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Sites of Glycation of βB_2-Crystallin by Glucose and Fructose

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We determined the sites of glycation of bovine βB_2-crystallin by glucose and fructose. After incubation with glucose or fructose, glycated tryptic peptides were purified by affinity chromatography/reverse-phase HPLC and identified by electrospray ionization mass spectrometry (ESIMS). The results gave evidence of glycation at lysine 10, 75, 100, 107, 120, 139, 167 and 171 by both glucose and fructose, while glycated lysine 119 and 47 or 67 were detected only after fructosylation. We conclude that glucose and fructose have similar glycation specificity. © 1996 Academic Press, Inc.

Sugar aldehydes or ketones can react with free amino groups of proteins to form reversible Schiff-base adducts which can further form stable ketoamine compounds called Amadori products and advanced glycation end-product (1). Since, after synthesis, lens crystallins remain throughout life, post-translational modifications such as glycation can occur. In fact, increased glycation in human diabetic cataract has been found (2) and glucose is thought to be the the cause of the cataract (3). In diabetic lenses, fructose is found to accumulate to levels higher than that of glucose (4) and react more rapidly with lens proteins than glucose in the late stage of Maillard reaction (5). However, it is not known whether these sugars have the same site specificity in the glycation process.

Beta-crystallin constitutes a group of polydisperse oligomeric structural proteins in the mammalian lens (6). About 40% of the crystallins in the bovine lens are β-crystallin. Together with α- and γ-crystallins, it gives high refractive index to the lens. Bovine β-crystallin is composed of basic (βB1, βB2 and βB3) and acidic (βA1, βA2, βA3 and βA4) subunits (7). All of them are from a multigene family. Beta B2-crystallin is the major β-crystallin subunit in the lens. Beta-crystallins also belong to the same superfamily as γ-crystallin. They have sequence homology and a similar two domain structure, but βB2-crystallin has 13 lysines (8) and a blocked N-terminus, while γB-crystallin only has 2 lysines and a free N-terminus (6). The glycation sites of major α- and γ-crystallin subunits have been determined by our work and others (9-12), but the glycation sites of β-crystallin have not been reported except what was reported earlier from the analysis of water-insoluble high molecular weight aggregates (13). Since β-crystallin is the most abundant crystallin in the lens, its glycation may be important in cataract formation.

MATERIALS AND METHODS

Preparation of bovine βL-crystallin. Decapsulated calf lenses were homogenized in 50 mM Tris-HCl (pH 7.4) containing 50 mM $NaHSO_3$, 20 mM EDTA and 0.02% NaN_3 and the homogenate was centrifuged at 10,000×g for

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Abbreviations: ESIMS, electrospray ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RP-HPLC, reverse phase-high performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TPCK, L-(1-tosylamino-2-phenyl) ethyl chloromethyl ketone; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

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30 min at 4°C. The supernatant was loaded on a 1.5×100 cm Sephacryl-S-300HR (Sigma, St Louis, MO) column and the proteins were eluted using the same buffer. Beta L-crystallin fraction was collected, concentrated and dialysed against PBS (pH 7.4) containing 0.02% NaN_3 and the purity was checked by SDS-PAGE.

In vitro glycation of βL-crystallin by glucose (glucosylation) and fructose (fructosylation). βL-crystallin (5 mg/ml) was incubated with 1 M glucose or fructose (Fisher, Pittsburgh, PA) in the presence of 50 mM sodium cynoborohydride (Aldrich, Milwaukee, WI) and 0.02% NaN_3 at 37°C for 5 days. After incubation, proteins were extensively dialysed against 0.1 M ammonium bicarbonate (pH 8.0).

Trypsin digestion of glycated βL-crystallin. Fructosylated and glucosylated βL-crystallin were heated at 100°C for 5 min. The proteins were then digested with 2% (W/W) TPCK treated trypsin (Sigma) for 4 hours at 37°C under N_2.

