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Structural elucidation of a novel lysine-lysine crosslink generated in a glycation reaction with L-threose

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Summary. A novel lysine-lysine crosslink was isolated from a reaction containing L-threose (a degradation product of ascorbic acid) and N-α-acetyllysine. The compound, after chromatographic purification, had a relative molecular mass of 628 as determined by fast atom bombardment spectrometry. Structural analysis by NMR spectroscopy (^{1}H, ^{13}C, COSY, HETCOR and DEPT) suggests that the compound is composed of 3 threose residues and 2 N-α-acetyllysines with an empirical formula of $C_{28}H_{45}N_4O_{12}$. The compound, referred to as threosidine (2,5-dihydroxy-5,6,7,8-tetrahydro-1,7-naphthyridinium as the core molecule) has an absorption maximum at 328 nm with an excitation and emission maxima at 328 nm and 402 nm respectively. Threosidine was stable upon acid hydrolysis, and it was not found in the reactions containing threose and amino acids other than N-α-acetyllysine. Threosidine was also not found in the reactions containing N-α-acetyllysine and sugars other than threose.

Keywords: Amino acids – Lysine – L-threose – Glycation – Crosslinking – AGE

Introduction

There is considerable interest in advanced glycosylation end products (AGEs), which are produced *in vivo* during the Maillard reaction. This reaction results from the interaction of sugars with protein amino groups (glycation), and the subsequent degradation of glycated proteins (Monnier, 1989). The formation of AGEs are thought to have health-related consequences, and it has been suggested that they may contribute to diabetic complications, as well as cataractogenesis.

Some of the well established AGEs include carboxymethyllysine or CML (Dunn et al., 1990; Reddy et al., 1995), pentosidine (Dyer et al., 1991; Sell and

Monnier, 1989), crosslines (Nakamura et al., 1992), lens Maillard compound or LM1 (Nagaraj and Monnier, 1992) and pyrraline (Nagaraj et al., 1996). Most of these AGEs have been isolated as a result of interactions between glucose and the ε-amino group of lysine, although other sugars would be expected to react as well.

In vitro studies in our laboratory have shown that ascorbic acid and its oxidation products react more rapidly with protein-bound lysine residues than glucose (Slight et al., 1990). It has also been shown that L-threose, the most reactive sugar among the breakdown products of ascorbic acid (Lopez and Feather, 1992), rapidly glycates basic poly amino acids or proteins (Prabhakaram and Ortwerth, 1994). Recently it has been shown that water-insoluble lens proteins isolated from cataractous human lens contain protein-bound UVA sensitizers, which resemble those formed in the *in vitro* reaction of ascorbic acid or its breakdown products with bovine lens proteins (Ortwerth et al., 1995). In a quest to investigate the possible ascorbate glycation crosslinks *in vivo*, the present paper describes the isolation and characterization of a crosslink isolated from an *in vitro* reaction containing L-threose and N-α-acetyllysine.

Experimental

Materials

N-α-acetyllysine, diethylenetriaminepentaaceticacid (DTPA) trifluoroaceticacid (TFA) and all other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO. L-threose was synthesized according to the method of Perlin (1962) using arabinitol and benzaldehyde as the precursors. Analytical (4.6 × 250 mm) and preparative (22 × 250 mm) HPLC columns were purchased from Vydac Separations Group, Hesperia, CA. Biogel P-2 resin (super fine) was obtained from Biorad Laboratories, Richmond, CA. All other reagents used were of the analytical grade or better.

Methods

Incubation conditions for N-α-acetyllysine and L-threose

N-α-acetyllysine and L-threose were incubated (2:1 molar ratio) at 37°C under sterile conditions in 0.1 M phosphate buffer, pH 7.0 containing 1.0 mM DTPA as the chelator. Aliquots withdrawn at different time periods from zero to one week were analyzed by RPHPLC on a Vydac C18 analytical column and also by TLC on silica plates (Whatman, Hillsboro, OR).

