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Determination of Dideoxyosone Precursors of AGEs in Human Lens Proteins

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Abstract

Dideoxyosones (DDOs) are intermediates in the synthesis of advanced glycation end products (AGEs), such as pentosidine and glucosepane. Although the formation of pentosidine and glucosepane in the human lens has been firmly established, the formation of DDOs has not been demonstrated. The aim of this study was to develop a reliable method to detect DDOs in lens proteins. A specific DDO trapping agent, biotinyl-diaminobenzene (3,4-diamino-*N*-(3-([5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyl]aminopropyl) benzamide) (BDAB) was added during in vitro protein glycation or during protein extraction from human lenses. In vitro glycated human lens protein showed strong reaction in monomeric and polymeric crosslinked proteins by western blot and ELISA. Glycation of BSA in the presence of BDAB resulted in covalent binding of BDAB to the protein and inhibited pentosidine formation. Mass spectrometric analysis of lysozyme glycated in the presence of BDAB showed the presence of quinoxalines at lysine residues at positions K1, K33, K96, and K116. The ELISA results indicated that cataractous lens proteins contain significantly higher levels of DDO than non-cataractous lenses (101.9±67.8 AU/mg protein vs. 31.7±19.5 AU/mg protein, $p<0.0001$). This study provides first direct evidence of DDO presence in human tissue proteins and establishes that AGE crosslink synthesis in the human lens occurs via DDO intermediates.

Keywords

Glycation; advanced glycation endproducts; dideoxyosones; human lens proteins; cataract

1. INTRODUCTION

The human lens proteins have negligible turnover rates and therefore accumulate post-translational modifications throughout life [1]. It is thought that these post-translational modifications cause the formation of high molecular weight protein aggregates, which diffract light in aged and cataractous lenses [1–4]. The major molecular mechanisms by which lens proteins are chemically modified include oxidation [5–7], glycation [8–10], carbamylation [11, 12], truncation [13–15], and deamidation [16–18].

Glycation occurs between amino groups on lysine and arginine residues in proteins with carbonyl compounds [19, 20]. The carbonyl compounds in the lens include glucose,

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fructose, methylglyoxal, glyoxal and ascorbate oxidation products [9, 10, 20]. A variety of stable protein adducts are generated by glycation and are collectively known as advanced glycation end products or AGEs. In the human lens, many AGEs have been reported, including pentosidine [9], glucosepane [10], K2P [21], N-carboxymethyl lysine [8], MOLD [22, 23], and argpyrimidine [24]. AGEs accumulate progressively in lens proteins during aging and are generally present at higher levels in cataractous lenses than in non-cataractous lenses. AGE levels are positively correlated with yellow/brown pigmentation, non-tryptophan fluorescence and crosslinked aggregates in aged and cataractous lenses, suggesting that AGEs play a central role in these changes [25, 26].

The glycation of lysine residues proceeds through the formation of an unstable aminoketose (Schiff's base), which rearranges to form a more stable structure known as the Amadori product. The Amadori product further reacts with neighboring lysine or arginine residues in proteins to form protein crosslinking AGEs. Although the role of the Amadori product is clear, the molecular mechanisms by which Amadori product generates AGEs are less clear. Several years ago, a novel pathway for Amadori-mediated AGE formation was discovered, which involved the transformation of the Amadori product into dideoxyosone (DDO) structures [10, 27]. DDO are dicarbonyl compounds produced from the long-range shift of the carbonyl groups (through enolization and dehydration) in the Amadori product [27]. These intermediates exhibit high reactivity towards guanidino groups of arginine residues in proteins, and these reactions produce lysine-arginine protein crosslinking AGEs. Prominent examples of crosslinking AGEs are glucosepane and pentosidine, which are produced from hexose- and pentose-derived DDOs [10, 28].

The observation that glucosepane is the dominant lysine-arginine crosslinking AGE in human skin collagen indicates that DDO are major precursors of AGEs in vivo [29]. DDO-mediated AGE synthesis in tissue proteins becomes even more apparent when the potential of other sugars, such as ribose and fructose, are taken into consideration [28]. These sugars have much higher glycation capacity than glucose and therefore are expected to produce significantly more DDO than glucose. In the human lens, in addition to sugars, ascorbate is a potential source of DDO. The ascorbate concentration is relatively high in the human lens (~2 mM), and it is readily oxidized during cataract formation to form highly reactive sugars, such as erythrulose and threose [30]. These sugars rapidly react with lysine residues on proteins and generate significant quantities of DDO. Taken together, it is conceivable that many carbohydrates in the lens can produce AGEs through DDO intermediates.

Despite the recognition that DDO could be major precursors of AGEs, neither the chemical pathways by which DDO generate AGEs nor their levels in tissues have been elucidated. Thus far, only two AGEs, glucosepane and pentosidine are known to be synthesized through DDO. These two AGEs probably account for a small fraction of AGEs that could be synthesized from DDO, as Lederer's group estimated that glucosepane accounted for only ~8% of the DDO in glycated proteins [10]. Thus, to understand the broader role of DDO in AGE synthesis in vivo, it is necessary to first understand the quantitative importance of DDO for AGE synthesis. We previously provided immunological evidence for DDO in glycated proteins [31]. A specific monoclonal antibody reacted with quinoxaline (a product of the reaction between protein-DDO and diaminobenzene), but an ELISA using this antibody was not sufficiently sensitive to detect DDO in tissue proteins.

This study was conducted 1) to identify DDO-bearing lysine residues in glycated proteins, 2) to develop a reliable and reproducible method for the detection and quantification of DDO in tissue proteins and 3) to understand the importance of DDO-mediated AGE formation in aging lenses and cataract formation.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

D-ribose ($\geq 99\%$ pure), D-glucose ($>99.9\%$ pure), pentafluorophenol ($\geq 99\%$), N-hydroxysuccinimide (98%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride ($\geq 97.0\%$), biotin ($\geq 99\%$), methylglyoxal (40% aqueous solution), trifluoroacetic acid (TFA; protein sequencing grade; $\geq 99.5\%$), bovine serum albumin, lysozyme, formic acid (LC-MS grade) and ammonium bicarbonate were purchased from Sigma Chemical (St. Louis, MO). Sequencing grade trypsin was from Roche (Mannheim, Germany). Dithiothreitol (DTT) was obtained from Roth (Karlsruhe, Germany). *tert*-Butyl-N-(3-aminopropyl)carbamate and di-*tert*-butyl dicarbonate were purchased from AK Scientific (Mountain View, CA). *N* $^{\alpha}$ -t-Boc lysine was obtained from Bachem Americas Inc. (Torrance, CA). 3,4-diaminobenzoic acid (99.9%) and 3,4-dinitrobenzoic acid (99.9%) were from Alfa Aesar (Ward Hill, MA). Acetonitrile (HPLC grade), N,N-dimethylformamide (99.9%, anhydrous), and formic acid (analytical grade, 88%) were from Fisher Scientific (Pittsburg, PA). De-ionized water (18 M Ω or greater) was used throughout this project. All buffers used in this study were treated with Chelex-100 resin (10.0 g/l, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA).

