Early Glycation Products Produce Pentosidine Cross-links on Native Proteins

NOVEL MECHANISM OF PENTOSIDINE FORMATION AND PROPAGATION OF GLYCATION*

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Bovine lens α-crystallin was immobilized on EAH-Sepharose gel and glycated using α-ribose. Incubation with 500 and 100 mM α-ribose for 2 and 15 days produced short-term glycated (STGP gel) and long-term glycated proteins (LTGP gel). Both STGP and LTGP gels produced oxygen free radicals. Hydroxyl radical production was twice that in STGP gel compared with the LTGP gel. Incubation with the glycated gels produced pentosidine in a mixture of N-α-acetylarginine + N-α-acetyllysine, bovine lens proteins (BLP), and lysozyme; the amounts measured with STGP gel were higher than those with LTGP gel. Reactive oxygen species scavengers decreased the formation of pentosidine. Pentosidine was also formed in BLP when incubated with water-insoluble proteins extracted from aged or brunescent human lenses. Early glycated proteins from aged or diabetic lenses were bound to a boronate affinity column, the protein-containing gel was incubated with BLP, and pentosidine was measured in the incubation mixtures. With this method we found that diabetic lens proteins produced more pentosidine on BLP than did aged lens proteins. Further investigation indicates that two and three carbon carbohydrates possibly formed from oxidative cleavage of early glycation products are involved in pentosidine formation. Based on our findings, we propose a novel pathway for pentosidine formation on native proteins from glycated proteins.

The Maillard reaction involves reaction of amino groups on proteins with aldehydes and ketones to produce advanced glycation end-products, or AGEs. The reaction products include amino acid cross-links, fluorophores, and chromophores on proteins (1, 2). AGEs can bind to specific receptors on cells to cause intracellular oxidative stress as well as the synthesis of growth factors and cytokines (3–6), and the end result is usually damage to the affected tissues (7, 8).

Many tissues have increased AGE content with aging, but increased AGEs are also associated with specific pathologies such as formation of cataracts (9), Alzheimer’s disease (10), amyloidosis (11), atherosclerosis (12), and diabetic retinopathy (13, 14). A direct role for AGEs in diabetes was proposed when it was found that AGE infusion into normal rats produced diabetic changes (15). In addition, pharmacological intervention to block the effects of AGEs appears to prevent certain complications of diabetes (16, 17).

Cataract formation is a multifactorial process. One mechanism by which glycation damages proteins is through production of reactive oxygen species (ROS), and various studies link the production of glycation products and the formation of ROS (18–22). Ample evidence indicates that ROS contribute to lens protein damage (23–25), but it is not clear how ROS could be produced within the lens. Recent studies suggest that lens pigments, in the presence (26) or absence of ultraviolet light (27), could generate ROS.

The Amadori product, the initial reaction product in the Maillard reaction, remains almost constant within normal aging lenses, but it occurs at much higher levels in diabetic and senile cataractous lenses (28, 29). Numerous studies document the formation of AGEs in lens proteins during aging and cataractogenesis (9, 30–32). In fact, Ortwerth et al. (33) found that ascorbate-derived AGEs can produce ROS, and human lenses contain relatively large amounts of ascorbate. Oxidation of ascorbate produces highly reactive compounds, including dicarbonyls that can form AGEs (34, 35).

We reasoned that, if glycation products are a source of ROS in the lens, continued formation of ROS could account for cumulative damage to lens crystallins during aging and cataractogenesis. However, we made the surprising discovery that glycated proteins can produce pentosidine, a protein crosslinking AGE on native unmodified lens proteins. Accordingly, we investigated the formation of pentosidine from early and advanced glycation products on α-crystallin, and we propose a pathway for pentosidine synthesis from short-chain carbohydrates derived from degradation of early glycation products.

