Chapter 4

Does 3OHKyn Crosslink Lens Proteins?

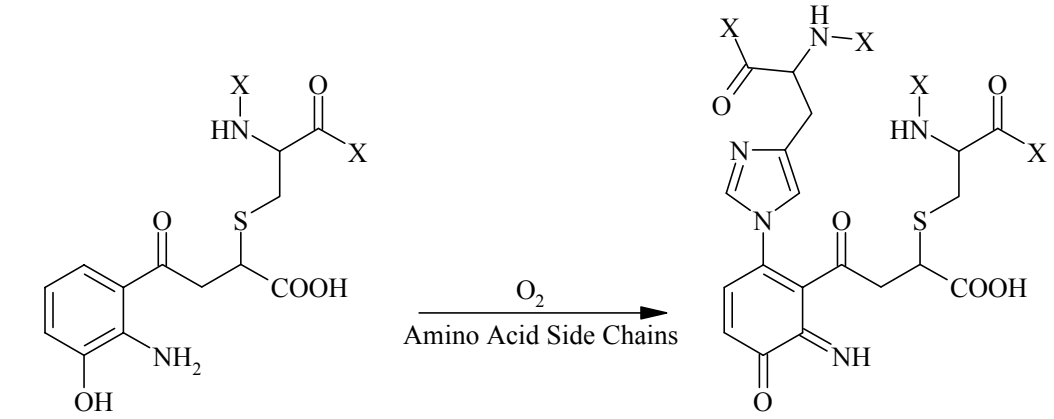
4.1 Introduction

The studies from Chapters 2 and 3 of this thesis have shown that 3OHKyn amino acid adducts are unstable at physiological pH, CLP oxidises when modified with 3OHKyn at pH 7.2, *i.e.* Met and Trp oxidation is observed analogous to the oxidations that occur in human cataractous lenses, and finally small amounts of 3OHKyn are attached to normal aged human lens protein. 3OHKyn is however, undetectable in human cataract lens proteins. These findings demonstrate that 3OHKyn may contribute to the onset of ARN cataract.

Crosslinking of lens polypeptides is also characteristic of ARN cataract.[166] Crosslinking involves disulphide bond linkages as well as various other types of crosslinking of unknown type. One possibility is that 3OHKyn could be involved in these crosslinks.

The hypothesis to be examined in this chapter, is that, 3OHKyn may crosslink proteins in human cataractous lenses. One way in which this could occur is via addition of nucleophilic amino acid residues to 3OHKyn amino acid adducts.

As a first step in testing this theory, 3OHKyn amino acid adducts were incubated in the presence of excess amino acids. The products were analysed by HPLC and mass spectrometry. CLP modified with 3OHKyn at pH 7.2 and pH 9.5 was also examined. An example of the type of crosslinks expected from the above incubations is shown in Scheme 4.1. The aromatic ring of 3OHKyn should oxidise and the quinoneimine be susceptible to nucleophilic attack of another amino acid, *para* to the carbonyl group.



e.g. Old Normal Lens Protein
(containing covalently bound 3OHKyn)

X - protein

e.g. Cataract Lens Protein

Scheme 4.1 Possible scheme for formation of 3OHKyn amino acid crosslinks.

4.2 Materials and Methods

4.2.1 Materials

All organic solvents and acids were HPLC grade (Ajax, Auburn, NSW, Australia). Milli-Q® water (purified to 18.2 MΩ/cm^2) was used in the preparation of all solutions. The amino acids (N-α-*t*-Boc-L-His, and N-α-*t*-Boc-L-Lys), formic acid, HCl (6 M, sequencing grade), 3OHKyn, guanidine HCl, TFA, thioglycolic acid and phenol were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Vivaspin (6 mL) protein concentrators (MW cut off 10,000 Da) were purchased from Vivascience.

4.2.2 Incubations with 3OHKyn-*t*-Boc-His and 3OHKyn-*t*-Boc-Lys

3OHKyn-*t*-Boc-His/3OHKyn-*t*-Boc-Lys (0.2 mg) was dissolved in 0.1 M phosphate buffer, pH 7.2 (4 mL). The α-amino-blocked amino acid N-α-*t*-Boc-His/N-α-*t*-Boc-Lys was added to the buffer in 20-fold molar excess (see Table 4.1), and chloroform (20 μL) was added to inhibit bacterial growth. The solutions were sealed, wrapped in foil and incubated for 12 days at 37^{0}C. Aliquots were taken every 3 days, and examined by HPLC.

Control experiments using 3OHKyn-*t*-Boc-His and 3OHKyn-*t*-Boc-Lys, without the addition of excess amino acids (see Table 4.1), were incubated in 0.1 M phosphate buffer as controls.

After the above reaction mixtures were incubated for 12 days, they were acidified with 1% (v/v) aqueous formic acid and purified through a Waters Vac 1cc 100 mg tC18 Sep Pak. The following mobile phase conditions were used: solvent A (aqueous 1% (v/v) formic acid) for 2 mL, then the sample was loaded and washed with 2 mL of solvent A to remove salts, then the sample was eluted with 1 mL solvent B (80% (v/v) ACN/H_20, 1% (v/v) formic acid). Samples were then freeze dried and hydrolysed in an evacuated hydrolysis tube with 6 M HCl (1 mL), thioglycolic acid (5% v/v) and phenol (1% w/v) for 24 hours at 110^{0}C. Following hydrolysis, the mixture was freeze dried, dissolved in 0.1% (v/v) TFA and purified by HPLC.

Table 4.1 Combination of incubations undertaken with the 3OHKyn amino acid adducts.

Crosslink Combination Incubations			
3OHKyn Adducts	**Excess Amino Acids**		
	N-α-*t*-Boc-His	**N-α-*t*-Boc-Lys**	**No Amino Acids**
3OHKyn-*t*-Boc-His	✓	✓	control
3OHKyn-*t*-Boc-Lys	✓	✓	control

4.2.3 Incubation of CLP Modified by 3OHKyn at pH 7.2

CLP (160 mg) or CLP modified by 3OHKyn at pH 7.2 (160 mg), or CLP modified by 3OHKyn at pH 9.5 (160 mg) was dissolved in 6 M guanidine HCl (5.6 mL) and 0.1 M phosphate buffer, pH 7.2 (2.4 mL), and chloroform (100 μL) was added to inhibit bacterial growth. The pH was adjusted to 7.2 with 4 M NaOH if necessary. The solutions were sealed, wrapped in foil and incubated (for 15 or 10 days) at 37^0C. Aliquots were taken for 3-D fluorescence analysis, acid hydrolysis and SDS-PAGE analysis.

Aliquots for 3-D fluorescence measurements were ultra-filtered (4^0C) in a Vivaspin concentrator (MW cut off 10,000 Da) to remove non-covalently bound material prior to analysis.

Aliquots for acid hydrolysis were ultra-filtered (4^0C) in a Vivaspin concentrator (MW cut off 10,000 Da) and the protein washed with 1 mL H_20 (6000 *g*, 4^0C). The protein was freeze dried and hydrolysed in an evacuated hydrolysis tube with 6 M HCl (1 mL), thioglycolic acid (5% v/v) and phenol (1% w/v) for 24 hours at 110^0C. Following hydrolysis, the mixture was freeze dried, dissolved in 0.1% (v/v) TFA and purified by HPLC. The filtrate was also examined by HPLC.

4.2.4 SDS-PAGE

See Section 3.2.18 for details.

4.2.5 HPLC

See Section 2.2.9 for details.

4.2.6 Mass Spectrometry

See Section 2.2.10 for details.

4.2.7 Tandem Mass Spectrometry (MS/MS)

See Section 2.2.11 for details.

4.2.8 Fluorescence and UV-visible Spectroscopy

See section 2.2.14 for details.

