Covalent Binding of Glycosyl Residues to β-Lactoglobulin: Effects on Solubility and Heat Stability

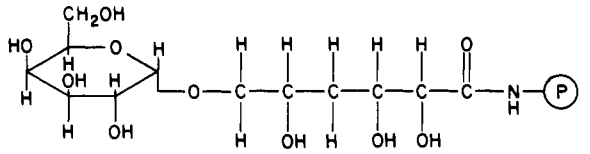
Naofumi Kitabatake,[1] Jean Louis Cuq, and Jean Claude Cheftel*

Gluconic or melibionic acids were covalently attached to amino groups of bovine β-lactoglobulin by using a water-soluble carbodiimide for the activation of carboxyl groups. The influence of reaction time and of the molar ratio of acid sugar to β-lactoglobulin in the glycosylation medium was investigated. Up to 8 mol of glycosyl residues could be attached per monomer of β-lactoglobulin. The synthetic glycoproteins displayed high solubility even at low ionic strength or at the isoelectric pH of native β-lactoglobulin. Heat stability was also improved by glycosylation. These improvements in functional properties increased with the extent of glycosylation. Melibionic acid was more effective than gluconic acid, on a molar basis.

Large amounts of milk whey are available in western countries, due to the increasing production of milk and of cheese. This has prompted the use of large-scale concentration and separation techniques for the manufacture of a whole range of undenatured protein concentrates and isolates, including single protein constituents, but these products are underutilized as well. Attempts have been made to further improve functional properties of milk and other proteins through physical and/or chemical treatments (Cheftel and Lorient, 1982; Morr, 1982; Evans and Gordon, 1980).

Although a number of glycosylation reagents are available to synthesize "neoglycoproteins" (Stowell and Lee, 1980), these have not been applied to milk whey proteins.

The present study was undertaken to artificially glycosylate β-lactoglobulin with gluconic acid or with melibionic



protein glycosylated with melibionate

acid by using water–soluble carbodiimide as an intermediate binding reagent in order to react the carboxyl group of the acid sugar with ε- or α-amino groups of the protein (Loengrenn et al., 1976).

Gluconic acid can be inexpensively produced by microbial fermentation, while melibionic acid has the advantage of being easily measured by a colorimetric method after it has attached covalently to the protein.

The effects of glycosylation on the solubility and heat stability of β-lactoglobulin have also been determined.

MATERIALS AND METHODS

Materials. Melibionic acid (6-*O*-α-D-galactosyl-D-gluconic acid) and gluconic acid (D-gluconic acid) were purchased from Sigma Chemical Co. as their potassium salts. Methyl α-galactoside (1-*O*-methyl α-D-galactopyranoside), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), Schiff's reagent (Fuchsin–sulfite reagent), and bovine serum albumin were also obtained from Sigma Chemical Co. 2,4,6-Trinitrobenzenesulfonic acid trihydrate (TNBS) was from Fluka AG. Periodic acid was purchased from Merck. β-Lactoglobulin (βLG) prepared by ion-exchange chromatography was a gift from Bel Industry (Vendome, France). It was almost free from other milk proteins, as checked by fast liquid chromatography (Pharmacia, Mono Q anion exchanger column, pH 6.7).

Glycosylation Procedure. Glycosylated βLGs were prepared by using various acid sugar to βLG ratios. The molar ratio of EDC to acid sugar was always equal to 1, unless otherwise specified. The acid sugar was dissolved in the βLG solution, and the aqueous solution of carbodiimide was then added. A typical coupling experiment with a molar ratio of 10 melibionates or gluconates per amino group of βLG in the reaction mixture was carried out as follows: 876 mg (2.19 mmol) of potassium melibionate or 512 mg (2.19 mmol) of potassium gluconate was added to a rapidly stirred 3.0-mL βLG solution (80 mg/mL, 0.219 mmol of amino groups). The pH was adjusted to 4.75 by using 1.0 N HCl. One milliliter of a solution of EDC (413 mg, 2.19 mmol) was then added dropwise over 30 min at room temperature, the pH being maintained at 4.75 by addition of 0.5 N HCl. After a reaction time of 3 h at room temperature (except for the experiments of Figure 1), 2 mL of 2 M sodium acetate buffer, pH 5.5, was added and the solution was then dialyzed extensively against 0.2 M NaCl at 4 °C. The first and last changes of dialysis solution contained 0.02% sodium azide in order to prevent microbial growth. Sodium azide was found not to influence glycosylation. The dialyzed solution of glycosylated βLG was used for subsequent experiments and analysis.

