Direct Evaluation of β-Lactoglobulin Lactosylation in Early Maillard Reaction Using an Antibody Specific to Protein-Bound Lactose

Tsukasa Matsuda,* Yasuko Kato, Kenji Watanabe, and Ryo Nakamura

A new analytical method using an antibody specific to protein-bound lactose has been developed for the evaluation of the extent of the early Maillard reaction between lactose and β-lactoglobulin. The specific antibody was prepared by immunizing a rabbit with ovalbumin lactosylated by the early Maillard reaction. The antibody reacted well with lactosylated β-lactoglobulin but not with the native protein. The reaction between the antibody and the lactosylated β-lactoglobulin was inhibited strongly by lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose) but not by lactose, galactose, and sucrose. The lactosylation of β-lactoglobulin with lactose in dry or aqueous system could successfully be monitored by two immunochemical methods: single radial immunodiffusion and enzyme-linked immunosorbent assay, using the sugar-specific antibody.

INTRODUCTION

The extent of the early Maillard reaction between lactose and milk proteins has been evaluated by several analytical methods mainly based on the measurement of available or reactive lysine from the nutritional point of view: in vivo assays using rats (Mottu and Mauron, 1967), in vitro enzymatic hydrolysis (Bujard et al., 1967), a reduction method (Hurrell and Carpenter, 1974), determination of free amino groups (Kakade and Liener, 1969). A high sensitivity would not be expected for the indirect analyses of the rate of the protein–lactose reaction by measuring the loss of available lysine, and the blocked lysine estimated by such methods seemed likely to contain lysine derivatives produced by reactions with not only lactose but also degradation products formed through Maillard reaction. Hence, it seemed impossible to adequately analyze the lactose-binding reaction with proteins at the first step of the Maillard reaction.

Finot et al. (1981) showed that furosine was formed by acid hydrolysis of ε-deoxyfructosyllysine and that biologically unavailable lysine could be estimated by chromatographic measurement of furosine in acid hydrolysates of glycosylated proteins. This furosine method has the advantage of being sensitive at very low levels of blocked lysine, but the chromatographic determination of furosine has seemed somewhat troublesome and time consuming.

In a previous study (Matsuda et al., 1985), the authors demonstrated that a lactose-specific antibody was produced by immunizing mice with lactosylated β-lactoglobulin produced by Maillard reaction and that the antibody reacted well with other proteins lactosylated by that reaction.

The experiments reported here were designed to determine whether the specific antibody directed against the lactose moiety of lactosylated proteins could be used as a specific reagent to estimate protein lactosylation caused at an early stage of the Maillard reaction and to investigate the lactose-binding reaction of proteins and the subsequent degradation of protein-bound lactose by immunochemical techniques using the sugar-specific antibody.

MATERIALS AND METHODS

Protein Lactosylation. Bovine β-lactoglobulin (LG) was lactosylated through the Maillard reaction in both dry and aqueous systems.

LG (Miles) and lactose were dissolved in distilled water (5 mg/mL each), and the pH of the solution was adjusted to 8.0 with dilute NaOH. After being freeze-dried, the powdered protein–sugar mixture was stored at 50 °C and 65% relative humidity for various periods (0–10 days).

Equal volumes of LG (6 mg/mL) and lactose (60 mg/mL) dissolved in phosphate buffer (I = 0.1, pH 7.0) were mixed in test tubes (1 mL/tube) and heated at 100 °C for various periods (0–120 min). Each sample was cooled in ice water immediately after the heat treatment.

Lactosylated ovalbumin (lac-OVA) was prepared by keeping a lactose–ovalbumin mixture, prepared as for LG–lactose, for 72 h under the same conditions as above.