EXPERIMENTAL PROCEDURES

Materials—Bovine lenses were obtained from Pel-Freez Biologicals, Roger, AR. EAH-Sepharose was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. Lysozyme, cytochrome c, Chelex-100, deferoxamine, α-ketoglutarate, luminol, thiourea, N-α-acetyllysine, and N-α-acetylarginine were obtained from Sigma Chemical Co. EDC and salicylate were purchased from Aldrich. Fuorsine standard was from NeoSystem, France. Normal and diabetic human lenses were obtained from the National Disease Research Interchange and the Cleveland Eye

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**SCHEME 1. Preparation of STGP and LTGP gels.** α-Crystallin was immobilized on EAH-Sepharose in the presence of EDC. It was then glycated with 500 mM D-ribose for 2 days in the case of STGP gel and with 100 mM for 15 days in the case of LTGP gel. The ST and LT glycated gels were incubated with BLP or lysozyme or amino acids. The supernatant was used for analysis.

**TABLE I**

| Experiment | Pentosidine Increase/Decrease | Control | STGP | LTGP |
|------------|-------------------------------|---------|------|------|
| Control    |                               | 0.0     | 1.2  | 20.6 |
| STGP       |                               | 31.8    | 11.2 | 64   |
| LTGP       |                               | 20.6    | 0.34 | 98   |

Effect of ± oxygen in the formation of pentosidine from STGP and LTGP gels

BLP (2 mg/ml in 50 mM phosphate buffer, pH 7.4) was incubated with 100 mg of STGP and LTGP gel at 37 °C in the presence and absence of oxygen for 3 days. Pentosidine in BLP was estimated by HPLC.

**Table**

**Formation of Hydrosol Radical from Glycated α-Crystallin—Hydrosol radical production by glycated gels (from α-crystallin) was measured by the salicylate hydroxylation method. Typically, 100 mg of STGP- or LTGP gel was incubated with salicylate. The amount of 2,3-dihydroxy benzoate formed was assayed by a spectrometric method (38) described by Bhuyan and Bhuyan (37).

Estimation of Pentosidine in Glycated Protein—STGP and LTGP gels (100 mg each) (from α-crystallin) were acid hydrolyzed in 6 N HCl for 16 h at 110 °C. The contents were dried in a Speed-Vac concentrator (Savant Instruments, Inc. Farmingdale, NY), rehydrated in 200 μl of water, and filtered through a 0.2-μm filter. Pentosidine was measured by the HPLC method as described previously (9) and quantified by comparison with a standard curve for synthetic pentosidine.

**Incubation of BLP or Lysozyme with STGP and LTGP Gels—** The STGP and LTGP gels (from α-crystallin or lysozyme) were washed 10 times with 50 mM sodium phosphate buffer (pH 7.4) before incubation. BLP or lysozyme (2 mg/ml) was incubated with the STGP- or LTGP gels (100 mg) in 50 mM phosphate buffer (pH 7.4) at 37 °C with gentle mixing for up to 7 days. The final volume of the reaction mixture was 1.0 ml. BLP incubated with unglycated α-crystallin (EAH-Sepharose bound) served as control. Aliquots (0.2 ml) were drawn at regular time intervals. In some experiments with α-crystallin immobilized gels, we added ROS quenchers (1.0 mM luminol, 5 mM α-ketoglutarate, 5 mM mannnitol), metal ion chelator (1 mM deferoxamine), or FeCl3 (100 μM) at the start of the reaction. Pentosidine was measured by HPLC.

**Incubation of N-α-Acetyllysyl + N-α-Acetylarginine with STGP and LTGP gels—** N-α-Acetyllysylamine (5 mM) and N-α-acetylarginine (5 mM) were incubated at 37 °C in 50 mM phosphate buffer (pH 7.4) as described above for BLP. Aliquots of the supernatant were taken after 3 and 7 days for pentosidine measurement.