Determination of the Extent of Melibionate Binding to βLG. Amounts of melibionate covalently bound to βLG were determined by the phenol–sulfuric acid method. The calibration curve was established with a βLG solution containing various amounts of methyl α-galactoside. A blank was made by reacting phenol with βLG. A total of 0.50 mL of 5% (w/w) phenol solution was mixed to 0.50 mL of sample solution, and 2.50 mL of concentrated H_2SO_4 was then added within 10–15 s. After incubation for 10 min at room temperature, the reaction mixture was well mixed by using a vortex mixer and further incubated for 10 min at 20 °C in a water bath. Absorbance was then read at 490 nm.

Determination of Protein Concentration and of the Number of Unsubstituted Amino Groups. βLG concentration was determined by the biuret method using crystallized bovine serum albumin as a standard.

The number of free (unsubstituted) amino groups in βLG was determined with the TNBS method according to Fields (1972), using the value of 19 200 M^{-1} cm^{-1} for the

Laboratoire de Biochimie et Technologie Alimentaires, Université des Sciences et Techniques, 34060 Montpellier, France.

[1]Present address: Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan.

0021-8561/85/1433-0125$01.50/0 © 1985 American Chemical Society

molar absorptivity of TNP–amino groups. The reaction of TNBS with unsubstituted amino groups was carried out at room temperature for 30 min in order to get a constant value.

Electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS)-containing buffer (13.5% acrylamide) was performed following the general procedure of Laemmli (1970). Acrylamide (3%) was used in the stacking gel. Protein solutions (2.0 mg/mL) were heated for 10 min at 80 °C and left overnight at room temperature in the presence of SDS (1%) and 2-mercaptoethanol (MSH) (5%) in order to complete protein denaturation and reduction. Protein bands were stained with Coomassie Brilliant Blue R-250 and glycosylated protein bands were stained with PAS (periodic acid, Schiff's reagent) according to Glossmann and Neville (1971).

Assessement of the Solubility of the Glycosylated Protein. The solubility of glycosylated βLG was assessed by turbidimetry of the protein solution at various ionic strengths and pH values. The turbidity of samples is given as percent transmittance at 500 nm with a 1-cm light path.

In order to assess the ionic strength dependency of protein solubility, the following procedure was used. The pH of dialyzed solutions of glycosylated or of native (control) βLG (both 0.2 M in NaCl) was adjusted to 5.2 with 1 N NaOH or 1 N HCl. Distilled water was gradually added dropwise to small volumes of the dialyzed solutions under gentle shaking (so as to avoid air bubble entrapment), and a given volume of 0.4 M NaCl solution was then immediately added to reach the desired ionic strength. After the samples were allowed to stand for 1 h at room temperature, the transmittance of samples was measured at 500 nm against distilled water. The percent transmittance values were constant after 30-min standing until 2 h. The final protein concentration and sample volume were 10 mg/mL and 3.0 mL, respectively, in all cases.

The influence of pH on the solubility at low ionic strength was measured as follows. The pH of the dialyzed solutions of glycosylated and native βLG was adjusted to the required value with 1 N NaOH or 1 N HCl. Addition of distilled water and of 0.4 M NaCl to the protein solution and transmittance measurements were performed as described above. The final protein and NaCl concentrations were 1.0 mg/mL and 20 mM, respectively.

Assessment of the Heat Stability of the Glycosylated Protein. The effect of heating temperature on the solubility of glycosylated and native βLG was determined as follows. The pH of the dialyzed protein solutions (0.2 M in NaCl) was adjusted to 7.0 with 1 N NaOH. Distilled water and a given volume of 0.4 M NaCl were added to reach a final protein concentration of 6.0 mg/mL and a final ionic strength of 0.2 M. Samples of this solution were then heated for 15 min at various temperatures in a water bath. After the samples were cooled with tap water, the transmittance was measured at 500 nm.

RESULTS AND DISCUSSION

Preparation of Glycosylated β-Lactoglobulin. The influence of reaction time on the extent of glycosylation by melibionate (for an initial melibionate/amino group molar ratio of 15) is shown in Figure 1. Both the number of melibionate residues covalently bound to βLG and the number of remaining TNBS-reactive amino groups of βLG were determined.

It can be seen that ca. 6 mol of melibionate is covalently bound per mol of βLG, after only 1-h reaction, while 7 mol is bound after a reaction time of 3 h.