Synthesis of threosidine

For preparative purpose, N-α-acetyllysine (5 g = 26.57 mmoles) and threose (1.6 g = 13.28 mmoles) were incubated for one week under conditions described as above. Following this, the reaction products were analyzed on a Vydac C18 preparative HPLC column. For HPLC, solvent A consisted of 0.1% TFA in water and solvent B consisted of 0.1% TFA in 100% acetonitrile. A linear gradient of 0–40% B was used over 40 minutes to elute the reaction products retained by the HPLC column. Absorbance of the fractions collected during HPLC was monitored at 210 nm and 328 nm using a dual wavelength detector.

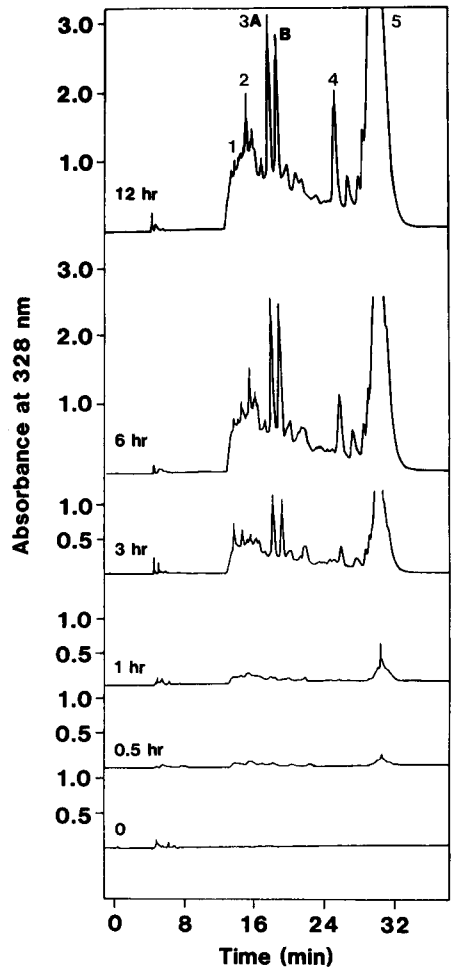


Fig. 1. Formation of threosidine (3A and 3B) and other reaction products with time, in a reaction containing N-α-acetyllysine and threose. Analytical HPLC of the individual reactions was performed as described in the methods section

Of the several compounds eluted from the preparative HPLC column, two compounds (labeled as 3A and 3B in Fig. 1) were stable to borohydride reduction and acid hydrolysis. Both these compounds were purified by repetitive injections on a preparative HPLC column until a single peak was obtained. Further purification was achieved by size exclusion chromatography of the threosidine on a Biogel P-2 column (2.5 × 100 cm) with 0.02 M phosphate buffer, pH 7.0 as the eluant. Absorbance of the fractions eluted from the Biogel column was monitored at 328 nm and those fractions with maximum absorbance were pooled and rechromatographed on the preparative HPLC column. The final yield was ~5–7 mg of threosidine from one gram of N-α-acetyllysine. Purity of the threosidine was monitored by analytical HPLC on a C18 column and also on silica gel TLC plates using different irrigants. For TLC, neutral (70% ethanol), acidic (acetic acid:n-butanol:water, 1:2:1) and basic (ammonia:n-propanol, 3:7) solvents were used.

Analysis of threosidine

Structural analysis of threosidine was achieved by measuring the UV-visible absorption maximum, fluorescence maxima, FAB-MS, MS-MS, ^{1}H, ^{13}C, ^{13}CDEPT, COSY, NOESY and HETCOR NMR spectra. FAB-MS and tandem mass spectrometry were carried out at the mass spectrometry facilities of Dr. Jean Smith, Purdue University, West Lafayette, IN and Dr. Todd Williams, University of Kansas, Lawrence, KS. NMR spectra were obtained in D_2O on a Bruker AC 500 MHz spectrometer at the NMR facility located in the Department of Chemistry of the University of Missouri.