**Pentosidine Formation under Anaerobic Conditions—** In some experiments, air-tight screw cap tubes with an inlet for argon were used to incubate 100-mg STGP- or LTGP gel with BLP under anaerobic conditions. Otherwise, incubation conditions were as indicated earlier. Argon was bubbled through the mixture for 20 min and the argon-air mixture was removed with a vacuum pump. The tubes were then tightly sealed and incubated at 37 °C for 3 days. Controls included nonglycated protein gels and glycated gels incubated without proteins. In addition, samples were also incubated simultaneously under aerobic conditions for comparison.

**Formation of Pentosidine from Water-insoluble Human Lens Proteins—** Water-insoluble proteins (WI) from non-diabetic aged (age: 67 years) or brunescent lenses (age: 76 years), were prepared as described by Shamsi et al. (39). 5 mg of WI was washed thoroughly five times with 1 ml of 50 mM sodium phosphate buffer (pH 7.4). After the final wash, 2.0 mg of BLP was added, and the sample was incubated at 37 °C for 7 days. Aliquots (0.2 ml) were taken at 3 and 7 days to measure pentosidine. Furosine content in the WI before and after incubation was measured by HPLC (Alltech, Deerfield, IL) using a furosine-dedicated column (40). For some incubations, the WI protein extract was reduced with sodium borohydride. Incubations of WI with sodium phosphate buffer alone served as a control.

**Binding of Early Glycation Products from Human Lenses to Affinity Matrix—** Water-soluble lens protein (WS) was isolated (34) from age- and weight-matched normal and diabetic (nuclear cataractous lenses from patients with type II diabetes, diabetes duration: > 10 years) human lenses. Two lenses (200–240 mg) from donors of 60–70 years of age were used for each sample. The samples were dialyzed against 50 mM sodium phosphate buffer (pH 7.8), and loaded onto a previously equilibrated (50 mM phosphate buffer) Affi-Gel-601 boronate column (Bio-Rad Laboratories, CA). The amount of protein bound to the column was calculated by subtracting the amount of unbound proteins.
from the amount loaded on the column. After binding of WS samples, the gel was thoroughly washed with buffer and then used in incubations with 2.0 mg of BLP in sodium phosphate buffer at 37 °C for up to 7 days. Some experiments used N-acetylarginine (5 mM) + N-acetylysyl (5 mM) in the place of BLP. In other experiments, the gel was reduced with sodium borohydride before incubation. Aliquots from the supernatant were assayed for pentosidine.

**Formation of Pentosidine from Short-chain Carbohydrates**—N-acetylarginine and N-acetylysyl were incubated with two and three carbon carbohydrates. N-acetylarginine and N-acetylysyl (5 mM each) were incubated in 50 mM phosphate buffer (pH 7.4) with 5 mM carbohydrate at 37 °C. Aliquots were drawn at 24 and 48 h, and pentosidine was measured after acid hydrolysis (6 N HCl, 110 °C, 16 h).

**Other Procedures**—SDS-PAGE used 15% gels under reducing conditions according to the method of Laemmli (41). Amino acid content of acid-hydrolyzed proteins was measured by the ninhydrin reaction (34), and protein was measured by the Bradford method using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

Sodium borohydride (SBH) reductions involved incubating 5 mg of protein with 5 mM SBH for 2 h at room temperature at pH 9.0 with slow stirring. Unreacted SBH was removed by washing the protein (WI).

**Statistics**—Mean differences were evaluated by one-way analysis of variance using StatView Software (SAS Institute, Inc., Cary, NC). Fisher’s protected least significant difference test was employed in these calculations. We considered a p value of < 0.05 statistically significant. In all figures the means that do not share a common superscript are statistically different at p < 0.05.

**RESULTS**

We found no pentosidine in control incubations where EAH-Sepharose immobilized proteins with or without glycation were incubated with buffer alone. These controls rule out the possibility of pentosidine leaching from the gel or the formation of pentosidine on the gel itself.