One mole of βLG contains 15 mol of ε-amino groups and 1 mol of α-amino group (Swaisgood, 1982). In the present

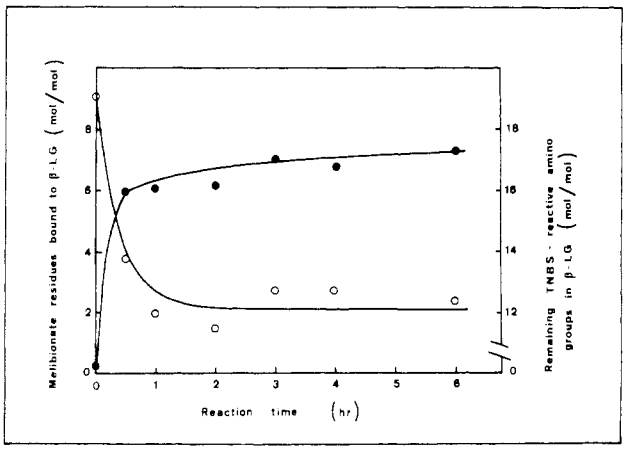


Figure 1. Covalent binding of melibionate and decrease of chemically reactive amino groups during reaction between β-lactoglobulin (βLG), potassium melibionate, and water-soluble carbodiimide (EDC). Amount of βLG in glycosylation medium, 240 mg; amount of melibionate, 1314 mg, 3.285 mmol; amount of EDC, 619.5 mg, 3.285 mmol; room temperature; pH 4.75; molar amount of amino groups in the initial glycosylation medium (calculated from the βLG sequence), 0.219 mmol.

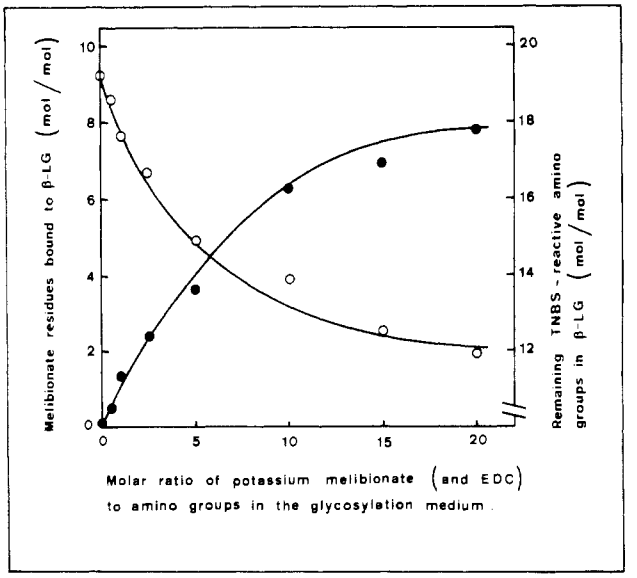


Figure 2. Effect of potassium melibionate concentration on the extent of melibionate binding to βLG and on the decrease of chemically reactive amino groups in βLG. Amount of βLG in glycosylation medium, 240 mg; molar amount of EDC in glycosylation medium equal to that of melibionate; reaction time, 3 h; room temperature; pH 4.75; molar amount of amino groups in the initial glycosylation medium, 0.219 mmol.

measurements, about 19 mol of TNBS-reactive amino groups were determined. This discrepancy probably results from the reaction of other amino acid side groups with TNBS (Fields, 1972) and from the higher color intensity of TNP-α-amino groups as compared to that of TNP-ε-amino groups (Fields, 1972). Since incorporation of melibionate into βLG appears to reach a plateau after 3 h (Figure 1), a reaction time of 3 h was adopted for all subsequent glycosylation experiments. In order to evaluate the influence of carbohydrate concentration in the reaction medium, glycosylation experiments were carried out with melibionate/amino group molar ratios varying from 0 to 20.

As shown in Figure 2, the number of melibionate residues covalently bound (and the number of TNBS-unreactive amino groups) per mol of βLG increased with the melibionate/amino group molar ratio in the reaction me-

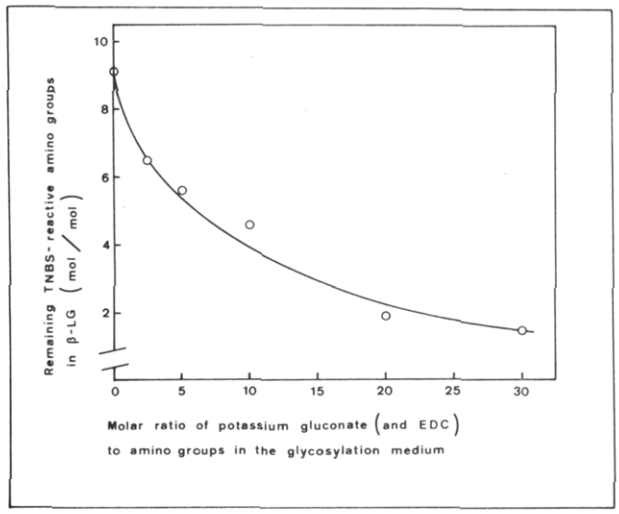


Figure 3. Effect of potassium gluconate concentration on the decrease of chemically reactive amino groups in βLG. Amount of βLG in the glycosylation medium, 240 mg; molar amount of EDC equal to that of gluconate; reaction time, 3 h; room temperature; pH 4.75; molar amount of amino groups in the initial glycosylation medium, 0.219 mmol.

dium, reaching an almost constant value of 7 for molar ratios of 15 and above.