Preparation of Antisera. The lac-OVA (500 μg) in 500 μL of phosphate-buffered saline (PBS) was emulsified with 500 μL of Freund's complete adjuvant (Difco). A rabbit was immunized intramuscularly with the antigen suspension, 0.5 mL in each rear thigh. The rabbit received two booster injections intraperitoneally with 500 and 50 μg of the antigen prepared as above 36 and 57 days after the first immunization, respectively (Lemieux et al., 1977). Bleeding was performed 10 days after the last injection, and the serum was separated and stored at −80 °C before use. Rabbit antiserum to LG was prepared similarly by subcutaneous injection of the antigen emulsified with Freund's complete adjuvant as described previously (Matsuda et al., 1985).

Immunochemical Methods. Immunoelectrophoresis was performed in 1.0% agarose gel (FMC Corp.) prepared with barbital buffer (I = 0.025). After electrophoresis of antigen (5–10 μg), the rabbit antiserum to LG or lac-OVA was allowed to diffuse from the trough between the wells. After incubation at 25 °C for 24 h, the gel plates were washed with PBS containing 0.1% NaN_3 for 3 days, stained with 5% Amido Black 10B in methanol/acetic acid (9:1, by volume), and destained with 2% acetic acid.

Single radial immunodiffusion (SRID) (Mancini et al., 1965) was used for the quantitative analysis for lactosylated LG (lac-LG). Antigens (0.5 μg) were loaded into wells bored in agarose gel (1.2% in PBS) that contained the antiserum to lac-OVA (1:30, by volume) and allowed to diffuse for 3 days. After washing with PBS, immunoprecipitin halos formed around the wells were stained with Amido Black as above.

The reaction between the sugar-specific antibody and lac-LG was measured also by enzyme-linked immunosorbent assay (ELISA) (Engval and Perhann, 1971). Flat-bottomed microtiter plates (Coster) were coated with lac-LG (10 μg/mL), and the anti-lac-OVA serum (10^{-5} diluted) was added to each well. After incubation for 3 h, the rabbit IgG antibody, which reacted with the plate-

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

0021-8561/85/1433-1193$01.50/0

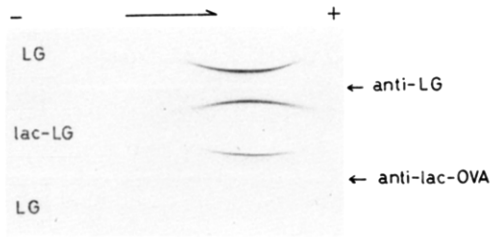

Figure 1. Immunoelectrophoresis of LG and lac-LG against antisera to lac-OVA and LG. The lac-LG was prepared by the storage of LG with lactose for 3 days in the dry system described in Materials and Methods.

binding antigen, was determined by using peroxidase-coupled goat anti-rabbit IgG (Cappel) as described previously (Matsuda et al., 1983). For the competitive inhibition assay, several concentrations of competitive inhibitor and the diluted antiserum were added to the wells of antigen-coated ELISA plates. The plates were incubated for 4 h, and the plate-binding antibody was determined as above.

Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (12.5% acrylamide) was performed according to the method of Laemmli (1970). Gel sheets were stained with 0.2% Coomassie Brilliant Blue R-250 and destained with 7% acetic acid.

Analytical Methods. Protein concentrations and the free amino group content were determined by the method of Lowry et al. (1951) and the fluorometric method using fluorescamine (Böhlen et al., 1973), respectively. Amino acid analysis was performed with a type JLC-6AH amino acid analyzer (JEOL, Tokyo) after hydrolysis at 110 °C for 24 h in 6 N HCl in evacuated, sealed tubes.

RESULTS AND DISCUSSION

The reactivity of LG and lac-LG with antibody to lac-OVA was examined by immunoelectrophoresis (Figure 1). The antiserum to LG formed a precipitin arc against both LG and lac-LG, whereas the antiserum to lac-OVA formed it against lac-LG but not against LG. The immunoprecipitation occurring between lac-LG and anti-lac-OVA serum indicated that a considerable amount of antibody directed against the sugar moiety of lac-OVA could be produced by immunizing a rabbit with lac-OVA. The specific antibody titer to the sugar moiety seemed to be much higher than that of mouse antiserum described previously (Matsuda et al., 1985), because no specific precipitation was observed in agarose gel using the mouse serum instead of the rabbit one.

