluorescent properties, and the structure of the fluorescent compound in the Maillard browning products has been determined to be 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (Pongor et al., 1984). Therefore, the fluorescent property of the OVA–sugar mixtures was investigated in the present study. Since the emission fluorescence spectra of the OVA–sugar mixtures showed maximum fluorescence at 415 nm (inset, Figure 3), the production of fluorescent compounds during storage in the OVA–mixtures was monitored by the emission fluorescence at 415 nm (Figure 3). The OVA–iso and OVA–mel systems produced much more fluorescent compounds than the other OVA–sugar systems. This result corresponded well with the results of brown colorization (Figure 2). However, the fluorescence of OVA–iso and OVA–mel was detected after only 1-day storage, and it reached a plateau after 15-day storage. The brown, on the other hand, increased almost linearly through the storage period tested. It seemed that the fluorescent products were produced earlier than the brown compound.

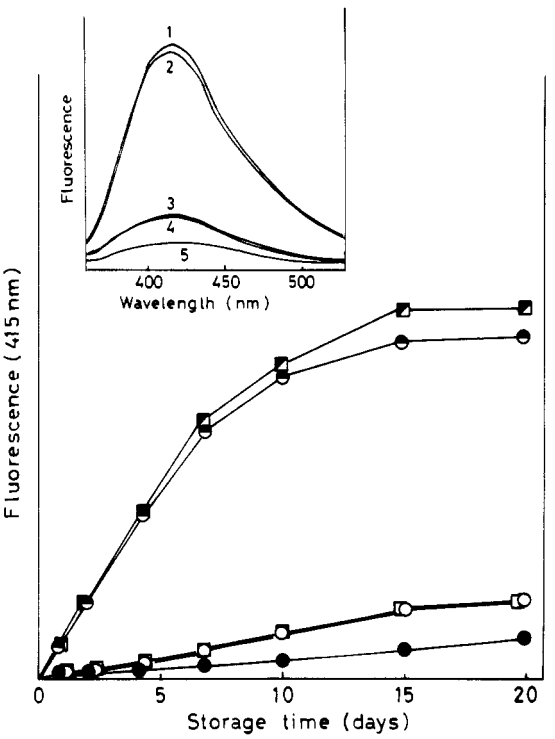

Figure 3. Time course of increasing fluorescence at 415 nm in the Maillard reaction of ovalbumin with maltose (●), cellobiose (○), isomaltose (◓), lactose (□), and melibiose (◩). The emission fluorescence spectra of samples stored for 15 days are shown in the inset. Key: (1) melibiose–, (2) isomaltose–, (3) lactose–, (4) cellobiose–, and (5) maltose–ovalbumin mixtures, respectively.

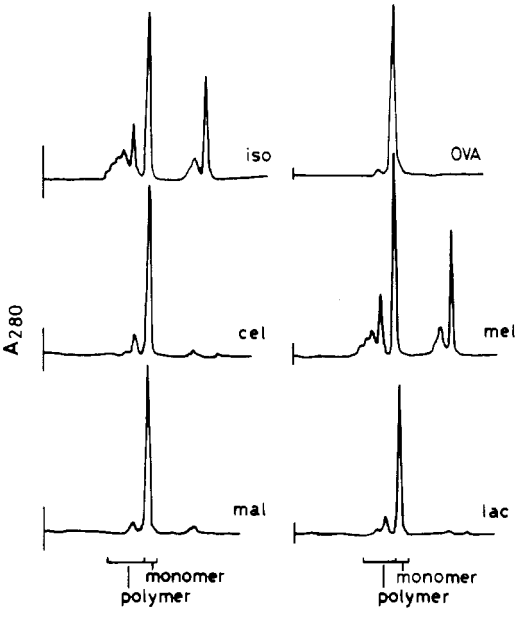

Figure 4. Protein polymerization of ovalbumin stored with maltose, cellobiose, isomaltose, lactose, and melibiose for 15 days as measured by gel permeation HPLC (Toyo Soda G-3000 SW column). Samples (15 μg of protein/50 μL) were loaded and eluted with PBS at a flow rate of 0.7 mL/min. The range of UV (280 nm) is 0.08.

