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Cysteine Is the Initial Site of Modification of α-Crystallin by Kynurenine

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Tryptophan metabolites, such as kynurenine, are spontaneously unstable at neutral pH. They undergo side-chain deamination yielding reactive α, β unsaturated ketones. In the lens, where these compounds act as UV filters, reaction of the breakdown products with lens proteins (crystallins) may be largely responsible for age-dependent colouration of this tissue. In previous research, where high pH (pH 9) was used to promote deamination and conjugation with lens protein, histidine, lysine, and cysteine residues were found to be modified. In this study we show that, at pH 7, site of reaction with the major lens chaperone α-crystallin, is the single cysteine residue of the αA subunit. This apparent selectivity has important ramifications because the cysteine-kynurenine adduct is itself unstable under physiological conditions.

Key Words: **kynurenine; protein modification; lens; ageing; cataract.**

The primary function of the lens of the eye is to transmit and focus light upon the retina. In common with lenses from other species, the human lens grows by building up layer after layer of fibre cells around the original core, which is present at birth [1]. Since no cells are lost as the younger cells envelop the older ones, the lens contains cells that were present even prior to the birth of the organism [2].

The high refractive index of the human lens can be attributed to the abundance and short-range packing of three families of structural proteins called crystallins [3, 4], which account for approximately 90% of the total lens protein [5]. The exceptionally long lifetime of the crystallins makes them susceptible to the accumulation of a variety of post-translational modifications that may affect their tertiary structure [6]. These include phosphorylation [7], deamidation of glutamine and asparagine residues [8–10], covalent modification of the ϵ-amino group of lysines [11] and truncation of the C-terminus [12] or N-terminus [8] of crystallin subunits.

With ageing, the lens also becomes progressively more yellow and fluorescent [13] and this appears to be largely due to interactions involving endogenous UV-filter substances [14, 15]. The lens contains a number of tryptophan-derived, coloured UV-filter compounds. Two of these compounds, kynurenine (Kyn) and 3-hydroxykynurenine *O*-β-D-glucoside have been shown to bind to nucleophilic amino acids, under basic conditions, via deamination of the aliphatic side chain [16]. At neutral pH, this is a relatively slow, non-oxidative process [17]. Recent work in our laboratory has identified individual Kyn-modified amino acids in acid hydrolysates of the aged lens [18], confirming that Kyn is an *in vivo* modifier of lens protein. The aim of this work was to establish the site(s) of Kyn modification on the polypeptide chains of the major lens protein, α-crystallin, at physiological pH.

MATERIALS AND METHODS

Materials

All organic solvents were HPLC grade. L-Kynurenine and pepsin (EC 3.4.23.1) were purchased from Sigma–Aldrich (St. Louis, MO). 3HKG was synthesised as previously described [19].

Fresh calf lenses, obtained from a local abattoir, were used in the preparation of whole lens protein. α-Crystallin was isolated from total calf lens protein by size exclusion chromatography on a Sephacryl S-300 column (800 × 26 mm) in 100 mM phosphate buffer, pH 7.2, then concentrated, dialysed and lyophilised for storage at −20°C.

αA-crystallin was isolated from total α-crystallin by semi-preparative HPLC on a 5-μm, C18 Hypersil column, (250 × 10 mm) using a linear H_2O/acetonitrile gradient (0.05% v/v TFA) of 0–80% acetonitrile over 40 min at a flow rate of 4.0 ml min^{-1}. The separated subunit fractions were collected, lyophilised and stored at −20°C until required.

Modification of αA- and Total α-Crystallins by Kynurenine

pH 9.5. αA-Crystallin (2 mg) and kynurenine (1 mg) were dissolved in 600 μl of 50 mM sodium carbonate buffer, pH 9.5. The

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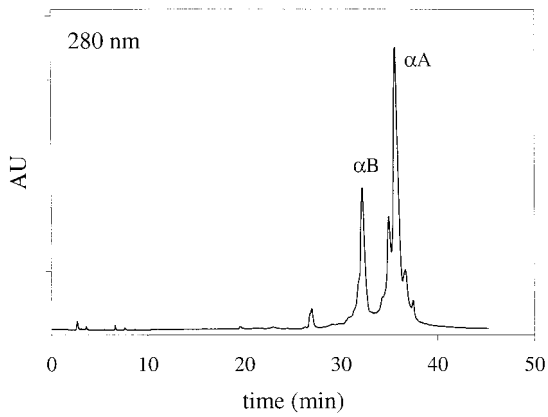

FIG. 1. Semipreparative reverse-phase HPLC separation of bovine α-crystallin into the constituent αA and αB subunits. Total α-crystallin was initially separated from bovine lenses by size exclusion chromatography, 20 mg of which was loaded onto the HPLC column to give the chromatogram shown. The αA protein collected in the second peak was subsequently used for modification by Kyn.