The results of experiments in which potassium gluconate was used instead of potassium melibionate are shown in Figure 3. Since protein-bound gluconate cannot be determined by the phenol–sulfuric acid colorimetric method, only the numbers of remaining TNBS-reactive amino groups in βLG have been determined. The maximal decrease in such groups appears to be close to 8, a value that is similar to that (7) found with melibionate. With gluconate, it was possible to raise the gluconate/amino group molar ratio to 30, and no precipitation took place during dialysis. However, some protein polymerization was found to occur in glycosylation experiments both wiith melibionate and with gluconate (see below).

In the absence of information of the three-dimensional structure of βLG, the location or state of the various lysine residues is not known. From the results given in Figures 1–3, it appears that 7 to 8 of the 16 amino groups may be exposed at the surface of the molecule or in a position readily available for glycosylation.

Electrophoretic Patterns of Glycosylated β-Lactoglobulin. MSH–SDS–PAGE patterns of βLG glycosylated with different melibionate/amino group molar ratios in the reaction medium are shown in Figure 4. Coomassie Brilliant Blue R-250 was used for staining protein bands in the gels of Figure 4, while Schiff's reagent was used for specific staining of glycoprotein bands.

As seen in Figure 4, a control sample of nonglycosylated βLG shows a single protein band, while glycosylated βLG samples display additional proteins species with lower mobility. From a plot of the mobility of the marker proteins (α-lactalbumin, L chain of immunoglobulin G, ovalbumin, bovine serum albumin, H chain of immunoglobulin G) vs. the log of their molecular weights, it appears that the band next to βLG is probably a dimer of βLG. Other high molecular weight bands are polymers of βLG. Since the protein samples were completely reduced by excess amounts of 2-mercaptoethanol before electrophoresis, this polymerization does not result from intermolecular disulfide formation. It is likely that some carboxyl groups of βLG were activated by EDC and reacted with amino groups of other protein molecules, thus resulting in intermolecular covalent cross-linking.

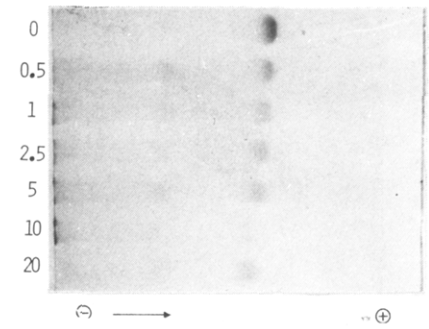


Figure 4. SDS–polyacrylamide gel electrophoretic patterns of βLG glycosylated with melibionate. Protein staining with Coomassie Brilliant Blue R-250. Numbers represent the molar ratios of potassium melibionate (and EDC) to amino groups in the initial glycosylation medium. The degree of glycosylation of each sample is shown in Figure 2.

When the extent of glycosylation of βLG increases (higher melibionate/amino group molar ratios being used in the reaction medium), the mobility of the main protein band decreases slightly with respect to that of native βLG (Figure 4). This may result from the increased molecular weight of glycosylated βLG as more melibionate residues are covalently bound.

The glycosylated βLG band is broader and more diffuse than that of the native βLG. This indicates that there is a polydisperse distribution of glycosylated species. The number of melibionate residues bound per mol of βLG, as determined in Figure 2, must therefore represent the average of various protein species with different extents of glycosylation.

Carbohydrate staining with Schiff's reagent is weak but gives interesting information: (1) native βLG is not stained, as can be expected since normal βLG is not a glycoprotein; (2) glycosylated βLG is stained, and the same mobility is observed for the bands stained with Schiff's reagent and for those stained with Coomassie Brilliant Blue; (3) the higher molecular weight protein bands, which correspond to polymers of βLG, are also stained by Schiff's reagent. This supports the data that show that βLG has indeed been covalently glycosylated with melibionate.

The extent of βLG polymerization has not been quantified but appears to increase with the extent of glycosylation. This is probably due to the higher concentration of EDC in the reaction medium.

When βLG is glycosylated with gluconate, identical gels were obtained, and some protein species with a molecular weight higher than that of native βLG were also observed by SDS–PAGE.

Solubility Characteristics of Glycosylated β-Lactoglobulin. *Influence of NaCl Concentration on Protein Solubility.* Native βLG cannot be dissolved in neutral solutions of low ionic strengths. At pH 5.2 (isoelectric pH) especially, the addition of salt(s) is necessary for solubilization (Grönwall, 1942).

