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# Specific detections of the early process of the glycation reaction by fructose and glucose in diabetic rat lens

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**Abstract The glycation reaction by fructose, as well as that by glucose, in control and diabetic rat lens was analyzed by using antibodies which specifically recognize adducts of lysine with fructose and with glucose. Levels of fructose adducts in diabetic rat lens were 2.5 times that of the control, and correlated with sorbitol levels. This was mainly due to enhanced glycation of β- and γ-crystallins by fructose under diabetic conditions. These data suggest that glycation by fructose may also play a role in cataract formation under conditions of diabetes and aging.**


*Key words:* Glycation; Polyol pathway; Fructation; Glucation; Crystallin; Amadori product

## 1. Introduction

The cross-linking of long-lived proteins, such as collagen and lens crystallins, correlates with aging and diabetic processes [1]. Glycation, which proceeds during normal aging, and at accelerated rates in diabetes, is involved in structural and functional alterations of proteins and other cellular components. The final products of the glycation reaction, referred to as advanced glycation end products (AGE), are fluorescent compounds, which increase under diabetic conditions as well as the aging process, and, hence, have been suggested to be a cause of these conditions [2].

Glucose is a major reducing substance in blood and is generally thought to be a major glycating agent in the body. Since the level of fructose is about 100-fold lower than that of glucose in most tissues, the involvement of fructose in the glycation reaction (fructation) is generally thought to be much less significant than glucose-induced glycation (glucation). In some tissues such as lens, kidney, and peripheral nerve, however, a polyol metabolizing pathway, which converts glucose to sorbitol via aldose reductase (AR) and then sorbitol to fructose by sorbitol dehydrogenase (SDH), is active [3]. Therefore fructose-induced glycation may also play a role in such tissues.

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*Abbreviations:* AGE, advanced glycation end products; ELISA, enzyme linked immunosorbent assay; STZ, streptozotocin; AR, aldose reductase; SDH, sorbitol dehydrogenase; RP-HPLC, reverse phased-high performance liquid chromatography; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; α-CHCA, α-cyanohydroxycinnamic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

It is well known that lens is one of the most susceptible tissues to the glycation reaction because the half life of lens proteins is very long and sugars also accumulate there at relatively high levels. The glycation of lens crystallins is thought to accelerate the aggregation of these proteins and to cause cataract formation via decreasing the transparency of the lens [4]. The concentration of fructose in the lens becomes equivalent to glucose concentration in severe cases of diabetes [5,6]. In addition, fructose is known to be a more potent glycating agent than glucose because the population of its acyclic (open chain) form, which is the reactive species, is approximately 10 times that of glucose [7]. However, studies dealing with the fructation of proteins in vivo are few in number [8] because there is no reliable and simple method to quantitate the fructated proteins.

We have recently established an antibody which specifically recognizes fructated proteins with no need for any preliminary treatment [9]. The use of this antibody, together with anti-hexitol lysine antibody which specifically recognizes the reduced form of an Amadori product formed in glucated proteins [10], enables the analysis of the early events in the glycation reaction by glucose and fructose to be carried out.

This paper describes the characterization of the early products in the glycation reaction of lens proteins in normal and diabetic rats, along with a comparison of the contributions to the glycation mode by glucose and fructose as this relates to cataract formation.

## 2. Materials and methods

### 2.1. Materials

Glucose and fructose were purchased from Nakarai Tesque (Kyoto, Japan). Sodium borohydride ($NaBH_4$) was obtained from Wako Pure Chemicals (Osaka, Japan). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from ZYMED Laboratories (San Francisco, CA, USA). Sephacryl S-300 was obtained from Pharmacia LKB Biotechnology (Tokyo, Japan). Polyvinylidene difluoride (PVDF) transfer membrane (Immobilon) was a product of Millipore (Bedford, MA, USA). Sodium cyanoborohydride ($NaBH_3CN$), *O*-phenylene-diamine dihydrochloride, TPCK-treated trypsin and ASP-N were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Preparation of lens homogenates from normal and streptozotocin (STZ)-induced diabetic rats