4.2.9 NMR Spectroscopy

See section 2.2.13 for details.

4.3 Results

4.3.1 Incubations with the 3OHKyn-*t*-Boc-His Adduct

Control Experiment with 3OHKyn-t-Boc-His

In an attempt to synthesise 3OHKyn crosslinked compounds, a control study was first undertaken. 3OHKyn-*t*-Boc-His was incubated at pH 7.2 for 12 days at 37^0C, in the absence of excess amino acids. Aliquots of the reaction mixture were taken every 3 days and examined by HPLC, and analysed by mass spectrometry. The HPLC chromatogram of the initial reaction mixture is shown in Figure 4.1A. The large peak eluting at 35 min is 3OHKyn-*t*-Boc-His.

Figure 4.1B is the HPLC profile of the reaction mixture after 12 days of incubation. Numerous peaks were observed. A large peak eluting at 32.5 min (peak 1) revealed a molecular ion of *m/z* 777 (Figure 4.2A). MS/MS of this ion (spectrum not shown) showed fragment ions at *m/z* 565, 487, 438, 263, 259, and 135. This compound could not be structurally identified on the basis of mass spectrometry data alone, since none of these fragment ions are typical of 3OHKyn UV filter adducts. The next major peak eluted at 33.5 min (peak 2). The molecular ion appeared to be *m/z* 650 (Figure 4.2B). The minor peak eluting at 34.9 min (peak 3) could not be identified from the mass spectrum (Figure 4.2C). This peak was also observed in Figure 4.1A, and this minor compound appears to be an impurity from the synthesis of 3OHKyn-*t*-Boc-His, which is also stable to oxidation. A small amount of 3OHKyn-*t*-Boc-His eluted at 35.4 min (labeled X on Figure 4.1B). The peak eluting at 39.7 min (peak 4) exhibited an ion of *m/z* 664 (Figure 4.2D), (a *m/z* 664 ion had been observed in Chapter 2 Section 2.3.8) and the MS/MS fragment ions (spectrum not shown) are identical to the compound that has been identified as 1,3,4,5-tetrahydro-11-(2-(N-α-*tert*-butyloxycarbonyl-histidyl)-(4-hydroxy-1,4-dioxo-butanyl)-1,5-dioxo-2H-pyrido(3,2-a)phenoxazine-3-carboxylic acid (Chapter 2). The peak eluting at 40.6 min (peak 5) also contained a molecular ion *m/z* 664 (spectrum not shown).

Figure 4.1C is the HPLC profile of the reaction mixture after 12 days of incubation, monitoring absorbance at 440 nm. Phenoxazone compounds absorb highly at 440 nm.[184] Examination of Figure 4.1B and Figure 4.1C shows that peaks 1, 2, 4 and 5 (Figure

4.1B) exhibit absorbances at both 360 and 440 nm. This indicates that these products may contain a phenoxazone within their structure. However, at this stage it is difficult to elucidate a structure for compounds based on mass spectral data alone.

Figure 4.3 shows a graph of the rate of formation of the unknown compounds in peaks 1, 2, 3, 4, and 5 over the 12 day incubation period. Compounds 1 and 2 steadily increase in yield over the 12 days, compound 3 remains constant, and compounds 4 and 5 decrease in yield after 3 days of incubation. 3OHKyn-*t*-Boc-His decreased steadily over time, and at 12 days only a small amount of this compound remained.

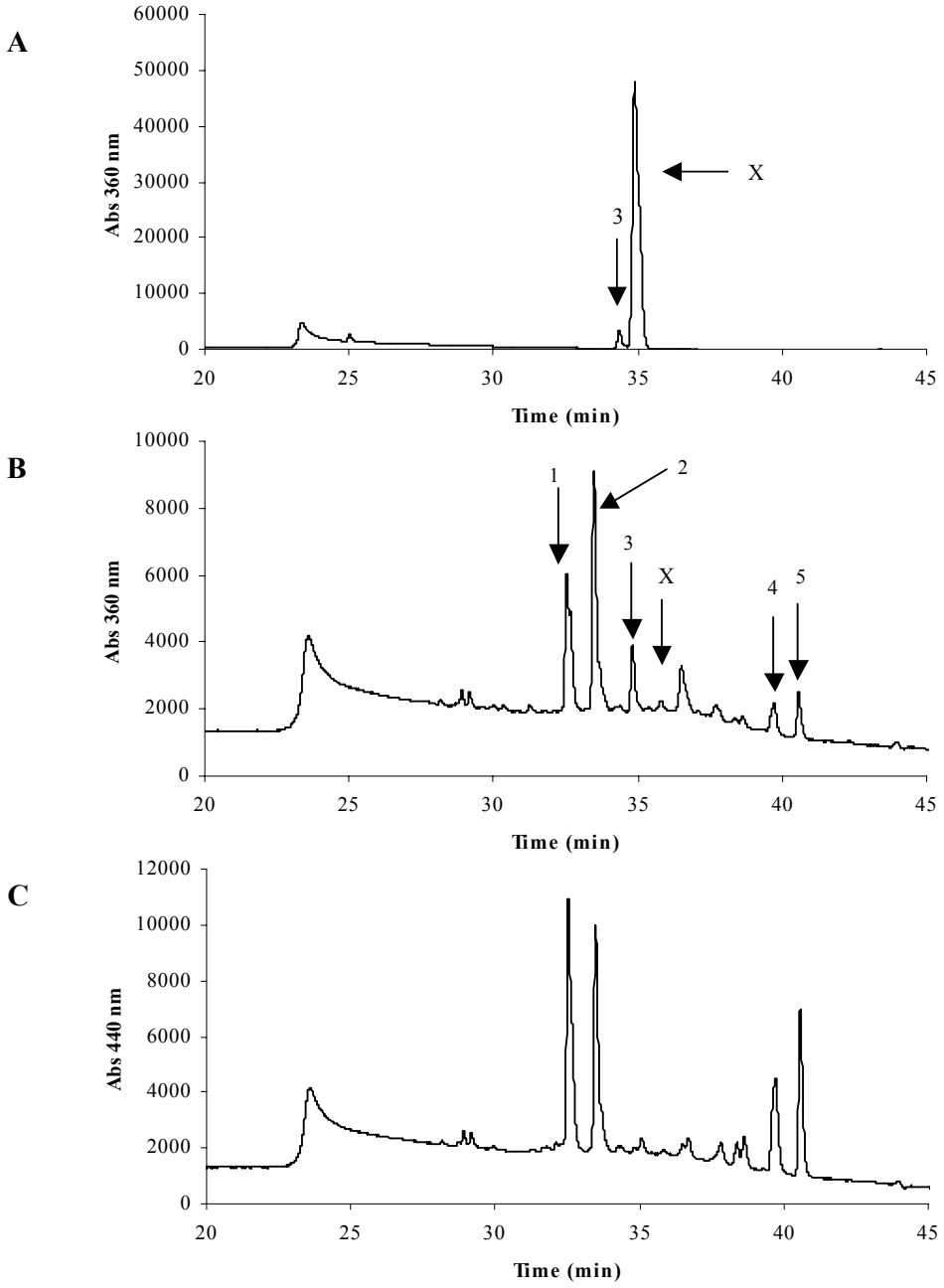


Figure 4.1 HPLC separation of 3OHKyn-*t*-Boc-His incubated at pH 7.2. X is 3OHKyn-*t*-Boc-His. Peak 3 is an impurity stable to oxidation. *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation. Arrowed peaks were collected for mass spectrometry. Absorbance monitored at 360 nm; *C*, Absorbance monitored at 440 nm.