Glycation of amino acids by L-threose

Amino acids, such as lysine, N-α-acetyllysine, N-ε-acetyllysine, N-α-acetylalanine, N-α-acetylarginine or N-α-acetylhistidine and also aminoguanidine or penicillamine were incubated with threose in a 2:1 molar ratio under the standard incubation conditions described as above. After 3 weeks the glycated amino acids were dried in Savant rotatory evaporator and then hydrolysed in 6N HCl at 110°C for 20 hrs. Amino acid analysis of acid hydrolyzed reaction products was performed (Gehrke et al., 1987) at the Agricultural Experiment Station of the University of Missouri and the values were represented in nmoles/mg amino acid.

Results and discussion

Advanced glycation end products (AGEs) are of interest for several reasons. Most of the AGEs isolated thus far can be used as markers for diabetic complications (John and Lamb, 1993). However, it is not yet known to what extent the AGEs contribute to either senile cataractogenesis (lens opacification) or diabetes. Both CML (Dunn et al., 1989; Reddy et al., 1995) and pentosidine (Dyer et al., 1991; Nagaraj et al., 1991; Sell and Monnier, 1989) represent the classical AGEs, but due to their minute levels in aged or diabetic tissues, they are currently used as senescence or glycation markers. Therefore, identification of true lysine-sugar-lysine crosslinks in nature and their biological significance are important areas of investigation at present. In this regard, we have isolated two crosslinks from an *in vitro* reaction mixture of threose and N-α-acetyllysine and describe the properties of one of these crosslinks, in the present report.

Analysis of the glycation products of N-α-acetyllysine and threose

When N-α-acetyllysine was incubated with threose and analyzed by RPHPLC, we observed the formation of several reaction products (Fig. 1). Retention times of the major peaks were 18, 19, 26 and 30 minutes and were designated as 3A, 3B, 4 and 5 respectively. We have used a dual wave length detector to monitor the absorbance of both reacted and unreacted reaction products eluted from the HPLC column. The selected wavelengths were at 210 nm or 328 nm. Pre-scanning of a 3 day reaction mixture of N-α-acetyllysine and threose on HPLC showed maximum absorbance of peaks 1, 2, 3 and 5 at 328 nm, whereas peak 4 showed a maximum absorbance at 300 nm. For consistency, we have selected 328 nm wavelength to monitor the fractions eluted from HPLC column.

Initially, at zero time, none of the retention compounds in the incubation mixture were bound to the RPHPLC column. However, reaction products accumulated within 30 minutes and were detectable by HPLC after 3 hrs. Maximum yield was obtained in 3 days with no further accumulation of the reaction products even after 6 weeks. Reduction of the incubation mixture with sodium borohydride prior to analysis caused peak 4 to disappear (data not shown). We also observed that either HPLC-purified peak 4 or peak 4 in an initial 3-days reaction mixture did not bind to a cation exchange (H^+ form) column. Additionally, both ^1H and ^{13}C NMR data on peak 4 (which was isolated in pure form by preparative HPLC on a Vydac C18 column) were identical to that reported for pyrrole (glycosyl formyl pyrrole-lysine, GFP) by Nagaraj and Monnier (1995). Rechromatography of the peak 5 with a gradient of 10–40% solvent B (0.1% TFA in 100% acetonitrile) in 60 minutes showed more than 10 products, which were not stable even at 4°C.

Properties of threosidine

Though we have purified both threosidine A and B (labeled as 3A and 3B in Fig. 1), we initially have used threosidine B (hereafter referred to as threosidine) for structural characterization. Threosidine has an extinction coefficient of $4.2 \times 10^3 M^{-1}cm^{-1}$ at 328 nm and was produced in 3–5% yield. The absorption spectrum of threosidine was recorded at pH values ranging from 2.0 to 12.0. No major changes were observed in the spectra, whether threosidine was dissolved in deionized water (neutralized with acid or base) or in 0.01 M phosphate buffer (used for pH values 3.0–9.0). The spectral properties were similar at pH values from 2.0 to 4.0; pH 5.0 to 8.0 and pH 9.0–12.0. Fig. 2A exhibits representative spectra recorded in acidic (pH 3.0), neutral (pH 7.0) and basic (pH 11.0) buffers. It is of interest to note that even the extensively acid hydrolyzed (24 hr in 6 N HCl at 110°C) threosidine retained its characteristic spectral properties between 210–400 nm. Spectral properties of threosidine were also not changed, when it was reduced with sodium borohydride (data not shown).