Diabetic rats developed by injection of STZ (50 mg/kg) via the intravenous route were kindly provided by Ono Pharmaceutical (Osaka, Japan). These animals were sacrificed at 8, 20, 24, and 28 weeks after STZ injection by decapitation under anesthesia with diethyl ether. Normal and STZ-induced diabetic rat lenses were homogenized in 1 ml of 50 mM phosphate-buffered saline (PBS), pH 7.4. The homogenates were ultracentrifuged at $100\,000 \times g$ at 4°C for 30 min. The supernatant was obtained and the protein concentration therein was determined using a protein assay kit (Bio-Rad, Yokohama, Ja-


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pan). Immunoplate Maxisorp was purchased from Nunc (Rochester, NY, USA).

*2.3. Quantification of glucated and fructated lens proteins by enzyme linked immunosorbent assay (ELISA)*

Fifty microliter aliquots of samples containing 5 μg protein were added to a 96-well immunoplate and incubated for 2 h at room temperature. For the quantification of glucation, samples were reduced by adding 10 μl of 0.1 M $NaBH_4$ solution as described previously [10]. The plate was washed four times with PBS containing 0.05% Tween-20 to remove unbound proteins and blocked with 1% bovine serum albumin (BSA) in PBS by incubation at room temperature for 1 h. The affinity purified polyclonal antibody raised against 1-hexitol lysine [10] diluted to 1:500 or that against fructated lysine [9] diluted to 1:250 with PBS was added and reacted at room temperature for 2 h. After washing four times, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution in PBS) was added and allowed to react for 2 h. Peroxidase activity was detected by adding 50 μl of an *O*-phenylenediamine dihydrochloride solution containing hydrogen peroxide. The reaction was stopped by the addition of 50 μl of 1 N $H_2SO_4$ to each well. The absorbance at 490 nm was determined with an immunoreader NJ200 (Intermed, Conshohocken, PA, USA) and amounts of glucated and fructated proteins were expressed arbitrary using glucated and fructated BSA as standards, respectively. The level of fructation and glucation for each of the crystallin species (α, β and γ) was quantitated in this manner.

*2.4. Gel filtration of control and STZ rat lens*

Samples containing ten control rat lenses and ten 20-week diabetic rat lenses were homogenized separately in 10 ml of buffer containing 0.05 M Tris, 0.5 M NaCl, 0.001 M EDTA, and 0.02% $NaN_3$, pH 7.4, at 4°C. The soluble portion was centrifuged at $100\,000 \times g$ for 30 min. After concentration by membrane filtration, the supernatants were fractionated into the αHM-, α-, βH-, βL- and γ-crystallin by gel filtration (2.5×90 cm, Sephacryl S-300) using the same buffer at a flow rate of 9 ml/h at 4°C. The electrophoretic profiles of these proteins were nearly the same for each chromatography.

*2.5. Detection of glucated proteins and fructated proteins by immunoblotting*

For the detection of fructated proteins by immunoblotting, we biotinylated anti-fructated lysine IgG using a protein biotinylation system (Amersham, Uppsala, Sweden), since the goat anti-rabbit IgG (the second antibody) bound non-specifically to rat γ-crystallins, although it did not cross-react with γ-crystallins in a native conformation [10]. Whole lens proteins were subjected to 15% SDS-PAGE and were transferred onto PVDF membranes under semi-dry conditions using a Trans-blot (Bio-Rad, Hercules, CA, USA). For Immunoblot analysis, by anti-hexitol Lys IgG, the blot was reduced with 0.1 M $NaBH_4$ for 2 h at room temperature prior to incubation with the IgG [10,11]. The membranes were blocked by incubation with 4% skimmed milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl) at room temperature for 1 h with gentle agitation, washed four times with TBS containing 0.05% Tween 20 for 15 min each time, and then incubated with the diluted (1:500) biotinylated anti-fructated lysine IgG at 4°C overnight. After washing, the samples were incubated with the horseradish peroxidase-conjugated streptavidin diluted to 1:2000 for 30 min at room temperature and then washed for 6 h. The chemiluminescence method was employed to amplify the signal using an ECL kit (Amersham, Uppsala, Sweden).