Protein polymerization during the storage with the disaccharides for 15 days was analyzed by HPLC. Figure 4 shows the elution profiles of each protein–sugar mixture. The apparent proportions of the polymer fraction were estimated from the peak area of the monomer and polymer fractions and are summarized in Table I. The protein polymerization in OVA–iso and OVA–mel systems was remarkable, though the polymerization was observed in

Table I. Apparent Proportion of Ovalbumin Polymers Formed by the Maillard Reaction during Storage for 7 and 15 Days with Maltose, Cellobiose, Isomaltose, Lactose, and Melibiose[a]

	monomer, %	polymer, %
	7-Day Storage	
OVA	92.5	7.5
OVA–mal	90.4	9.6
OVA–cel	85.7	14.4
OVA–iso	74.1	25.9
OVA–lac	85.5	14.5
OVA–mel	77.4	22.6
	15-Day Storage	
OVA	87.4	12.6
OVA–mal	84.3	15.7
OVA–cel	81.7	18.3
OVA–iso	52.3	47.7
OVA–lac	82.9	17.1
OVA–mel	56.0	44.0

[a] Peak area was measured by an integrator of Shimadzu Type C-R3A.

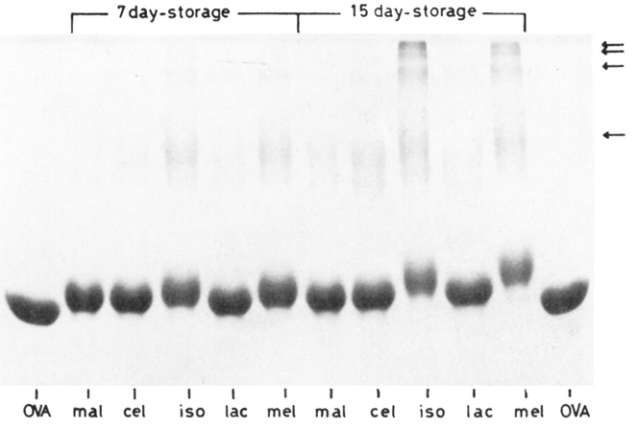

Figure 5. SDS–polyacrylamide gel electrophoresis of ovalbumin stored with maltose, cellobiose, isomaltose, lactose, and melibiose stored for 7 and 15 days. Cross-linked proteins are indicated by arrows.

all systems after 7-day storage. After 15-day storage, about half of the protein was polymerized in OVA–iso and OVA–mel systems, whereas the polymerization in the other sugar systems was much lower and almost the same as in the nonsugar (OVA only). Two peaks eluting from the HPLC column later than the OVA monomer were observed in OVA–iso and OVA–mel systems. These might contain some decomposed products formed in the advanced Maillard reaction. The chemical nature of these degradation products is of interest, and studies of structure and chemical composition are now in progress.

Protein polymerization induced by the Maillard reaction was analyzed by SDS-PAGE (Figure 5). Some protein bands with small mobility corresponding to polymerized cross-linked OVA appeared in OVA–iso and OVA–mel systems after 7-day storage. The formation of polymerized OVA was more remarkable for these two systems after 15-day storage, suggesting that isomaltose and melibiose accelerated not only brown and fluorescent product formations but also protein cross-linking more strongly than the other sugars. On the other hand, the polymers detected by HPLC analysis were almost never observed in SDS-PAGE patterns of OVA–mal and OVA systems. The high molecular weight compounds observed in the SDS–PAGE could be proteins covalently cross-linked through an amino–carbonyl reaction since the samples were treated with SDS and 2-ME. Long-time storage at 50 °C might induce protein denaturation followed by protein aggregation through noncovalent bonds and/or an intermolecular disulfide bridge, since OVA has four free sulfhydryl groups per molecule. Thus, protein polymers detected by HPLC analysis (Figure 4) might be formed partially by such hydrophobic and/or sulfhydryl group interaction on the basis on slight protein denaturation with the reaction. Isomaltose and melibiose will probably promote the latter stage reaction, and it may form more protein polymers.

