Immunological detection of fructated proteins *in vitro* and *in vivo*

Nobuko MIYAZAWA*[1], Yoshimi KAWASAKI*[1], Junichi FUJII*, Myint THEINGI*, Ayumu HOSHI*, Rieko HAMAOKA*, Akio MATSUMOTO*, Naofumi UOZUMI*, Tadashi TESHIMA† and Naoyuki TANIGUCHI*[2]

*Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0891, Japan, and †Peptide Institute, Protein Research Foundation, 4-1-2 Ina, Mino, Osaka 562-0015, Japan

An antibody has been raised against fructated lysine in proteins by immunizing fructated lysine-conjugated ovalbumin in rabbits. The affinity-purified antibody specifically recognized proteins incubated with fructose but not with other reducing sugars such as glucose, galactose or ribose, as judged by immunoblotting and ELISA techniques. Competitive binding to this antibody was observed specifically by fructated lysine but not by glucated lysine, glucose, fructose or lysine. The antibody binds specifically to fructated lysine residues in the protein but not to borohydride-reduced material or advanced glycation end products, indicating that the antibody recognizes only the reducing, carbonyl-containing forms produced in the early stage of the fructation reaction. When BSA was incubated with various concentrations of fructose, the reactivity of the antibody increased in a dose- and time-dependent manner. When soluble proteins prepared from either normal or streptozotocin-induced diabetic rat eyes were analysed by ELISA with this antibody, an increase in the reactive components was observed as a function of aging as well as under diabetic conditions. Western blotting analysis showed that lens crystallin reacted highly with this antibody. Because fructose is biosynthesized largely through the polyol pathway, which is enhanced under diabetic conditions, and lens is known to have a high activity of enzymes in this pathway, this antibody is capable of recognizing fructated proteins *in vivo*. Thus it is a potentially useful tool for investigating two major issues that seem to be involved in diabetic complications, namely the glycation reaction and the polyol pathway.

INTRODUCTION

Glycation occurs during normal aging and at accelerated rates in diabetes, and is involved in structural and functional alterations of proteins and other cellular components. Glycation has been suggested as a factor in the pathogenesis of diabetic complications [1] because reducing-sugar-derived metabolites in the glycation reaction are responsible for the modification and cross-linking of proteins. The cross-linking of long-lived proteins such as collagen and lens crystallin is correlated with the aging and diabetic processes. Furthermore glycation alters the activity of some enzymes such as Cu,Zn-superoxide dismutase [2–4], carbonic anhydrase [5], aldehyde reductase [6] and sorbitol dehydrogenase [7]. Reactive oxygen species are produced in conjunction with the glycation of proteins, especially metal-containing enzymes such as Cu,Zn-superoxide dismutase, which might lead to protein fragmentation and DNA damage [8,9]. The final products of the glycation reaction, called advanced glycation end products (AGEs), increase under diabetic conditions and during the aging process, and have been suggested as a cause of diabetic complications and aging [1].

Glucose is a main blood sugar and is therefore thought to be a major glycating agent in the body. In some tissues, such as lens, kidney and peripheral nerve, a polyol-metabolizing pathway, converting glucose to sorbitol by aldose reductase and then sorbitol to fructose by sorbitol dehydrogenase, is active and is thought to participate in diabetic complications [10]. Because the accumulation of sorbitol is thought to have a role in the pathogenesis of diabetic complications in these tissues, extensive studies on aldose reductase have been performed to elucidate a causal connection between the activity of this enzyme and diabetic complications [11].

In terms of diabetic complications, the glycation hypothesis and the polyol pathway hypothesis are usually discussed independently. Because the level of fructose is much lower than that of glucose in normal tissues, the involvement of fructose in glycation reactions is generally thought to be less important than glucose-induced glycation. However, in tissues such as eyes and peripheral nerves, in which the polyol pathway is active, the concentration of fructose approaches the levels of glucose [12]. The resultant fructose is known to be a stronger glycating agent than glucose because the population of its acyclic (open-chain) form, which is the reactive species, reacts with protein amino groups at a rate approx. 10-fold that of glucose [13]. A report describing the use of a sorbitol dehydrogenase inhibitor for minimizing diabetic complications [14] suggests that fructose might also induce similar complications. In addition, fructose has been shown to be a building block of some AGEs [15]. Although several groups are currently investigating fructose-initiated glycation in diabetic conditions [16–18], an adequate method of distinguishing fructation from glucation is not available [19].

Here we report the establishment of a novel antibody that specifically recognizes fructose–protein adducts and the use of this antibody to detect fructated protein in diabetic as well as normal rat tissues. This technique should aid in clarifying the roles of the polyol pathway and its relationship to the glycation reactions in diabetic complications.

EXPERIMENTAL

Materials

Glucose, fructose, galactose and lysine were purchased from Nakarai Tesque (Kyoto, Japan). $NaBH_4$, 1,4-dioxane, D-ribose,

Abbreviations used: AGEs, advanced glycation end products; Boc-Lys, N^{α}-t-butoxycarbonyl-L-lysine; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS.

[1] These authors contributed equally to the work.