The reaction between lac-LG and the sugar-specific antibody was characterized by ELISA competitive inhibition analysis using several sugars. Figure 2 shows the inhibition profile of the reaction by lactose and lactulose (4-*O*-β-D-galactopyranosylfructofuranose) (Sigma). Lactulose strongly inhibited the antigen–antibody reaction, but lactose and some other sugars (galactose, sucrose, glucose) did not. No antibody binding was detected for the serum incubated with 10 μmol of lactulose, indicating that the antibody with reactivity to lac-LG was highly specific to ε-deoxylactulosyllysine (lactulosyllysine) of lac-LG, an Amadori rearrangement compound produced in an early stage of the Maillard reaction (Finot et al., 1981). Thus, the sugar-specific antibody could be used as a unique reagent for the estimation of lactulosyllysine in Maillard reaction products from lactose and protein.

In the previous study, inhibition of the reaction between lac-LG and mouse-specific antibody by galactose and lactose was observed (Matsuda et al., 1985), but the reaction with rabbit antibody was not inhibited by these

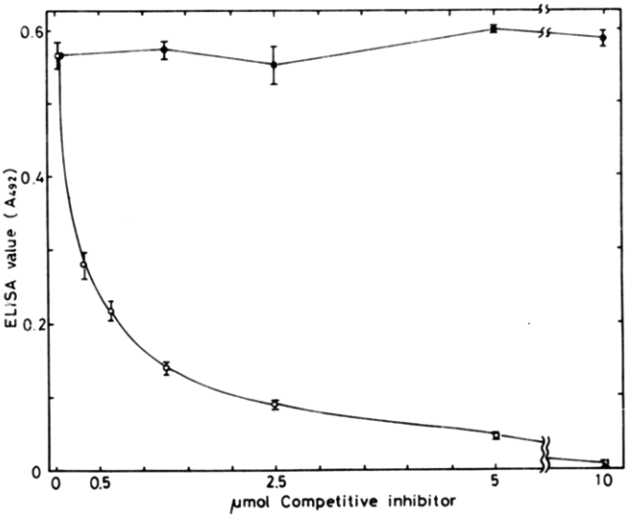

Figure 2. Competitive inhibition of the reaction between lac-LG and the sugar-specific antibody by lactose (●) and lactulose (○). The 10^{-5} diluted antiserum to lac-OVA (50 μL) and 100 μL of the sugar solution containing various amount of each sugar were added to the wells coated with lac-LG (3-day storage). The specific antibody bound to the coated antigen represents an ELISA value (mean of two determinations) measured after the enzyme reaction for 50 min.

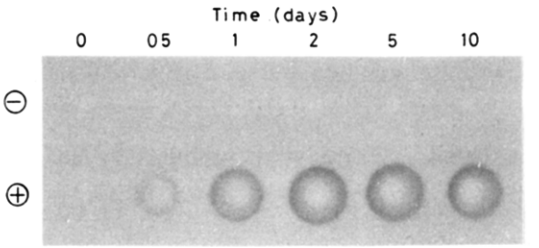

Figure 3. SRID of LG stored with (+) and without (-) lactose for various periods (0–10 days) in the dry system.

sugars in the present study. This discrepancy between the results of antibody binding reaction was considered due to the difference in the binding affinity and/or binding specificity; the rabbit antibody prepared in the present study seemed to have rather high specificity to the lactulosyllysine moiety of lactosylated proteins.

The LG that reacted with lactose during storage (0–10 days) in the dry system was measured by SRID. As shown in Figure 3, the specific precipitin halo was detected after 12-h storage, increased gradually in size, and had the maximum after 2 days storage. It decreased slightly in size during further storage. The samples stored for less than 12 h also were examined, but little or no precipitin halo was observed for the samples stored for less than 10 h. The square of the diameter of an immunoprecipitin halo formed around the well is proportional to the amount of antigen added to the well (Mancini et al., 1965). Hence, the change in halo size could be regarded as that of lactosylated protein amount in the samples tested.