Lysine and arginine residues after the Amadori rearrangement in OVA–sugar systems could not be restored to the amino acids by acid hydrolysis. The damage was measured by amino acid analysis after acid hydrolysis (Table II). The Amadori rearrangement compound deoxyketosyllysine, in OVA with lactose stored for 7 and 15 days, was quantitated by the methods of Bujard and Finot (1978) and Finot et al. (1981). The contents of Amadori rearrangement compounds in the maltose, isomaltose, cellobiose, and melibiose systems were determined with lactosyllysine measurement. The reactive lysine (free lysine plus Schiff base) of the OVA–iso and OVA–mel systems stored for 15 days was slightly lower than that in the other systems. The sum of deoxyketosyllysine and reactive lysine ($A + B$, Table II), which corresponded to the sum of free lysine, Schiff base, and the Amadori rearrangement compound, in OVA–iso and OVA–mel was obviously lower than that of the other systems. The $A + B$ value for the OVA–mal system was almost the same as

Table II. Evaluation of Reactive Lysine and Deoxyketosyllysine and Arginine of Maillard-Reacted Ovalbumin Stored with Maltose, Cellobiose, Isomaltose, Lactose, and Melibiose by Amino Acid Analysis after Acid Hydrolysis

	mol/mol of protein					
	Lys[a,d]	furosine[a,d]	reactive[b] Lys (A)	deoxyketosyl–lysine[c], (B)	$A + B$	Arg
			7-Day Storage			
OVA	19.8	0.0	19.8	0.0	19.8	15.0
OVA–maltose	16.4	1.7	14.2	5.4	19.6	15.0
OVA–cellobiose	15.5	1.8	13.3	5.6	18.9	15.0
OVA–isomaltose	13.2	1.7	11.2	5.1	16.3	11.5
OVA–lactose	14.9	1.9	12.6	5.8	18.4	14.9
OVA–melibiose	12.9	1.8	10.7	5.6	16.3	11.8
			15-Day Storage			
OVA	20.1	0.0	20.1	0.0	20.1	15.0
OVA–maltose	15.4	2.5	12.4	7.6	19.9	14.9
OVA–cellobiose	14.4	2.4	11.4	7.4	18.8	15.0
OVA–isomaltose	13.0	1.9	10.6	5.9	16.5	9.6
OVA–lactose	14.1	2.2	11.8	6.9	18.7	15.2
OVA–melibiose	12.9	1.7	10.8	5.2	16.0	9.0

[a] Determination by amino acid analysis. [b] [Reactive lysine] = [free lysine] + [Schiff base] = [lysine] − 0.4[deoxyketosyllysine]. [c] [Deoxyketosyllysine] = 3.1[furosine]. [d] The variance was ±0.2 mol.