[2] To whom correspondence should be addressed (e-mail proftani@biochem.med.osaka-u.ac.jp).

and 2,4,6-trinitrobenzenesulphonic acid sodium salt were obtained from Wako Pure Chemical Industries (Osaka, Japan). N^{α}-t-butyoxycarbonyl-L-lysine (Boc-Lys), ovalbumin, BSA and *o*-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. Horseradish peroxidase-conjugated goat anti-(rabbit IgG) was purchased from Zymed Laboratories (San Francisco, CA, U.S.A.). PD-10 Sephadex G-25M and activated CH-Sepharose 4B columns were obtained from Pharmacia LKB Biotechnology. Nitrocellulose transfer membrane was a product of Schleicher & Schuell.

Synthesis of N^{ε}-fructated Boc-Lys

Fructated Boc-Lys was synthesized by the same method as described for the preparation of glucated Boc-Lys [20]. Boc-Lys (5 mmol) and D-fructose (25 mmol), dissolved in 50 ml of methanol, were incubated in an oil bath at 65 °C for 50 min. Fructated Boc-Lys was detected on a TLC plate after detection with ninhydrin. After the removal of methanol by evaporation, the fructated product was purified by adsorption on a Dowex 50 (H^+) column and elution with a gradient of 0.2 M pyridinium acetate (pH 3.1) to 2.0 M pyridinium acetate (pH 5.0). Boc was removed by hydrolysis with a large excess of aqueous trifluoroacetic acid for 30 min at room temperature.

MS

To confirm the identification of the adducts of Boc-Lys with fructose, MS was performed. Matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS) was performed on a Voyager-RP equipped with a delayed-extraction system (PerSeptive Biosystems, Framingham, MA, U.S.A.). A 1 μl sample containing the fructated Boc-Lys fractionated by reverse-phase HPLC was placed on the flat surface of a stainless steel plate, mixed with 1 μl of the saturated matrix solution (α-cyano-4-hydroxycinnamic acid; Aldrich Chemical Co.) and dried in air. The ions were generated by irradiation of the sample area with the output of a nitrogen laser (337 nm).

Production of antiserum and affinity purification of the anti-(fructated lysine) IgG

Fructated lysine (0.57 mg) was conjugated with 30 mg of ovalbumin. After the inoculation of 100 μg of fructated lysine conjugated with ovalbumin in Freund's complete adjuvant into lymph nodes, each rabbit received subcutaneous injections of 100 μg of the same antigen four times over a period of 2 weeks. Each animal was bled on the 10th day after the last injection. The immunoglobulin fraction of rabbit antiserum, which had been prepared by $(NH_4)_2SO_4$ fractionation, was dissolved in 10 ml of 50 mM PBS, then dialysed against the same buffer for 24 h. An affinity column was prepared by coupling fructated lysine to activated CH-Sepharose 4B in accordance with the manufacturer's instructions. The antiserum in PBS was loaded on the affinity column (1 cm × 7 cm) equilibrated with 10 bed vol. of the dialysis buffer at 30 min intervals. Unbound proteins were washed out with 7 bed vol. of the starting buffer, and the bound protein was then eluted with 7 bed vol. of 100 mM glycine/HCl, pH 2.8. The pH of the eluted fractions was immediately adjusted to 7.4 with 2 M Tris. The remaining proteins were eluted with PBS (pH 7.4) containing 10 % (v/v) dioxan. The absorbance of all fractions was measured at 280 nm; peak fractions were checked by ELISA on fructated lysine–BSA-coated immunoplates. The antibody-containing fractions were concentrated and then dialysed against PBS, pH 7.4.

Preparation of glycated BSA with several sugars

BSA (2 mg/ml) in 50 mM PBS was mixed with 100 mM D-glucose, fructose, galactose or ribose. The mixture was sterilized by filtration through a 0.2 μm pore-size nitrocellulose filter (Advantec) and then incubated at 37 °C for 7 days under aerobic conditions. At the end of the incubation all samples were subjected to gel filtration on a PD-10 column to remove the excess sugars. Control BSA was incubated without sugar under the same conditions.

Quantification of free amino groups

After incubation with various sugars, free sugars were removed as above. Then 1 ml of glycated BSA (1 mg/ml) in 4 % (w/v) $NaHCO_3$, pH 8.5, was reacted with 1 ml of 0.1 % 2,4,6-trinitrobenzenesulphonic acid sodium salt at 37 °C for 2 h. The samples were solubilized by the addition of 1 ml of 10 % (v/v) SDS followed by 0.5 ml of 1 M HCl. The absorbance at 345 nm was measured and the quantities of free amino groups were calculated from $\epsilon_{354} = 1.4 \times 10^4\ M^{-1} \cdot cm^{-1}$.

Preparation of tissue homogenates from normal and streptozotocin-induced diabetic rats

Rats in which diabetes had been induced by intravenous injection with streptozotocin were kindly provided by Ono Pharmaceutical Co. At 9, 20, 24 and 28 weeks after streptozotocin injection, these animals were killed by decapitation under anaesthesia with diethyl ether. Normal and streptozotocin-induced diabetic rat tissues were homogenized in 2 vol. of 50 mM PBS containing 1 mM *p*-amidinophenylmethanesulphonyl fluoride hydrochloride, 10 mM benzamidine and 10 ml of antipain (10 μg/ml). The homogenates were ultracentrifuged at 100 000 ***g*** and 4 °C for 1 h. Supernatant fractions were taken and the protein concentration was measured with a protein assay kit (Bio-Rad).