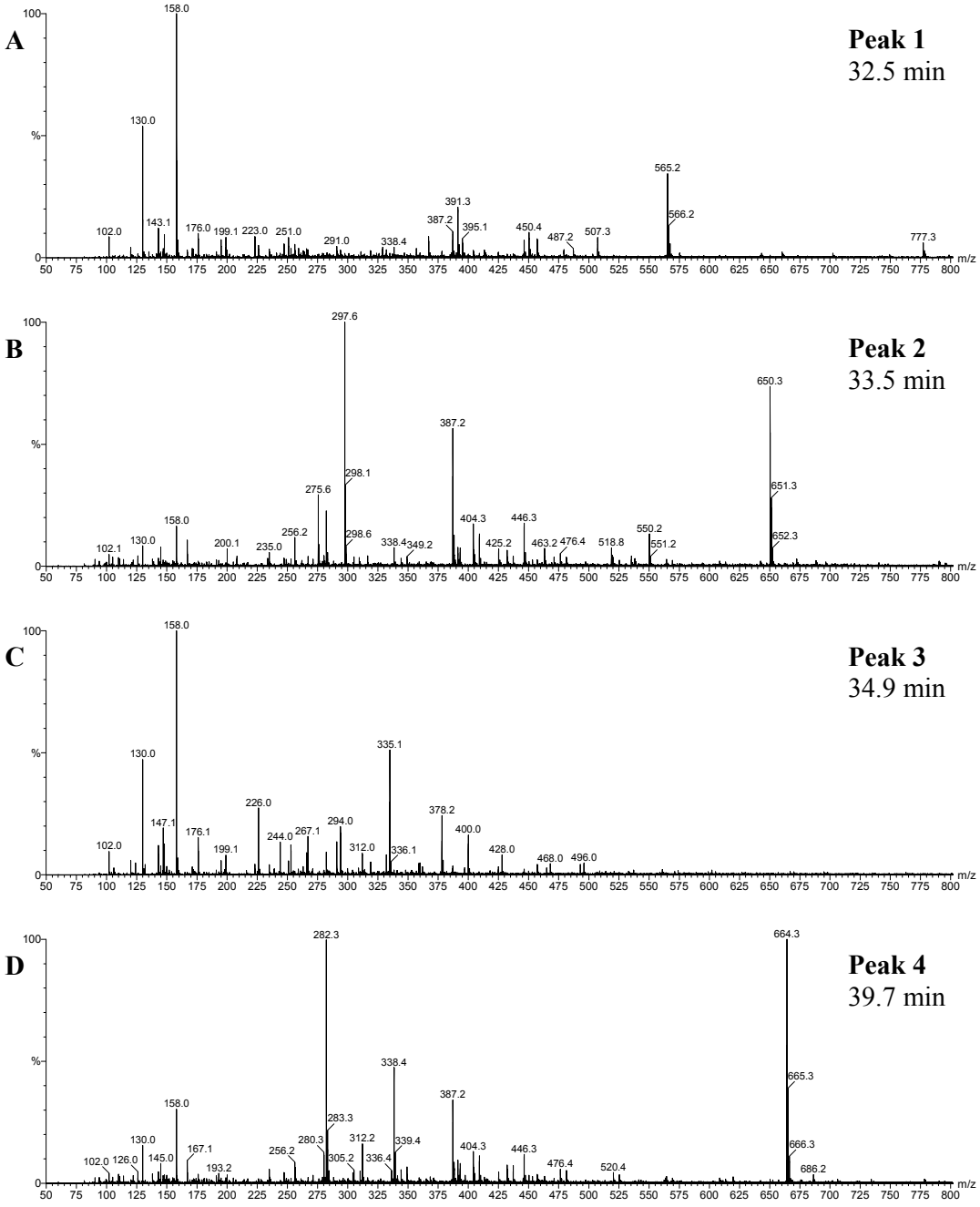


Figure 4.2 ESI mass spectra of peaks eluting from HPLC chromatogram in Figure 4.1B.

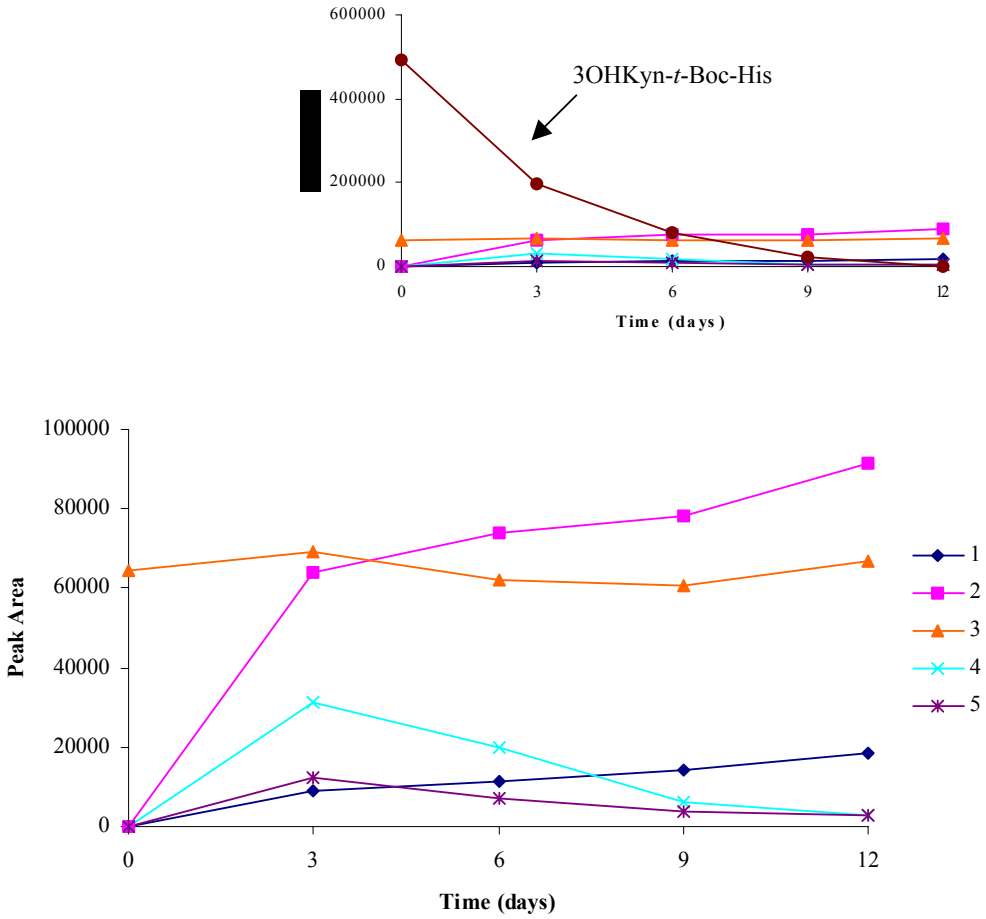


Figure 4.3 Rate of formation of unknown products in peaks 1, 2, 3, 4, and 5 from Figure 4.1B. The inset shows the rate of loss of 3OHKyn-*t*-Boc-His, in relation to the formation of products. Peak 3 is an impurity from the synthesis of 3OHKyn-*t*-Boc-His, which is stable to oxidation.

Following the 12 day incubation period, the remaining mixture was washed through a Sep Pak to remove the salts from the mixture. The mixture, which eluted from the column, was dried and hydrolysed with acid and antioxidants, since this method could also be used for detecting crosslinked compounds in lens proteins. The hydrolysate was separated by HPLC, and the peaks were monitored at 360 nm and 440 nm, since phenoxazones absorb at 440 nm. The HPLC chromatogram of the hydrolysed reaction mixture of 3OHKyn-*t*-Boc-His following 12 days of incubation is shown in Figure 4.4. Figure 4.4A shows the absorbance monitored at 360 nm, and Figure 4.4B shows the absorbance monitored at 440 nm.