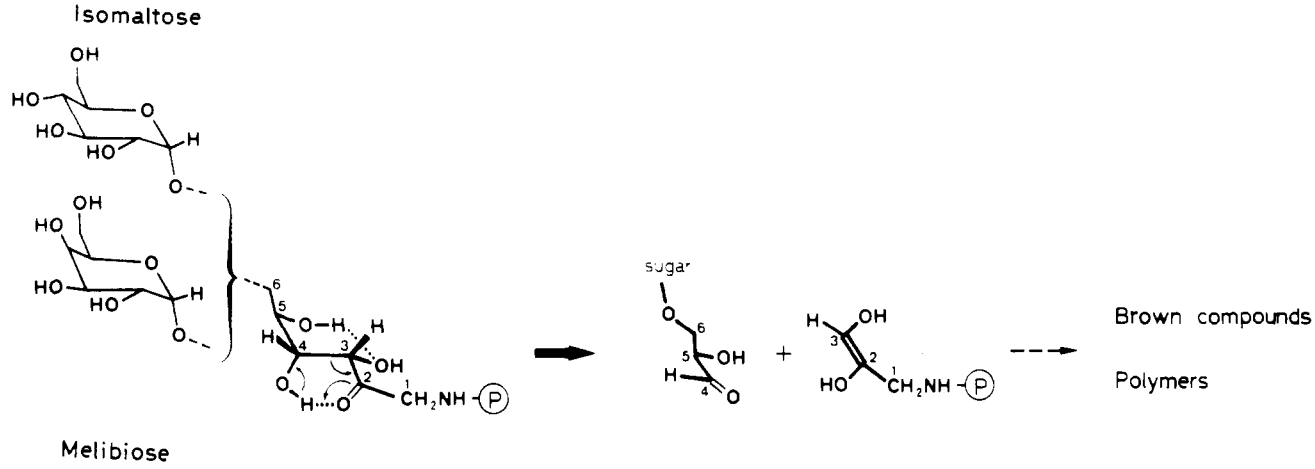

Figure 6. Conceivable cleavage mechanism of isomaltose- and melibiose–OVA Amadori compounds. P = protein.

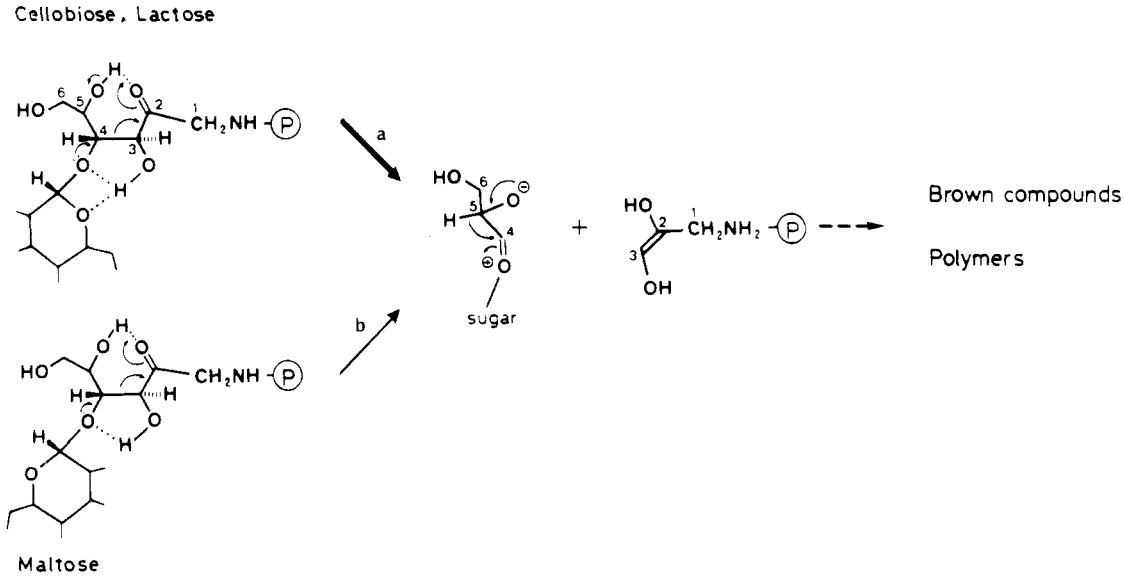

Figure 7. Probable cleavage reactions via the bidentate hydrogen-bonding intermediate of the lactose- and cellobiose–OVA complexes (path a) and unfavorable hydrogen-bonding intermediate of the maltose–OVA complex (path b). P = protein.

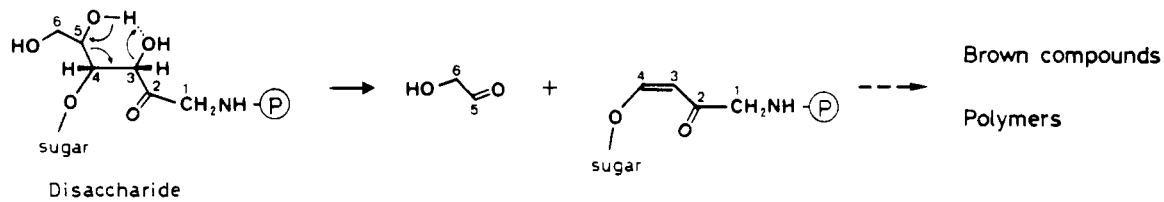

Figure 8. Plausible cleavage mechanism via the hydrogen-bonding intermediate with disadvantageous conformation to form a part of the brown color formation from disaccharide–ovalbumin complexes. P = protein.

that of OVA stored without sugars used as the control. Arginine damage was remarkable in the OVA–iso and OVA–mel systems but not in the other sugar systems. The low $A + B$ values and arginine content in the OVA–iso and OVA–mel systems were attributed to the more rapid degradation to Amadori compounds.