Purification of glycated peptides. Glycated peptides were purified using a 1×10 cm column packed with GLYC-AFFIN resin (ISOLAB Inc, Akron, OH), instead of Affi-gel 601 as described in a previous communication (9). The trypsin digested peptides were loaded on the column pre-equilibrated with 0.05 M HEPES/NaOH (pH 8.5) buffer containing 10 mM $MgCl_2$ and 0.02% NaN_3, and the unglycated peptides were washed out with the same buffer (14). The glycated peptides were eluted with 0.1 M sorbitol (Sigma) in the same buffer. Then, the peptides were separated by RP-HPLC on a MICROSORB-MV C_{18} column (Rainin Instrument Co. Inc. Emeryvill, CA). A linear gradient from 0 to 100% B was applied in 200 min at a flow rate of 0.5 ml/min and the peaks were collected for mass spectrometric analysis. Mobile phase A consisted of 0.1% TFA in water and mobile phase B consisted of 0.1% TFA in acetonitrile.

Electrospray ionization mass spectrometry (ESIMS). The molecular masses of the peptides present in each of the fraction isolated by affinity chromatography/RP-HPLC were determined by ESIMS (Micromass Platform Quadrupole Mass Spectrometer, Manchester, UK). The instrument was calibrated with NaI over a range of 300-2000 u to a mass accuracy of 0.3 u. The sample was delivered to the mass spectrometer in a 50% acetonitrile and water solution at a flow rate of 5 μl/min. Data were processed with MassLynx 2.0 software.

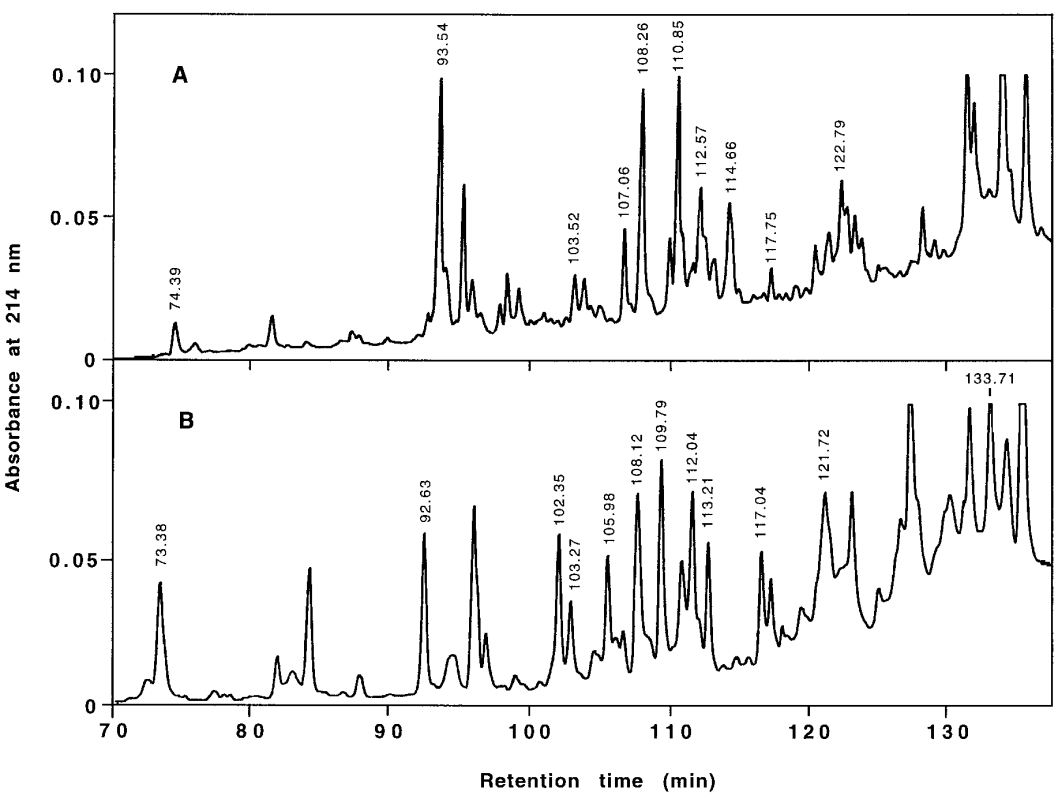

FIG. 1. RP-HPLC chromatograms of affinity purified glycated peptides. A. Glucosylated peptides. B. Fructosylated peptides. Only the peaks containing glycated peptide(s) from βB_2-crystallin identified by mass spectrometry are marked with retention time.