The protein lactosylation in the dry system was analyzed also by ELISA (Figures 4 and 5). In Figure 4, the ELISA value, the square of the halo diameter of SRID, and the value for residual free amino group are plotted against the storage time (0–10 days). The lactosylation profile monitored by ELISA had the maximum after the 2-day storage, in good agreement with the results of SRID. The decrease in both the ELISA value and the precipitin halo size after 5-day storage or more suggested that the lactulosyl moiety of lac-LG was converted into degradation products with the progress of the Maillard reaction. The value for re-

Table I. Evaluation of Lactulosyllysine and Reactive Lysine of Lactosylated β-Lactoglobulin by Amino Acid Analysis after Acid Hydrolysis

mol/mol of protein	storage time, h						
	0	6	12	24	48	120	240
furosine[a]	0.0	0.14	0.41	0.72	1.17	1.44	1.44
lactulosyllysine[b]	0.0	0.5	1.3	2.2	3.2	4.5	4.5
lysine[a]	14.9	14.8	14.2	13.4	12.8	9.7	9.3
reactive lysine[c]	14.9	14.5	13.7	12.5	11.5	7.9	7.5
lactulosyllysine + reactive lysine	14.9	15.0	15.0	14.7	14.7	12.4	12.0

[a] Determination by amino acid analysis. [b] [Lactulosyllysine] = 3.1[furosine]. [c] [Reactive lysine] = [free lysine] + [Schiff's base] = [lysine] − 0.4[lactulosyllysine] (Finot et al., 1981).

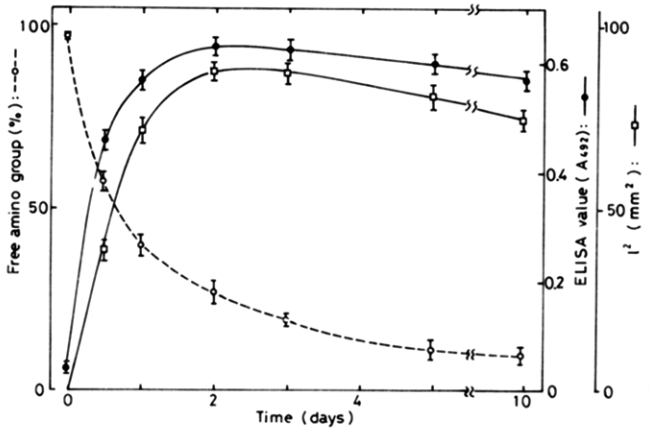

Figure 4. Lactosylation profile of LG stored with lactose for various periods (0–10 days) in the dry system as measured by ELISA, SRID, and free amino group determination. The square of the diameter (l^2) was calculated from the halos in SRID, and the free amino group content represents the percent ratio to that of native LG. The incubation time for the ELISA enzyme reaction was 30 min. Each value is the mean of two determinations.

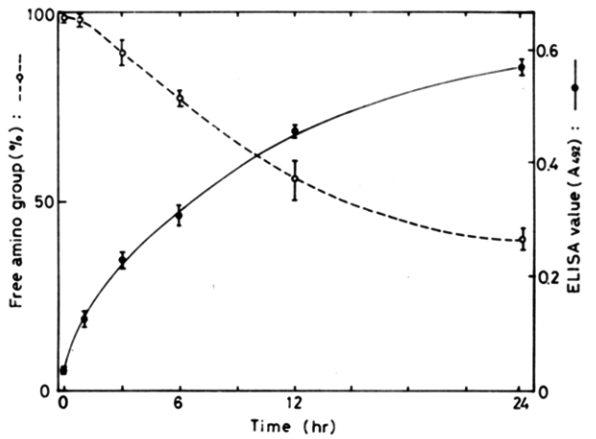

Figure 5. Lactosylation profile of LG stored with lactose for various periods (0–24 h) in the dry system as measured by ELISA and free amino group determination. Each value represents the mean of two determinations.

sidual free amino group was less than about 30% even after the 2-day storage and further decreased to about 10% with increase in the storage time. The profile of the free amino group decrease did not completely correspond to that of the lactosylated protein increase as measured by ELISA and SRID. This could be ascribed to the degradation of lactulosyllysine residues mentioned above and to the amino group blocking by degradation products formed during the Maillard reaction.