Fluorescence and brown color developments of the OVA–iso and OVA–mel systems were clearly different from those of the other OVA–disaccharide systems. Brown color intensities and protein polymerization of the OVA–iso and OVA–mel systems were comparable to those of the OVA–glucose system; the absorbance at 420 nm of the mixture (1 mg of OVA plus 0.5 mg of glucose) stored for 10 days was 0.23, and glucose induced protein cross-linking to a similar extent (Kato et al., 1988). These results suggest that isomaltose and melibiose have the same characteristic as monosaccharide and glucose in the reaction with OVA. Thus, the OVA–iso and OVA–mel systems may be quickly degradated to aldehyde compounds through the concerted transition state, cis–chair, chair conformation, proposed for the OVA–monosaccharide system (Kato et al., 1986) (Figure 6). In these cases, terminal pyranoside, glucose, and galactose groups bonded at the C-6 OH of the glucopyranose do not block further degradation.

In OVA–mal, OVA–cel, and OVA–lac systems, any further degradation reaction was retarded at the Amadori rearrangement product. These disaccharides have free hydroxy groups at C-3, C-5, and C-6 positions but a glycosidic bond with other sugars at the C-4 OH position. Since the C-4 OH groups of these disaccharides are blocked by another sugar moiety, they are difficult to cleave via a concerted reaction mechanism proposed in the degradation of the OVA–iso and OVA–mel complexes. However, they were slowly decomposed and gradually showed a

brown color on storage over time. After the formation of the Amadori rearrangement products, OVA–lac and OVA–cel complexes exhibited the same behavior in fluorescence and polymer formation and the same brown color, occurring slightly faster than in the OVA–mal system. The faster reaction of the OVA–lac and OVA–cel complexes may be attributed to the activation by a bidentate hydrogen bonding between C-3 OH and the acetal oxygen atom (Figure 7, path a). However, in the OVA–mal complex with a 1,4-linkage, unfavorable hydrogen bonding took place only between C-3 OH and the glycoside oxygen atom by steric hindrance (Figure 7, path b). For this reason, the OVA–mal system may be most stable in the formation of brown color and fluorescence. A part of the brown color formation from these OVA–disaccharide systems may have resulted in cleavage between a C-4–C-5 bond of the hydrogen-bonding intermediate with a disadvantageous conformation (Figure 8).

Registry No. Lac, 63-42-3; mal, 69-79-4; cel, 528-50-7; iso, 499-40-1; mel, 585-99-9.

LITERATURE CITED

Böhlen, O; Stein, S.; Dairman, W.; Untenfriend, S. Fluorometric Assay of Proteins in the Nanogram Range. *Arch. Biochem. Biophys.* **1973**, *155*, 213–220.

Bujard, E.; Finot, P. A. Mesure de la Disponibitité et du Blocage de la Lysine Dans les Laits Industries. *Ann. Nutr. Aliment.* **1978**, *32*, 291–305.

Burton, H. S.; McWeeny, D. J. Non-Enzymatic Browning Reactions: Consideration of Sugar Stability. *Nature (London)* **1963**, *197*, 266–268.

Finot, P. A.; Deutsh, R.; Bujard, E. The Extent of the Maillard Reaction during the Processing of Milk. In *Maillard Reaction in Food*; Eriksson, C., Ed.; Pergamon Press: GBR, 1981; pp 345–355.

Kato, Y.; Watanabe, K.; Sato, Y. Effect of Some Metals on the Maillard Reaction of Ovalbumin. *J. Agric. Food Chem.* **1981a**, *29*, 540–543.