The solubility of glycosylated βLG at pH 5.2 has been studied as a function of NaCl concentration. As shown in Figure 5, native βLG is soluble at a concentration of 1.0 mg/mL of 0.2 M NaCl. Transmittance decreases at lower NaCl concentrations. βLG glycosylated with melibionate at a low extent behaves similarly to native βLG. Highly glycosylated βLG, however, remains soluble at much lower ionic strengths. For example, when the glycosylation medium contains a molar ratio of melibionate to amino groups above 10, the glycosylated protein (to which more than six residues of melibionate are covalently bound per mole) remains soluble down to a concentration of NaCl equal to 20 mM.

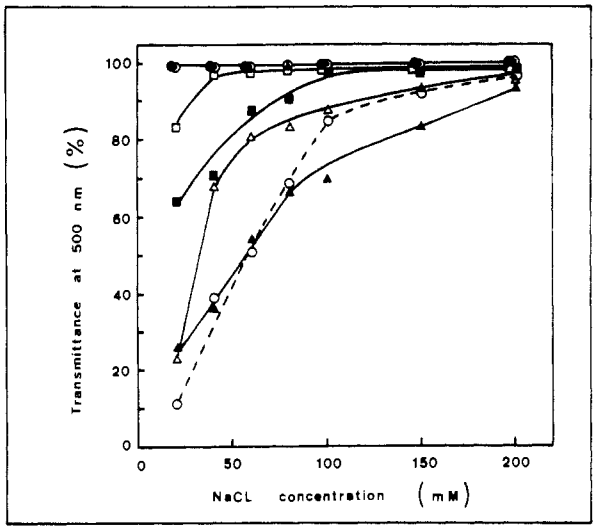


Figure 5. Effect of NaCl concentration on the solubility of βLG glycosylated with melibionate. Protein concentration, 1.0 mg/mL; pH 5.2. The extent of glycosylation of each sample is shown in Figure 2. Molar ratio of potassium melibionate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 0.5 (▲); 1.0 (△); 2.5 (■); 5 (□); 10 (●); 20 (⊙).

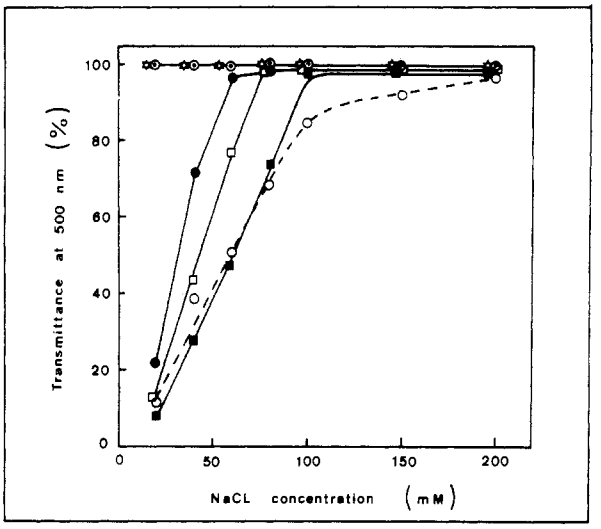


Figure 6. Effect of NaCl concentration on the solubility of βLG glycosylated with gluconate. Protein concentration, 1.0 mg/mL; pH 5.2. The extent of glycosylation of each sample is discussed in the text. Molar ratio of potassium gluconate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 2.5 (■); 5 (□); 10 (●); 20 (⊙); 30 (☆).

A similar behavior is noted when βLG is glycosylated with gluconate (Figure 6). A close comparison of Figures 5 and 6, taking also into account the results of Figures 2 and 3, indicates that gluconate is less effective than melibionate for enhancing βLG solubility at low ionic strength provided the comparison is made at equal extents of glycosylation. This may simply reflect the fact that melibionate is a disaccharide and brings more hydrophilicity than gluconate.

Influence of pH on Protein Solubility. The enhanced solubility of glycosylated βLG at low ionic strength observed at pH 5.2 (Figures 5 and 6) could be partly caused by a change in the isoelectric pH of the protein following substitution of amino groups with melibionate or gluconate. For this reason the pH–solubility profile, or more exactly the pH–turbidity profile, of glycosylated βLG has been investigated at low ionic strength (20 mM NaCl) over a wide range of pH. The protein concentration is kept at