A peak eluting at 24.7 min (peak 1') in Figure 4.4A was identified as 3OHKyn-His, since the ion *m/*z 363 in Figure 4.5A yielded the fragment ions (spectrum not shown) for authentic 3OHKyn-His. This peak was not observed in Figure 4.4B since 3OHKyn-His does not absorb at 440 nm. A major doublet (peak 2') eluted at 29.0 min, and absorbed highly at 360 and 440 nm, and had a molecular ion of *m/z* 550 (Figure 4.5B). Peak 3' (32.5 min) also eluted as a doublet, and exhibited an abundant ion at *m/z* 487 (Figure 4.5C). Finally peak 4' eluted at 34.7 min, and had an observed molecular ion of *m/z* 764 (Figure 4.5D). The structures of these peaks could not be elucidated from the mass spectral data.

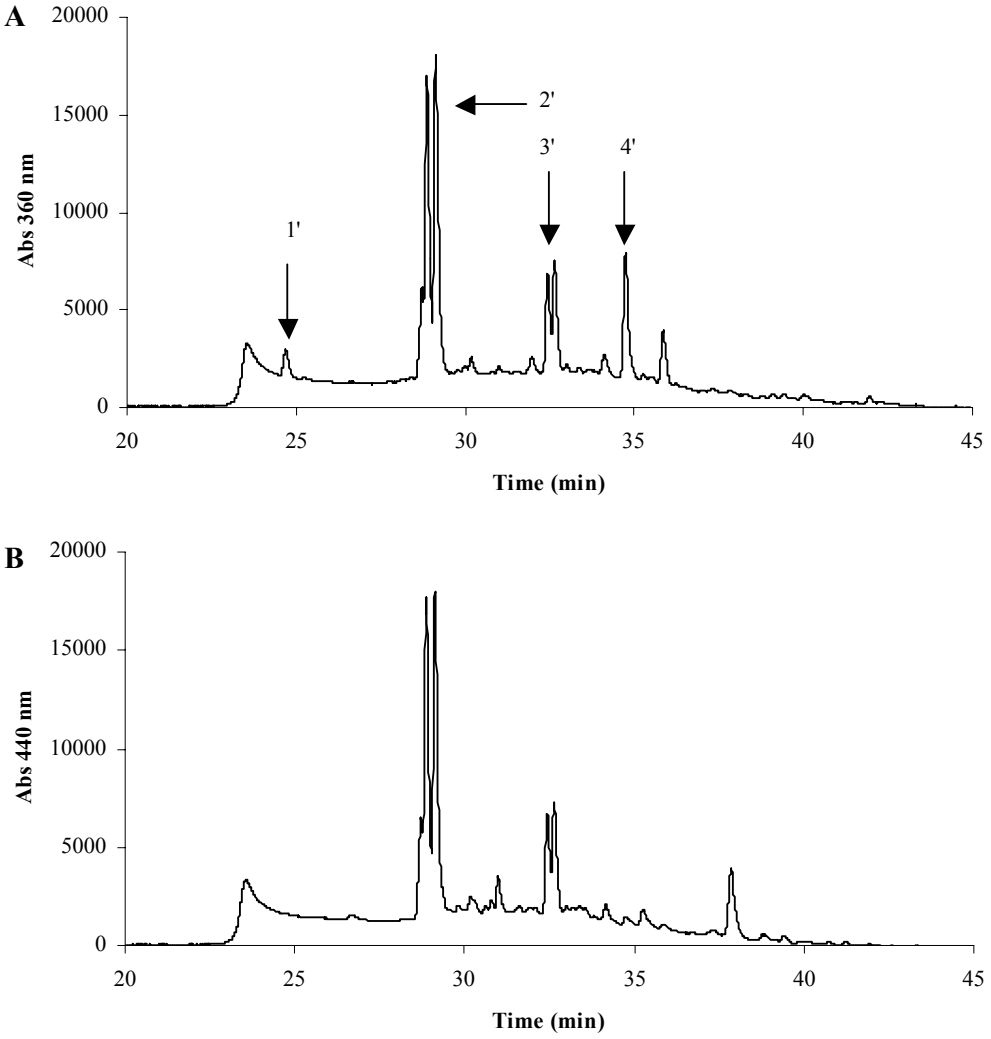


Figure 4.4 HPLC of the acid digest of 3OHKyn-*t*-Boc-His following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm. Peaks 1, 2, 3, and 4 were collected for mass spectral analysis. *B*, Absorbance monitored at 440 nm.

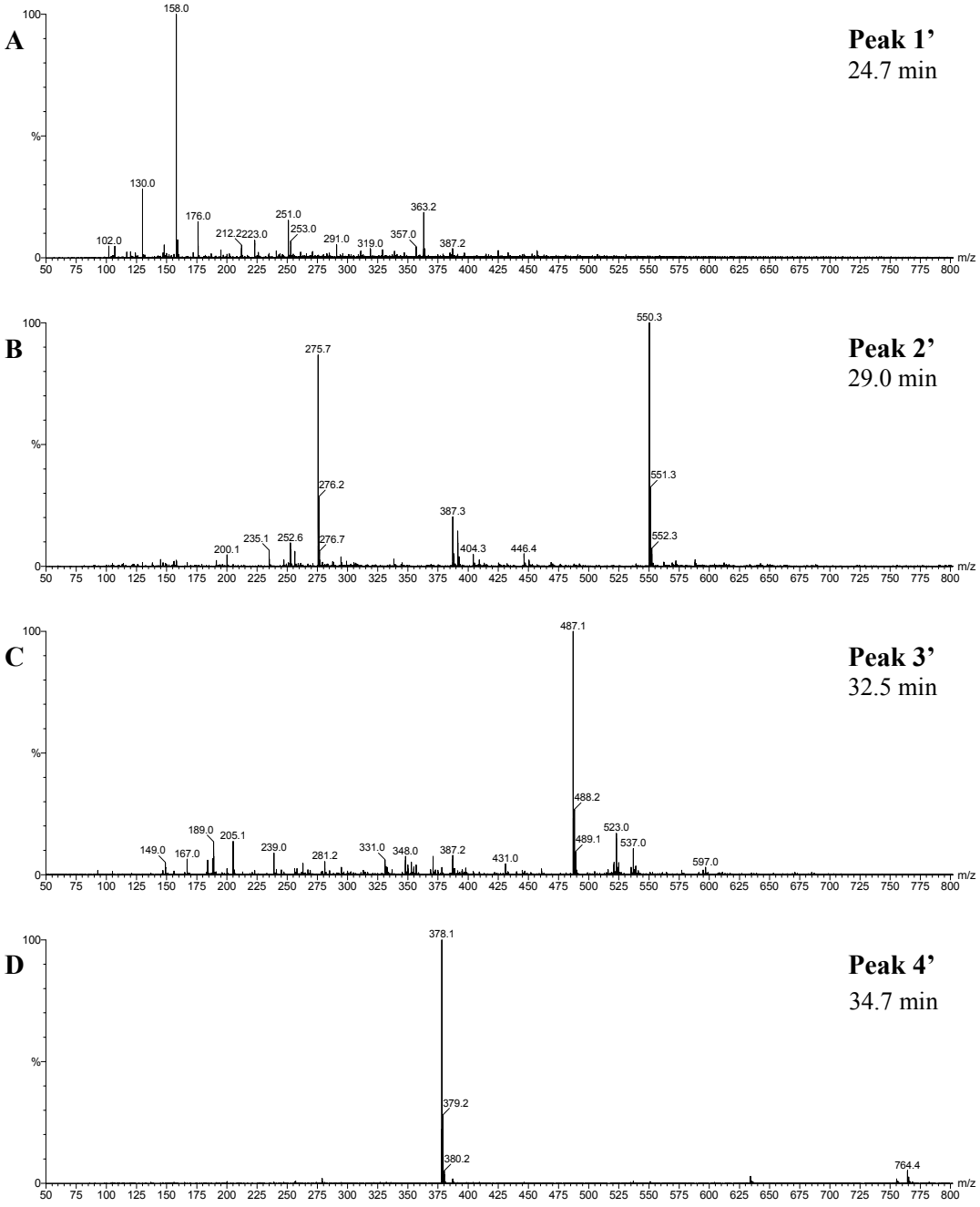


Figure 4.5 ESI mass spectra of peaks eluting from the HPLC chromatogram in Figure 4.4A.