Unlike pentosidine (Sell and Monnier, 1989; Dyer et al., 1991), LM1 (Nagaraj and Monnier, 1992) or pyridinoline (Fujimoto, 1977) in which lysine is involved in crosslinking, threosidine did not show any quenching or changes in spectral properties with a change in pH. The slight spectral shifts in the wavelength at lower (pH 3.0) and higher (pH 11.0) pH values suggest the presence of exchangeable H^+ and OH^- ions in threosidine. Fluorescence spectra of threosidine exhibited an excitation maximum at 328 nm and emission maximum at 402 nm in 0.01 M phosphate buffer, pH 7.0 buffer (Fig. 2B). Similar values were obtained in acidic (pH 3.0) or basic (pH 11.0) buffers (data not shown).

Threosidine was auto fluorescent in long-wavelength UV light and it was visualized as a single spot on TLC plates when neutral (70% ethanol), acidic (acetic acid:n-butanol:water, 1:2:1) and basic (ammonia:n-propanol, 3:7) solvents were used as irrigants (data not shown). When these TLC plates were

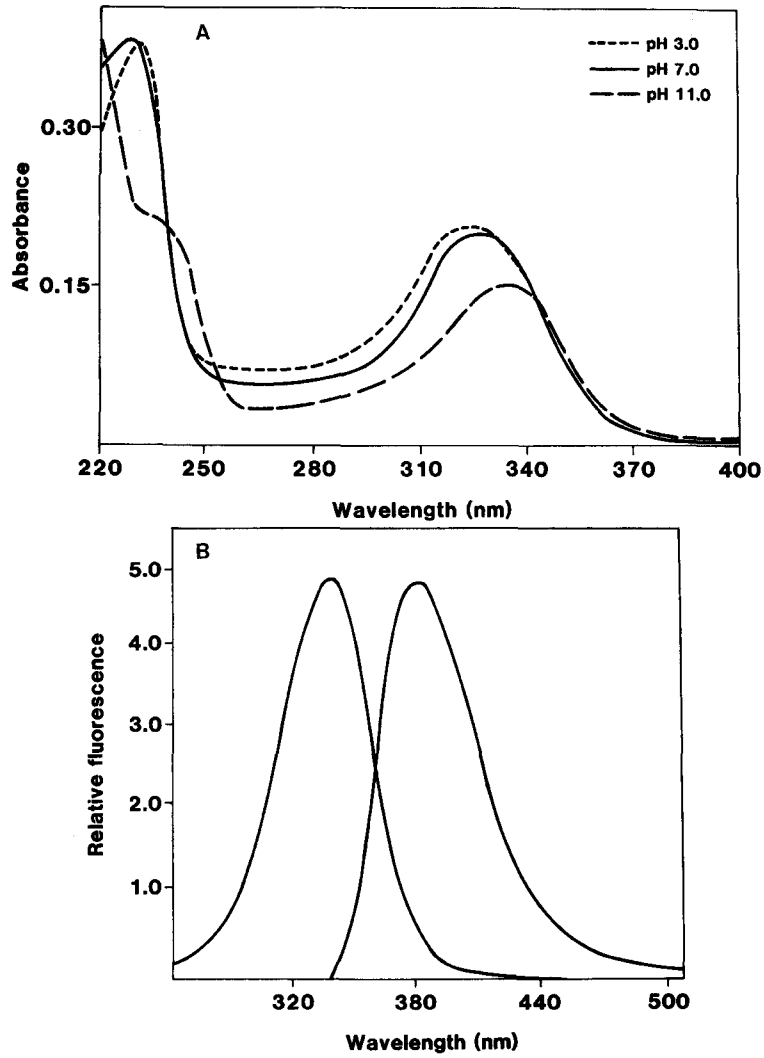


Fig. 2. UV-absorption spectra (**A**) and fluorescence spectra (**B**) of the purified threosidine in water. UV-absorption spectra were recorded at different pHs as described in methods section. Fluorescence spectra were recorded at pH 7.0 in 0.02 M phosphate buffer

sprayed with ninhydrin (0.5% in 100% acetone) threosidine did not react with ninhydrin. Threosidine did not react even with the most sensitive free amino group reactant, fluorescamine, which is routinely used to determine free amino groups (Udenfriend et al., 1972). These results suggest the lack of free amino groups in threosidine.