*2.6. Isolation of tryptic peptide from fructated γ-crystallins by reverse phase-high performance liquid chromatography (RP-HPLC)*

After 6 days incubation with 100 mM fructose and 100 mM $NaBH_3CN$, approximately 100 μg of the γ-crystallins were dissolved in 500 μl of 50 mM Tris-HCl, pH 7.4, and digested with TPCK-treated trypsin at a protein to enzyme ratio of 50:1 for 24 h at 37°C. The fructated peptides were separated by RP-HPLC (LC6A, Shimadzu, Kyoto, Japan) on a Cosmosil $C_{18}$ column (4.6×250 mm, Nacalai Tesque, Kyoto, Japan), at a flow rate of 0.8 ml/min, with a gradient of 5–80% B over 60 min. Solvent A (0.05% TFA in $H_2O$); solvent B (0.05% TFA in acetonitrile). Peptides were detected by measuring absorbance at 280 nm. Each peak was collected, lyophilized and then subjected to further analysis.

*2.7. Mass spectrometrical analysis of fructated peptide*

Tryptic peptides, purified from the RP-HPLC, were dissolved in 1 μl of 50% acetonitrile, 0.05% TFA, mixed with 1 μl of saturated α-cyanohydroxycinnamic acid (α-CHCA; Aldrich, Milwaukee, WI, USA) dissolved in 5% acetonitrile, 0.05% TFA, and applied to a sample plate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Voyager-RP equipped with a delayed-extraction system (Perceptive Biosystems, Flamingham, MA, USA) with an $N_2$ gas discharge laser (337 nm).

*2.8. Amino acid sequencing and amino acid analysis*

The isolated tryptic peptide was subjected to amino acid sequencing in a gas-phase protein sequencer (ABI-477A, Applied Biosystems, Drive Foster, CA, USA). PTH-amino acids were quantitated by RP-HPLC (Applied Biosystems). The peptide was also hydrolyzed in 6 N HCl at 100°C for 24 h under vacuum. Amino acid analysis was carried out with an automatic amino acid analyzer (L-8500, Hitachi, Tokyo, Japan).

*2.9. Measuring sorbitol content in rat lenses*

The sorbitol content of rat lens was determined using a method described previously [13], by measuring the enzymatic formation of NADH from NAD with sorbitol dehydrogenase.

## 3. Results

Fig. 1a shows quantitative data for the levels of glucated proteins in normal and diabetic rat lens, as assayed by ELISA using an anti-hexitol lysine antibody, which specifically recog-