Incubation of 3OHKyn-t-Boc-His with excess N-α-t-Boc-His

The objective in this section was to form crosslinked compounds, in which, 3OHKyn was crosslinked to other nucleophilic amino acids. Therefore, 3OHKyn-*t*-Boc-His was incubated with a 20-fold molar excess of N-α-*t*-Boc-His at pH 7.2 for 12 days. Aliquots of the reaction mixture were again taken every 3 days, and purified by HPLC, and analysed by mass spectrometry. The HPLC chromatogram of the initial reaction mixture is shown in Figure 4.6A. The large peak eluting at 35.2 min is authentic 3OHKyn-*t*-Boc-His. Figure 4.6B is the HPLC profile of the reaction mixture after 12 days of incubation. This profile was surprisingly, essentially identical to that of Figure 4.1B. In Figure 4.6B peaks eluted at 32.5 min, 33.5 min, 34.9 min, 39.7 min and 40.6 min. Mass spectral analysis of each of these peaks (spectra not shown) showed that they were each, the same as those shown in Figure 4.2.

Similarly, the reaction mixture after 12 days of incubation was applied to a Sep Pak, and the 80% ACN eluate was dried, and acid hydrolysed with antioxidants. Peaks from the HPLC were monitored at 360 nm and 440 nm. The HPLC chromatogram of the hydrolysed reaction mixture of 3OHKyn-*t*-Boc-His incubated with excess N-α-*t*-Boc-His is shown in Figure 4.7. Figure 4.7A exhibits the absorbance monitored at 360 nm, and Figure 4.7B exhibits the absorbance monitored at 440 nm. The HPLC profiles of Figure 4.7 are essentially identical to those of Figure 4.4. In Figure 4.7A peaks eluted at 25.0 min, 28.6 min, 32.5 min and 34.7 min. Mass spectral analysis of each of these peaks (spectra not shown) showed that they were each the same as those shown in Figure 4.5.

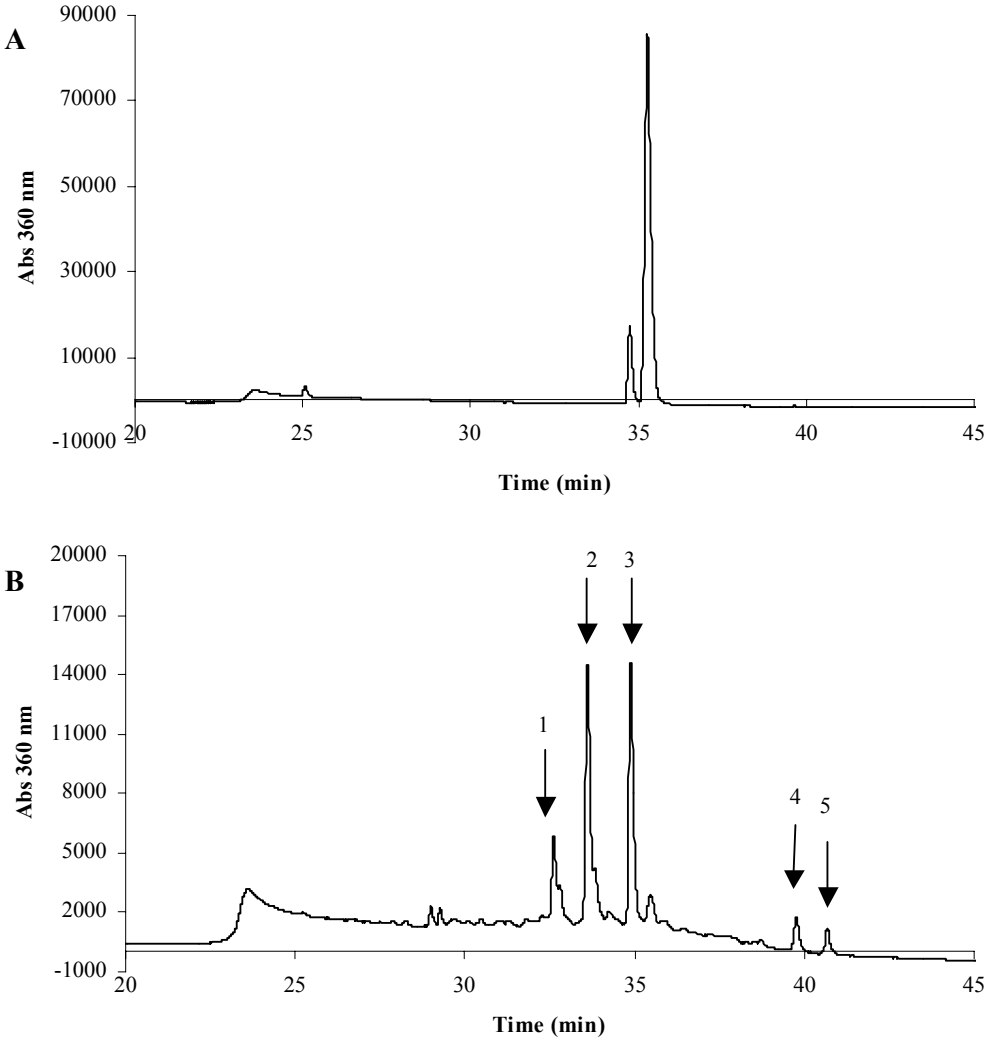


Figure 4.6 Incubation of 3OHKyn-*t*-Boc-His with a 20-fold molar excess of N-α-*t*-Boc-His for 12 days. HPLC chromatograms of, *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation. Peak 1 eluted at 32.5 min, peak 2 at 33.5 min, peak 3 at 34.9 min, peak 4 at 39.7 min and peak 5 at 40.6 min (identical to Figure 4.1B).