solution was placed under argon, sealed and incubated at 37°C for 48 h. Modified protein was isolated from the reaction mixture using a 1 cc, Sep-Pak Vac C18 cartridge (Waters). Briefly, 300 μl was loaded onto a Sep-Pak which had previously been prepared by washing with 2 × 1 ml volumes of methanol and H_2O. The column was washed with 2 ml of H_2O, and the unreacted Kyn was eluted with 3 ml of 20% acetonitrile/H_2O, 1% formic acid. αA-Crystallin was eluted with 1 ml of 80% acetonitrile/H_2O, 1% formic acid. This final eluant was used for mass spectral analysis.

pH 7.0. The pH 7.0 incubations were performed in 5 ml of 100 mM phosphate buffer. Total α-crystallin (100 mg) was weighed in to each of two glass vials. Kynurenine (10 mg) was added to one of the vials whilst the other was used as a control. The pH of the solutions was not affected by the protein or Kyn. 20 μl of chloroform was added to the solutions as an antibacterial, which were placed under argon, sealed and incubated at 37°C for 14 days. After 7 days, 100 μl of each reaction mixture was removed and prepared for MS analysis using a Sep-Pak as above. After 14 days, approximately 15 mg of the protein was purified from each reaction vial using the semipreparative HPLC method described under Materials subsection; however, a 30-min gradient was used and the αA and αB peaks were combined prior to lyophilisation.

SCR-3HKG was prepared at pH 9.0 in the same way as the αA-Kyn adduct and was purified using semipreparative HPLC.

TABLE 1

Predicted and Observed Masses for Kyn-Modified αA-Crystallin

Subunit	Predicted mass	Observed mass
αA	19832	19833
$αA_p$	19912	19910
αA + Kyn	20024	20025
$αA_p$ + Kyn	20104	20105
αA + 2 Kyn	20216	20215
$αA_p$ + 2 Kyn	20296	20297
αA + 3 Kyn	20408	20409
$αA_p$ + 3 Kyn	20488	—

Stability Studies of SCR-3HKG Using HPLC-MS

Approximately 50 μg of the purified SCR-3HKG adduct was dissolved in 100 μl of either Tris or ammonium bicarbonate buffer, pH 8.1. An aliquot (20 μl) was removed at 0 and 24 h for analysis by microbore HPLC (Applied Biosystems, Model 172 Separation System, CA) using an Alltech Alltima 250 × 2.1 mm, C18 column. Samples were eluted using an acetonitrile gradient in aqueous 1% (v/v) formic acid and a flow rate of 200 μl min^{-1}. The gradient was linear from 0 to 80% acetonitrile in 40 min. Samples were monitored at 360 nm and by in-line electrospray ionisation mass spectrometry (HPLC-MS). Mass spectra were acquired on a VG Quattro II triple quadrupole mass spectrometer (VG Biotech Ltd., Altincham, Cheshire, UK) in positive ion mode with a skimmer potential of 28 V and a source temperature of 140°C.

Identification of Kyn-Modified Peptic Digest Fragments

Kyn-modified α-crystallin, (2.0 mg) was digested with pepsin (1:100 w/w) in 2 ml of 10 mM HCl for 14 h. The digest mixture was separated on a C18, 250 × 4.6 mm, 5 μm, 100 Å HPLC column

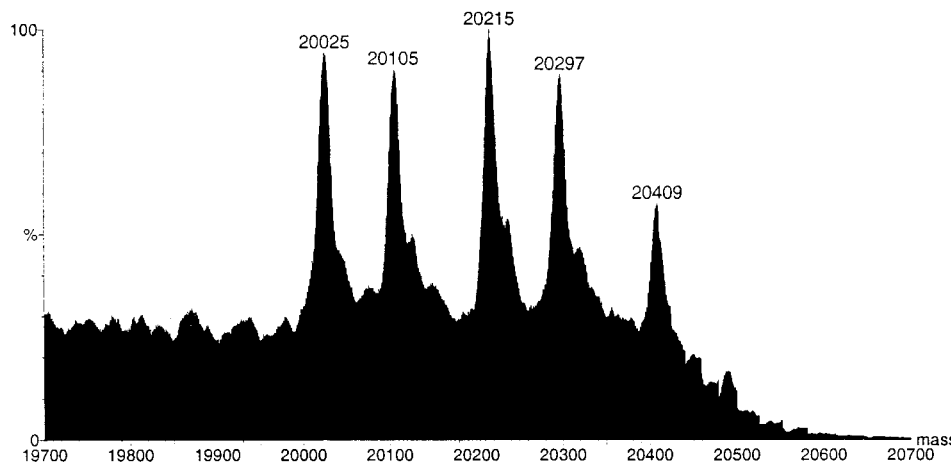

FIG. 2. Transformed mass spectrum of αA-crystallin after incubation with Kyn for 48 h at pH 9.5. The peak masses indicated that covalent addition of Kyn to the protein subunits had occurred.