**Pentosidine Content of Immobilized Glycated α-Crystallin**—Pentosidine was measured by HPLC and expressed as nanomoles per gram of gel. LTGP had nearly twice the amount of pentosidine (4.8 ± 0.3 nmol/g of gel) as compared with STGP (2.5 ± 0.1 nmol/g of gel). We were unable to quantify the Amadori product of the ribose reaction with protein amino groups, because there are no specific assays for it. Measurements of pentosidine indicate that LTGP accumulated more AGEs than did STGP. However, as suggested by the studies of Booth et al. (42), we expected STGP to accumulate more Amadori product under the incubation conditions of our experiments.

**Superoxide Anion and Hydroxyl Radicals from Immobilized Glycated α-Crystallin**—We used superoxide dismutase-inhibitable reduction of cytochrome c as a measure of superoxide anion. Fig. 1A shows that superoxide anion was minimal with unmodified α-crystallin (coupled to gel) but was significant in glycated α-crystallin (coupled to gel). SOD inhibited superoxide anion production from both STGP and LTGP gels. Based on the data, we calculated that the amount produced by STGP gel and
LTGP gel was 9.5 ± 2.1 nmol/h/g of gel and 7.8 ± 2.0 nmol/h/g of gel, respectively. Hydroxyl radical production was twice that in STGP gel (6.2 ± 2.3 μmol/h/g of gel) compared with the LTGP gel (3.0 ± 0.9 μmol/h/g of gel) (Fig. 1B).

Pentosidine Formation in BLP Incubated with Glycated α-Crystallin—The STGP and LTGP gels prepared from incubation of EAH-Sepharose-α-crystallin with ribose were washed thoroughly before every incubation to remove loosely bound glycated proteins and any breakdown products formed during storage. The gels were then incubated with BLP. Pentosidine was measured after acid hydrolysis of an aliquot of the incubation mixture. HPLC chromatograms showed pentosidine as a well-resolved peak from samples in which BLP incubated with STGP and LTGP gels (Fig. 2A). Fig. 2B shows that the pentosidine content in both incubations nearly doubled at 7 days compared with the amount at 3 days. The STGP gel produced two to three times as much pentosidine as did the LTGP gel (p < 0.05). These results indicate that pentosidine is produced from carbohydrates derived from glycated proteins, and the early glycation products (Amadori and Schiff’s base) are the precursors.

Effect of ROS Quenchers and a Metal Ion Chelator on Pentosidine Formation—STGP and LTGP gels were incubated with BLP for 7 days in the presence or absence of indicated ROS quenchers or deferoxamine or FeCl3. As can be seen in Fig. 3, addition of α-ketoglutarate (a scavenger of H2O2) most effectively inhibited pentosidine formation in BLP incubated with STGP gels; pentosidine was approximately half that in samples incubated without the ROS quencher. Luminol, an O2· scavenger, only marginally inhibited pentosidine formation, and thiourea, an OH· scavenger, inhibited it by ~30%. The effects were not as great in incubations with LTGP gels, but α-ketoglutarate and thiourea caused significant (p < 0.05) inhibition. The metal ion chelator, deferoxamine, was ineffective. Addition of 100 μM FeCl3 enhanced pentosidine formation in both STGP and LTGP gels. The increase was 17% with STGP and 73% with LTGP gel. These results suggest that H2O2 is an important constituent in pentosidine synthesis from glycated proteins.

Effect of Lack of Oxygen—To assess the importance of oxygen in the formation of pentosidine, we did some incubations in the absence of air, and the results were similar to those in Fig. 3B. These results indicate that oxygen is not a critical factor in pentosidine formation.

Table II: Formation of pentosidine from short-chain carbohydrates

| Short-chain carbohydrates | Pentosidine 1 day | Pentosidine 2 days |
|---------------------------|------------------|-------------------|
| Glyceraldehyde           | 26.86            | 28.08             |
| Oxalic acid              | 34.50            | 35.86             |
| Glyoxalic acid           | 0.03             | 0.12              |
| Methylglyoxal            | 2.65             | 2.81              |
| Hydroxy acetone          | 3.05             | 3.26              |
| Lactic acid              | 3.25             | 3.48              |
| Glycerol                 | 3.45             | 3.68              |

![Fig. 3. Effect of ROS quenchers on the formation of pentosidine.](image)

![Fig. 4. A, covalent cross-linking of BLP on incubation with STGP and LTGP gels.](image)
absence of oxygen. Table I shows that pentosidine was decreased –64 and 98% in STGP and LTGP gels in the absence of oxygen, which indicates that oxidative reactions mediated by ROS are necessary for pentosidine formation.