Kato, Y.; Watanabe, K.; Sato, Y. Effect of Maillard Reaction on Some Physical Properties of Ovalbumin. *J. Food Sci.* **1981b**, *46*, 1835–1839.

Kato, Y.; Matsuda, T.; Kato, N.; Watanabe, K.; Nakamura, R. Browning and Insolubilization of Ovalbumin by the Maillard Reaction with Some Aldohexoses. *J. Agric. Food Chem.* **1986**, *34*, 351–355.

Kato, Y.; Matsuda, T.; Kato, N.; Nakamura, R. Browning and Protein Polymerization Induced by Amino–Carbonyl Reaction of Ovalbumin with Glucose and Lactose. *J. Agric. Food Chem.* **1988**, *36*, 806–809.

Laemmli, V. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature (London)* **1970**, *227*, 680–685.

Lewis, V. M.; Lea, C. H. A Note on the Relative Rates of Reaction of Several Reducing Sugars and Sugar Derivatives with Casein. *Biochem. Biophys. Acta* **1950**, *4*, 532–534.

Marshall, R. D.; Neuberger, A. Hen's Egg Albumin. In *Glycoproteins*; Gottshalk, A., Ed.; Elsevier: Amsterdam, 1972; pp 732–761.

Pomeranz, Y.; Johnson, J. A.; Schllenberger, J. A. Effect of various Sugars on Browning. *J. Food Sci.* **1962**, *27*, 350–354.

Pongor, S.; Ulrich, P. C.; Bensath, F. A.; Cerami, A. Aging of Proteins: Isolation and Identification of Fluorescent Chromophore from the Reaction of Polypeptides with Glucose. *Proc. Natl. Acad. Sci.* (U.S.A.) **1984**, *81*, 2684–2688.

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A Method for the Determination of the Main Metabolites of Malathion in Biological Samples

Berit Muan* and Janneche U. Skåre

A simple method has been developed for the quantification and identification of the main metabolites of malathion in biological samples. These metabolites are malathion α-monoacid, malathion diacid, *O,O*-dimethyl phosphorothioate, *O,O*-dimethyl phosphorodithioate, and desmethylmalathion. Following sorbent extraction using Bond Elut extraction columns containing anionic bonded-phase silica sorbent, the extract is methylated and quantified by use of gas–liquid chromatography equipped with a nitrogen–phosphorus selective detector. Gas–liquid chromatography—mass spectrometry is used to identify the metabolites. Recoveries of the metabolites from urine at fortification levels of 20–160 μg/mL ranged from 82 to 127% with CV <20. From feces fortified with 2–20 μg/g of the metabolites, the recoveries ranged from 18 to 66%, with CV <30.

The organophosphorus compound malathion, *O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate, is a broad-spectrum insecticide with low mammalian toxicity, which is widely used as an agricultural insecticide and as an ectoparasitic agent in both animals and humans. Malathion is activated to its oxygen analogue malaoxon, which is responsible for the toxic effects of the compound (Hayes, 1982). Degradation of malathion to inactive metabolites results from hydrolytic cleavage of one or two of the carboxyethyl ester groups, giving rise to malathion α-monoacid (MCA) or malathion diacid (DCA), respectively (Chen et al., 1969; Bradway and Shafik, 1977). Additional metabolites arise from cleavage of the P–S and S–C linkages, giving *O,O*-dimethyl phosphorothioate (DPT) and *O,O*-dimethyl phosphorodithioate (DPDT), respectively, and by demethylation, giving desmethylmalathion (DMM) (Bhagwat and Ramachandran, 1975; Nomeir and Dauterman, 1978).

A few methods for the extraction, purification, and quantification of these metabolites in different materials have been reported. However, some of these involve analysis of only the alkyl phosphate metabolites (Shafik and Enos, 1969; Shafik et al., 1971, 1973; Lores and

Department of Pharmacology and Toxicology, The Norwegian College of Veterinary Medicine, Post Box 8146 Dep., 0033 Oslo 1, Norway.

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