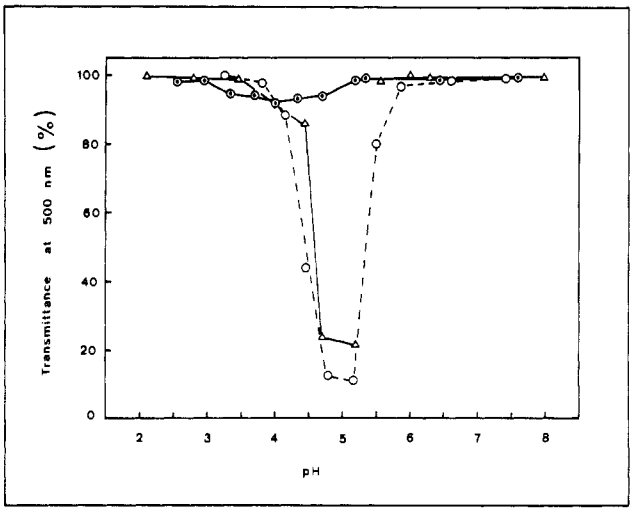


Figure 7. Effect of pH on the solubility of βLG glycosylated with melibionate. Protein concentration, 1.0 mg/mL; NaCl concentration, 20 mM. The extent of glycosylation of each sample is shown in Figure 2. Molar ratios of potassium melibionate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 1 (△); 20 (⊙).

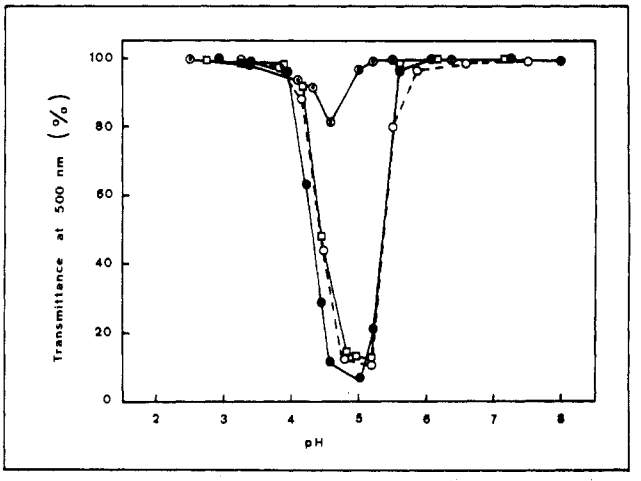


Figure 8. Effect of pH on the solubility of βLG glycosylated with gluconate. Protein concentration, 1.0 mg/mL; NaCl concentration, 20 mM. The extent of glycosylation of each sample is discussed in the text. Molar ratios of potassium gluconate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 5 (□); 10 (●); 20 (⊙).

1.0 mg/mL, as for the experiments previously reported (in Figures 5 and 6).

The pH–turbidity profiles of βLG glycosylated with melibionate and with gluconate are shown in Figures 7 and 8, respectively. The solution of native βLG becomes turbid at pHs close to 5 but remains clear above pH 6 or below pH 4. The solution of βLG glycosylated with melibionate at a high extent of glycosylation remains clear over the whole pH range studied (pH 2–8). A slight decrease in transmittance taking place around pH 4.3 suggests that the isoelectric point of βLG is somewhat shifted by glycosylation (Figure 7).

The pH–turbidity profile of βLG glycosylated with gluconate indicates similar effects (Figure 8). When only 4 mol of gluconate is attached per mol of βLG (molar ratio of gluconate to amino group in the glycosylation medium = 10), the pH–turbidity profile remains almost the same as that of native βLG. When 3 mol of gluconate is bound per mol of βLG (above-mentioned molar ratio = 5), the pH–turbidity profile indicates higher solubility, close to that obtained by the covalent binding of 1 mol of meli-

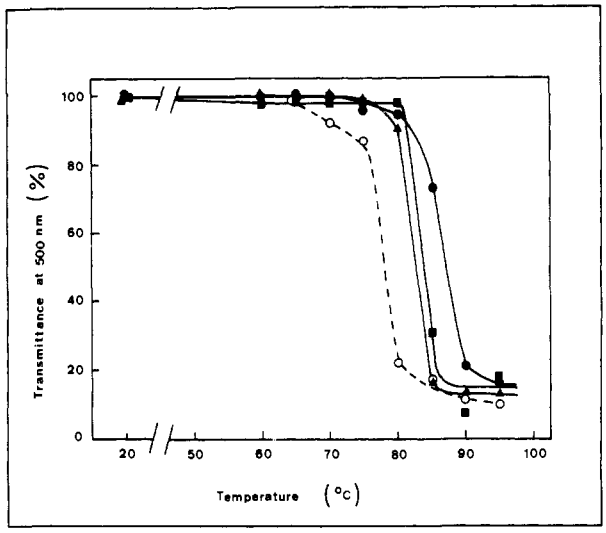


Figure 9. Effect of heating temperature on the solubility of βLG glycosylated with melibionate. Protein concentration, 6.0 mg/mL; NaCl concentration, 0.2 M; pH 7.0. Each sample was heated for 15 min before turbidity measurement. Molar ratios of potassium melibionate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 0.5 (▲); 2.5 (■); 10 (●).

