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Lysine Loss and Polymerization of Bovine β-Lactoglobulin by Amino Carbonyl Reaction with Lactulose (4-*O*-β-D-Galactopyranosyl-D-fructose)

Tsukasa Matsuda,*,† Yasuko Kato,‡ and Ryo Nakamura†

Department of Food Science and Technology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan, and Women's College of Tokaigakuen, Tenpaku-ku, Nagoya 468, Japan

Loss of lysine, protein polymerization, and production of fluorescent substances were investigated for bovine β-lactoglobulin stored with lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) at 50 °C and 65% relative humidity for 10 days as a model system for dried milk and milk products supplemented with lactulose. Reactivity of lactulose with protein amino groups was much lower than that of lactose, whereas the lactulose induced the protein polymerization and production of fluorescent substances not less strongly than lactose did. These results suggested that the effect of the amino carbonyl reaction between lactulose and milk proteins on protein nutritive value, i.e., lysine loss, was not very serious but that proteins reacted with lactulose were easily polymerized by cross-linking through lactulose–lysine amino carbonyl adducts.

INTRODUCTION

The amino carbonyl reaction occurs between reducing sugars and protein amino groups, with subsequent changes in the structure of "advanced" reaction derivatives (Reynolds, 1963; Namiki, 1988). Food proteins reacted with reducing sugars through the amino carbonyl reaction have been reported to have good functional properties such as high solubility, heat stability, and emulsifying activity (Tybor et al., 1973; Morales et al., 1976; Kato et al., 1978). On the other hand, amino carbonyl products formed at advanced stages of the reaction are responsible for a decrease in the nutritive value of the protein and some adverse physiological effects (Finot et al., 1981; Finot, 1990).

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) was first detected by Adachi (1985) in the whey from heated milk, although it has not been detected in raw milk. The presence of lactulose in heated milk is explained by the fact that lactose is converted into lactulose by the rearrangement of aldoses to ketoses at high temperature or in alkaline solution. It has been reported that heat-sterilized liquid milk formulas used for infant feeding contained lactulose representing 1–5% of the total carbohydrate (Berrnhart et al., 1965). When milks for infant feeding were heated before feeding, lactulose was produced in amounts that increased with heating time.

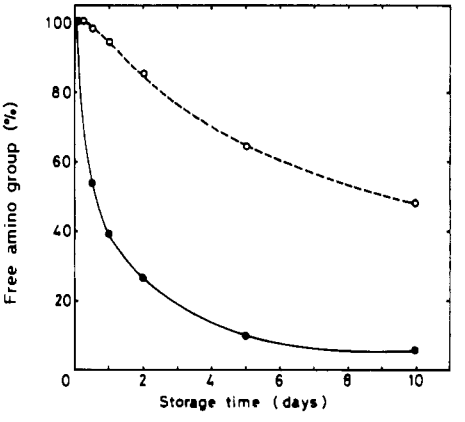

Figure 1. Decrease in free amino group of β-lactoglobulins stored with lactulose (○) and lactose (●). The free amino group was measured by the fluorescamine method (Böhlen et al., 1973).

Lactulose is regarded as the factor responsible for the growth of bifidus flora in bottle-fed infants. The addition of 1.2–1.5% lactulose to the diet induced an increase in the *Bifidobacterium bifidum* population of the large intestine and a lowering of its pH (Mendez and Olano, 1979).

* Author to whom correspondence should be addressed.
† Nagoya University.
‡ Women's College of Tokaigakuen.

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Table I. Lysine, Arginine, and Furosine Content[a] of β-Lactoglobulins (LG) Stored with Lactulose and Lactose for Various Periods

	LG–lactulose				LG–lactose				LG
amino acid	0 days	2 days	5 days	10 days	0 days	2 days	5 days	10 days	10 days
lysine	15.0	15.1	13.4	12.1	15.0	12.4	8.9	8.5	15.1
arginine	3.0	3.1	3.1	2.9	2.9	3.1	2.8	2.8	2.8
furosine	ND[b]	ND	ND	ND	ND	1.3	1.9	2.0	ND

[a] Moles/mole of the protein. [b] ND, not detected.