The lactosylation at an earlier stage was measured, and the profile as monitored by ELISA and determination of free amino group is shown in Figure 5. The ELISA showed the presence of a trace amount of lactosylated protein,

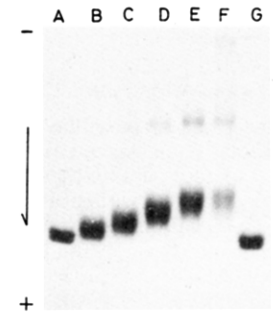

Figure 6. Polyacrylamide gel electrophoresis of LG stored with lactose in the dry system for various periods (0–10 days). The lanes A–F are for samples stored for 0, 0.5, 1, 2, 5, and 10 days, respectively. The lane G represents a sample stored for 10 days without lactose.

which could not be detected by SRID; thus, it was demonstrated that lactose reacted with LG during storage in the dry state for only 1 h.

The protein lactosylation and the subsequent polymerization during the dry-system storage were examined by $NaDodSO_4$ gel electrophoresis, and the electrophoretogram is shown in Figure 6. The electrophoretic mobility of monomer protein decreased gradually, and the protein band corresponding to dimer appeared with increase in the storage time. These changes in electrophoretic properties corresponded to the change in the free amino group content rather than to that of the lactosylated protein determined by ELISA and SRID (Figure 4). This suggested that the increase in molecular weight of monomer protein during the storage was caused by reaction with not only lactose but also lactose degradation products, which seemed to react also with amino acid residues other than lysine (Okitani et al., 1984).

The lactulosyllysine content of LG lactosylated during the dry-state storage was quantitated by the determination of furosine after acid hydrolysis according to the method of Finot et al. (1981). Table I shows the results of amino acid analysis and the evaluated lactulosyllysine and the reactive lysine contents. The profile of lactulosyllysine formation detemined by the furosine method corresponded relatively well to the lactosylation profile evaluated by SRID and ELISA (Figures 4 and 5). The lactulosyllysine content of LG stored for less than 12 h was about one residue per protein molecule or less. Hence, it is reasonable that the LG stored for less than 12 h formed little or no precipitin halo in SRID, because more than two antibody binding sites per antigen unit are required for the immune complex precipitation. It was demonstrated that ELISA could detect such a trace amount (less than 1 mol/mole of protein) of lactulosyllysine of lactosylated proteins (see Figure 5).

Protein lactosylation by heating with lactose in the aqueous system was examined by SRID, ELISA, and free amino group determination. No immunoprecipitin halo was formed in SRID by lactose–LG solution heated at 100

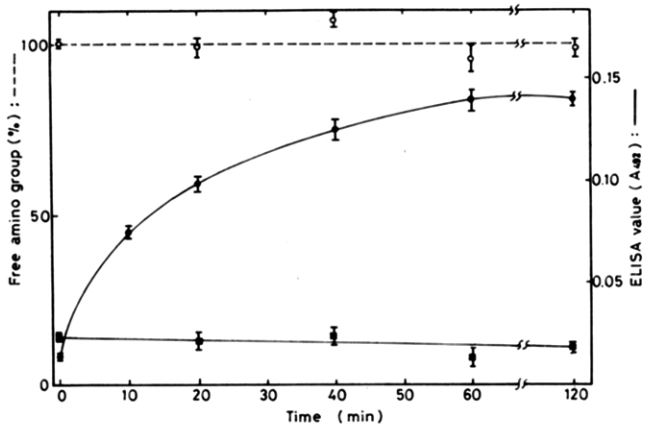

Figure 7. Lactosylation profile of LG heated with lactose in the aqueous system at 100 °C for various periods (0–120 min) as measured by ELISA (●) and free amino group determination (○). The ELISA value for LG heated without lactose (■) is also shown for comparison. The incubation time for ELISA enzyme reaction was 50 min. The free amino group value represents the percent ratio to that of native LG. Each value represents the mean of two determinations.