**Covalent Cross-linking of BLP Incubated with STGP and LTGP Gels**—SDS-PAGE revealed that BLP underwent covalent cross-linking upon incubation with glycated gels. We found cross-linked proteins of molecular mass 40–45 kDa in both STGP and LTGP gel incubated proteins (Fig. 4A). STGP gels incubated with BLP produced higher levels of these proteins than comparable incubations with LTGP gels, suggesting that covalent cross-linking possibly occurred as a result of pentosidine and other AGEs in BLP. Results of densitometric analysis showed that, after 7 days of incubation, STGP gel produced nearly three times as much cross-linked proteins as did the LTGP gel (Fig. 4B).

**Lysozyme and N-α-Acetylsarnine + N-α-Acetyllysinine Incubated with Gels**—The amount of pentosidine formed from lysozyme after 3 and 7 days of incubation with STGP gel was $9.7 \pm 1.3$ and $13.2 \pm 0.9$ pmol/μmol of amino acids, whereas in LTGP gel measured at the same time, it was $2.5 \pm 0.8$ and $5.3 \pm 0.6$ pmol/μmol of amino acids (Fig. 5A). Again, the amounts measured in STGP gel were higher than those in LTGP gel. The pentosidine levels in the STGP gel samples after incubation with BLP were about half those measured in BLP incubated under similar conditions.

In another set of experiments, gels were incubated with $N$-α-acetylarginine + $N$-α-acetyllysine instead of BLP. Arginine and lysine are precursor amino acids for pentosidine, and we found that significant amounts of pentosidine were produced in these incubations. The levels were 30–44% higher following incubation with STGP gel than with the LTGP gel (Fig. 5B). Taken together, these results show that pentosidine formation is not specific to BLP alone and that it forms in other proteins as well.

**Pentosidine in BLP Incubated with Glycated Lysozyme**—Lysozyme was immobilized on EAH-Sepharose and glycated using α-crystallin (EAH-Sepharose-bound) served as control. Means that do not share a common superscript letter are statistically significant at $p < 0.05$.

**Formation of Pentosidine in BLP Incubated with Water-insoluble Human Lens Proteins**—To determine whether these lens proteins could mimic the effects of in vitro glycated proteins, we incubated WI proteins from aged or brunescent lenses with BLP. This experiment also helped us to establish that glucose-derived early glycation products produce pentosidine. We washed the WI thoroughly with water before incubation to remove all soluble protein and incubated them with BLP (2 mg/ml) for 3 and 7 days. Analysis of BLP after incubation revealed that proteins from aged lenses produced nearly 3-fold higher levels of pentosidine (Fig. 7A and B) than proteins from brunescent lenses. Sodium borohydride treatment of WI before incubation with BLP almost completely suppressed pentosidine formation (Fig. 7B). Furosine content of WI protein of aged lens proteins could mimic the effects of in vitro glycated proteins, we incubated WI proteins from aged or brunescent lenses with BLP. This experiment also helped us to establish that glucose-derived early glycation products produce pentosidine. We washed the WI thoroughly with water before incubation to remove all soluble protein and incubated them with BLP (2 mg/ml) for 3 and 7 days. Analysis of BLP after incubation revealed that proteins from aged lenses produced nearly 3-fold higher levels of pentosidine (Fig. 7A and B) than proteins from brunescent lenses. Sodium borohydride treatment of WI before incubation with BLP almost completely suppressed pentosidine formation (Fig. 7B). Furosine content of WI protein of aged lenses could mimic the effects of in vitro glycated proteins, we incubated WI proteins from aged or brunescent lenses with BLP. This experiment also helped us to establish that glucose-derived early glycation products produce pentosidine. We washed the WI thoroughly with water before incubation to remove all soluble protein and incubated them with BLP (2 mg/ml) for 3 and 7 days. Analysis of BLP after incubation revealed that proteins from aged lenses produced nearly 3-fold higher levels of pentosidine (Fig. 7A and B) than proteins from brunescent lenses. Sodium borohydride treatment of WI before incubation with BLP almost completely suppressed pentosidine formation (Fig. 7B). Furosine content of WI protein of aged lenses could mimic the effects of in vitro glycated proteins, we incubated WI proteins from aged or brunescent lenses with BLP. This experiment also helped us to establish that glucose-derived early glycation products produce pentosidine. We washed the WI thoroughly with water before incubation to remove all soluble protein and incubated them with BLP (2 mg/ml) for 3 and 7 days. Analysis of BLP after incubation revealed that proteins from aged lenses produced nearly 3-fold higher levels of pentosidine (Fig. 7A and B) than proteins from brunescent lenses. Sodium borohydride treatment of WI before incubation with BLP almost completely suppressed pentosidine formation (Fig. 7B).
Glycation Products Produce Pentosidine Cross-links