Detection of fructated proteins by immunoblotting

Samples were subjected to SDS/PAGE [10 % (w/v) gel], and the separated proteins were transferred to nitrocellulose membranes under semi-dry conditions with the use of Trans-blot (Bio-Rad). The membranes were blocked by incubation with 4 % (v/v) skimmed milk in TBS [10 mM Tris/HCl (pH 7.4)/0.15 M NaCl] at room temperature for 1 h with gentle agitation, washed four times with 10 mM TBS containing 0.05 % (v/v) Tween 20 for 15 min each, then incubated with the diluted [1:(300–500)] polyclonal anti-(fructated lysine) IgG at 4 °C overnight. After being washed, the samples were incubated with horseradish peroxidase-conjugated goat anti-(rabbit IgG) diluted to 1:2000 for 2 h at room temperature and then washed for 2 h. Rat lens proteins on the membranes were reacted with the biotinylated anti-(fructated lysine) IgG (1:500 dilution). After being washed, they were incubated with horseradish peroxidase-conjugated streptavidin (1:2000 dilution) for 30 min. The chemiluminescence method was employed to amplify the signal with an enhanced chemiluminescence kit (Amersham).

Reverse-phase HPLC analysis

The synthesized fructated Boc-Lys was separated by reverse-phase HPLC (Shimadzu LC6A) at a flow rate of 0.8 ml on a Cosmosil C_{18} column (4.6 mm × 250 mm; Nacarai Tesque), and peak fractions were collected. A gradient system formed between solvent A [0.05 % (v/v) trifluoroacetic acid in water] and solvent B [0.05 % (v/v) trifluoroacetic acid in acetonitrile] was used. The programme used was as follows: 0–10 min, 5 % solvent B;

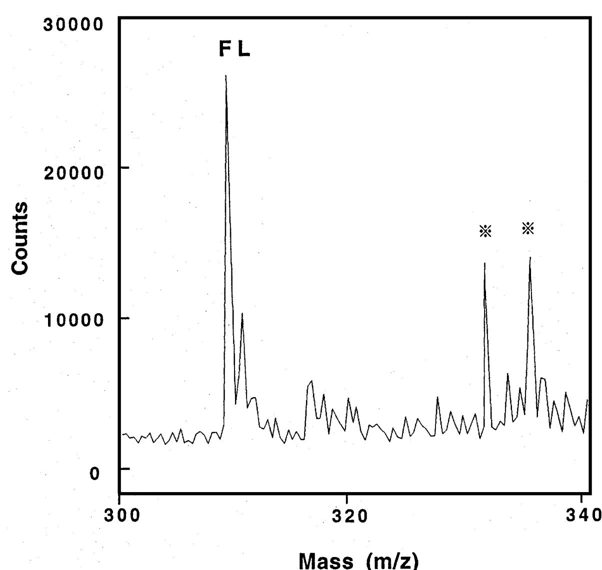


Figure 1 MALDI–TOF-MS of fructated lysine used as an antigen

The molecular mass of synthesized fructated lysine (FL) was evaluated by MALDI–TOF-MS. The mass of the main peak was 309.178. Asterisked peaks are from the matrix.

50 min, 80% solvent B; 60 min, 80% solvent B. Fructated Boc-Lys was detected by its absorbance at 214 nm.

Quantification of fructated proteins by ELISA

Aliquots (50 μl) of samples were added to a 96-well immunoplate (Nunc) and incubated at room temperature for 2 h. The plate was washed four times with PBS containing 0.05% (v/v) Tween-20 to remove unbound proteins, then blocked with 1% (w/v) BSA in PBS by incubation for 1 h. The affinity-purified polyclonal antibody, diluted to 1:500 with PBS, was added and left to react overnight at 4 °C. After four washes, horseradish peroxidase-conjugated goat anti-(rabbit IgG) (1:2,000 dilution in PBS) was added and left to react for 2 h at room temperature. Peroxidase activity was detected by the addition of 50 μl of an *o*-phenylenediamine dihydrochloride solution containing H_2O_2. The reaction was terminated by the addition of 50 μl of 1 M H_2SO_4 to each well. The absorbance at 490 nm was determined with an Immuno-reader NJ200 (Intermed). A paired Student's *t*-test was used to compare the significance of differences between data.

Measurement of fluorescence

The fluorescence of BSA incubated with either 100 mM fructose or 100 mM glucose was determined at excitation/emission wave-