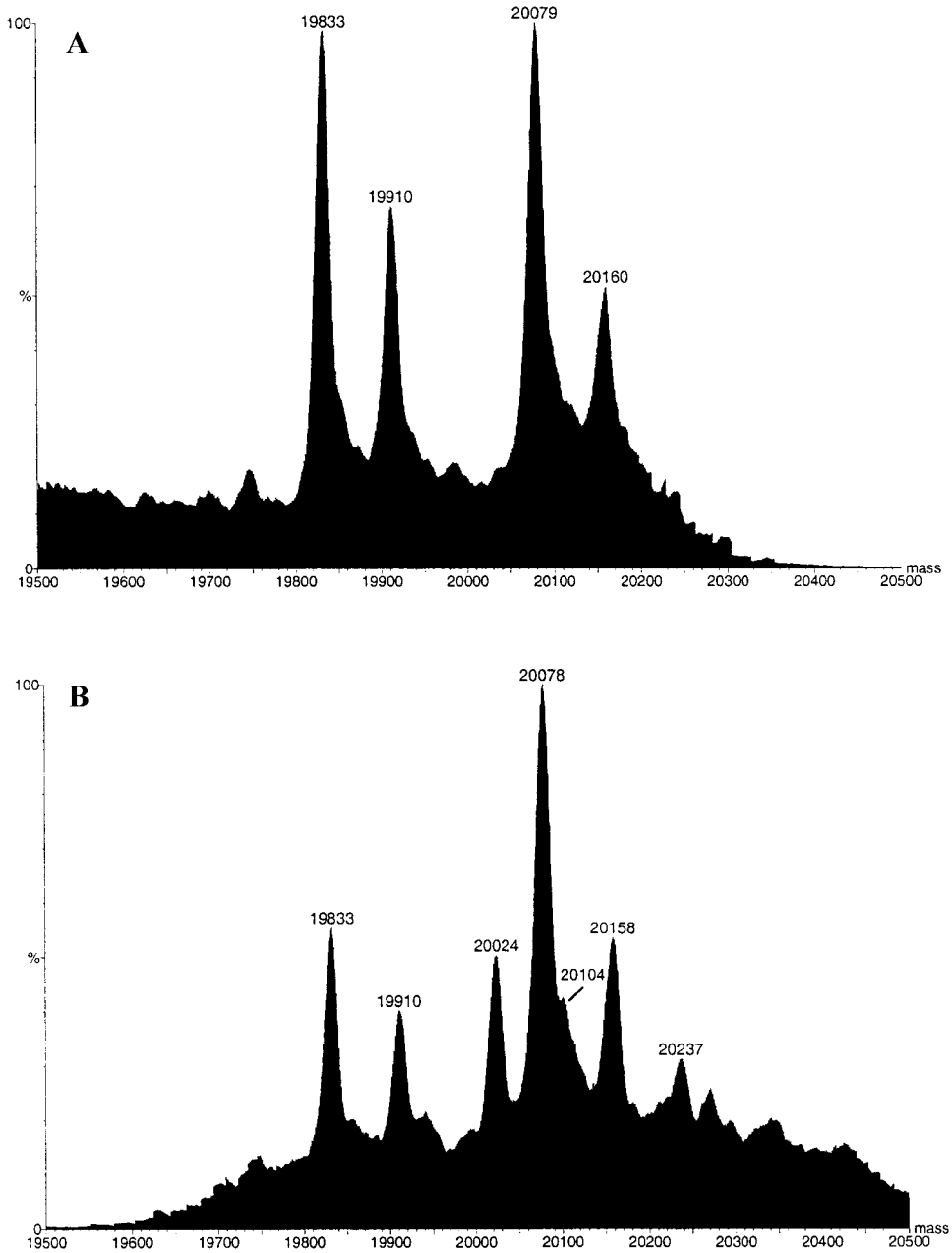

FIG. 3. Transformed mass spectra of total α-crystallin after 7 days of incubation at pH 7.0 in the absence (A) or presence (B) of Kyn. Inclusion of Kyn in the sample produced two peaks which were not observed in the control, at masses 20,024 and 20,104 (B). These peaks suggested that a single Kyn moiety had attached to the to the αA and αA_p subunits. Peaks of mass 20,078, 20,158, and 20,237 Da in B arose from the native, singly- and doubly-phosphorylated αB subunits, respectively.