2.2. Synthesis of ribated lysine

Ribated lysine was prepared by incubating *N* $^{\alpha}$ -t-Boc lysine with D-ribose and was purified on Dowex 50X4 in the pyridinium form as previously described [32].

2.3. Synthesis of N-(3-((3,4-diaminobenzyl)amino)propyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (BDAB)

Biotin pentafluorophenyl ester was synthesized following a previously published procedure [33]. 3,4-bis((*tert*-butoxycarbonyl) amino)benzoic acid (**1**) was synthesized as described by Tilley et al. [34], except that 2.2 molar excess of di-*tert*-butyl dicarbonate over 3,4-diaminobenzoic acid was added to the reaction mixture, which was incubated for 16 h at room temperature. After another addition of di-*tert*-butyl dicarbonate (0.5:1 ratio of di-*tert*-butyl dicarbonate: 3,4-diaminobenzoic acid), the mixture was incubated for another 120 h, yielding 90.5% 3,4-bis((*tert*-butoxycarbonyl)amino)benzoic acid. The succinimide ester of 3,4-bis((*tert*-butoxycarbonyl) amino)benzoic acid (**2**) was prepared following the procedure of Uchida et al. [35]. The biotinylated handle, N-(3-aminopropyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide formate salt, (**3**) was obtained using the two-step procedure described by Boudi et al. [36], except that biotin pentafluorophenyl ester was used instead of the biotin/*i*Bu-O-COCl/triethylamine mixture, which yielded 83.5% of the product.

Products (**2**) (1.7 mmol, 0.77 g) and (**3**) (1.9 mmol, 0.65 g) were suspended in 7 ml of a mixture of water:N,N-dimethylformamide (5:2) in the presence of 1.9 mmol of triethylamine and stirred under argon for 16 h at ambient temperature. The mixture was then evaporated to dryness under a vacuum. The target compound, di-*tert*-butyl 4-(((3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl) amino)methyl)-1,2-phenylene)dicarbamate (**4**), was re-crystallized from this preparation with an ether:ethyl acetate mixture to yield 0.93 g (86.2% yield). This product (100 mg, 0.16 mmol) was further de-protected with neat trifluoroacetic acid (1.0 ml at room temperature for 1 hour with stirring) and evaporated to yield 68 mg (85%) of crude N-(3-((3,4-diaminobenzyl)amino)propyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**5**). This preparation was dissolved in water, lyophilized and re-dissolved in a minimal volume of 50 mM sodium phosphate buffer (pH 7.0); the compound was then purified by a semi-preparative RP-HPLC (R_t = 19.5–21.5 min) to yield impurity-free BDAB (25 mg) as a white amorphous powder (99% pure by HPLC analysis). The following

conditions were used for BDAB purification: Prodigy C₁₈ HPLC semi-preparative column (10×250 mm; Phenomenex, Los Angeles, CA); solvent A, 0.5% formic acid in water; solvent B, 0.5% formic acid in acetonitrile, linear gradient of 1%–99% B, 0–35 min; 99% B at a flow rate 2.5 ml/min, 35–45 min; and the effluent absorbance was monitored at 270 nm.

The MS analysis (ESI+) of this compound showed [M+H]⁺ m/z = 435.33 Th, which agreed well with the proposed structure. The ¹H NMR spectrum showed the following characteristics: 7.20 (d, J = 2 Hz, 1H), 7.14 (dd, J₁ = 8 Hz, J₂ = 2 Hz, 1H), 6.68 (d, J = 8 Hz, 1H), 4.46 (ddd, J₁ = 8 Hz, J₂ = 4.8 Hz, J₃ = 1 Hz, 1H), 4.27 (dd, J₁ = 7.6 Hz, J₂ = 4.4 Hz, 1H), 3.37 (t, J = 6.8 Hz, 2H), 3.26 (t, J = 6.8 Hz, 2H), 3.18 (m, 1H), 2.90 (dd, J₁ = 12.8 Hz, J₂ = 5.2 Hz, 1H), 2.68 (d, J = 12.4 Hz, 1H), 2.22 (t, J = 7.2, 2H), and 1.78–1.39 (8H).

2.4. Synthesis of 3,4-dinitro-N-(3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)benzamide (BDNB)

To synthesize BDNB, we first synthesized 3-(3,4-dinitrobenzamido)propan-1-aminium chloride (**6**) as follows: 3,4-dinitrobenzoic acid (1.06 g, 5 mmol) was dissolved in a mixture of anhydrous ethanol and tetrahydrofuran (20 ml, 1:1) and cooled to 0°C; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1.15 g, 6 mmol) was then added. After 30 min of stirring at 0°C, *tert*-butyl (3-aminopropyl)carbamate (0.89 g, 5.1 mmol in 5 ml of ice-cold anhydrous ethanol) was added to the reaction mixture, which was stirred at 0°C for another hour and then at room temperature overnight. After solvent evaporation, the reaction mixture was dissolved in 100 ml of chloroform and washed with a saturated solution of sodium bicarbonate, brine, 5% acetic acid and water. The organic layer was dried over magnesium sulfate and filtered. The combined aqueous extract was re-extracted three times with 50 ml of petroleum ether, dried with magnesium sulfate filtered, combined with the chloroform extract and evaporated to yield the bright yellow target compound (1.5 g, 81.4%). This preparation was (1 g, 2.72 mmol) was suspended in 20 ml of 4 N HCl in dioxane, stirred for 30 min at room temperature, evaporated and used without further purification.