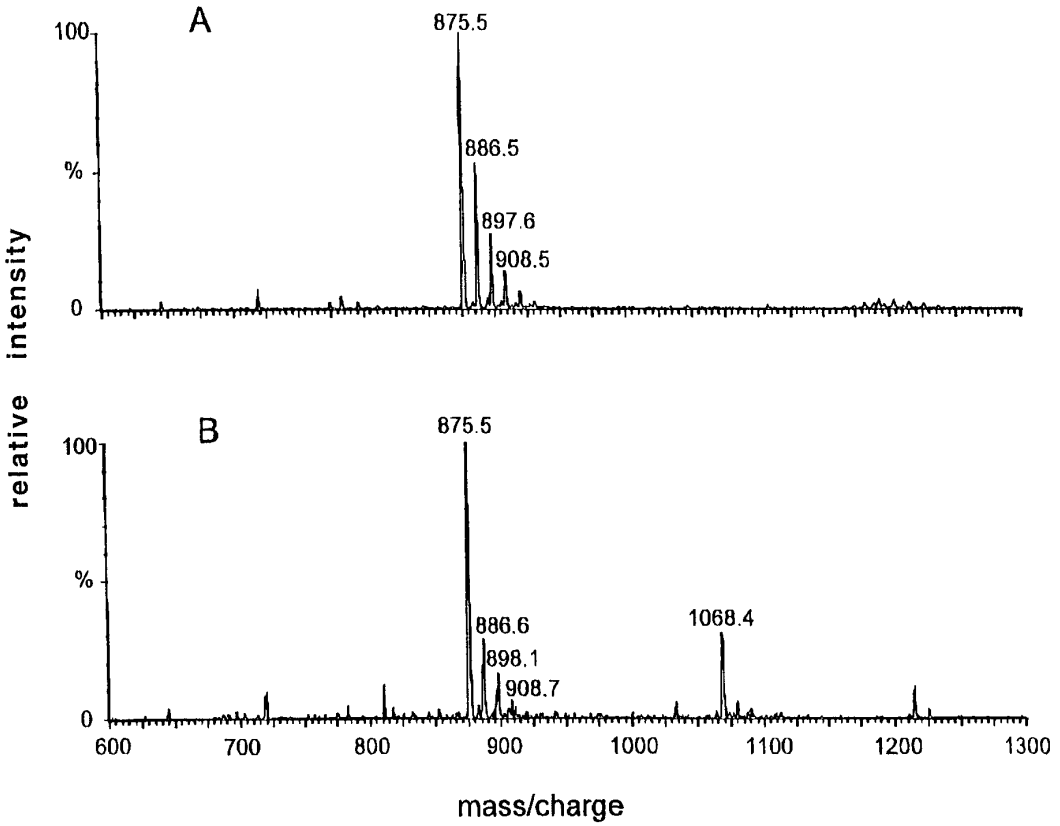

FIG. 2. Electrospray ionization mass spectra showing peaks due to βB_2 tryptic peptide 68-80 modified by incubation in A) glucose and B fructose. The fact that trypsin did not cleave at Lys 75 supports this position as a site of modification. The mass/charge of 875.5 can be attributed to the doubly charged (MH_2^{2+}) ion of peptide 68-80 (MW 1585) with an additional 164 u due to glycation. The extra peaks to the right of the principal peak are due to sodium replacing hydrogen in the sugar moiety, adding 22 u. For the doubly charged ion, this mass difference is seen as an additional 11 u.