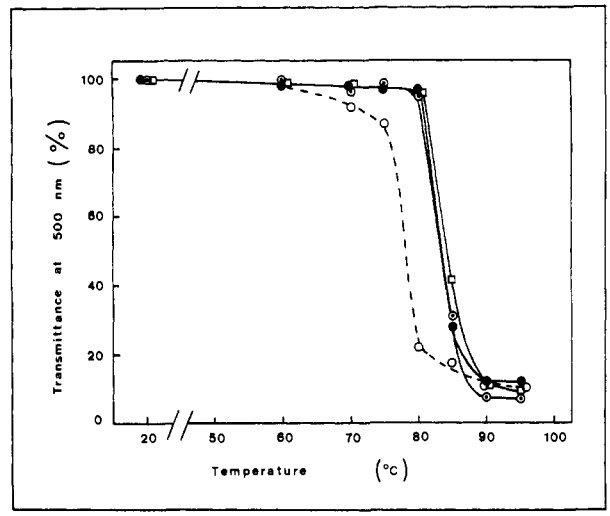


Figure 10. Effect of heating temperature on the solubility of βLG glycosylated with gluconate. Protein concentration, 6.0 mg/mL; NaCl concentration, 0.2 M; pH 7.0. Each sample was heated for 15 min before turbidity measurement. Molar ratios of potassium gluconate (and EDC) to amino groups in the initial glycosylation medium: 0 (○); 5 (□); 10 (●); 20 (⊙).

bionate. In these experiments also, melibionate therefore appears to be more effective than gluconate for enhancing solubility at low ionic strength and a pH close to 5.

Sung et al. (1983) have prepared phosphorylated soy protein and examined the changes in pH–turbidity profiles. The solubility minimum was found to be shifted to acidic pHs, and this shift increased with the extent of phosphorylation. However, a solubility minimum was still observed, even at high extents of phosphorylation. It therefore appears that glycosylation modifies protein solubility in a manner remarkably different from that of phosphorylation.

Influence of Heat on Protein Solubility. It is well-known that heat induces aggregation, and eventually gelation, of βLG, depending on the conditions of pH, ionic strength, and protein concentration. It was of interest to investigate whether the heat sensitivity of glycosylated βLG is similar or different from that of native βLG. For this, a solution of glycosylated βLG (6 mg of protein/mL of 0.2 M NaCl adjusted to pH 7) is heated for 15 min at different temperatures, and the transmittance of the solution is determined after cooling. The NaCl concentration and the pH of the solution were selected in order to cancel the effects of low ionic strength and of isoelectric pH on protein solubility.

Results of experiments using βLG glycosylated with melibionate and with gluconate are shown in Figures 9 and 10, respectively. The solution of native βLG becomes turbid above 70 °C, and its transmittance decreases to ca. 50% at 77 °C. In the case of βLG glycosylated with melibionate, the temperature–turbidity profile is shifted toward higher temperatures by about 5 °C. Similar results are observed in the case of βLG glycosylated with gluconate, but higher extents of glycosylation are needed in order to obtain the same shift. This confirms the conclusion drawn from previous experiments that glycosylation with gluconate is less effective than with melibionate, on a molar basis, to enhance protein solubility in environmental conditions that tend to promote aggregation.

It is known that the heat stability of proteins generally increases when sugars are added (at high concentrations, close to 1 M) to the protein solution [e.g., Lee and Timasheff (1981)]. The addition of a small amount of free sugar (1.67 or 16.7 μmol of glucose or melibiose) to a solution of βLG (6 mg/mL) was investigated as a control experiment. The glucose or melibiose concentration corresponds to that of gluconate or melibionate attached to βLG by glycosylation. No heat stabilization effect was noted in the control experiment. This confirms that the covalent binding of a small number of glycosyl residues to βLG is specifically responsible for the increased heat stability.

Glycoproteins usually possess higher solubility and heat stability and are catabolized in vivo less rapidly than their nonglycosylated counterparts (Holcenberg et al., 1975; Marsh et al., 1977; Marshall, 1978). However, when beef liver catalase was glycosylated by attachment of lactose, resistance to inactivation by proteolysis was increased but not heat stability (Wasserman and Hultin, 1982).

The present study shows that the covalent binding of glycosyl residues to βLG increases heat resistance and promotes higher solubility at low ionic strength or at the isoelectric pH of the native protein. These effects are probably due both to the change in net charge of βLG and to the introduction of hydrophilic residues.