Product (**6**) (0.183 g, 0.6 mmol), biotin pentafluorophenyl ester (0.205 g, 0.5 mmol [33]) and triethylamine (83.6 mg, 0.6 mmol) were suspended in 3 ml of N,N-dimethylformamide and stirred overnight, dried under vacuum and chromatographically separated on silica gel using a chloroform:methanol system to yield 150 mg (52%) of 3,4-dinitro-N-(3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)benzamide (**7**).

The MS analysis (ESI+) of Product (**7**) showed [M+H]⁺ m/z = 471.42 Th, which agreed well with the proposed structure. The ¹H NMR spectrum showed the following characteristics: 8.79 (d, J = 2 Hz, 1H), 8.05 (dd, J₁ = 9.2 Hz, J₂ = 2 Hz, 1H), 7.09 (d, J = 9.2 Hz, 1H), 4.48 (ddd, J₁ = 8 Hz, J₂ = 5.2 Hz, J₃ = 0.8 Hz, 1H), 4.28 (dd, J₁ = 8 Hz, J₂ = 4.8 Hz, 1H), 3.49 (m, 2H), 3.34 (m, 1H), 2.91 (dd, J₁ = 12.8 Hz, J₂ = 4.8 Hz, 1H), 2.68 (d, J = 13.2 Hz, 1H), 2.23 (t, J = 7.2 Hz, 2H), 1.90 (m, 2H), and 1.76–1.42 (8H).

2.5. Reaction of BDAB with methylglyoxal (MGO)

To a 1-ml sample containing 50 μM BDAB in 50 mM Chelex-100-treated phosphate buffer (pH 7.1) with 0.1 mM DTPA, 0.025 ml of 2.0 mM MGO solution was added, and the reaction was followed spectrophotometrically (Beckman DU-800) for 60 min at 25°C and with spectra collection at 10-min intervals. An aliquot (100 μl) of the reaction mixture was injected onto an analytical HPLC column (Prodigy C₁₈ ODS3; 4.6×250 mm; Phenomenex); solvent A was 0.5% formic acid in water, solvent B was 0.5% formic acid in acetonitrile, and the following gradient program was used: 1–70% B, 0–40 min; 70–100% B, 40–43 min; 1.0 ml/min flow rate; and monitoring at λ=320 nm (Jasco Model UV-970; Japan).

To purify the major adduct of the BDAB and MGO reaction, 2.0 ml of the reaction mixture was lyophilized, reconstituted in 500 μ l of water and injected into an HPLC semi-preparative column (Prodigy C₁₈ ODS3; 10 $\times$ 250 mm) under the conditions described for BDAB purification, and the eluate was monitored at λ =320 nm (see above). A major peak was observed at R_t =26.5–28.5 min, which was collected and lyophilized. The material was submitted for structural analysis (see the Results section for details).

2.6. Reaction of glycosylated BSA with BDAB and BDNB

BSA (1 mg/ml) was incubated with 1 mM ribose and either BDAB or BDNB (0 to 50 μ M) in 0.1 M sodium phosphate buffer (pH 7.4), filtered through a 0.2- μ m filter and incubated for 7 days at 37°C. The protein samples were dialyzed against 20 mM HEPES buffer (pH 7.4) containing 1.0 mM DTPA, 100 mM NaCl, and 1% Triton X-100 for 24 h with one buffer change after 16 h. The samples were further treated with SDR HyperD detergent removal chromatography resin (300 μ l of the manufacturer-supplied suspension) (Pall Corp., Port Washington, NY) packed in a centrifugal device (Spin-X centrifuge tube filter; 0.45 μ m Nylon membrane; Costar, Corning Inc., Corning, NY) and incubated for 5 min at room temperature with periodic vortexing. After the incubation, the suspension was centrifuged at 800 g for 5 min at 4°C, and the filtrate was collected. The protein concentration in the filtrate was estimated by the BCA method using BSA as the standard.

The incorporation of BDAB and BDNB into glycosylated BSA was determined by an ELISA. Briefly, 5 μ g of protein in 50 μ l of 50 mM sodium carbonate buffer was dispensed into each well of a 96-well plate and incubated at 4°C overnight. The wells were washed three times with phosphate buffered saline containing 0.1% Tween-20 (PBST), blocked with 5% non-fat dry milk (NFD) in PBST for 1 h at room temperature, washed three times with PBST and incubated for 1 h at 37°C with 50 μ l of an avidin-horseradish peroxidase conjugate (Bio-Rad; 1:25,000 dilution in 5% NFD in PBST). The wells were then washed three times with PBST and incubated with 100 μ l of 3,3',5,5'-tetramethylbenzidine H₂O₂ solution (Sigma, St. Louis). After 30 min, the HRP reaction was stopped by the addition of 25 μ l of 2 N H₂SO₄, and the absorbance of the product was measured at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA).

2.7. Preparation of BDAB-incorporated glycosylated lysozyme

Lysozyme (10 mg/ml) and BDAB (TFA salt; 22 mg, 33.2 μ moles) in the presence or absence of D-glucose (18 mg, 100 μ moles) in phosphate buffer (1.0 ml, 0.1 M, pH 7.1) containing 0.1 mM DTPA was filtered through a 0.22- μ m membrane filter into a sterile tube, which was flushed with argon. Toluene (10 μ l) and chloroform (10 μ l) were added to the reaction mixture with a sterile syringe via a septum. The tops of the tubes were sealed with melted wax, and the mixtures were incubated for 1 month in the dark at 37°C. After the incubation, the protein samples were purified on a semi-preparative column (Vydac C₁₈; 10 $\times$ 250 mm, 218TP510) using a linear gradient system: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; 1% B, 0–5 min; 1–70% B, 5–45 min; 70–100% B, 45–48 min; 100% B, 48–58 min; 2.5 ml/min flow rate; and effluent monitoring at λ =270 nm. Both unmodified lysozyme and glycosylated BADB-treated lysozyme eluted as one symmetrical peak with R_t = 25.3–26.2 min. This product was lyophilized and submitted for MS analysis (see below).