Other methods. Protein concentrations were determined by the method of Bradford (15). SDS-PAGE was performed according to the method of Laemmli (16) using a 12% separating gel and a 4% stacking gel under reducing condition.

RESULTS

Glycated βL-crystallin was digested with trypsin and loaded on the affinity column. After extensive washing, a single peak was eluted from the affinity column by 0.1M sorbitol. The constituent peptides present in the glycated fraction were further separated by RP-HPLC (Fig. 1).

The molecular masses of peptides isolated by affinity chromatography and RP-HPLC were determined by ESIMS. Fig. 2 A and B show the ESIMS spectra of peptides containing glycated Lys 75 by glucose and fructose. Table I and Table II provide the molecular masses of the glycated peptides and the identities of the glycated lysines formed from glycation with glucose and fructose, respectively. The known molecular masses of tryptic peptides of βB2-crystallin were used to identify glycated peptides showing increase of 164 Da for the attached glucose or fructose (after reduction with $NaBH_3CN$ glycated moieties formed from both glucose and fructose will produce the same mass shifts). In both glucosylated and fructosylated βB2-crystallin, lysine residues 10, 75, 100, 107, 120, 139, 167 and 171 were found glycated. Lysine

TABLE I
Mass Spectromeric Analysis of Glucosylated βB_2-Crystallin

Peak I.D. (RT. min)	Molecular weight Calculated	Found	Segment	Sequence	Glycation site
74.39	1858	2022	1–17	ASDHQTQAGKPQPLNPK	K10
93.54	2151	2314	168–187	GDYKDSGDFGAPQPQVQSVR	K171
103.52	2195	2360	89–107	RTDSLSSLRPIKVDSQEHK	K100
	2039	2204	90–107	TDSLSSLRPIKVDSQEHK	K100
107.06	2219	2384	101–119	VDSQEHKITLYENPNFTGK	K107
108.26	1585	1749	68–80	GEQFVFEKGEYPR	K75
110.85	2344	2509	120–139	KMEVIDDDVPSFHAHGYQEK	K120
112.57	2872	3201*	120–144	KMEVIDDDVPSFHAHGYQEKVSSVR	K120 & K139
114.66	2744	2909	121–144	MEVIDDDVPSFHAHGYQEKVSSVR	K139
117.75	1426	1590	160–171	GLQYLLEKGDYK	K167
122.79	3094	3424*	160–187	GLQYLLEKGDYKDSGDFGAPQPQVQSVR	K167 & K171

* Two glucose moieties (328 u)

119 and Lys 47 or 67 was found only in the fructosylated βB2-crystallin. When two lysines occurred in a peptide and one of them was at the C-terminus (peak I. Ds. 74.39, 103.52, 107.06, 110.85 and 117.75 min in Table I and peak I. Ds. 73.38, 102.35, 103.27, 105.98, 109.79, 117.04 and 133.71 min in Table II) the fact that trypsin will not cleave immediately after a glycated lysine made it easier to conclude that the C-terminal lysine is not the glycated site in that peptide. When one of the lysine is at the N-terminus and the other is an internal residue or both are internal residues, there are two possibilities: 1) both the sites were glycated, therefore trypsin did not cleave after these sites (peak I. Ds. 112.57 and 122.79 min of Table I and peak I. Ds. 112.04 and 121.72 min of Table II). In fact, a mass shift of about 328 confirmed the presence of two glucose or fructose moieties; 2) since the presence of acidic residues (e. g. Asp. and Glu.) immediately before or after a lysine significantly decreases the