Fig. 7. A and B, formation of pentosidine in BLP incubated with WI proteins from aged and brunescent cataractous human lenses. Washed WI proteins (5 mg) were incubated with BLP (2 mg/ml) as described in the text. C and D, furosine content of WI before and after incubation with BLP. Furosine was estimated by HPLC. For some experiments, WI was reduced by SBH before incubation with BLP. Unreacted SBH was removed by washing proteins several times with buffer.

and brunescent lenses was estimated before and after incubation. The amount of furosine in WI of aged and brunescent lenses were similar (~500 pmol/μmol of amino acids) (Fig. 7C) but was completely degraded after 7 days of incubation (Fig. 7D). As expected, sodium borohydride treatment resulted in the reduction of the Amadori, and therefore, furosine was not detected in these samples (Fig. 7D). These results show that proteins glycated in vivo can produce pentosidine on nonglycated proteins and that early glycation products are pentosidine precursors.

Incubation with Affinity Matrix-bound Early Glycation Products—We calculated that 18.5 and 28.8 mg of protein from early glycation products were bound to a boronate affinity gel from incubations with aged and diabetic lenses. The gel was then incubated with BLP or a mixture of N-α-acetylarginine + N-α-acetylysine. As Fig. 8 shows, the bound proteins produced pentosidine in BLP. The amounts formed from diabetic lens proteins were higher than those formed by aged lenses after either 3 or 7 days of incubation. Similarly, when a mixture of N-α-acetylarginine + N-α-acetylysine was added to the matrix-bound diabetic lens proteins, pentosidine formation was nearly twice that from comparable samples from aged lenses. Neither control incubations of BLP with gel, but without bound lens proteins, nor incubations with amino acids or BLP produced pentosidine. We concluded that diabetic lenses produce more pentosidine than nondiabetic lenses of comparable age, and that early glycation products promote pentosidine formation.

Pentosidine Formation from Short-chain Carbohydrates—To determine which of the fragments derived from early glycation products might serve as precursors of pentosidine, we incubated two and three carbon carbohydrates with a mixture of N-α-acetylarginine + N-α-acetylysine and measured the amount of pentosidine formed. Among the carbohydrates tested, glyceraldehyde produced the highest levels of pentosidine followed by glycolaldehyde and glyoxal (Table II). 1H NMR (D2O) and UV absorption spectra of the product from glyceraldehyde were fully compatible with those reported for ribose-derived pentosidine (43) (data not shown). In the case of glyceraldehyde and glyoxal, spectroscopic characterization is necessary to confirm the formation of pentosidine.