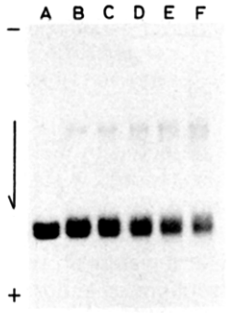

Figure 8. Polyacrylamide gel electrophoresis of LG heated with lactose in the aqueous system for various periods (0–120 min). The lanes A–F correspond to samples heated for 0, 10, 20, 40, 60, and 120 min, respectively.

°C for 0–120 min, and no decrease in free amino group content was observed even in the sample heated for 120 min. As shown in Figure 7, however, protein lactosylation was detected clearly by ELISA. The ELISA value increased with an increase in the heating time, reached the maximum after 60 min, and remained nearly constant for 120 min. The ELISA values for the protein lactosylated by heating in the aqueous system were rather lower than that of protein lactosylated in the dry system (Figure 5), and no detectable decrease in free amino groups was caused by heating in spite of the showing of protein lactosylation by ELISA. The high temperature such as 100 °C might accelerate the degradation of protein-bound lactose and the reaction of lactose with amino acid residues other than lysine.

Figure 8 shows that electrophoretogram of LG lactosylated by heating in the aqueous system. The band of monomer protein became blurred with increase in heating time, but no considerable decrease in electrophoretic mobility was observed. The protein band corresponding to dimer protein had already appeared in the electrophoretogram of sample heated for 10 min. The protein polymerization probably caused by reaction with lactose degradation products seemed to be promoted for the Maillard reaction in the aqueous system at high temperature.

In the previous study, a specific antibody against protein-bound glucose was not detected (Matsuda et al., 1985). Indeed it is uncertain whether antibodies specific to sugars other than lactose could be produced by immunizing animals with glycosylated proteins. Attempt to produce specific antibodies against some other sugars are in progress.

Registry No. Lactulose, 4618-18-2; lactulosyllysine, 34326-63-1.

LITERATURE CITED

Böhlen, P.; Stein, S.; Dairman, W.; Udenfriend, S. *Arch. Biochem. Biophys.* **1973**, *155*, 213–220.
Bujard, E.; Handwerck, V.; Mauron, J. *J. Sci. Food Agric.* **1967**, *18*, 52–57.
Engval, E.; Perhann, P. *Immunochemistry* **1971**, *8*, 871–874.
Finot, P. A.; Deutsch, R.; Bujard, E. In "Progress in Food and Nutritional Science—Maillard Reaction in Food"; Eriksson, C., Ed.; Pergamon Press: Oxford, 1981; Vol. 5, pp 345–355.
Hurrell, R. F.; Carpenter, K. J. *Br. J. Nutr.* **1974**, *32*, 589–604.
Kakade, M.; Liener, I. E. *Anal. Biochem.* **1969**, *27*, 273–280.
Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
Lemieux, R. U.; Baler, D. A.; Bundle, D. R. *Can. J. Biochem.* **1977**, *55*, 507–512.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
Mancini, G.; Vaerman, J.; Carbonara, A. O.; Heremans, J. F. *Immunochemistry* **1965**, *2*, 235–254.
Matsuda, T.; Kato, Y.; Watanabe, K.; Nakamura, R. *J. Food Sci.* **1985**, *50*, 618–621.
Matsuda, T.; Watanabe, K.; Nakamura, R. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 75–81.
Mottu, F.; Mauron, J. *J. Sci. Food Agric.* **1967**, *18*, 57–62.
Okitani, A.; Cho, R. K.; Kato, H. *Agric. Biol. Chem.* **1984**, *48*, 1801–1808.

Received for review May 3, 1985. Accepted August 28, 1985.