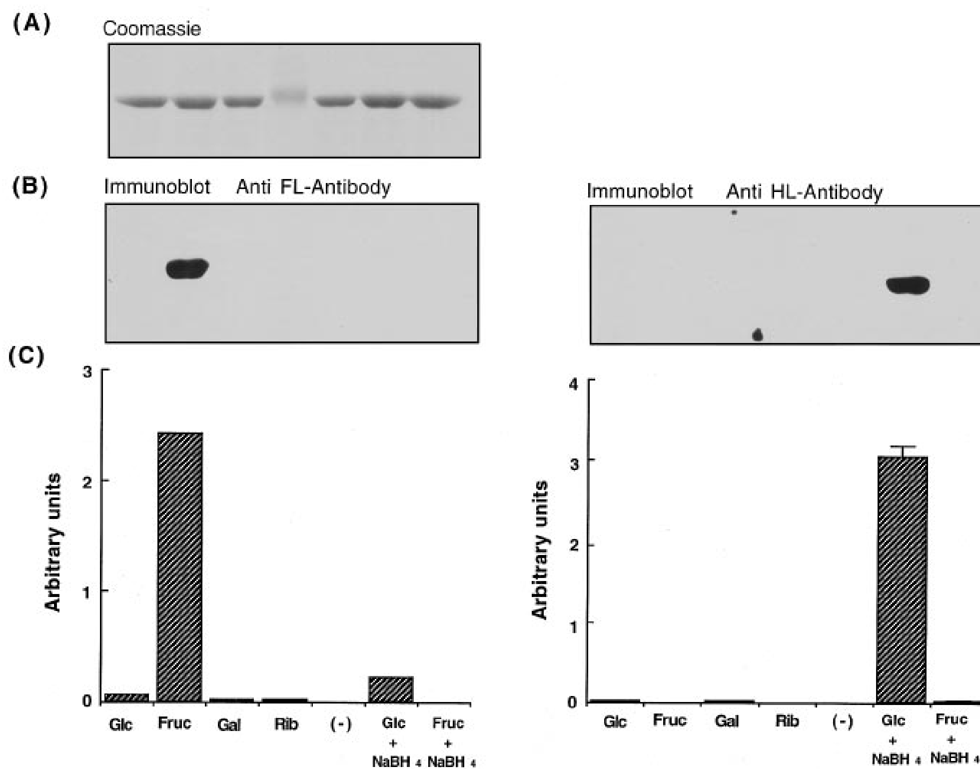


Figure 2 Specificity of the anti-(fructated lysine) antibody

Coomassie Blue staining (**A**) and immunoblotting (**B**) of BSA incubated with several different sugars. BSA was incubated with 100 mM glucose, fructose (Fruc), galactose, and ribose. BSA that had been incubated with glucose or fructose was then reduced with $NaBH_4$; 2 μg of each protein was separated by SDS/PAGE [10% (w/v) gel], followed by transfer to a nitrocellulose membrane. Anti-(fructated lysine) (anti-FL) antibody (left panel) or anti-(hexitol lysine) (anti-HL) antibody (right panel) was used as the first antibody at a 1:500 dilution. Quantification of BSA that had been incubated with several different sugars was also performed by ELISA with these antibodies (**C**). Each sample was glycated to the same extent by adjusting the free amino groups with non-glycated BSA and used for this experiment.

lengths of 370 and 440 nm respectively with an F-4000 fluorescence spectrophotometer (Hitachi).

RESULTS

Chemical structure of the antigen

We have chemically synthesized fructated lysine as an antigen for polyclonal antibody as described in the Experimental section. The purity of the antigen was examined by TLC and NMR [20], which showed that approx. 60 % of Boc-Lys in the antigen is fructated (results not shown). The 40 % impurity was mainly unreacted Boc-Lys, and the resulting free Lys after the hydrolytic removal of the Boc group was not antigenic, as described below. MALDI–TOF-MS of the antigen confirmed that the adduct was that of lysine with fructose and showed no high-molecular-mass material of more than 309 Da (Figure 1). Therefore this antigen contained mainly fructated Boc-Lys and free lysine, and was virtually free of AGE-like compounds.