(Microsorb, Rainin, CA) using a linear 0–80% acetonitrile gradient (0.05% v/v TFA). The eluant was monitored for fluorescence at ex 370/em 490 nm (HP 1046A, programmable fluorescence detector, Hewlett–Packard) and UV-absorbance at 229 nm (SD 2100 UV–Vis variable wavelength, ICI Instruments). The collected fluorescent peaks were lyophilised and approximately 10% of this material was rechromatographed by the HPLC-MS method described (under Stability studies of SCR-3HKG using HPLC-MS). Tandem mass spectrometry (MS/MS) was used to identify the peptide responsible for the coloured peaks in the HPLC-MS chromatograms. Samples were dissolved in 50% aqueous acetonitrile, 1% formic acid, and delivered by syringe pump at 5 μl min^{-1}. MS/MS experiments were acquired with a collision energy of 15 V and an Argon gas collision cell pressure of 3.5×10^{-3} mbar. Theoretical fragmentation series were generated using the BioLynx module of MassLynx.

RESULTS

Modification of αA-Crystallin by Kyn at pH 9.5

Previous work in our laboratory [16] identified four tryptic peptides in total α-crystallin which were modi-

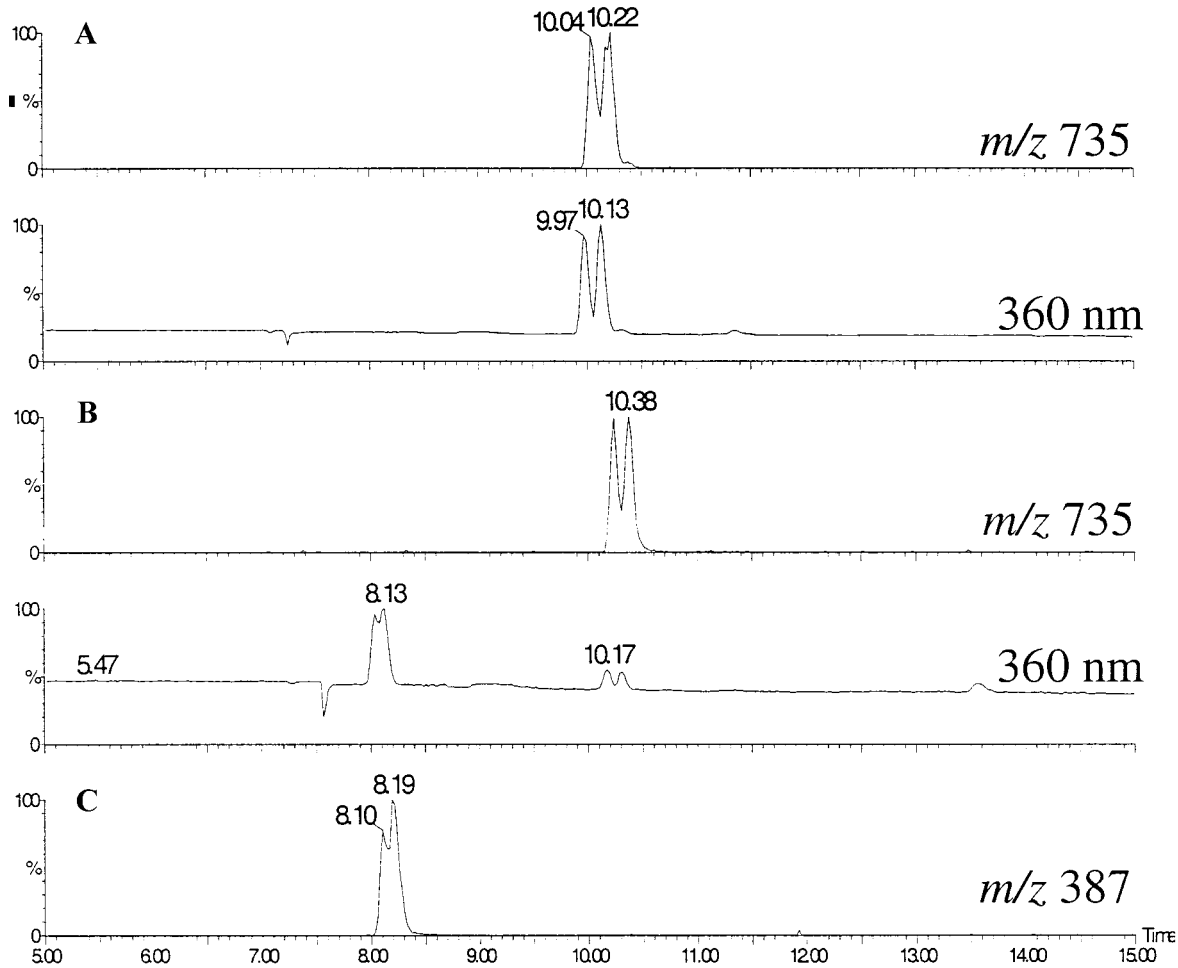

FIG. 4. LC–MS chromatograms of the purified 3HKG adduct, SCR-3HKG after 0 h (A) and 24 h (B) incubation in ammonium bicarbonate buffer, pH 8.1. At time zero, the selected ion chromatogram for SCR-3HKG (*m/z* 735) and the UV trace show that the compound is a relatively pure, diastereoisomer, as indicated by the doublet peaks. After 24 h (B), a major peak was observed in the UV profile which corresponded to a product of the same mass as 3HKG (C), at *m/z* 387, indicating that the compound was unstable under the incubation conditions.

fied by Kyn at pH 9.0. Of these, one originated from αA- and three from αB-crystallin. It was suggested that His, Lys and Cys, were the likely sites of covalent attachment of Kyn under the basic, nonoxidative conditions described.