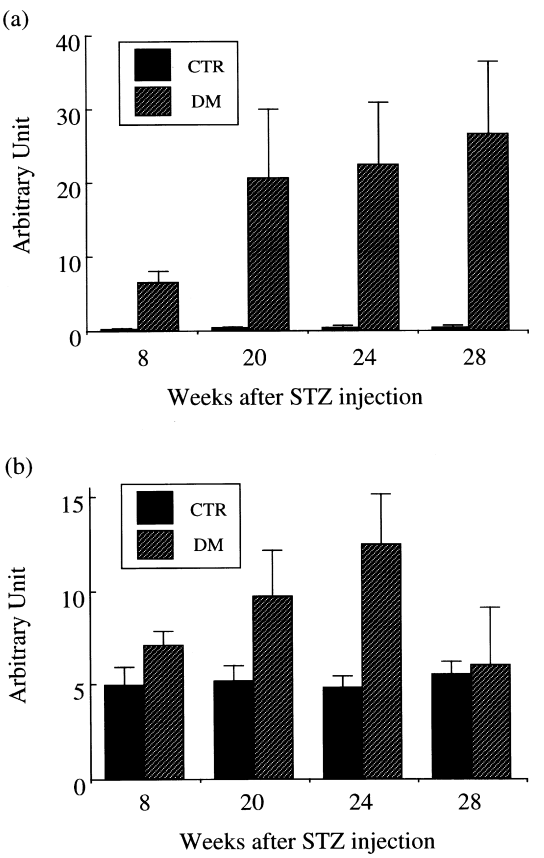


Fig. 1. ELISA analysis of levels of fructated or glucated lens proteins in control and diabetic rats. Each lens was prepared individually from four controls and four diabetic rats at 8, 20, 24 and 28 weeks after STZ injection and homogenized. After ultracentrifugation, 5 μg of soluble proteins were subjected to ELISA using the anti-hexitol lysine antibody (a) or the anti-fructated lysine antibody (b). CTR: control; DM: diabetes.

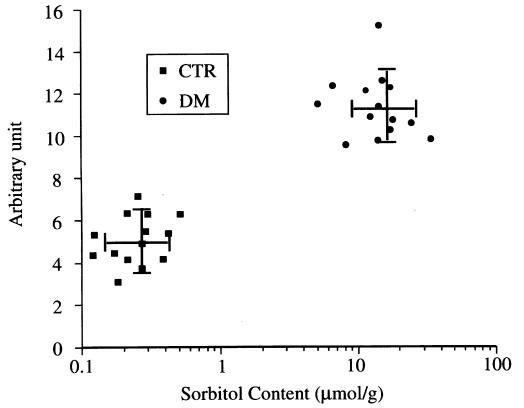


Fig. 2. Correlation between sorbitol content and fructation in rat lenses. The sorbitol content and levels of fructated proteins in rat lenses were compared in control and diabetic groups. CTR: control; DM: diabetes.

nizes the glucated proteins after reduction with sodium borohydride. The levels of glucated proteins increased and reached a plateau at 20 weeks, when cataract formation was obvious and severe.

The fructation reaction, however, started to advance at 8 weeks, reaching a maximum level at 24 weeks, and then decreased considerably at 28 weeks (Fig. 1b). Since only soluble proteins after ultracentrifugation were used for the ELISA assay, this suggests that fructated proteins became insoluble at an advanced stage of diabetes. As described below, the solubility may be decreased, as the result of the accelerated formation of cross-linked insoluble AGEs in fructated proteins vs. glucated proteins.

Since fructose is mainly produced by the polyol metabolizing pathway and sorbitol is a direct precursor of fructose, sorbitol contents in lens were measured and compared with levels of fructated proteins in rat lenses sacrificed 4 weeks after STZ injection (Fig. 2). The levels of sorbitol were significantly higher in diabetic rats ($16.08 \pm 7.70$ μmol/g), compared to those in control rats ($0.27 \pm 0.12$ μmol/g) ($P < 0.01$). The levels of fructated proteins were correlatively higher in diabetic rats ($11.47 \pm 1.46$) than those in control rats ($5.01 \pm 1.20$) ($P < 0.01$) and correlated to the levels of sorbitol. These data clearly demonstrate that fructose, produced through the polyol metabolizing pathway is actually involved in the fructation of lens proteins.

To characterize the fructation and glucation reaction comparatively at advanced stages of glycation, total lens proteins were incubated either with 100 mM glucose or with 100 mM fructose for 7 days at 37°C, and the fluorescent materials corresponding to AGE were then quantitated by fluorescence spectrophotometry (data not shown). While the levels of fluorescence in the glucated proteins increased only about 30% compared with the control, a significant elevation, about 10 times that of the control, was observed in fructated proteins. These data, which are consistent with our previous report [9], clearly show that the glycation by fructose proceeds at an accelerated rate compared with the same concentration of glucose.