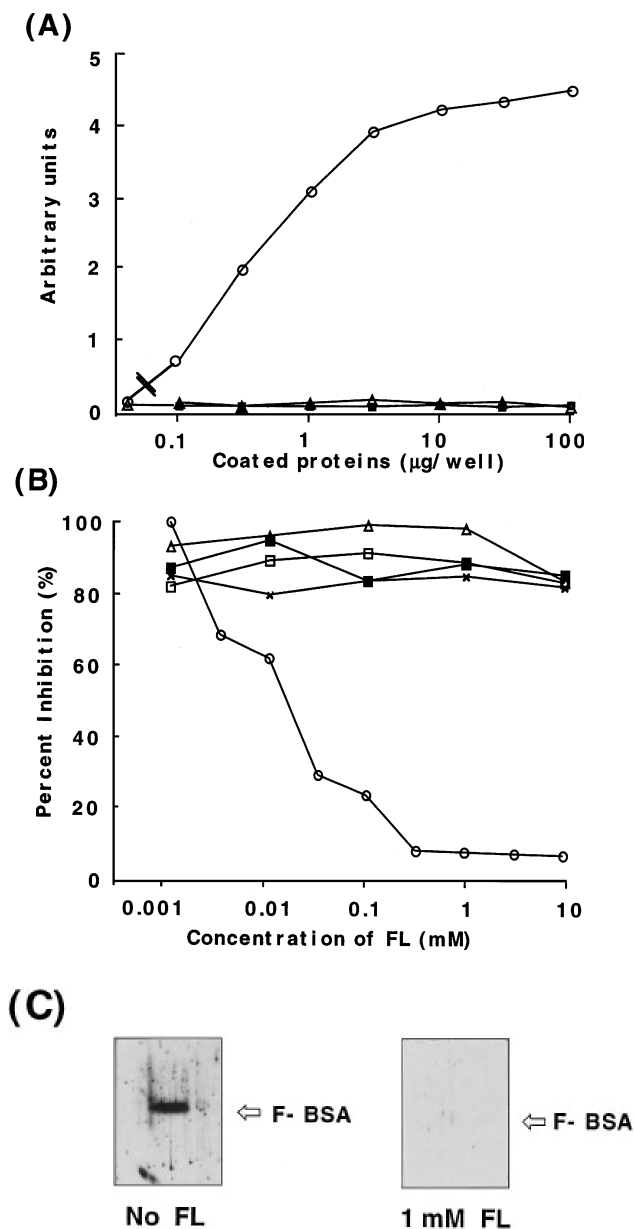


Figure 3 Competitive inhibition by sugars and lysine of binding of antibody to fructated BSA

(**A**) Dose-dependent curve of glycated BSA in ELISA using anti-(fructated lysine) antibody. Coated proteins were fructated BSA (○), glucated BSA (△) and non-glycated BSA (■). (**B**) ELISA analysis of fructated BSA (10 μg/well) was carried out using the anti-(fructated lysine) antibody in the presence of varying concentrations of competitors, glucose (×), fructose (■), lysine (□), glucated lysine (△) and fructated lysine (○). Glucated lysine was that synthesized previously [20]. Fructated lysine (FL) was the same as used in Figure 1. The experiments were performed in triplicate. (**C**) Immunoblot analysis of fructated BSA (100 ng) was performed with anti-(fructated lysine) antibody in the absence or presence of 1 mM fructated lysine (FL).

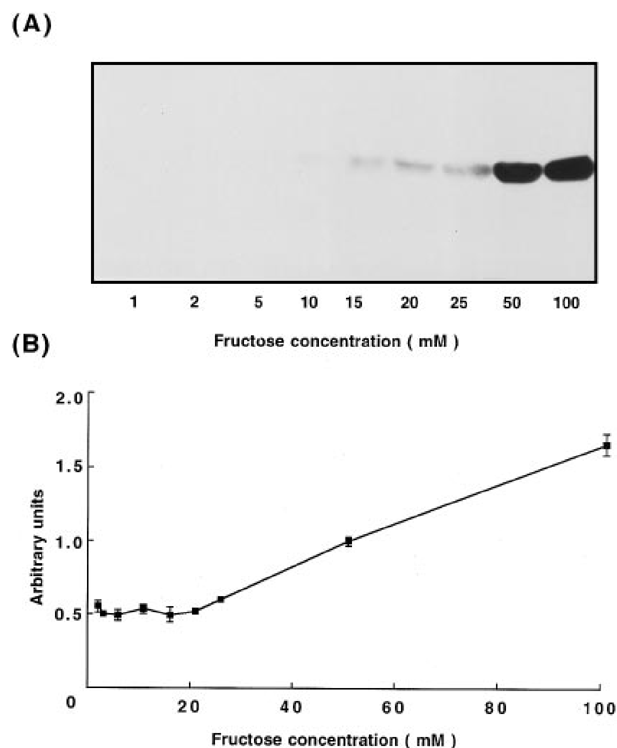


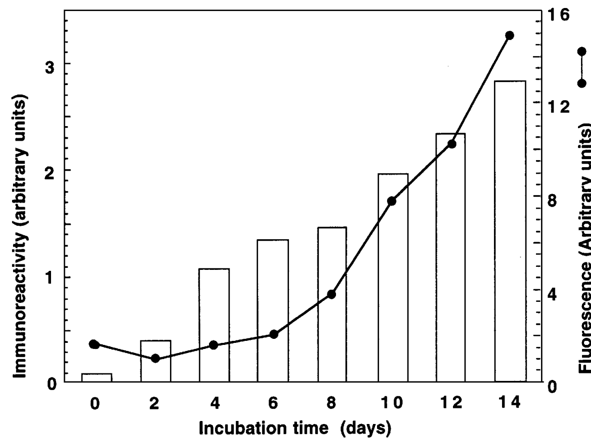
Figure 4 Dose dependence for the formation of fructated BSA

BSA (2 mg/ml) was incubated with various concentrations of fructose for 8 days at 37 °C. Immunoblot data of 2 μg of the BSA are shown (**A**). A 50 μg sample was subjected to ELISA analysis with anti-(fructated lysine) antibody (**B**). Means $\pm$ S.D. for triplicate experiments are shown.

Specificity of the anti-(fructated lysine) antibody

We raised polyclonal antibodies against fructated lysine and purified the IgG fraction from rabbit sera by affinity chromatography on a fructated lysine-conjugated CH-Sepharose 4B column. The specificity of the antibodies was examined by immunoblotting and ELISA (Figure 2) and was found to be specific for BSA incubated with fructose but not with glucose, galactose, ribose or reduced fructated BSA. The antigen used for the immunization was not reduced. The IgG reacted only to non-reduced fructated BSA and failed to recognize the reduced fructated BSA. In contrast, the anti-(hexitol lysine) antibody [20] reacted only to $NaBH_4$-reduced glucated BSA.