2.8. Mass spectrometric relative quantification of BDAB incorporation into glycosylated lysozyme

One milligram of protein (from lysozyme + BDAB reaction mixture or native protein purified by HPLC) was dissolved in 175 μ l of deionized water. LC-MS/MS analyses were carried out on a Dionex (Idstein, Germany) Ultimate 3000 UPLC system, comprising a

SRD-3400 degasser, an HPG-3400 RS binary pump, a WPS-3000 TRS autosampler and a TCC-3000 RS column compartment. The UPLC was connected to a 4000 QTrap mass spectrometer (ABSciex, Darmstadt, Germany). A 2- μ l aliquot of the protein solutions was injected and separated on a Waters Acquity BEH 300 C₁₈ column (2.1 $\times$ 100 mm, 1.7 μ m) with the following linear gradient: 0–5 min, 5% B; 5–10 min, 5–90% B; solvent A, 0.1% formic acid in water; and solvent B, acetonitrile. Before each injection, the system was equilibrated for 5 min with 5% solvent B. An enhanced mass spectrum scan was performed using the following parameter settings: de-clustering potential of 50 V, collision energy of 10 V, temperature of 550 $^{\circ}$ C, gas 1 at 60 psi, gas 2 at 75 psi, curtain gas at 30 psi, ion spray voltage of 5500 V, scan rate of 1000 Da/sec, and CAD gas set at medium). Because only the 8-fold, 9-fold, and 10-fold charged species were detected in purified preparations of lysozyme incubated with glucose and BDAB, the MS scan range was set to m/z 1200–1900 Da.

The relative amount of BDAD -modified lysozyme was calculated using the following equation:

$$\text{Relative amount [\%]} = \frac{\text{Intensities (BDAB – modified protein signals)}}{\text{Intensity (native protein signal) + Intensities (Glc – and/or BDAD – modified protein signals)}} \times 100$$

2.9. Mass spectrometric localization of BDAB incorporation into glycosylated lysozyme

One milligram of protein (from lysozyme + BDAB reaction mixture or native protein purified by HPLC) was dissolved in 125 μ l of deionized water. A 10- μ l aliquot corresponding to 80 μ g of the protein was mixed with 10 μ l of 50 mM ammonium bicarbonate buffer (pH 8.0). To this solution, 10 μ l of trypsin (0.8 μ g in 1 mM HCl) was added, and the solution was incubated at 37 $^{\circ}$ C for 16 h. Following enzymatic digestion, 5 μ l of 1 M DTT was added, and the reaction was incubated for 30 min at room temperature. Prior to LC-MS analysis, the sample volume was adjusted to 500 μ l with deionized water and filtered through a PVDF (0.2 μ m) membrane filter. Aliquots of the digested samples (20 μ l) were injected and separated on a Waters Acquity BEH 300 C₁₈ column (2.1 $\times$ 100 mm, 1.7 μ m) with the following linear gradient: 0–5 min, 5% B; 5–55 min, 5–50% B; 50–55.5 min, 50–95% B; 55.5–60 min, 95% B; solvent A, 0.1% formic acid in water; and solvent B, acetonitrile. Before each injection, the system was equilibrated for 5 min with 5% solvent B. First, a Q1 scan was used to determine the sequence coverage, which was 98.4% (MS parameters: de-clustering potential of 100 V, collision energy of 5 V, temperature of 500 $^{\circ}$ C, gas 1 at 50 psi, gas 2 at 50 psi, curtain gas at 30 psi, ion spray voltage of 5500 V, scan range of m/z 200–1500 Da, and cycle time of 2 sec).

For the identification of BDAB-modified peptides, an enhanced resolution (ER) scan was performed, which was centered on the following m/z values: 1148.5 and 574.8 (peptide_{mod} AA 1–5); 1534.6 and 767.8 (peptide_{mod} AA 6–14); 909.9 and 606.9 (peptide_{mod} AA 115–125); 1074.1 and 808.8 (peptide_{mod} 22–45); 1166.8 and 933.6 (peptide_{mod} 74–112); 1042.1 and 868.5 (peptide_{mod} 74–112); 1003.1 and 752.6 (peptide_{mod} 74–97); and 1173.5 and 782.7 (peptide_{mod} 97–112). The scans were performed at open resolution of Q1, which allowed for a scan window of 30 m/z for each signal.

The other MS parameters for ER detection were the same as those used for the Q1 scan, except for the scan rate, which was 250 Da/sec. The identified modified peptides were further submitted to MS/MS analyses in the enhanced product ion (EPI) scan mode. Fragment ions of the parent ions at m/z 574.8, 752.5, 606.9, and 805.8 were scanned in the range of m/z 50–2000. The source parameters were the same as those used for the ER scan,

except for the collision energy, which was dependent on the parent ion: 30 V (m/z 574.8), 35 V (m/z 606.9), 40 V (m/z 752.6), and 40 V (m/z 805.5).

2.10. Inhibition of pentosidine synthesis by BDAB in human lens proteins (HLP)

Non-cataractous human lenses were obtained from the National Diseases Research Interchange, Philadelphia, and complied with the tenets of the Declaration of Helsinki. Proteins were extracted from five pooled non-cataractous human lenses (age 20–30 years) by homogenization in 5 ml PBS and centrifugation at 20,000 g for 30 min at 4°C. The supernatant, which contained the water-soluble proteins (HLP), was dialyzed against 2 L of PBS for 24 h. HLP (1 mg/ml) was incubated for 7 days at 37°C in 0.1 M sodium phosphate buffer (pH 7.4) with 1 mM ribose and in the presence or absence of 0–1 mM BDAB. All reaction mixtures were sterile-filtered with 0.2- μ m syringe filters prior to incubation. After incubation, the samples were dialyzed against 2 L of 10 mM phosphate buffer (pH 7.4) for 24 h. Aliquots of the protein samples were hydrolyzed with 6 N HCl for 16 h at 110°C, dried and suspended in water and assayed for pentosidine, as previously described [32]. The free amino acid concentration in the samples was estimated by the ninhydrin assay [37].

Ribated lysine was prepared by incubating N^{α} -t-Boc lysine with D-ribose and was purified on Dowex 50 $\times$ 4 in the pyridinium form as previously described [32].