Rat lens crystallins are composed of three major classes, α, β, and γ. To characterize their susceptibility to glycation by glucose and fructose independently, immunoblot analysis (Fig. 3) and quantitation by ELISA (Fig. 4) were carried out using both the anti-hexitol lysine antibody and the anti-fructated lysine antibody. Levels of glucation and fructation of γ-crystallins were originally high even in normal lens, and were further increased in diabetic lens. The level of glycation of β-crystallins was lower than the γ-crystallins in normal lens, but increased markedly in diabetic rats in terms of both glucation and fructation. Thus, the major contribution to glycation occurred in the β-crystallins, suggesting that β-crystallins may play an important role in the formation of cataract as the result of diabetic complications.

γ-Crystallins, in which the glycated fraction was removed by passing through a boronate column, was incubated with 100 mM fructose and $NaBH_3CN$ for 6 days. After TPCK-tryptic digestion, the resulting peptides were separated on RP-HPLC (data not shown), and each fraction was subjected to mass spectrometric analysis. We detected two candidate peptides which could have been modified by one or two moles of fructose. Since these fractions contained some impurity, they were re-chromatographied on RP-HPLC after digestion with ASP-N, to give one pure peptide, although the other one could not be recovered due to its low content in the fraction. The molecular mass of the peptide (1021.1, Fig. 5) suggested that the pure protonated peptide corresponded to the protonated N-terminal peptide (Gly-1 to Glu-7, the calculated mass: 857) with an excess of 164. The minor peptide likely contained two moles of bound fructose at the α-amino group of Gly-1 and the ε-amino group of Lys-2. Amino acid analysis identified 6 amino acids which exactly matched the residues

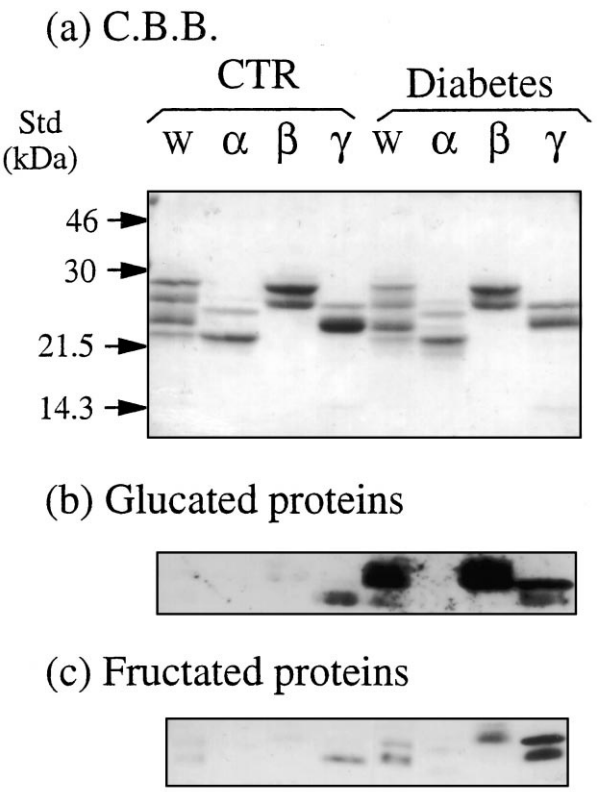


Fig. 3. Immunoblot analysis of glucated and fructated lens proteins in control and diabetic rats. Five μg of the crystallins isolated at 20 weeks after STZ injection were subjected to immunoblot analysis using the anti-hexitol lysine antibody (b) and anti-fructated lysine antibody (c). W: whole lens proteins; C.B.B.: Coomassie brilliant blue; Std: standard marker.