To characterize the antigenic epitope in the fructated BSA, we examined the inhibitory effect of the sugar and/or lysine moiety on the antibody reactivity by ELISA. As shown in Figure 3, only lysine incubated with fructose inhibited the immunoreactivity of this antibody to fructated BSA; free fructose, glucose or lysine



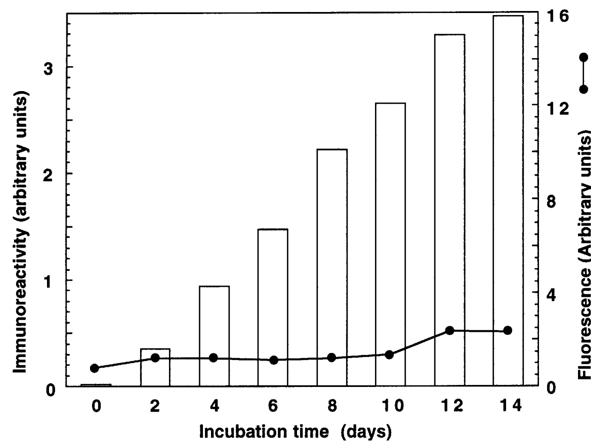
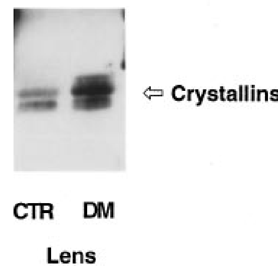


Figure 5 Time-dependent changes in fructated and glucated proteins and fluorescent materials

BSA (2 mg/ml) was incubated with 100 mM fructose (**A**) or glucose (**B**) for various durations. After removal of free sugars by passage through PD 10 columns, levels of fructated and glucated BSA were measured by ELISA with the anti-(fructated lysine) antibody (**A**) and the anti-(hexitol lysine) antibody (**B**) (open columns) respectively. The fluorescent intensities of these materials (solid lines) were also measured at excitation and emission wavelengths of 370 and 440 nm respectively.



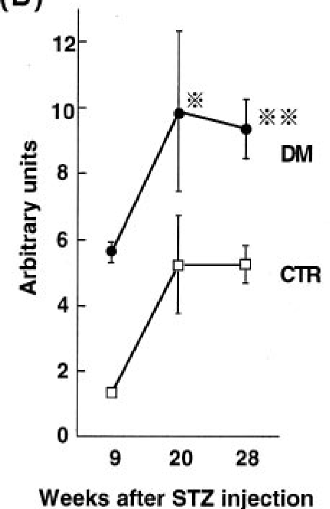


Figure 6 Immunoblot and ELISA analyses of levels of fructated proteins in eyes from control and diabetic rats

Eyes were prepared from three control (CTR) and diabetic (DM) rats at 9, 20 and 28 weeks after streptozotocin injection and homogenized. After ultracentrifugation, soluble proteins were subjected to immunoblot (**A**) and ELISA (**B**) with anti-(fructated lysine) antibody as described. Means $\pm$ S.D. for triplicate assays are shown. $^{*}P < 0.05$; $^{**}P < 0.005$.

did not, indicating that the sugar–amino acid adduct was the epitope. Therefore an epitope of this antibody would correspond to adducts of fructated lysine in the glycated protein.

The nature of the glycation reaction by fructose *in vitro*

Although the glycation reaction by glucose has been extensively investigated [1,2], that by fructose is only poorly understood. To characterize the fructation reaction we incubated BSA with various concentrations of fructose for 8 days at 37 °C and quantified the levels of fructated BSA by ELISA (Figure 4). Fructated proteins were increased in a dose-dependent manner and were clearly detected at levels of fructose higher than 25 mM; saturation was not reached even at 100 mM. We also examined the time-dependent changes of fructation with 100 mM fructose and glucation with 100 mM glucose at 37 °C (Figure 5). The materials that reacted to anti-(fructated lysine) antibody increased gradually and continued to increase even at 14 days, which was similar to the glycation reaction with glucose [20]. Thus the glycation reaction with fructose was found to proceed for at least 2 weeks in the presence of a large excess of fructose. However, a marked difference was observed in fluorescence intensities, corresponding to levels of AGEs, between samples incubated with fructose and glucose. The formation of AGEs in fructated BSA was much faster than that in glucated BSA, as reported previously [12].

To exclude the possibility that anti-(fructated lysine) antibody also recognized AGE, we examined the reactivity of the antibody to glucated BSA containing AGEs, which were formed by incubation with glucose for several weeks. AGE-derived fluorescence was detected at 4 weeks of incubation and further increased 24-fold at 10 weeks. The anti-(hexitol lysine) antibody recognized the glucated BSA containing AGE at 4 and 10 weeks of incubation, probably because a significant amount of early products still existed. However, no reactivity of anti-(fructated lysine) antibody to glucated BSA containing AGE was detectable, indicating that AGE produced by incubation with glucose was not an epitope of this antibody.

Detection of fructated proteins in eyes from control and streptozotocin-induced diabetic rats

We then applied this antibody to the detection of fructated proteins in streptozotocin-induced diabetic rat eyes as well as in control samples. Soluble proteins from the eyes of control and streptozotocin-induced diabetic rats at different times were analysed by ELISA and immunoblotting. Materials reactive to this antibody were significantly elevated in diabetic rat eyes during the aging process, not only in diabetic but also in control rats (Figure 6), in which augmentation of the polyol pathway and the resulting accumulation of fructose have been reported [12]. Reactive materials to anti-(hexitol lysine) antibody were also increased in eyes during the aging process in both normal

and diabetic rats; they were more prominent in diabetic rats (Figure 6b).