Structural elucidation of threosidine

Mass spectrometry analysis of threosidine

The relative molecular mass of threosidine was determined by FAB-MS and MS-MS (Fig. 3A and B). These data show a predominant molecular ion at 629

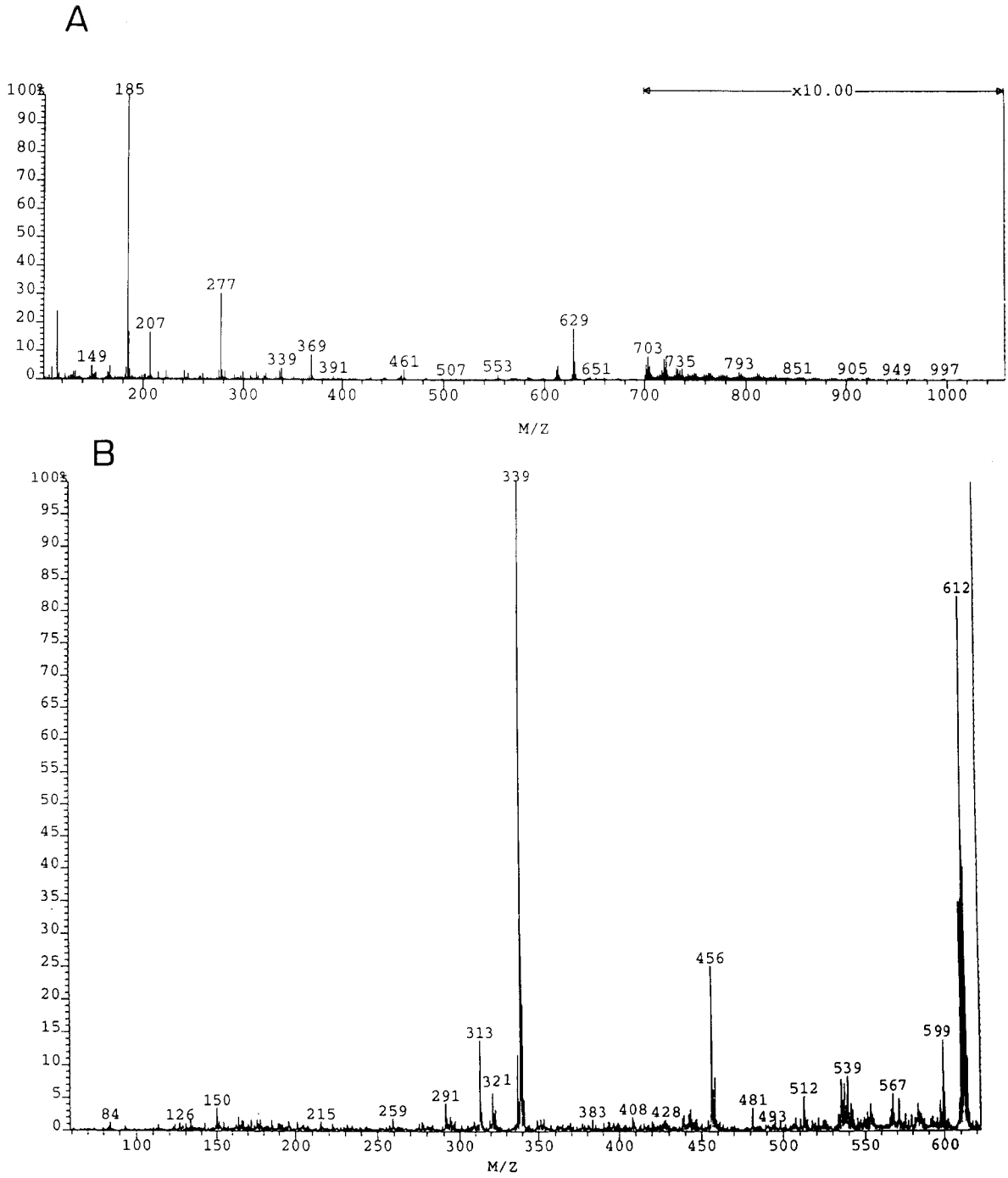


Fig. 3. FAB-MS (**A**) and MS-MS (**B**) of threosidine

with the exact mass determined as 629.3031 and it is equivalent to an empirical formula of $C_{28}H_{45}N_4O_{12}$. This corresponds to a chemical reaction between 3 threose molecules and 2 molecules of N-α-acetyllysine minus six water molecules. The molecular ions other than 629 mainly represent glycerol matrix

(Fig. 3A). MS-MS of threosidine showed molecular ions correspond to 612 (loss of OH at the anomeric carbon), 456 (loss of lysine from ε-$NH_{2\ group}$), 339 (loss of N-α-threulose N-α-acetyllysine from the saturated ring), 291 (the threulose-N-α-acetyllysine component). In addition, FAB-MS of the acid hydrolysate of threosidine B displayed molecular ions at m/z 585 and 541, suggesting the loss of 1 and 2 acetyl groups respectively, from the parent compound (data not shown).

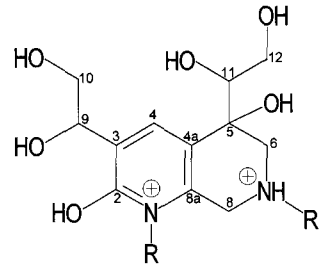
NMR analysis of threosidine

Based upon a combination of two-dimensional and various NMR spectra described as in methods section, the structure of threosidine B has been determined. The ^{1}H and ^{13}C NMR resonances (Table 1) are consistent with the structure proposed in figure 4. The DEPT spectrum showed that 6 quaternary, 3 methine, 13 methylene, 4 methyl and 4 unprotonated carbons are