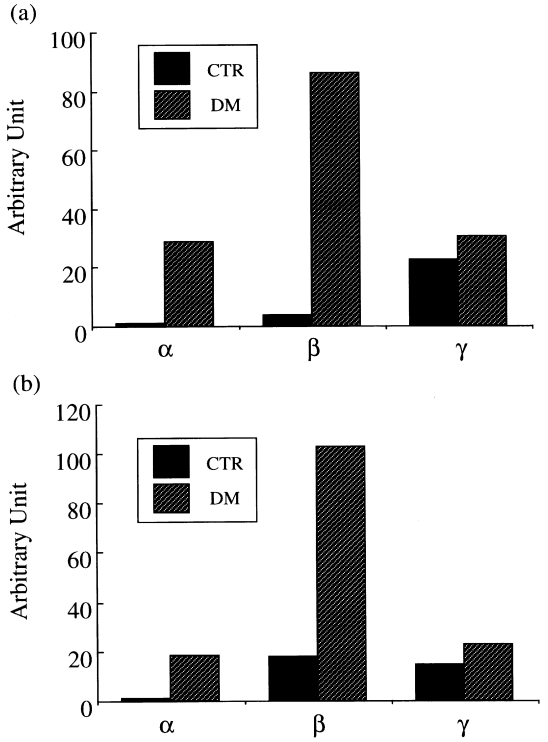


Fig. 4. ELISA analysis of the levels of fructation and glucation in crystallins isolated from control and diabetic rat lenses. After gel filtration, each fraction (α, β, γ) was collected and subjected to ELISA using the anti-hexitol lysine antibody (a) or the anti-fructated lysine antibody (b). CTR: control; DM: diabetes.

Lys-2 to Glu-7 in γ-crystallins at molar ratios from 0.7 to 1.2 and a trace amount of glycine, the N-terminal amino acid residue (data not shown). These collective data suggest that fructation mainly occurred at the α-amino group of the N-terminal Gly.

## 4. Discussion

Aldose reductase, a rate-determining enzyme of the polyol metabolizing pathway, is a target of a drug designed to ameliorate diabetic complications [12]. It has been demonstrated that such drugs are actually effective in suppressing cataract formation in lens [13]. A generally accepted mechanism by which the drug exerts its effect is through suppression of the formation of sorbitol, a sugar alcohol which penetrates the cell membrane with difficulty and, therefore, contributes to the elevation of osmolarity within cells. Rat lens, however, expressed fairly large amounts of AR but quite low levels of SDH compared with other tissues, and hence this accumulation of sorbitol in lens would be caused by low SDH activity. A recent study demonstrated that an inhibitor of SDH also was capable of improving the diabetic complications by an unknown mechanism [14]. Since SDH converts sorbitol to fructose, a more potent glycating sugar than glucose, the glycation of proteins by fructose may be significant in tissues with high polyol metabolizing activity.

Lens represents one of the tissues in which fructose accumulates at a level approximately equivalent to glucose under diabetic conditions and the half-life of its major structural proteins, crystallins, is quite long [6]. Thus glycation by the accumulated fructose would also cause defects in crystallin function and consequently a serious damage to lens function [15]. However, since there was no adequate method to distinguish glucation from fructation, no reliable data to assess the roles of glucation and fructation in cataract formation in vivo has been presented. Quite recently we developed a specific antibody against fructated proteins [9]. This, together with our anti-hexitol lysine antibody [10], enabled us to analyze precisely and independently the role of glucation and fructation in vivo.

Glycation occurred in significant levels even in control rat lens. The Amadori product accumulated at very high levels in diabetic rat lens 8 weeks after STZ injection. Compared to Amadori products, the early products reacted with fructose, also referred to as a Heyns product, and did not accumulate to as great an extent, although AGE formation as judged by measuring fluorescent materials was much higher than glucation. This would be due to the acceleration of AGE formation from the Heyns product vs. the Amadori product, the result of which is to keep Heyns products at lower levels. Our data showed that the fructation of lens proteins correlates to the sorbitol content, as evidenced by the control group vs. the diabetic rat lenses. These data support our hypothesis that the fructation of lens proteins increases under conditions in which the polyol metabolizing pathway is active.