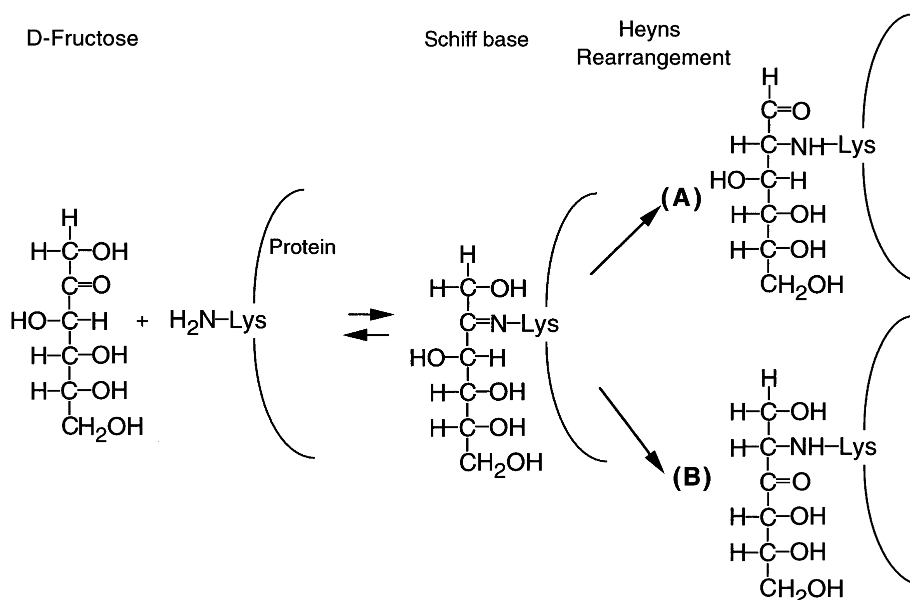
To identify which proteins were fructated in these tissues, we performed an immunoblot analysis with this antibody (Figure 6a). The second antibody, goat anti-(rabbit IgG), alone bound to the same lens proteins, possibly γ-crystallin as judged by the abundance in the fraction and the molecular sizes in both normal and diabetic rats. Non-specific binding observed in γ-crystallin would be due to a structural similarity to rabbit immunoglobulin [21]. However, the increased signals corresponding to β_L-crystallin observed in diabetic rat lens were suppressed by the presence of authentic fructated lysine (Y. Kawasaki, J. Fujii, N. Miyazawa, A. Hoshi, A. Okado, Y. Tano and N. Taniguchi, unpublished work). This suggests that β_L-crystallin is a major fructated crystallin isoform in diabetes.

DISCUSSION

Augmentation of the glycation reaction and of the polyol pathway under hyperglycaemic conditions are two major hypotheses for the origin of diabetic complications. However, there is little apparent connection between these hypotheses [12]. Glycation reactions involve reducing sugars, which have aldehyde or ketone groups, as reactants. Fructose is mainly generated through the polyol pathway in the body [10], and in addition is a stronger glycating sugar than glucose because the population of the open-chain form is much higher than that of glucose under physiological conditions [13]. We therefore hypothesized that the fructation of proteins might have a role in diabetic complications, especially in tissues with high polyol-metabolizing activity. To demonstrate this hypothesis *in vivo* we established an antibody capable of recognizing fructated proteins. This antibody specifically recognized fructated BSA but not adducts with other sugars or borohydride-reduced fructated protein, as evidenced by ELISA and immunoblotting (Figure 2). The binding was inhibited only by fructated lysine, but not by either fructose or lysine alone, indicating that a fructated form of lysine is the epitope that this antibody recognizes (Figure 3).

We have examined the fructation of BSA *in vitro* by using this antibody and found that the fructation reaction did not saturate at 100 mM fructose even after incubation for 7 days. We also examined levels of fructated proteins in eyes from diabetic rats induced by the administration of streptozotocin. A significant elevation of fructated proteins was detected (Figure 6). The fructated proteins seem to be lens crystallins on the basis of the molecular size. Fructose accumulates to a level higher than that of glucose in the lens of diabetic animals [11,22]; the glycation reaction advances more rapidly for fructated proteins than for glucated proteins as judged by measuring fluorescent cross-linked proteins.