Because glyceraldehyde was the major precursor, we have proposed a pathway for pentosidine formation from this compound (Fig. 9). First, condensation of the aldehydic group of glyceraldehyde with the guanidino group of arginine and dehydration yields 1. Elimination of a molecule of formaldehyde yields 2. Oxidation of 2 followed by condensation with a molecule of lysine gives 4, which by cyclization and reaction with a second molecule of glyceraldehyde forms 6. Intramolecular cyclization of 6 followed by elimination of two molecules of water yields pentosidine. In fact, a structure similar to the proposed intermediate 6 has been reported from the reaction of short-chain carbohydrates with arginine and lysine (44).

DISCUSSION

We reasoned that Amadori products could accumulate in the lens, where proteins are long-lived. These products could continually generate the reactive oxygen species and damage lens proteins. Other investigators had shown that, when high amounts of D-ribose are used for glycation, proteins accumulate the Amadori product, and its degradation is inhibited (42, 45). Therefore, we used a novel preparation in which α-crystallin was coupled to EAH-Sepharose and glycated to enrich the lens proteins with Amadori products or AGEs.

Our studies confirm the formation of O2·− and OH· radicals from the glycated proteins. Previous studies from other laboratories demonstrated O2·− production from early glycation products that include 1,2- and 2,3-enolization of the Schiff’s base and oxidation of the enolate anion (46–49). We expected higher levels of O2·− from the short-term glycated α-crystallin (500 mM ribose, 2 days) than the long-term glycated α-crystallin (100 mM, 15 days), but we observed only modest increases. This may be due to the presence of early glycation products in the long-term glycated α-crystallin.

A puzzle was the production of OH· from glycated proteins. This radical could be produced via the Fenton reaction from H2O2. Phosphate buffer has trace amounts of cupric and ferric ions. To remove metal ions we passed our buffers through a...
Fig. 8. Formation of pentosidine in BLP from affinity matrix-bound early glycation products of human lenses. Early glycation products from WS extracts of diabetic and aged lenses were bound to a boronate affinity gel by passing through the column. The matrix was extensively washed with sodium phosphate buffer, and 100 mg of gel was incubated with 2 mg of BLP or with a mixture of N-acetyllysinine + N-acetyllysine (5 mM each) for 3 and 7 days as described in the text. We used 100 μg of BLP and 833 nmol of amino acids as pentosidine measurements. The results are expressed as picomoles of pentosidine per gram of gel.

Chelex-100 column, but this may not have removed all trace metal ions. In fact, a recent study showed that buffers passed through Chelex-100 resin retain some cupric ions (50). This may have initiated the Fenton reaction and produced OH⁻. Additionally, lens proteins themselves may have initiated the Fenton reaction. It is known that lens proteins can bind metal ions in vivo (51), which raises the possibility that the α-crystallins from bovine eyes may have redox-active metal ions bound to it.

We found cross-linked proteins of 40-45 kDa in bovine lens proteins that were incubated with glycated proteins, and lens proteins incubated with short-term glycated proteins had higher levels than those incubated with long-term glycated proteins. These observations suggest that the cross-linked proteins are produced by reactions mediated primarily by early glycation products, not likely by AGEs. The finding that pentosidine is formed in BLP, that was incubated with glycated α-crystallin, supports this view. In addition, Araki et al. (30) demonstrated immunoreactivity with 40- to 45-kDa proteins in aged and cataractous lenses with antibodies against glucose-derived AGEs. SDS-PAGE studies of lens proteins showed that aged and cataractous lenses have proteins of 40-45 kDa. Spec-
In the body of the text, it is noted that some therapeutic interventions may be effective in diabetes and aging, suggesting potential strategies for these conditions. The text also mentions that pentosidine, a glycation cross-link, may play a role in these processes.

The authors propose a novel pathway for pentosidine formation from glyceraldehyde, which they describe in detail. They acknowledge the contributions of various researchers and institutions, including the University of Michigan and Case Western Reserve University.

References are provided at the end of the text, citing a variety of sources from various fields such as chemistry, biology, and medicine. The list of references includes works by authors like Brownlee, Vlassara, and Monnier, among others.
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