2.11. Western blot detection of DDO in glycosylated HLP

HLP (1 mg/ml) was incubated with 5 M ribose for 24 h at 37°C in 0.2 M sodium phosphate buffer (pH 7.4) and dialyzed for 48 h against PBS. It has been shown that incubation of proteins with 5 M ribose for this period of time enriches them with Amadori product [38]. Sterile solutions of both native and ribose-modified HLP (1 mg/ml) were incubated in the presence or absence of BDAB (1 mM) for 16 h at 37°C. After exhaustive dialysis against 50 mM phosphate buffer (pH 7.0), the protein concentrations in the samples were measured. Aliquots of these preparations containing equal amounts of protein were loaded onto a 2 ml monomeric avidin column (Pierce monomeric avidin kit; Thermo Scientific, Rockford, IL) and incubated for 1 h at room temperature. The protein was eluted with 50 mM sodium phosphate buffer (pH 7.4, 12 ml) containing 2 mM biotin (12 ml) at 1 ml/min flow rate and monitored by absorbance at $\lambda=280$ nm. The UV-absorbing fractions (both bound and unbound) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.4), concentrated using an Ultrafree-MC filtering device (<10 kD cut off; Millipore Corp., Bedford, MA) and analyzed by western blotting. After SDS-PAGE on a 12% gel, proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk (NFD) for 16 h at 4°C and washed three times with PBS-Tween 20. The membrane was then incubated with a 1:5,000 dilution of avidin-horseradish peroxidase conjugate (Bio-Rad) in 5% NFD. The proteins were visualized using a chemiluminescence detection reagent (Thermo Scientific, Rockford, IL).

2.12. Measurement of DDO in human lens proteins

Cataractous lens nuclei were collected during extracapsular cataract surgery. Each lens (non-cataractous and cataractous lenses, aged between 42–95 years) was cut into three equal parts. One part of the lens was homogenized in 0.1 M phosphate buffer, 0.1 M NaCl, and 1 mM EDTA (pH 7.4) containing 300 μ M BDAB (500 μ l); the second part of the lens was homogenized as above except that BDNB was used in place of BDAB. The third part was homogenized in buffer without either BDAB or BDNB. The lens homogenates were incubated for 3 hrs at 37°C, lyophilized and stored at -80°C until use.

Lyophilized samples were suspended in 0.5 ml of 20 mM HEPES buffer (pH 7.4) containing 1.0 mM DTPA, 100 mM NaCl, and 1% Triton X-100 and sonicated for 6 min at 40%

amplitude (Digital Sonifier Model S-450D; Branson Ultrasonics Corp., Danbury, CT). The resulting suspension was centrifuged at 20,000g for 30 min at 4°C, and the supernatant was collected and treated with SDR HyperD solvent-detergent removal chromatography resin (Pall Corp., Port Washington, NY) as described above. The protein content in the samples was determined using the Bio-Rad BCA Reagent Kit and with BSA as the standard. The samples were then analyzed for DDO (as BDAB adduct) by ELISA. The microplate wells were coated with 5 µg protein/well in 50 mM sodium carbonate buffer (pH 9). Other details of the ELISA procedure are same as described above.

3. RESULTS

3.1. Reaction of BDAB with MGO

In the human lens, several glycation precursors are present. They include glucose and ascorbate oxidation products (threose and erythrulose), and each one could form structurally different DDOs on lysine residues [10, 25]. We designed a trap that was able to react with all DDOs in proteins regardless of which sugar they are derived from. The 3,4-diaminobenzoate moiety within BDAB upon reaction with DDO forms quinoxalines, while the terminal biotin is available as a reporter tag for interaction with avidin (Fig. 1).

To determine if BDAB could efficiently trap DDO, we first reacted BDAB with a dicarbonyl compound, MGO. Our data showed that MGO and BDAB reacted rapidly to form a conjugated product with $\lambda_{\text{max}}=319$ nm (Fig. 2A). HPLC of the reaction mixture showed that nearly 98% of the BDAB was consumed by the reaction with the concomitant formation of a product with $R_t=24.8$ min (Fig. 2B). Purification of this compound by HPLC followed by the mass spectroscopy analysis revealed that it contained a positively charged ion with $m/z=471.4$ (ESI; $[M+H]^+$). The ^1H NMR spectrum of this compound showed that it was mixture of two regioisomers (RI) **1** and **2**: 8.90 (s, 0.6H, RI1), 8.88 (s, 0.3H, RI2), 8.53 (d, $J=1.6$ Hz, 0.5H, RI1), 8.46 (d, $J=1.6$ Hz, 0.25H, RI2), 8.22 (dd, $J_1=8.8$ Hz, $J_2=2$ Hz, 0.6H, RI1), 8.18 (dd, $J_1=8.8$ Hz, $J_2=2$ Hz, 0.3H, RI2), 8.13 (d, $J=8$ Hz, 0.3H, RI2), 8.07 (d, $J=8$ Hz, 0.6H, RI1), 4.47 (ddd, $J_1=8$ Hz, $J_2=4.8$ Hz, $J_3=0.8$ Hz, 1H), 4.40 (dd, $J_1=8$ Hz, $J_2=4.4$ Hz, 1H), 3.49 (t, $J=6.8$ Hz, 2H), 3.20 (m, 1H), 2.91 (dd, $J_1=12.8$ Hz, $J_2=4.8$ Hz, 1H), 2.68 (d, $J=12.8$ Hz, 1H), 2.24 (t, $J=7.2$ Hz, 2H), and 1.89-1.41 (8H). The resonances around 8–8.9 ppm were from the quinoxaline moiety, and the resonances within 4.4–4.47 region were characteristic of the CH-N protons in the biotin structure. The MS and ^1H NMR data together with the UV spectrum of this compound suggested that 3-methyl-N-(3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido) propyl) quinoxaline-6-carboxamide (BDAB-quinoxaline) was the product of the reaction between BDAB and MGO. Mass spectral data agreed with the calculated m/z (471.21) for $\text{C}_{23}\text{H}_{30}\text{N}_6\text{O}_3\text{S}$ (Fig. 2C).

3.2. Reaction of BDAB with rebated N^{α} -Boc-lysine and Amadori-HLP

Similar to MGO, incubation of BDAB with rebated N^{α} -Boc-Lys (ribose-derived Amadori compound) led to *in situ* trapping of DDO (N^6 -(2-hydroxy-4,5-dioxopentyl)-lysine) with the formation of a quinoxaline derivative, as shown from the UV spectra taken over 16 h. At a 10-fold molar excess of BDAB over rebated Boc-Lys, the reaction proceeded at 37°C to form adduct with a $\lambda_{\text{max}}=319$ nm (Fig. 3A), which indicated the formation of quinoxaline. These data agreed with the results from the Amadori-enriched HLP experiment, which also showed the formation of quinoxaline (Fig. 3B). The BDAB-reacted glycosylated HLP was passed through a monoavidin column. Our results showed nearly 7% of the total protein loaded onto the column was retained on the column (Fig. 3C). Amadori-enriched HLP incubated alone or native HLP incubated in the presence of BDAB did not bind to the column, suggesting that the reaction was specific for DDO that was covalently bound to

HLP. Western blotting of the protein eluted from the monoavidin column showed monomeric subunits of DDO and polymeric aggregates of crystallins (Fig. 3D).