In order to more closely examine the effect of pH on this reaction, bovine α-crystallin was separated into its αA and αB subunits using semipreparative HPLC (Fig. 1). This method permits rapid and complete separation of the subunits with αB eluting at around 32 min followed by the major subunit, αA at 36 min. Although αA eluted as a broad multiplet, mass spectral analysis revealed that only native αA and its singly phosphorylated species (αA_p) were present in the peak, the heterogeneity most likely arising from slight differences in aggregation state under the HPLC conditions used.

The isolated αA was incubated with Kyn under argon, as described, for 48 h at pH 9.5. Adjustment to this pH was required to solubilise the αA-crystallin. The mass spectrum of αA after reaction with Kyn is depicted in Fig. 2. The two native species of αA-crystallin, αA and αA_p have masses of 19,832 and 19,912 Da, respectively [20]. In contrast, Kyn-modified αA contained 5 major species of higher molecular weight. Analysis of these peaks revealed that they arose from the addition of one, two and three 192 Da units, to each native species, respectively. A mass of 192 Da corresponds to the covalent addition of a deaminated Kyn moiety to the protein [16]. These results are summarised in Table 1. A comparison of the predicted and observed masses indicates that stoichiometric modification of αA by Kyn was observed by mass spectrometry of the whole protein. Due to the nonphysiological pH used in this reaction, identification of the sites of attachment was not pursued.

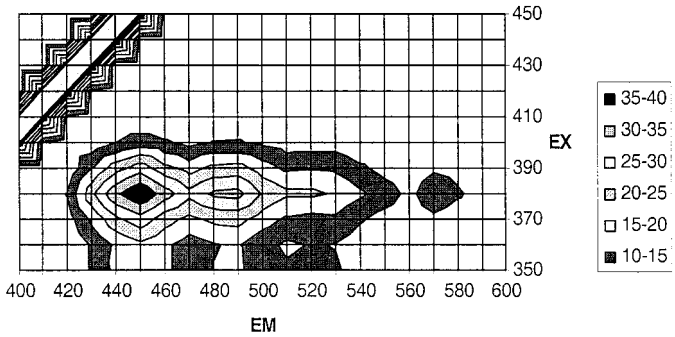

FIG. 5. 3D fluorescence spectrum of a 200 μg ml^{-1} solution of Kyn-modified α-crystallin in 100 mM phosphate buffer, pH 7.0. The covalent addition of Kyn to the protein resulted in both a shift of the EX/EM maxima of Kyn as well as an increased intensity.

Modification of αA-Crystallin by Kyn at pH 7.0

Modification of lens proteins by Kyn at neutral pH has not previously been examined in detail. Kyn deamination and conjugation with protein is known to be much more facile under basic conditions [16, 17]. Having ascertained the stoichiometry of binding at pH 9.5 for αA-crystallin, total α-crystallin was reacted with Kyn at pH 7.0 to compare the extent of reaction at physiological pH.

Mass spectra of total α-crystallin after 7 days of incubation in the absence and presence of Kyn are depicted in Fig. 3. The control incubation (Fig. 3A) exhibited four major peaks corresponding to the native and phosphorylated forms of both αA and αB subunits. This spectrum was identical to that obtained on the original unreacted α-crystallin (not shown). When Kyn was included in the incubation mixture, a new species was observed at mass 20,024 Da (Fig. 3B), corresponding to the addition of a single deaminated Kyn moiety to the αA subunit of α-crystallin (see Table 1). The peak at 20,237 Da arose from a diphosphorylated form of αB, however there was no evidence of significant attachment of Kyn to αB.

Although αA and αB are homologous proteins, inspection of the subunit amino acid sequences revealed that αA-crystallin contains one Cys, at residue 131, and αB-crystallin contains no Cys residues. Since Kyn had previously been observed to bind to either His, Lys or Cys residues at pH 9 [16], the observed modification pattern, and the distribution of Cys in α-crystallin suggested that this may be the residue to which Kyn was bound at pH 7. From examination of mass spectra, it appeared that ~50% of αA had been modified by Kyn after 7 days and ~60% after 14 days.