We have previously reported that slight differences in the chemical structure of the reducing sugars resulted in large differences in the production of characteristic amino carbonyl adducts formed at the advanced stages of the reaction (Kato et al., 1986, 1989). Thus, the amino carbonyl reaction of lactulose with a ketose-type reducing end was expected to be different from that of the other sugars with an aldose-type reducing end. The objective of the present study is to characterize the amino carbonyl reaction of bovine β-lactoglobulin with lactulose by comparing it with that of the protein–lactose system.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin (3× crystallized) and lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) were purchased from Sigma Chemical Co. (St. Louis, MO), and lactose was from Wako Pure Chemicals (Osaka, Japan). Fluorescamine (4-phenylspiro-[furan-2(3*H*),1′(3′*H*)-isobenzofuran]-3,3′-dione) was obtained from Hoffman-La Roche Inc. (Nutley, NJ).

Sample Preparation. The β-lactoglobulin was reacted with lactulose or lactose as follows: The protein and each sugar were dissolved in distilled water (5 mg/mL each), and the pH of the solution was adjusted to 7.5 with dilute NaOH. After being freeze-dried, the powdered protein–sugar mixture was kept at 50 °C and 65% relative humidity for various periods (0–10 days) to accelerate the amino carbonyl reaction.

Determination of Free Amino Group. Free amino groups of proteins were determined by the fluorometric method using fluorescamine according to the procedure of Böhlen et al. (1973). Fifty microliters of the protein solution (300 μg/mL) was mixed with 3.0 mL of 50 mM sodium phosphate buffer, pH 8.0. While the solution in a test tube is being vigorously shaken on a vortex-type mixer, 0.5 mL of fluorescamine in dioxane (300 μg/mL) is rapidly added. The fluorescence was measured by a Jasco FP-550A spectrofluorometer with excitation at 390 nm and emission at 475 nm.

Amino Acid Analysis. The lysine, arginine, and furosine were determined by a JEOL HPLC LC300 system for amino acid analysis after acid hydrolysis at 110 °C for 24 h in 6 N HCl in evacuated sealed tubes. Since pure furosine was not available, the color factor for lysine was used for the furosine analysis.

Size Exclusion HPLC Analysis. The protein polymerization induced by the reaction with sugars was analyzed by a size exclusion HPLC system equipped with a column of TSK-gel G3000SW (7.4 × 600 mm, Tosoh, Tokyo). The column was equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. The samples were eluted with the same buffer at a flow rate of 0.7 mL/min, and the elution profiles were monitored by the absorbance at 280 nm. The peak area was determined by a Shimadzu C-R3A integrator.

Gel Electrophoresis. The sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (12.5% acrylamide) was performed according to the method of Laemmli (1970). The gel sheets were stained with 0.2% Coomassie Brilliant Blue R-250.

Fluorescence Measurement. The protein stored with each sugar was dissolved in 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. The protein concentration was 2.5 mg/mL. Fluorescent compounds produced by the amino carbonyl reaction were analyzed by a Jasco FB-550A spectrofluorometer with excitation at 340 nm according to the method of Ponger et al. (1984).

RESULTS AND DISCUSSION

The reactivity of lactulose with protein amino groups was examined by measuring the remaining free amino groups of the protein and compared with that of lactose. As shown in Figure 1, the amino group content decreased gradually, and about 50% of the amino groups were blocked after the reaction with lactulose for 10 days. The reaction rate of lactulose with the protein amino groups appeared to be much slower than that of lactose. The semilog plots of the free amino group content did not give a straight line, indicating that the reaction did not obey first-order kinetics. The time required for the 50% blocking of the amino groups was 10 days for lactulose, whereas it was only 1 day for lactose. The reducing side of lactulose is a ketohexose, fructose, whereas lactose has an aldohexose, glucose. Hence, the relatively slow reaction rate of lactulose, which was in contrast with that of lactose, would be due to the keto group reactivity which was lower than that of the aldehyde group.

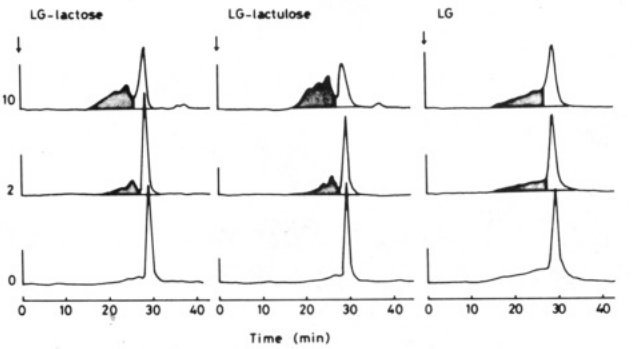

Figure 2. Protein polymerization by the reaction with lactulose and lactose as measured by size exclusion HPLC. The shaded areas were regarded as the polymer fraction. The protein stored in the absence of sugars (LG) was analyzed as a control.