It remains to be investigated whether this introduction of carbohydrate residues also promotes changes in other functional properties of interest in food systems: viscosity, water absorption, gelation, and emulsification. Changes in these properties take place when βLG is phosphorylated or esterified (Woo et al., 1982; Mattarella and Richardson, 1983; Woo and Richardson, 1983). Attachment of glucose, fructose, or lactose to casein through reductive alkylation was found to modify nutritional value, but functional properties were not investigated (Lee et al., 1979). In papers published after the present study was completed, Waniska and Kinsella (1984a,b) report the glycosylation of βLG with maltose or glucosamine, using the cyclic carbonate or the carbodiimide method, respectively. Up to 32 residues of maltose or 16.6 residues of glucosamine were covalently attached. The viscosity of maltosyl and glucosaminyl derivatives of βLG increased as the mass of the carbohydrates covalently linked to βLG increased. Changes in hydrophobicity and ultraviolet difference spectra were also determined.

ACKNOWLEDGMENT

We thank E. Dumay for her advice and comments. We gratefully acknowledge the help of the Ministère des Re-

lations Extérieures, Paris, for a postdoctoral fellowship attributed to one of us (N.K.).

Registry No. Gluconic acid, 526-95-4; melibionic acid, 21675-38-7.

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Received for review May 23, 1984. Accepted October 10, 1984.

Characterization of Bacon Odor and Other Flavor Components from the Reaction of Isovaleraldehyde and Ammonium Sulfide

Chi-Kuen Shu,* Braja D. Mookherjee, Henry A. Bondarovich, and Myrna L. Hagedorn

Characterization of the reaction of isovaleraldehyde and ammonium sulfide by gas chromatography–mass spectrometry revealed several components including the characteristic fried bacon compounds 2,4,6-triisobutyl-4*H*-1,3,5-dithiazine and 3,5-diisobutyl-1,2,4-trithiolane. A novel pyridine, 2-isobutyl-3,5-diisopropylpyridine, has also been identified by additional proton NMR analysis. Mechanisms of formation of those components are described.

It has been well-known that Maillard reaction and Strecker degradation play a very important role in food systems to provide flavor components using amino acids and sugars. The products from such reactions can further react with each other or react with the degradation products to form additional flavor components. Usually, amino acid generates the corresponding aldehyde with one carbon less through Strecker degradations (Schonberg and Moubacher, 1952), and sulfur-containing amino acid is degraded to different components including ammonia and hydrogen sulfide (Mulders, 1973).

Wiener (1972) reported the reaction between isovaleraldehyde and ammonium sulfide producing fried bacon odor but did not mention which components were responsible for such flavor. Now we are reporting the characterizations of the components of the reaction mixture including the ones that possess the fried bacon aroma.

EXPERIMENTAL SECTION

Preparation of the Reaction Mixture. Isovaleraldehyde was mixed with aqueous 22% ammonium sulfide (Mallinckrodt Inc., St. Louis, MO) in a 1:1 molar ratio at 15 °C for 2 h. The reaction mass was then extracted with hexane. The hexane extract was washed with water, dried, and concentrated.

Analysis. The concentrated material was analyzed by gas chromatography–mass spectrometry (GC–MS) on a 3% SE-30 stainless steel column (10 ft × $^1/_8$ in.) programmed from 70 to 210 °C at 4 °C/min. The mass spectrometer was a Kratos MS-50 operated at 70 eV. The peaks were trapped from GC for organoleptic evaluation and for nuclear magnetic resonance (NMR) analysis. The proton NMR spectrum was obtained from Varian XL-100 operated at 100 MHz in trichlorofluoromethane with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Figure 1 represents the GC chromatogram of the reaction mixture. Table I shows the components identified along with the major mass spectral fragments.

Peaks 12 and 13 were organoleptically evaluated to possess the fried bacon aroma and were identified as 3,5-diisobutyl-1,2,4-trithiolane and 5,6-dihydro-2,4,6-triisobutyl-4*H*-1,3,5-dithiazine respectively. The detailed information regarding the identification, the synthesis, and the organoleptic properties of these two compounds has already been published (Shu et al., 1980, 1981).

The mechanism of the formation of these two components can be described by the mechanism shown in Figure 2. Hydrogen sulfide and ammonia were derived from ammonium sulfide. Isovaleraldehyde reacted either with H_2S to form the intermediate compound I or with NH_3 to form the intermediate compound II. When 2 mol of the intermediate compound I were involved via oxidation and a loss of H_2S, 3,5-diisobutyl-1,2,4-trithiolane was generated. When both intermediate compounds were interacted with a loss of 3 mol of H_2S, 5,6-dihydro-2,4,6-triisobutyl-4*H*-1,3,5-dithiazine was generated. In the literature, similar

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0021-8561/85/1433-0130$01.50/0 © 1985 American Chemical Society