Proteolysis of Kyn-Modified α-Crystallin

Earlier work in our laboratory with the synthetic tripeptide SCR revealed that it was readily modified by the UV-filter, 3-hydroxykynurenine *O*-β-D-glucoside (3HKG). 3HKG has the same amino acid side chain as Kyn, deaminates and binds to proteins in the same manner. The HPLC purified, coloured product however, was found to be unstable to conditions normally used for tryptic digestion. This was readily demonstrated by analysing a small amount of the SCR-3HKG adduct by LC-MS prior to and after incubation in ammonium bicarbonate buffer, pH 8.1, for 24 h. The resultant chromatograms (Fig. 4) showed that SCR-3HKG was degraded, and this was found to be due to release of the 3HKG moiety. Figure 4A represents the single-ion chromatogram (SIC) for SCR-3HKG at *m/z* 734.6, as well as the 360 nm UV profile, prior to incubation. The diastereoisomers of the compound presumably gave rise to doublet peaks in each profile, since the mass spectra of both peaks were identical. Profiles for the sample after 24 h incubation (Fig. 4B) revealed the same doublet in the SIC; however, the intensity of the signal had decreased by an order of magnitude for an identical sample load, implying that the product had decomposed. This was reflected in the UV profile where a major peak was produced at 8.13 min which corresponded to released 3HKG. It was interesting to note that although it is the deaminated form of 3HKG which

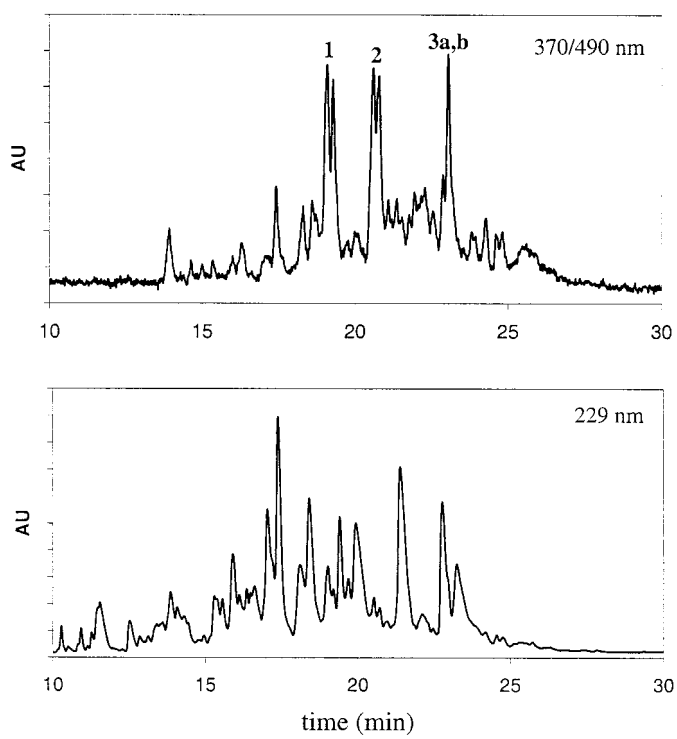

FIG. 6. HPLC chromatogram of 14-day Kyn-modified α-crystallin. The protein was digested with pepsin for 14 h prior to analysis. The lower profile monitored at 229 nm reflects the extent of protein digestion obtained with pepsin. The upper fluorescence profile exhibits numerous peaks, three of which (numbered), were collected for further analysis.