3.3. Reactions of BDAB and BDNB with glycated BSA

Next, we determined the ability of various concentrations of BDAB and BDNB to trap DDO on glycated BSA. BSA (1 mg/ml) and ribose (1 mM) were incubated with BDAB or BDNB (0, 5, 10 and 50 μ M) for 7 days at 37°C, and the samples were analyzed by ELISA. The DDO trapping efficiency of BDAB increased with concentration; the DDO level detected with 50 μ M BDAB was approximately 6-fold higher when compared to the DDO level with 5 μ M BDAB. At these concentrations, BDNB showed no reaction with glycated BSA. To determine the non-specific binding of BDAB, we incubated BSA and BDAB in the absence of ribose. Although SDR-Hyper-D resin treatment removed most of the BDAB non-specifically bound to BSA, a small fraction of BDAB was still bound. Non-specific binding increased with the BADB concentration; however, it was 20- and 4-fold lower when compared to BDAB binding to glycated BSA at 10 and 50 μ M BDAB, respectively. Western blotting of the incubated samples showed strong positive signals with BSA glycated in the presence of BDAB (Fig. 4B), and the signal strength increased as the BDAB concentration increased. The non-specific binding of BDAB to non-glycated BSA was apparent only with the highest concentration of BDAB. Taken together, these results indicate that most of the binding of BDAB to glycated BSA occurs through specific reaction with DDO; however, a small fraction of the reaction resulted from non-specific interaction.

3.4. Inhibition of pentosidine formation by BDAB

If BDAB could trap DDO intermediates, it should also inhibit AGE formation during glycation. This hypothesis was tested using HLP and ribose as the glyating agent, and we measured the formation of pentosidine as a representative marker for AGEs. As expected, glycation of HLP by ribose led to the formation of pentosidine (1.7 pmole/ μ mole amino acid after 7 days of incubation) (Fig. 5). The presence of BDAB in the reaction mixture decreased the levels of pentosidine in a concentration-dependent manner. At 50 μ M BDAB, the pentosidine level was 0.46 pmole/ μ mole amino acid, which was 73% lower than in its absence. At 1 mM BDAB, pentosidine synthesis was completely blocked.

3.5. Mass spectrometric identification of DDO in glycated lysozyme

To further confirm the formation of quinoxaline from the reaction of DDO in glycated proteins with BDAB, lysozyme was incubated in the presence of glucose and BDAB in 0.1 M Chelex-100-treated phosphate buffer (pH 7.1) and analyzed by LC-MS. Table 1 gives an overview of detected species and their intensities in the spectrum. We detected both Amadori and quinoxaline adducts in the modified protein. The relative amount of BDAB-modified (quinoxaline) lysozyme was calculated by dividing the sum of the intensities of BDAB-modified protein signals by the sum of the intensities of native, glucose- and BDAB-modified protein signals. Such an approach for relative quantification has been proposed by Kislinger et al. [39] and has been successfully applied for the relative quantification of protein modifications on peptide [39] or proteins level [40]. Basic principle of this data treatment is the assumption that limited glycation does not influence the electro spray ionization properties of a protein, which means that the ESI-MS response of native and modified species is identical and thus reflects their concentration in the sample [41]. Incubating lysozyme in the presence of 100 mM glucose and BDAB in 0.1 M Chelex-100-treated phosphate buffer (pH 7.1) at 37°C for 30 days showed the presence of BDAB-modified lysozyme (3.5%, see Table 1). The need to consider all charge states of the protein for quantitative evaluation of ESI-MS data has been already reported by Brock et al. [42] and is confirmed by our results.

While MS analysis of intact proteins allows for a rapid evaluation of the distribution of the BDAB modification in the protein, MS analysis on peptide level yields monoisotopically resolved signals and therefore more accurate masses, and thus the identification of the BDAB modification by the mass shift is more reliable. Furthermore, by fragmenting the peptides in the triple quadrupole, sensitive localization of the modification in the amino acid sequence is possible. To this end, lysozyme, native or incubated in the presence of glucose and BDAB, were partially digested with trypsin and subsequently analyzed by LC-MS/MS (Fig. 6). The lysozyme sequence coverage was 98.4%. In the first step, an enhanced resolution scan was performed, which provided sensitive detection of the modifications combined with increased resolution. In this manner, we were able to detect a mass shift of 542.2 Da in peptides AA 1–5, 22–45, 74–97, and 115–125, indicating the formation of the BDAB adduct (quinoxaline) at lysines 1, 33, 96, and 116, respectively (see Table 2). In the control (lysozyme incubated with BDAB in the absence of glucose), these signals were not detected. At lysine residues 13 and 97, the BDAB adduct (if present) was below the detection limit of the applied MS method (data not shown).

The detected modified peptides were submitted to fragmentation in an enhanced product ion scan. The appearance of characteristic y- and/or b-ions facilitated the assignment of the detected signals to the corresponding amino acid sequences of the parent lysozyme peptides. Figure 7 gives an example of the MS/MS spectrum of the peptide at m/z 606.9. This signal bears a charge of +3, as confirmed by enhanced resolution scanning, and corresponded to the peptide with a monoisotopic mass of 1817.8 Da (AA 115–125, modified with dideoxyosone trapped by BDAB; see Table 1). In addition to y-ions 1–9, which confirmed the expected amino acid sequences, signals that were characteristic for BDAB adducts (data not shown) could be detected. These results clearly demonstrate the incorporation of the BDAB moiety into glycosylated lysozyme and the formation of respective quinoxaline adducts.