Table I shows the contents of lysine, arginine, and a lysine derivative, furosine, of the sugar–protein complexes as measured by amino acid analysis after the acid hydrolysis. The lysine content slightly decreased after the storage with lactulose for 5 or 10 days. However, the decrease in the lactulose–protein system was not so remarkable as compared with that of the lactose–protein system. There was no significant change in the arginine content in either systems. Furosine, which is a lysine derivative produced by acid hydrolysis from Amadori compounds, was not detected in the lactulose–protein system.

Lactose, which reacted with lysine, produces an Amadori rearrangement product, ε-deoxylactulosyllysine (Heyns and Paulsen, 1959), whereas lactulose does not. Lactulose would produce a Heyns rearrangement compound by the reaction with lysine. The Heyns product, ε-deoxylactosyllysine, would not produce the lysine derivative, furosine, after acid hydrolysis (Finot et al., 1981). The results from the amino group and amino acid analyses suggested that the loss of protein-bound lysine caused by the reaction with lactulose was not very serious even as compared with that caused by the reaction with lactose, which has much lower reactivity with amino groups than that of glucose and galactose (Kato et al., 1986, 1989).

Protein polymerization and cross-linking caused by the reaction with lactulose were investigated by means of size

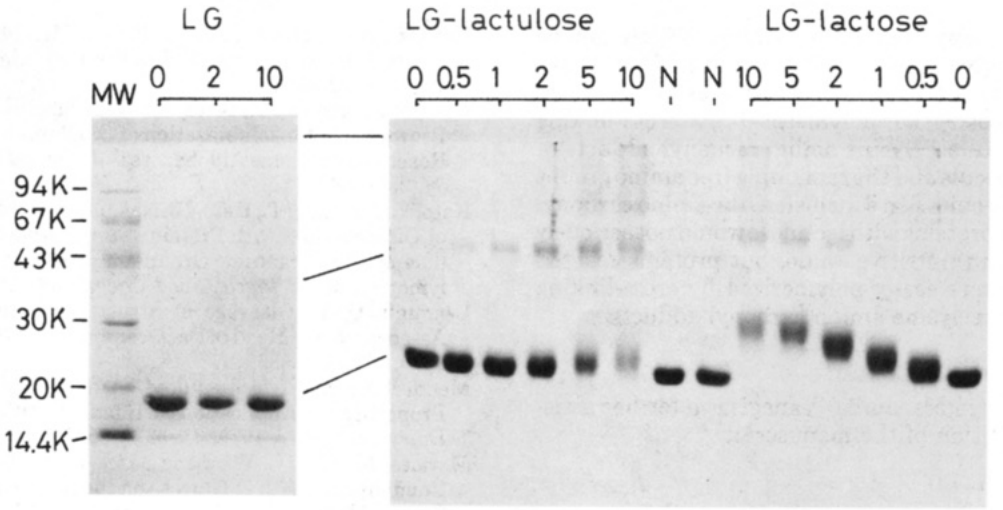

Figure 3. NaDodSO$_4$ gel electrophoresis of β-lactoglobulins stored with lactulose and lactose. Native β-lactoglobulin (N) and the protein stored in the absence of sugars (LG) also were analyzed for comparison. Molecular weight markers (MW) are given.

Table II. Apparent Proportion of Protein Polymers Calculated on the Basis of the Peak Area of HPLC Elution Patterns

	time, days	polymer, %	polymer/monomer ratio
LG–lactulose	2	44	0.79
	10	67	2.03
LG–lactose	2	29	0.41
	10	57	1.33
LG	2	36	0.57
	10	44	0.82

exclusion HPLC and NaDodSO$_4$ gel electrophoresis. Figure 2 shows the elution profiles of the proteins reacted with lactulose or lactose for 0, 2, and 10 days. Several peaks corresponding to protein polymers were observed for the proteins reacted with sugars. The protein stored without sugar also appeared to contain some aggregated forms. Apparent proportions of protein monomer and polymers were estimated by measuring each peak area. The results are summarized in Table II. The polymer/monomer ratio of the protein–lactulose complex was higher than that of the protein–lactose complex in either case of storage for 2 or 10 days. The protein polymerization appeared to be caused easily even by the reaction of lactulose with a small number of protein amino groups. The apparent proportion of polymers for the β-lactoglobulin–lactose system appeared to be higher than that of the ovalbumin–lactose system reported previously (Kato et al., 1989). This could be explained by the fact that even β-lactoglobulin itself formed some polymers after storage in the absence of sugars.