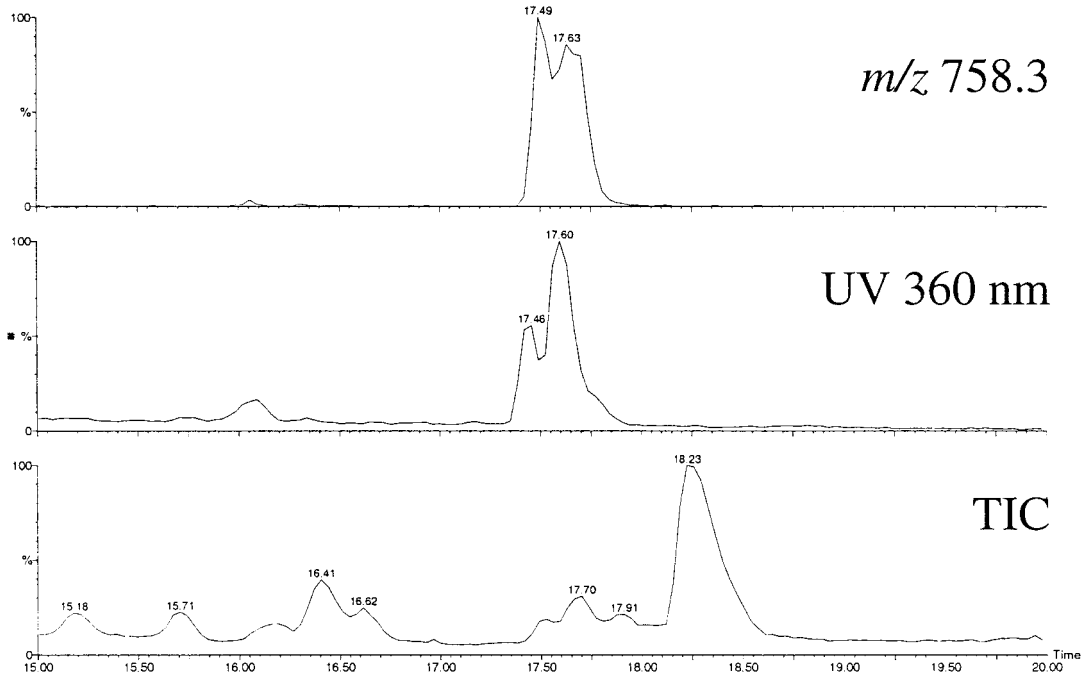

FIG. 7. LC–MS chromatogram of the 19-min peak labelled in Fig. 6. The UV profile exhibited a major 360-nm absorbing peak at 17.60 min, which corresponded to a peak in the TIC at 17.70 min. The major ion under this peak had an *m/z* of 758.3, as indicated in the selected ion chromatogram (top).

is bound to SCR [16], upon release in the ammonium bicarbonate buffer the reactant was reaminated to give the original 3HKG, as evidenced by the SIC for *m/z* 387 (Fig. 4C). A similar amination procedure was employed in the synthesis of 3HKG [19]. When the same experiment was conducted in Tris buffer (i.e., a buffer not containing ammonia), the unsaturated side chain, α, β-ketoalkene was evolved (not shown).

Studies in our laboratory have led to the synthesis of Kyn adducts of Cys, His and Lys, and confirmed both the instability of Kyn-Cys to neutral pH, and the stability of the Kyn adducts to acid pH [18]. For example, 50% of Kyn-Cys was found to be degraded after 18 h when incubated in pH 7.2 phosphate buffer at 37°C [18]. Thus the non-specific protease, pepsin, was employed to digest the Kyn-modified α-crystallin rather than trypsin which requires basic pH for activity.

In separate studies, we have shown that Kyn-His, Kyn-Lys and Kyn-Cys are coloured and fluorescent, with similar fluorescence spectra. In comparison, Kyn itself is barely fluorescent, while Kyn-modified α-crystallin (200 μg ml^{-1}) exhibits marked fluorescence maxima (Fig. 5). In order to specifically detect Kyn-modified peptides we took advantage of this fluorescence behaviour.

The desalted protein (2.0 mg) was digested with pepsin for 14 h at pH 2.0, to achieve maximum cleavage, after which the mixture was separated by RP-HPLC. The UV and fluorescence chromatograms of the pepsin digest mixture are shown in Fig. 6. The 229 nm UV profile (lower) is indicative of extensive proteolysis. The upper fluorescence chromatogram exhibited a number of peaks, three of which stood out as major components indicative of Kyn modification. These peaks, numbered 1, 2, and 3, were collected from 1.8 mg of total digest protein and lyophilised for subsequent analysis by LC-MS (Fig. 7).

The TIC trace (lower) for the LC-MS of peak 1 from Fig. 6, revealed that the collected material was still quite heterogeneous. The 360 nm UV trace (centre) however consisted of a small peak at 16.1 min and a major (doublet) peak at 17.60 min. The major ion under the corresponding portion of the TIC (17.70 min peak) was found to have a *m/z* of 758.3. The SIC for this species is shown in the upper trace.

The remainder of the sample was used for MS/MS analysis in an attempt to identify the sequence of this Kyn-modified digest fragment. The resultant spectrum (Fig. 8) displayed a b-ion series which corresponded to the sequential loss of the residues Ser-Leu-Ser from the molecular ion. This sequence corresponds to residues 132–134 of bovine αA-crystallin, which follow Cys131. Calculations for potential peptides around a Kyn-modified Cys131, having a *m/z* of 758.3, revealed that the fragment Ser-X-Ser-Leu-Ser-Ala (where X = Kyn modified Cys) would have the correct mass.

The major ion series from a theoretical MS/MS fragmentation of such a peptide is shown below.