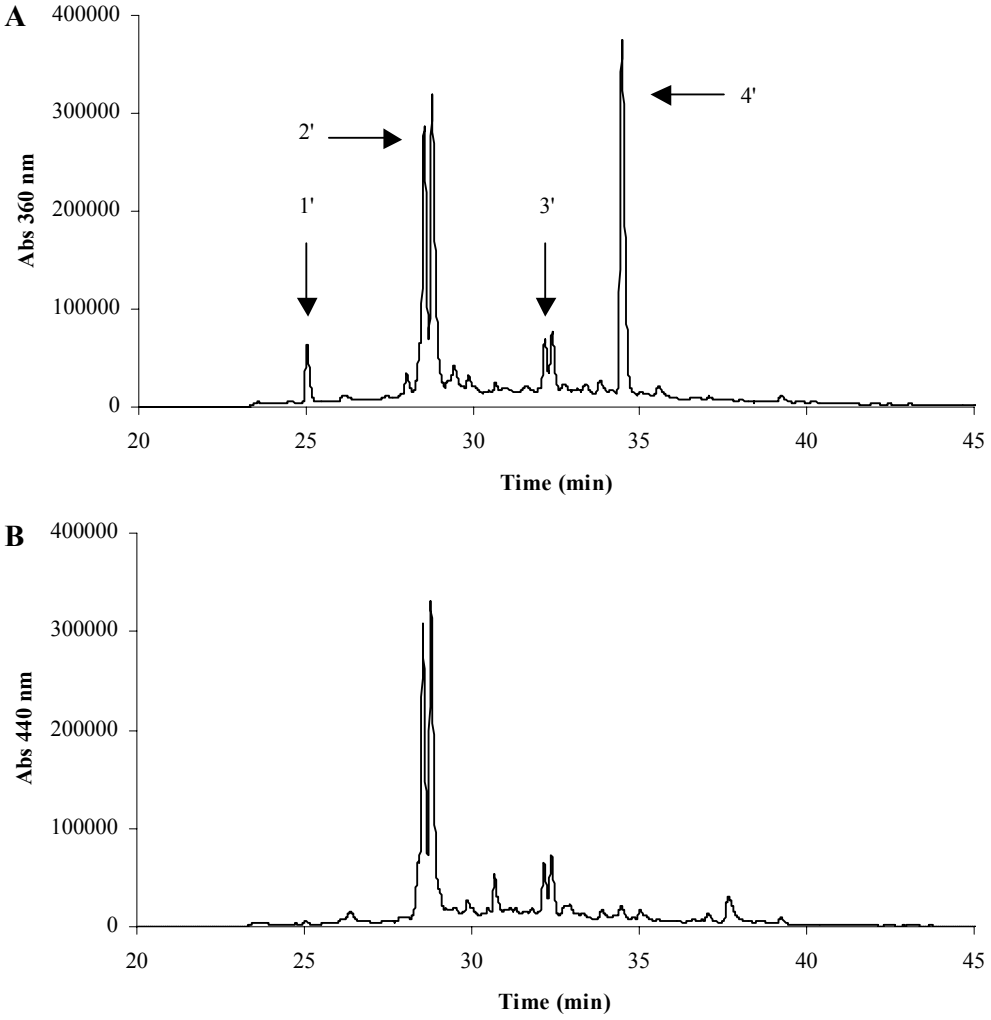


Figure 4.7 HPLC of the acid digest of 3OHKyn-*t*-Boc-His with a 20-fold molar excess of N-α-*t*-Boc-His following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm. Peak 1 eluted at 25.0 min, peak 2 at 28.6 min, peak 3 at 32.5 min and peak 4 at 34.7 min. *B*, Absorbance monitored at 440 nm.

Incubation of 3OHKyn-t-Boc-His with excess N-α-t-Boc-Lys

3OHKyn-*t*-Boc-His was also incubated with a 20-fold molar excess of N-α-*t*-Boc-Lys at pH 7.2 for 12 days. Aliquots of the reaction mixture were again taken every 3 days, and purified by HPLC, and analysed by mass spectrometry. The HPLC chromatogram of the initial reaction mixture is shown in Figure 4.8A. The large peak eluting at 35.2 min is authentic 3OHKyn-*t*-Boc-His. Figure 4.8B is the HPLC profile of the reaction mixture after 12 days of incubation. This profile is essentially identical to that of Figure 4.1B and 4.6B. In Figure 4.8B, peaks eluted at 32.3 min, 33.2 min, 34.5 min 39.9 min and 40.3 min. Mass spectral analysis of each of these peaks (spectra not shown) showed that each spectrum was the same as the spectra shown in Figure 4.2.

Similarly, the reaction mixture after 12 days of incubation was applied to a Sep Pak, and the 80% ACN eluate was dried and acid hydrolysed with antioxidants. Peaks from the HPLC were again monitored at 360 nm and 440 nm. The HPLC chromatogram of the hydrolysed reaction mixture of 3OHKyn-*t*-Boc-His incubated with excess N-α-*t*-Boc-Lys is shown in Figure 4.9. Figure 4.9A exhibits the absorbance monitored at 360 nm, and Figure 4.9B exhibits the absorbance monitored at 440 nm. The HPLC profiles of Figure 4.9 are essentially identical to those of Figures 4.4 and 4.7. The peak eluting at 25.3 min in Figure 4.9A is 3OHKyn-His. In Figure 4.9A, major peaks also eluted at 28.7 min, 32.5 min and 34.6 min. Mass spectral analysis of each of these peaks (spectra not shown) showed that the spectra were the same as those shown in Figure 4.5.

In summary, these findings suggest that the presence of a 20-fold molar excess of N-α-*t*-Boc-His or N-α-*t*-Boc-Lys, seemed to have no effect on the products that formed on incubation of, 3OHKyn-*t*-Boc-His in the presence of air.

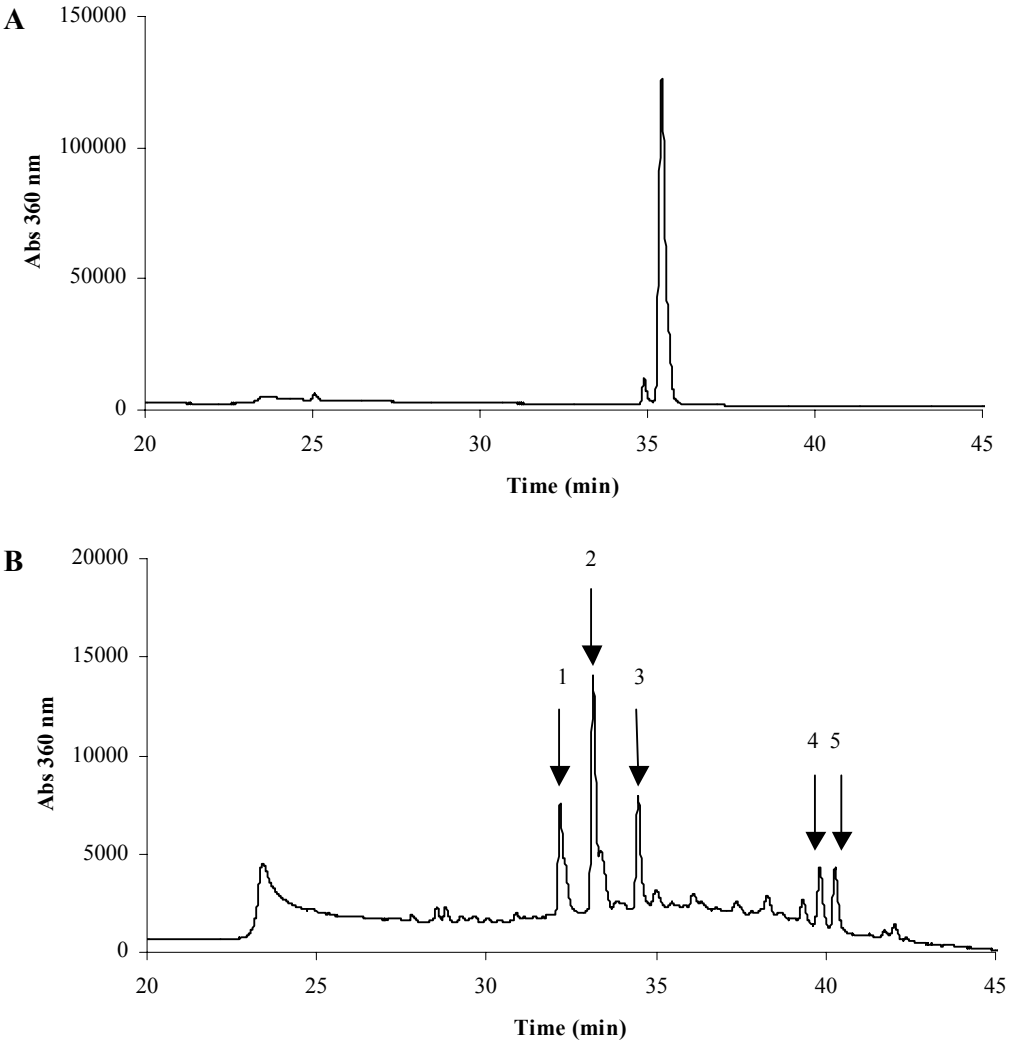


Figure 4.8 Incubation of 3OHKyn-*t*-Boc-His with a 20-fold molar excess of N-α-*t*-Boc-Lys for 12 days. HPLC chromatograms of, *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation. Peak 1 eluted at 32.3 min, peak 2 at 33.2 min, peak 3 at 34.5 min, peak 4 at 39.9 min and peak 5 at 40.3 min (~identical to Figure 4.1B).