present in threosidine. Based upon the NMR spectroscopy, the core molecule of threosidine is designated as 2,5-dihydroxy-5,6,7,8-tetrahydro-1,7-naphthyridinium. The connectivity of protons in the lysine side chains and in the core molecule of threosidine was determined by two dimensional ^{1}H-^{1}H correlation (COSY) NMR spectroscopy. These data showed the connectivity of protons in the core molecule as H9 (4.31 ppm) to H10 (4.27 ppm) and H10b

Table 1. ^{1}H and ^{13}C (ppm) resonances for threosidine

Position*	^{1}H	^{13}C
1	–	–
2	–	156.44
3	–	131.45
4	7.94	129.99
4a	–	126.8
5	–	83.9
6	3.58, 3.60	62.35
7	–	–
8	3.65, 3.66	56.07
8a	–	153.24
9	4.31	71.91
10	3.43, 4.27	60.99
11	4.3	71.31
12	3.45, 4.28	60.47
Position on lysine (R^1/R^2)*		
1′	–	175.39, 174.01
2′	4.24, 4.26	52.03, 52.27
3′	1.69, 1.73; 1.74, 1.75	24.2, 24.7
4′	1.41, 1.42	21.42, 21.38
5′	1.76, 1.78; 1.80, 1.82	29.72, 29.72
6′	3.32, 3.35; 4.64, 4.70	54.9, 55.77
7′	–	174.12, 175.29
8′	1.94, 1.96	21.9, 21.61

*Position corresponds to the numbering on threosidine (Fig. 4).



R = $CH_2CH_2CH_2CH_2CH(COOH)NHCOCH_3$

Fig. 4. Structure of threosidine

to H10a (3.43 ppm) and also from H11 (4.30 ppm) to H12 (4.28 ppm) and H12b to H12a (3.45 ppm). The COSY spectrum also confirmed the connectivity of protons in the lysines (R) from H2′ (4.24 ppm) to H3′ (1.69 ppm), H3′ to H4′ (1.41 ppm) and H5′ (1.76 ppm) to H6′ (3.32 ppm) for the first set of lysine; H2′ (4.26 ppm) to H3′ (1.74 ppm), H3′ to H4′ (1.42 ppm), H4′ to H5′ (1.80 ppm) and H5′ to H6′ (4.64 ppm) for the second set of lysine.