3.6. Detection of DDO in human lens proteins

Having established by LC-MS that BDAB reacts with DDO and forms quinoxaline in a glycosylated protein and an ELISA for detecting DDO in glycosylated proteins, we investigated the formation and the effect of cataract on DDO in human lens proteins. We used brunescant cataracts (highly pigmented at the center of the lens) and non-cataractous transparent lenses from donors between the ages of 45 and 96 years. Our data on glycosylated BSA showed that non-specific binding of BDAB disproportionally increased with increasing BDAB concentrations from 10 to 50 μ M (Fig. 4) and with a BSA concentration of 1 mg/ml. Extrapolating results from 10 μ M BDAB (and 1 mg/ml of BSA), we used 300 μ M BDAB during lens homogenization, which was based on the assumption that the average protein concentration in the adult human lens is about 100 mg. Because we homogenized a third of the lens (~30 mg protein), we used 300 μ M BDAB in the buffer. The DDO levels were significantly higher in cataractous lenses (approximately three-fold) than in non-cataractous lenses (Fig. 8A). The levels in non-cataractous lenses were 14–62 AU/mg of protein with an average of 31.7 AU/mg of protein (n=10). In cataractous lenses, the range was 17–245 AU/mg of protein with an average of 101.9 AU/mg of protein (n=44), which was statistically highly significant (p<0.0001, Student's unpaired t-test). Interestingly, in cataractous lenses, an age-associated increase in DDO was found (Fig. 8B); the regression analysis of the data showed an increase of 1.43 AU/mg of protein/year ($r^2=0.039$).

To determine if the DDO levels correlated with AGEs, we measured pentosidine formation in the same lens proteins. The pentosidine level in cataractous lenses was 1.81 ± 0.39 pmoles/ μ mole amino acid (n=37), which was approximately five-fold higher than in non-cataractous lenses (0.35 ± 0.08 pmoles/ μ mole amino acid; n=9) (Fig. 8C).

DISCUSSION

The purpose of this study was to develop a robust method for the detection and quantification of DDO intermediates of glycation in human proteins. Lens proteins are among the longest-lived proteins in humans, and they accumulate many AGEs throughout life [43]. In fact, almost all AGEs described in human tissues have been found in human lenses. Thus, the lens is an ideal system to study the mechanisms of AGE formation *in vivo*.

There are two general types of reactions in glycation. The first type encompasses the reaction of carbonyl compounds with lysine residues, and second type is the reaction of carbonyl compounds with arginine residues in proteins. AGEs that crosslink amino acids are generally derived through the glycation of lysine residues, which, through the formation of an Amadori product, form lysine-lysine and lysine-arginine crosslinked structures. However, the mechanisms of the conversion of the Amadori product into AGEs are poorly understood. Spontaneous long-range carbonyl shift in an Amadori compound was recently described [27]. This transformation occurs non-enzymatically and produces terminal dioxo products. For example, if the glycation sugar is a pentose, such as ribose, a 4,5-dioxo product is generated on glycated lysine (Fig. 1). The dicarbonyl structure thus formed could react with neighboring arginine and lysine residues in proteins to produce lysine-arginine and lysine-lysine crosslinked structures. Because these dicarbonyl structures are present within proteins, they cannot be metabolized by dicarbonyl metabolizing enzymes, such as glyoxalase and aldose reductase [44, 45]. Thus, once formed, DDO are destined to eventually form crosslinking AGEs in proteins.

The half-life of DDO in proteins is not known but is probably influenced by the microenvironment in which it resides, the sugar from which it is derived, the local pH and proximal amino acids. Thus, DDO in proteins is not likely to be a stable intermediate. Indeed, it must be chemically trapped for detection and quantification. Aminoguanidine has been used for this purpose, but it was found to be sluggish when compared to diaminobenzene [46]. Because diaminobenzene was a better trapping agent, we modified it by adding a reporter biotin molecule, which could be detected and quantified by avidin-conjugated horseradish peroxidase. The fact that BDAB, but not BDNB, could form quinoxalines with methylglyoxal, ribated lysine and ribated HLP underscored the specificity of the reaction of BDAB with DDO. Furthermore, mass spectrometric data for glycated lysozyme clearly showed quinoxalines in lysine residues (Table 1). The detection of quinoxaline in four out of six lysine residues (K1, K33, K96, and K116) indicated that DDO is a major intermediate in AGE formation. One example for this was the total inhibition of pentosidine synthesis in glycated BSA.

In the human lens, DDO can be generated through glycation of several sugars. Our DDO trap is not able to discriminate between these DDOs; therefore, our ELISA did not allow us determine the absolute amounts of DDO in lens proteins. However, our data clearly showed that there is an age-associated increase in DDO in cataractous lenses, and the levels in cataractous lenses are significantly higher than non-cataractous lenses, which suggest that AGE formation through DDO is a major mechanism of cataract formation.

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Abbreviations

AGEs	advanced glycation endproducts
DDOs	dideoxyosones
BDAB	3,4-diamino- <i>N</i> -(3-{{5-(2-oxohexahydro-1 <i>H</i> -thieno[3,4- <i>d</i>]imidazol-4-yl)pentanoyl}aminopropyl)benzamide
BDNB	<i>N</i> -(3-(3,4-dinitrobenzylamino)propyl)-5-(2-oxo-hexahydro-1 <i>H</i> -thieno[3,4- <i>d</i>]imidazol-4-yl)pentanamide
HLP	human lens proteins
MGO	methylglyoxal
TFA	trifluoroacetic acid
DTT	dithiothreitol

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Fig. 1. Trapping of DDO by BDAB and BDNB

The insert shows the structures of the BDAB and BDNB chemical traps.

Fig. 2. Spectrophotometric and RP-HPLC analysis of the reaction between methylglyoxal and BDAB

MGO (50 μ M) and BDAB (50 μ M) were reacted for 1 h, and spectra were taken at 10-min intervals for 60 min (A). The reaction mixture at 0 and 30 min was injected into an HPLC. The peak at $R_t = 26.5$ – 28.58 min was collected, lyophilized and analyzed by mass spectrometry and ^1H NMR (B). The structure of the product (quinoxaline derived from the reaction between methylglyoxal and BDAB) is shown in panel C.

Fig. 3. Reaction of BDAB with ribated Lys and Amadori-HLP

UV-spectra of the reaction mixture of ribated N^{α} -Boc-Lys (10 μ M) and BDAB (100 μ M) in 100 mM sodium phosphate buffer (pH 7) taken over a period of 16 h at 37°C in 1-hour intervals (A). UV-spectra of HLP (1 mg/ml) incubated with 1 mM ribose (Amadori-HLP) and 2 mM BDAB for 16 h at 37°C. The control was Amadori-HLP in the absence of BDAB (B). Affinity separation of Amadori-HLP and Amadori-HLP (1 mg/ml) incubated with 2 mM BDAB on a monoavidin column (C). The column-bound protein was eluted with 2 mM biotin, dialyzed and subjected to western blotting (10 μ g/lane) using avidin-HRP (D). The left panel shows the Coomassie blue-stained gel and the right panel of the western blot.