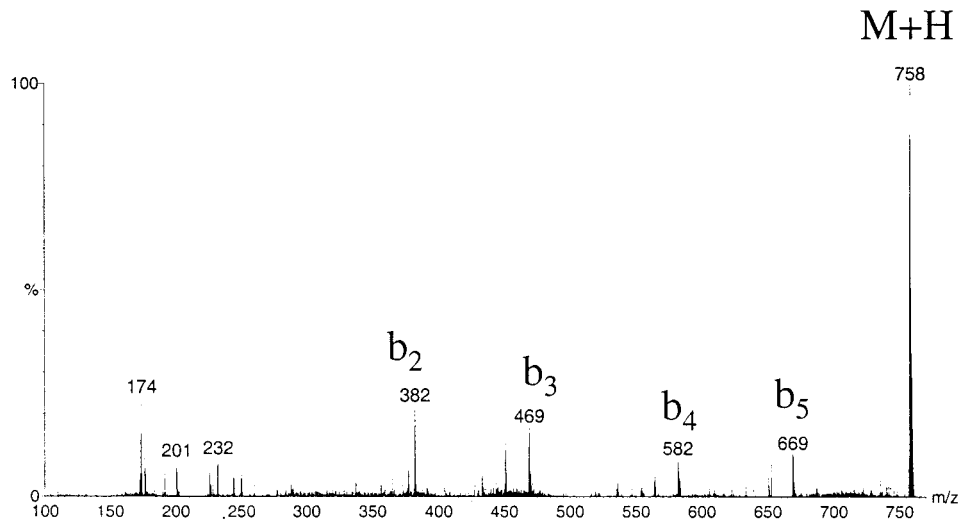

FIG. 8. MS/MS spectrum of the molecular ion at *m/z* 758.3 (Fig. 7). The ion was collisionally dissociated and gave rise to a b series of fragment ions (labelled) which corresponded to a peptide of sequence SXSLSA, where X indicates a Kyn-modified Cys residue.

b	88.0	382.1	469.1	582.2	669.3	—
	1	2	3	4	5	6
	Ser	X	Ser	Leu	Ser	Ala
	6	5	4	3	2	1
y″	758.3	671.3	377.2	290.2	177.1	90.1

Matching ions in the spectrum are appropriately labelled (Fig. 8). In the b series, fragment ions b_2 to b_5 are present in the spectrum. The y″ series is represented only by the molecular ion (y''_6). This is unusual in peptide fragmentation, but may be explained by a disturbance of the normal y″ series, due to the Kyn modification at Cys131. The ion at *m/z* 174.0 represents loss of water from the deaminated Kyn molecule, whereas loss of water from b_3 and b_4 accounts for the ions at *m/z* 451.2 and *m/z* 564.1, which is symptomatic of a Ser containing peptide.

A similar analysis of peaks 2 and 3 of the peptic digest (Fig. 6) permitted identification of these modified peptide fragments. Peak 2 was found to arise from the peptide LSXSLSA, whereas peak 3 was resolved into two coloured products by LC-MS, arising from the peptides XSLSADGML and LSXSLSADGM. These results are summarised in Table 2.

The detection of four Cys-Kyn containing peptides reflects the known lack of specificity of pepsin. However the sequence XSLSA is common to each peak, and since Cys is the only nucleophilic amino acid in these sequences, this, together with the MS/MS data, confirms that Cys131 is the site of *in vitro* modification in bovine α-crystallin by Kyn at physiological pH.

DISCUSSION

The results presented in this work highlight the importance of pH in directing the specificity of reaction of the UV-filter compound Kyn with protein. A previous study under more basic conditions suggested that a number of sites on bovine α-crystallin were modified by Kyn, including His and Lys residues [16]. In this work, a similar experiment revealed that purified αA-crystallin was modified by up to 3 Kyn molecules per protein subunit after 48 h (Table 1). When the experiment was repeated at neutral pH, however, with total α-crystallin, only a single modification was observed. This was despite quite major modification (~60%) of the protein by Kyn. The site of this modification was elucidated by peptide mapping using pepsin and LC-MS. This protease was used since Cys-Kyn adducts were found to be labile under the conditions used for site-specific proteases such as trypsin [18]. Cys 131 of the αA subunit of α-crystallin was identified as the site of modification by Kyn under neutral conditions. This reaction occurs under non-oxidative conditions, such as are found in the normal human lens.

The use of acidic digest conditions represents a novel approach to mapping post-translational modifications

TABLE 2

Pepsin Digest Sequence Data for Kyn-Modified α-Crystallin[a]

Peak number[b]	Fluorescent peptide			Identification	
1	S	XSLSA	—	αA	130–135
2	LS	XSLSA	—	αA	129–135
3a	—	XSLSA	DGML	αA	131–139
3b	LS	XSLSA	DGM	αA	129–138

[a] X refers to Cys131 which has been covalently modified by Kyn resulting in a net increase in mass of 192 Da.
[b] Peak numbers refer to Fig. 6.

in the lens, and may be useful in the identification of modified Cys residues in proteins from normal and cataractous human lenses. The pH of the normal lens is approximately 6.9 [21], thus the lability of the Cys-Kyn adduct in the lens may lead to other, as yet uncharacterised, modifications of lens proteins. This process of binding to and releasing from protein thiols may also contribute to an age-dependent decrease in the thiol content of crystallins.

ACKNOWLEDGMENT

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