Specificity of threosidine formation

Specificity of the ε-amino group of lysine in the formation of threosidine

When some selected amino acids were reacted with threose under standard incubation conditions and analyzed by a C18 RPHPLC column, we observed the formation of threosidine exclusively in the reaction containing N-α-acetyllysine and threose and also to some extent in the reaction containing lysine and threose. Since threosidine has a specific retention time on Vydac C18 analytical column (under the elution conditions described as in the methods section) and since threosidine is also resistant to acid hydrolysis (hydrolysis in 6 N HCl at 110°C), we routinely performed analytical HPLC and amino acid analysis to determine threosidine levels in the reactions containing amino acids and threose. As evidenced by HPLC and amino acid analysis, no products related to threosidine have been observed, when N-ε-acetyllysine, N-α-acetylalanine, N-α-acetylarginine, N-α-acetylhistidine, aminoguanidine or penicillamine were incubated with threose (2:1 molar ratio of amino acid:threose). These results indicate that the ε-amino group of lysine is selectively involved in the formation of threosidine (data not shown).

In order to determine the formation of threosidine in the presence of competing amino groups, N-α-acetyllysine was incubated with threose in presence of N-α-acetylalanine, N-ε-acetyllysine, N-α-acetylarginine, N-α-acetylhistidine, aminoguanidine or penicillamine and analyzed by both HPLC and amino acid analysis. The obtained results showed that acetylated alanine or histidine did not prevent threosidine formation. However, to some extent N-ε-acetyllysine, N-α-acetylarginine, aminoguanidine or penicillamine were able to prevent completely the production of threosidine. This may be due to

the rapid reactivity of threose with the available amino groups and thus favoring the possible formation of hetero crosslinks among glycated amino acids. One such example is pentosidine, a well characterized hetero crosslink formed between arginine and lysine during glycation (Dyer et al., 1991; Sell and Monnier, 1989). Our results also show that aminoguanidine, a well known inhibitor of AGEs (Brownlee, 1994; Hirsch et al., 1995) or penicillamine completely inhibit the formation of threosidine, suggesting that threosidine is an advanced glycation endproduct in nature.

Do all sugars generate threosidine?

A variety of sugars were incubated with N-α-acetyllysine, under incubation conditions similar to those employed for threose and N-α-acetyllysine. Threosidine was not observed in reactions containing N-α-acetyllysine and either glucose, 3-deoxyglucosone, glyceraldehyde or ribose (data not shown). However, a small amount of threosidine was formed in the reaction of N-α-acetyllysine and erythrose, which also is a tetrose. Although threose can be formed during the degradation of ascorbic acid, dehydroascorbic acid or diketogulonic acid (Lopez and Feather, 1992), we did not find threosidine, when these sugars were reacted with N-α-acetyllysine. This may be the result of the fact that the dehydroascorbic acid or diketogulonic acid may have reacted with the N-α-acetyllysine prior to the generation of threose from these sugars. Therefore, formation of threosidine is exclusively dependent on the presence of threose and N-α-acetyllysine in the reaction. We also observed that threosidine can be produced in incubations containing water alone, 0.1 M phosphate buffer, pH 7.0, or in phosphate buffer without DTPA, and reactions devoid of oxygen (data not shown). These results indicate the lack of an oxidation requirement for the formation of threosidine.

In conclusion, all the above data establish the formation of a novel lysine-lysine crosslink, threosidine, in a reaction containing N-α-acetyllysine and threose. Our preliminary results indicate the presence of threosidine A (and to some extent threosidine B) in various proteins glycated by threose. We are currently involved in the structural analysis of threosidine A, and are also establishing assays for threosidine from aged or diabetic lens proteins. It has been reported that AGEs accumulate in tissues proteins with age and at an accelerated rate during hyperglycemia in diabetes (Brownlee, 1994; Monnier, 1989). Since glycation and oxidation reactions concurrently lead to the production of AGEs, evaluation of the possible AGEs *in vivo* will be advantageous in determining the role of AGEs in the pathogenesis of diabetic complications or senile cataractogenesis.

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