TABLE II
Mass Spectromeric Analysis of Fructosylated βB_2-Crystallin

Peak I.D. (RT. min)	Molecular weight Calculated	Found	Segment	Sequence	Glycation site
73.38	1858	2022	1–17	ASDHQTQAGKPQPLNPK	K10
92.63	2151	2314	168–187	GDYKDSGDFGAPQPQVQSVR	K171
102.35	2195	2361	89–107	RTDSLSSLRPIKVDSQEHK	K100
103.27	2039	2248*	90–107	WDSWTSSRRTDSLSSLRPIKVDSQEHK	K100
	1524	1732*	108–120	ITLYENPNFTGKK	K119
105.98	1585	1749	68–80	GEQFVFEKGEYPR	K75
	2219	2428*	101–119	VDSQEHKITLYENPNFTGK	K107
108.12	1585	1793*	68–80	GEQFVFEKGEYPR	K75
109.79	2344	2553*	120–139	KMEVIDDDVPSFHAHGYQEK	K120
112.04	2872	3081*	120–144	KMEVIDDDVPSFHAHGYQEKVSSVR	K120 or K139
	2872	3244#	120–144	KMEVIDDDVPSFHAHGYQEKVSSVR	K120 & K139
113.21	2744	2953*	121–144	MEVIDDDVPSFHAHGYQEKVSSVR	K139
117.04	1426	1634*	160–171	GLQYLLEKGDYK	K167
121.72	3094	3467#	160–187	GLQYLLEKGDYKDSGDFGAPQPQVQSVR	K167–K171
133.71	3684	3849	42–75	ETGVEKAGSVLVQAGPWVGYEQANCKGEQFVFEK	K47 or K67

* Two Na adducts (+44 u.) plus one fructose moiety (+164 u.).
\# Two Na adducts (+44 u.) plus two fructose moieties (+328 u.).

rate of hydrolysis by trypsin, in some instances the peptide bonds immediately after the lysine were not cleaved, even when the lysines were not glycated (peak I. Ds. 112.04 and 133.71 min of Table II).

DISCUSSION

The potential glycation sites of βB2-crystallins are the ϵ-amino groups of the 13 lysines. Among them, eight were glycated by both glucose and fructose. Glycated Lys 119 and Lys 47 or 67 were found only after fructosylation. These results suggest that an aldohexose and a ketohexose have nearly the same site specificity for glycation.

In this study, βL-crystallin, not the βB2-crystallin subunit, was used for glycation and subsequent identification of glycation sites. Although strong homology between βB2- and other β-crystallin subunits exists (17), there is no risk that the same glycated peptide may come from different β-crystallin subunits because mass spectrometry permits us to distinguish which β-crystallin subunit each peptide comes from. Additional glycated peptides could have been generated from other β-crystallin subunits, but they were too small to be detected. Since our focus was on βB2-crystallin, no attempt was made to isolate sufficient quantities of the other peptides. There is a possibility that βB2 peptides containing Cys residues could form disulfide bonds between Cys 37 and Cys 66 during the digestion and peptide isolation process, creating large peptides that were not recovered after HPLC. Disulfide bonds could also have been formed with Cys containing peptides from other β-crystallin subunits, resulting peptides that were not recognized. However, it should be pointed out that significant amounts of a glycated peptide containing free Cys 66 were identified (Table II, peak I. D. 133.71 min) and, based on the mass, glycation could have occured at Lys 47 or 67. It is also noteworthy that glycation of βB2-crystallin is less specific, as evident from the fact that a relatively large proportion of the lysines were glycated as compared to α-crystallin under the same condition (9).

In the previous work, we have successfully purified glycated peptides from α- and γ-crystallins using Affi-gel 601 which is a polyacrylamide gel containing boronate as a functional group. But when this method was applied to βL-crystallin, we failed to obtain significant amounts glycated peptides. The reason for this is unknown, but could be due to a relatively slow glycation of various lysine residues of β-crystallin (1 M sugars were essential to obtain significant glycation). Glyc-Affin resin (agarose with m-aminophenyl boronic acid) has a different structure and seems to have a better affinity for glycated peptides. This increased sensitivity was found useful in isolating glycated peptides of βB2-crystallin in sufficient quantities.

So far, all the glycation sites of the major lens crystallins (αA-, αB-, βB2- and γB-crystallin) have been determined through *in vitro* glycation studies (9-12). We hope that these findings will help further investigation on the identification of the *in vivo* glycated site(s).

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