Fig. 4. Detection of DDO in glycated BSA

ELISA and western blot of the reaction mixtures of BDAB and BDNB (both at 0–50 μ M) with BSA (1 mg/ml), which had been incubated with ribose (1 mM) in PBS at 37°C for 7 days under sterile conditions. The incubated samples were dialyzed, treated with SDR-Hyper-D resin to remove non-specifically bound BDAB and analyzed by ELISA (top) and western blotting (bottom).

Fig. 5. BDAB inhibits pentosidine synthesis in glyated HLP

HLP (1.0 mg/ml) was incubated with ribose (1.0 mM) and 0–1 mM BDAB for 7 days at 37°C. After the incubation, samples were dialyzed and treated with SDR-Hyper-D resin, hydrolyzed using 6 N HCl at 110°C and analyzed for pentosidine by HPLC. The data shown are the average of two independent experiments.

Fig. 6. Peptide mapping of lysozyme

Q1 scan of partially digested lysozyme incubated with 100 mM glucose, 33.2 mM BDAB, and 0.1 mM DTPA in 100 mM Chelex-100 treated phosphate buffer (pH 7.1). Signals corresponding to lysozyme tryptic peptides are indicated.

Enhanced product ion scan of the signal at m/z 606.93 ($z=3$), detected in partially digested lysozyme that was heated with 100 mM glucose, 33.2 mM BDAB, and 0.1 mM DTPA in 100 mM Chelex-treated phosphate buffer (pH 7.1). Characteristic y -ions of the peptide backbone are displayed in the spectrum. Signals that correspond to the quinoxaline-BDAB adduct are marked with an asterisk. The sequence of the corresponding peptide (AA 115–125, monoisotopic mass: 1817.8 Da) and the theoretical fragmentation pattern are shown.

Fig. 8. The DDO levels are higher in cataractous lenses than in non-cataractous lenses
 The DDO levels in human lenses were measured with an ELISA. Microplate wells were coated with human lens proteins extracted in the presence of BDAB, blocked with NFDM and incubated with avidin-HRP and then with an HRP substrate. The results are expressed as absorbance units (AU) per 5 μ g of protein. The relationship between age and DDO levels in human non-cataractous (red squares) and cataractous (black circles) lenses is shown in (A), and $r^2=0.069$ and $p<0.0001$ for cataractous lenses. The DDO levels in normal and cataractous lenses are shown in (B). The pentosidine levels in the same lenses are shown in (C). The sample numbers are shown in parenthesis above the bars. * $p<0.0001$.

Table 1

Relative quantification of BDAB-modified lysozyme.

Species	Charged form			
	8+	9+	10+	Sum
Lysozyme	65.6	353.7	131.6	550.9
Lysozyme + 1 Amadori	53.0	327.9	496.1	877.1
Lysozyme + 2 Amadori	24.0	140.8	253.4	418.1
Lysozyme + 3 Amadori	nd	24.6	47.7	72.3
Lysozyme + quinoxaline (BDAB adduct)	nd	15.8	23.8	39.7
Lysozyme + Amadori + quinoxaline	nd	9.6	20.1	29.7
Relative amount of quinoxaline-lysozyme [%]	-	2.9	4.5	3.5

Lysozyme was incubated with 100 mM glucose, 31.2 mM BDAB, and 0.1 mM DTPA in 100 mM chelex-treated phosphate buffer pH 7.0. Relative quantification was performed on protein level relating intensities of ESI-MS signals of BDAB-modified species to the sum of intensities of all lysozyme species (for details see text). Intensities are displayed as 1×10^3 cps. nd: not detected.

Table 2

Identification of quinoxaline (the product of the reaction between DDO and BDAB) in glycosylated lysozyme by LC-mass spectrometry

Nr	Rt	AA	Sequence	Calculated mass [Da]	Calculated m/z (z)	Observed m/z
1	4.1	1-5	KVFGK	605.4	606.4 (1)	606.3
1*	19.2	1-5 *	K*VFGR	1147.6	1148.6 (1)	1148.5
2	5.8	2-5	VFGR	477.3	478.3 (1)	478.2
3	15.8	6-13	CELAAMK	835.4	836.4 (1)	836.3
4	6.4	15-21	HGLDNYR	873.4	437.7 (2)	437.8
5	26.5	22-33	GYSLGWVCAAK	1267.6	634.8 (2)	634.8
6*	31.9	22-45 *	GYSLGWVCAAK*FESNFTQATNR	3219.5	805.9 (4)	805.9
7	15.5	34-45	FESNFTQATNR	1427.6	714.8 (2)	714.8
8	23.3	46-61	NTDSTDYGIQNSR	1752.8	877.4 (2)	877.4
9	19.3	62-68	WWCNDGR	935.4	468.7 (2)	468.7
10	1.4	69-73	TPGSR	516.3	517.3 (1)	517.3
11	34.0	74-96	NLCNIPCSALLSSDITASVNC AK	2336.1	779.7 (3)	779.8
12*	33.6	74-97 *	NLCNIPCSALLSSDITASVNC AK*K	3006.4	752.6 (4)	752.6
13	28.4	98-112	IVSDGNGMNAWVAWR	1674.8	838.4 (2)	838.3
14	1.1	113-114	NR	288.2	289.2 (1)	289.2
15	21.1	115-125	CKGTDVQAWIR	1275.6	638.8 (2)	638.8
15*	24.8	115-125 *	CK*GTDVQAWIR	1817.9	606.9 (3)	607.0
16	22.1	117-125	GTDVQAWIR	1044.5	523.3 (2)	523.4
17	1.3	126-128	GCR	334.1	335.1 (1)	335.2
18	5.7	126-129	GCRL	447.2	448.2 (1)	448.2

Peptides and lysine residues modified with quinoxaline-BDAD are marked with an asterisk. Nr: Number (referring to numbering in the chromatogram in Figure 6). Rt: retention time (referring to Figure 6). AA: Amino acid residues.