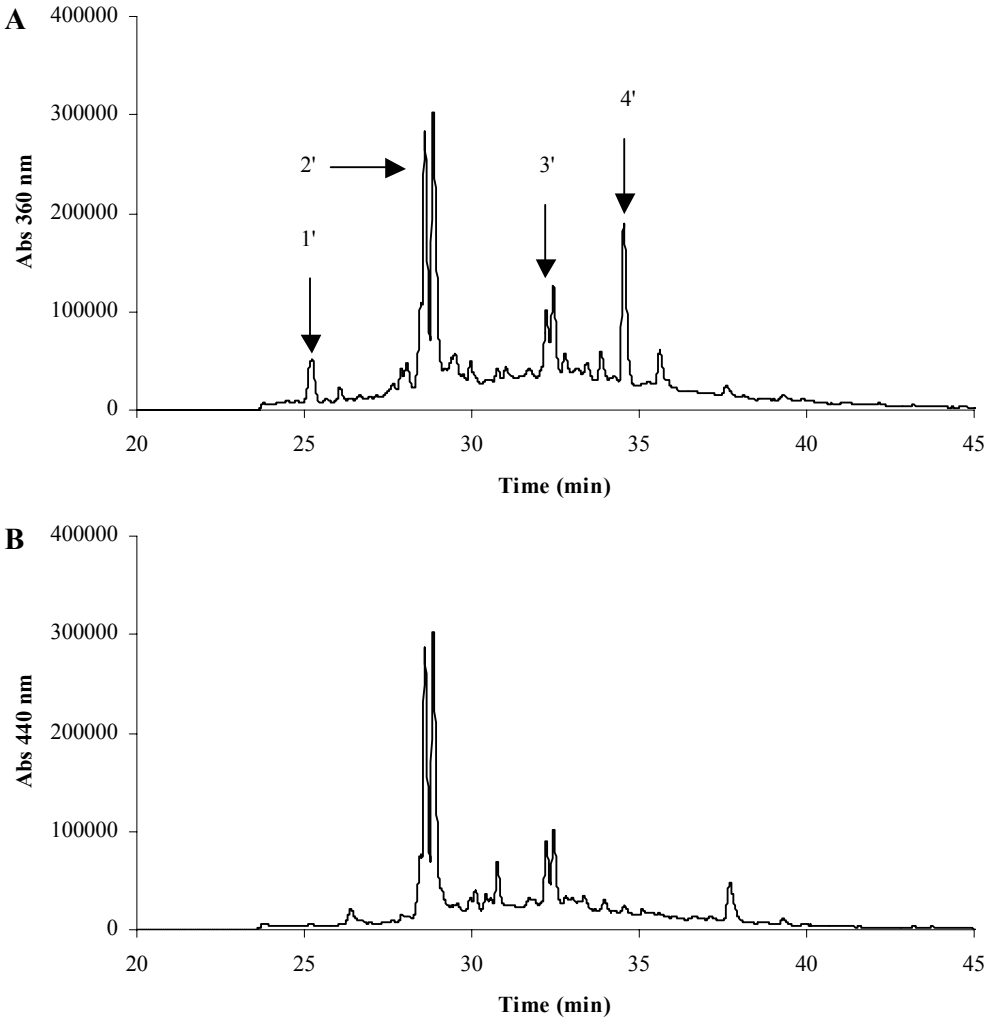


Figure 4.9 HPLC of the acid digest of 3OHKyn-*t*-Boc-His with a 20-fold molar excess of N-α-*t*-Boc-Lys following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm. Peak 1 eluted at 25.3 min, peak 2 at 28.7 min, peak 3 at 32.5 min and peak 4 at 34.6 min. *B*, Absorbance monitored at 440 nm.

Analysis of the Peak Eluting as a Doublet (Peak 2') from the HPLC Chromatograms of the Hydrolysed Reaction Mixtures

Analysis of the reactions involving 3OHKyn-*t*-Boc-His, both in the absence and presence of excess amino acids, all showed that following acid hydrolysis of the final reaction mixture, a prominent doublet (apparently a diastereoisomer) eluted at approximately 29 min, and absorbed at 360 and 440 nm. This compound was investigated further. The molecular ion of the doublet was identified, *m/z* 550 (Figure 4.5B). MS/MS (Figure 4.10) of this ion yielded fragment ions at *m/z* 506 (loss of 44 Da), 395 (further loss of 111 Da) and 351 (further loss of 44 Da).

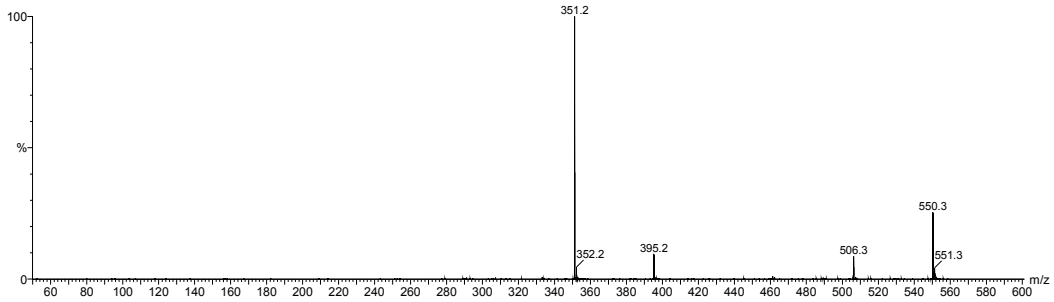


Figure 4.10 MS/MS spectrum of the molecular ion *m/z* 550, eluting as a doublet in the hydrolysed HPLC profiles, involving 3OHKyn-*t*-Boc-His incubations.

Following the 12 days of incubation of each reaction, the HPLC profiles (Figure 4.1B, 4.6B and 4.8B) all showed a large peak eluting at approximately 33.4 min (peak 2), with a molecular ion of *m/z* 650 (Figure 4.2B). Since this is a major peak, and also has a mass difference of 100 Da from the hydrolysed peak (*m/z* 550), it was assumed that the loss of 100 Da was a result of a *t*-Boc group attached to His, since acid readily cleaves the *t*-Boc group.[209] Therefore, in order to demonstrate that these two peaks were the same compound (± *t*-Boc group), the peak eluting at 33.4 min (peak 2) in Figures 4.1B, 4.6B and 4.8B, was collected, dried down to a minimum volume, and 6 M HCl was added, and the sample was incubated for 20 hours at 37^0C (these conditions cleave *t*-Boc groups[209]). Following this short incubation period, the sample was adjusted to pH 4 with NaOH, and examined by HPLC. A doublet eluted at 29 min (Figure 4.11), and mass spectral analysis of this doublet showed that the molecular ion was *m/z* 550

(spectrum not shown), and MS/MS of this ion yielded fragment ions *m/z* 506, 395 and 351 (spectrum not shown).