When the glycation reaction was analyzed by immunoblotting at the levels of the molecular species of crystallins, the glycation of γ-crystallins was higher than other forms. However, in a diabetic lens, the β-crystallins rather than γ-crystallins were found to be more susceptible to glycation by both glucose and fructose as judged by the ELISA assay. This difference may reflect the tertiary structures of crystallin isoforms. While antibodies react with antigenic proteins in non-denaturing conditions by ELISA, only denatured forms are detected by immunoblotting. In fact, goat anti-rabbit IgG reacted highly with γ-crystallins in immunoblots, but barely reacted in the ELISA assay. Although several reports concerning glycation of α-crystallin in relation to its chaperone

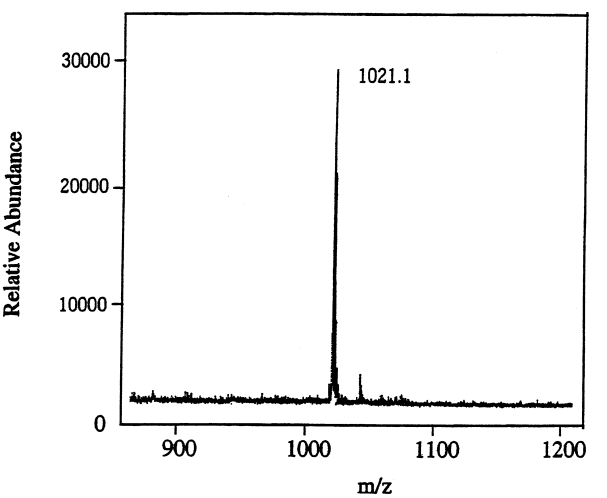


Fig. 5. Analysis of fructated peptides in γ-crystallins by MALDI-TOF-MS. After incubation of 100 μg of γ-crystallins with 100 mM fructose, tryptic peptides were separated by RP-HPLC and then re-chromatographed on RP-HPLC after digestion with ASP-N. The resultant peptide was analyzed with MALDI-TOF-MS. The obtained mass values (1021.1) correspond to the mass of the protonated ($MH^+$), amino-terminal peptides Gly-1 to Glu-7 with an excess of 164.

activity have appeared [15], susceptibility to the glycation reaction was much less than the other two isoforms. Thus, the β- and γ-crystallins might be more important in cataract formation in terms of diabetic complications. Since the tertiary structure of α-crystallin has not yet been determined, the mechanism by which the different crystallin isoforms react remains unclear.

The fructation detected by this antibody would be expected to occur at the ε-amino group of lysine residues because fructated lysine was the antigen [9]. Our data showed that β- and γ-crystallins were susceptible to glycation by fructose, and Zhao et al. [16] showed the sites of glycation in β-crystallin by fructose. Although Pennington et al. [17] and Smith et al. [18] reported the sites of glycation in γ-crystallin by fructose, we further investigated the γ-crystallin situation.

It is not possible to distinguish fructose-derived from glucose-derived AGEs at this time because they probably share a common structure with respect to fluorescence, and no specific method to distinguish them has yet been developed. Therefore it is of importance to evaluate the contribution of glucation and fructation in the glycation reaction at an early stage. We herein demonstrated for the first time that the fructation of crystallins may also contribute to cataract formation by measuring the early products of the fructation reaction with antifructated lysine antibody. It is known that mice hardly develop cataract in the eyes, and that the enzymes involved in the polyol metabolizing pathway are much lower than they are in rats. However, transgenic mice overproducing AR develop cataract and accumulate sorbitol in the lens like the rat [19]. This approach is also applicable to other tissues such as peripheral nerves and kidney and will be useful in evaluating the involvement of the polyol metabolizing pathway in diabetes mellitus and aging.

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