The early glycation products for the fructation of proteins are different from those of glucose. Fructation results in the formation of Heyns rearrangement products [12] in which the C-2 carbon in fructose is bound to the ϵ-amino group of lysine and proceeds by two alternative steps, one involving C-1 and the other with the participation of C-3 as in Scheme 1. In the present study the antibody recognized only fructated proteins that had not been reduced with borohydride, but not glucated proteins, suggesting that the antibody recognizes only the unreduced Heyns product(s) even though it is currently unclear whether or not one or both of the Heyns products are recognized by our antibody. Glycation by fructose is usually underestimated by common glycation assays such as the serum fructosamine assay [19]. The accumulation of a fructated protein adduct has also been demonstrated by McPherson et al. [16] but their method, employing the HPLC analysis of phenylthiocarbamoyl derivatives of the glycated protein followed by the calculation of the position of C-1 and C-2 of covalently bound hexose to the protein, is rather complicated and would be difficult to apply to the detection of fructated proteins *in situ*. The present method is simpler and more convenient than theirs for the quantitative detection of fructated proteins. Because there is no specific, reliable method to detect glycation by fructose, even though its involvement in glycation is evident [23], our immunological method, which specifically recognizes fructation, could prove to be useful. A preliminary use of this antibody was applied to a cell



Scheme 1 Heyns rearrangement of fructated proteins

culture system in which the role of fructose in the induction of oxidative stress and apoptosis were examined, and was found to be useful for the detection of fructated proteins [24]. Furthermore the use of a combination of anti-(hexitol lysine) antibody and anti-(fructated lysine) antibody permits the differentiation of glucation from fructation at an early stage of glycation, which would also be beneficial for clinical diagnosis and could provide the basis for a determination of whether or not an inhibitor of the polyol pathway should be administered.

This work was supported in part by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture, Japan, Research Grant (9A-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare, Japan, and Ono Pharmaceutical Co. We thank Ono Pharmaceutical Co. for providing us with streptozotocin-induced diabetic rats.

REFERENCES

1 Monnier, V. M. (1989) in The Maillard Reaction in Aging, Diabetes and Nutrition (Baynes, J. W. and Monnier, V. M., eds.), pp. 1–22, Alan R. Liss, New York
2 Taniguchi, N. (1992) Adv. Clin. Chem. **29**, 1–59
3 Arai, K., Iizuka, S., Tada, Y., Oikawa, K. and Taniguchi, N. (1987) Biochim. Biophys. Acta **924**, 292–296
4 Arai, K., Maguchi, S., Fujii, S., Ishibashi, H., Oikawa, K. and Taniguchi, N. (1987) J. Biol. Chem. **262**, 16969–16972
5 Kondo, T., Murakami, K., Ohtuka, Y., Tsuji, M., Gasa, S., Taniguchi, N. and Kawakami, Y. (1987) Clin. Chim. Acta **166**, 227–236
6 Takahashi, M., Lu, Y., Myint, T., Fujii, J., Wada, Y. and Taniguchi, N. (1995) Biochemistry **34**, 1433–1438
7 Hoshi, A., Takahashi, M., Fujii, J., Myint, T., Kaneto, H., Suzuki, K., Yamasaki, Y., Kamada, T. and Taniguchi, N. (1996) Biochem. J. **318**, 119–123
8 Ookawara, T., Kawamura, N., Kitagawa, Y. and Taniguchi, N. (1992) J. Biol. Chem. **267**, 18505–18510
9 Kaneto, H., Fujii, J., Suzuki, K., Kasai, H., Kawamori, R., Kamada, T. and Taniguchi, N. (1994) Biochem. J. **304**, 219–225
10 Gabbay, K. H. (1975) Annu. Rev. Med. **26**, 521–536
11 Tomlinson, D. R., Stevens, E. J. and Diemel, C. T. (1994) Trends Pharmacol. Sci. **15**, 293–297
12 Suárez, G. (1989) in The Maillard Reaction in Aging, Diabetes, and Nutrition (Baynes, J. W. and Monnier, V. M., eds.), pp. 141–162, Alan R. Liss, New York
13 Bunn, H. F. and Higgins, P. J. (1981) Science **213**, 222–224
14 Tilton, R. G., Chang, K., Nyengaard, J. R., Van den Enden, M., Ido, Y. and Williamson, J. R. (1995) Diabetes **44**, 234–242
15 Grandhee, S. K. and Monnier, V. M. (1991) J. Biol. Chem. **266**, 11649–11653
16 McPherson, J. D., Shilton, B. H. and Walton, D. J. (1988) Biochemistry **27**, 1901–1907
17 Suárez, G., Rajaram, R., Oronsky, A. L. and Gawinowicz, M. A. (1989) J. Biol. Chem. **264**, 3674–3679
18 Pennington, J. and Harding, J. J. (1994) Biochim. Biophys. Acta **1226**, 163–167
19 Ahmed, N. and Furth, A. (1992) J. Clin. Chem. **38**, 1301–1303
20 Myint, T., Hoshi, S., Ookawara, T., Miyazawa, N., Suzuki, K. and Taniguchi, N. (1995) Biochim. Biophys. Acta **1272**, 73–79
21 Hay, R. E., Woods, W. D., Church, R. L. and Petrash, J. M. (1987) Biochem. Biophys. Res. Commun. **146**, 332–338
22 Cheng, H.-M., Hirose, K., Xiong, H. and Gonzalez, R. G. (1989) Exp. Eye Res. **49**, 87–92
23 Dills, Jr., W. L. (1993) Am. J. Clin. Nutr. **58**, 779S–787S
24 Kaneto, H., Fujii, J., Myint, T., Islam, K. N., Miyazawa, N., Suzuki, K., Kawasaki, Y., Nakamura, M., Tatsumi, H., Yamasaki, Y. and Taniguchi, N. (1996) Biochem. J. **320**, 855–863

Received 22 June 1998/10 August 1998; accepted 11 September 1998