To investigate covalent protein cross-linking, the protein–sugar complexes were analyzed by means of NaDodSO$_4$ gel electrophoresis under a reducing condition. As shown in Figure 3, the stained band corresponding to the dimeric protein (about 36 kDa) was clearly observed for the protein–lactulose complex even after half a day of storage. The band intensity of the protein dimer and polymers for each sample of the protein–lactulose complexes was stronger than that of the corresponding sample of the protein–lactose complex. This was in agreement with the result from the HPLC analysis (Figure 2). Thus, the protein polymerization caused by the reaction with lactulose proceeded faster than that of lactose during the storage time. It seems interesting that the amino group blocking by lactulose was not very remarkable (Figure 1); contrary to this, the protein polymerization by the reaction

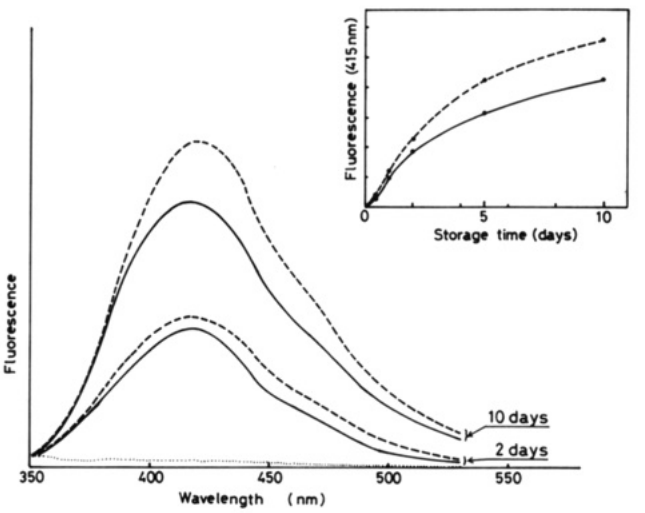

Figure 4. Fluorescence spectra of β-lactoglobulins stored with lactulose (- - -) and lactose (—) for 2 and 10 days. The time course of increasing fluorescence at 415 nm is shown in the inset. The spectrum of the protein–lactulose mixture without the storage (···) is shown for comparison.

with lactulose was remarkable. Although a faint band corresponding to the dimeric protein was observed for the protein stored in the absence of sugars, the band intensity was much weaker than that of proteins stored with sugars. The β-lactoglobulin became progressively heavier in the presence of lactose, whereas in the presence of lactulose the protein was converted to a distinct polymer. As demonstrated by the amino group analysis (Figure 1), more lactose than lactulose seemed to attach to the protein, resulting in the increase in apparent molecular mass of the protein. This would explain the slower electrophoretic mobility of the lactose–protein complexes.

Ponger et al. (1984) isolated a fluorescent compound from the advanced Maillard products and identified it as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole. This compound showed a typical fluorescence around 400–450 nm. The protein reacted with lactulose also produced fluorescent compounds, which gave a typical fluorescence spectrum shown in Figure 4. The relative fluorescence intensity of the protein–lactulose complex was somewhat higher than that of the protein–lactose complex.

Heyns products derived from lactulose and protein amino groups would have an aldehyde group newly formed by the rearrangement, and proteins reacted with lactu-

lose still have many free amino groups, which can be reaction counterparts for aldehyde groups of lactulose–protein complexes. Hence, the proteins reacted with lactulose would easily be polymerized by cross-linking between the lactulose–lysine amino carbonyl adducts of one protein molecule and the remaining free amino groups of the other molecules. In conclusion, the amino carbonyl reaction of milk proteins with lactulose would not seriously affect the protein nutritive value, but proteins reacted with lactulose were easily polymerized by cross-linking through lactulose–lysine amino carbonyl adducts.

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Registry No. Lactulose, 4618-18-2; lysine, 56-87-1.