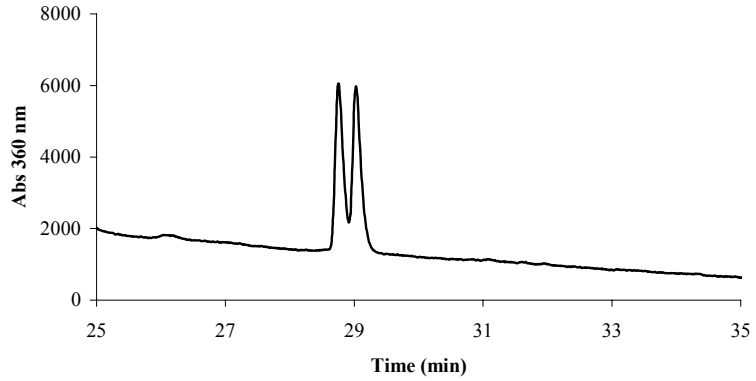


Figure 4.11 HPLC chromatogram of peak 2 (Figures 4.1B, 4.6B and 4.8B) following deprotection with acid.

As further support that the compound with the molecular ion *m/z* 650 (peak 2 Figures 4.1B, 4.6B and 4.8B) was indeed the precursor for the doublet with the molecular ion *m/z* 550 (peak 2' Figures 4.4A, 4.7A and 4.9A), a UV-visible spectrum of each (Figure 4.12) exhibited identical spectra, showing that they contained an identical chromophoric group. The UV-visible spectra also showed that these compounds absorbed most strongly at 408 nm and that 360 and 440 nm, that were used to monitor the peaks from the hydrolysates, were relatively minor wavelengths for the absorption of light.

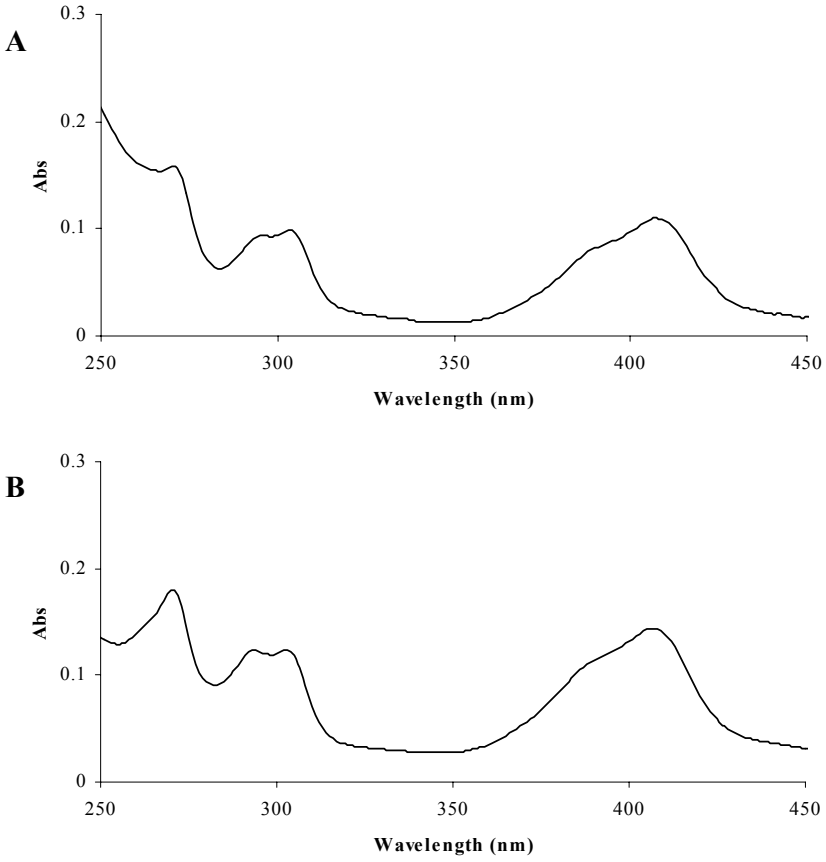


Figure 4.12 UV-visible spectra. *A*, Peak 2 (Figures 4.1B, 4.6B and 4.8B) (*m/z* 650); *B*, Peak 2' (Figures 4.4A, 4.7A and 4.9A) (*m/z* 550).

Phenoxazone compounds absorb at 440 nm on the UV-visible spectrum, however the diastereoisomer (doublet, peak 2') had a maximal absorbance at approximately 408 nm (Figure 4.12B), therefore it was of interest to try to determine the structure of this compound. A large-scale reaction was undertaken, and the product was purified by HPLC, and analysed by nano-NMR spectroscopy. The proton NMR spectrum is shown in Figure 4.13. The compound was analysed by the nano probe, since only 1 mg of sample was obtained after multiple semi-prep HPLC purification. The proton NMR was run for 2 hours, and after this time the spectrum showed the presence of numerous peaks in the aromatic region (*i.e.* 6.8 – 8.8 ppm), and there were various other peaks present. In particular, a large singlet was observed at 3.7 ppm but this was found to be a low molecular weight contaminant in the sample. A gCOSY experiment was undertaken however, after 6 hours of running the experiment there were no peaks present in the spectrum. The unknown compund could not be structurally elucidated from this proton NMR spectrum.

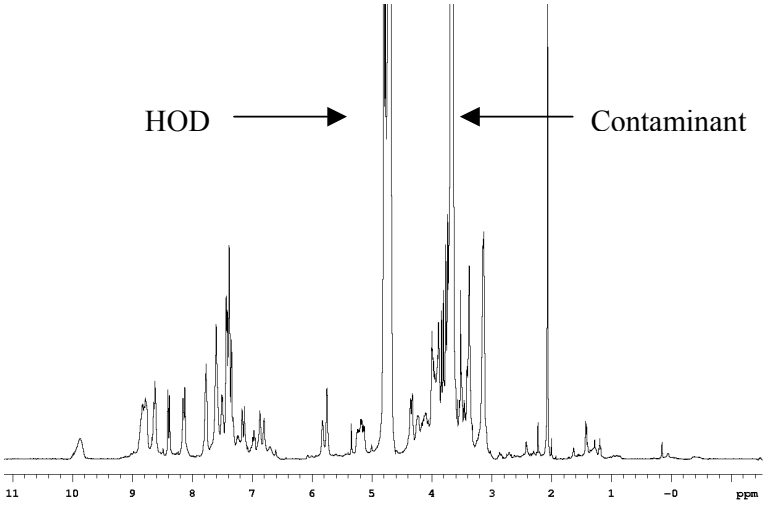


Figure 4.13 Proton NMR spectrum of peak 2' (Figures 4.4A, 4.7A and 4.9A) (*m/z* 550).

4.3.2 Incubations with the 3OHKyn-*t*-Boc-Lys Adduct

Control Experiment with 3OHKyn-t-Boc-Lys

Incubations were also undertaken with the 3OHKyn-*t*-Boc-Lys adduct, under the same conditions as those for 3OHKyn-*t*-Boc-His. A control experiment involving 3OHKyn-*t*-Boc-Lys was first undertaken. The HPLC chromatograms are shown in Figure 4.14. Figure 4.14A is the HPLC chromatogram of the initial reaction mixture. The peak eluting at 35.7 min is 3OHKyn-*t*-Boc-Lys. Figure 4.14B is the HPLC chromatogram of the reaction mixture after 12 days of incubation. The peaks eluting between 30 and 40 min have a low absorbance. The y-axis of these two chromatograms shows that the compounds eluting in Figure 4.14B are very low absorbing (*i.e.* compounds are all found close to the baseline of the chromatogram) at 360 nm. These compounds could not be identified.

The reaction mixture following 12 days of incubation was acid hydrolysed and the HPLC separation is shown in Figure 4.15. Numerous peaks eluted at 360 nm (Figure 4.15A), and very few low absorbing compounds eluted at 440 nm (Figure 4.14B). Previous studies have shown that 3OHKyn-*t*-Boc-Lys is unstable (Chapter 2), and in agreement with this, the compound was not observed after 12 days of incubation at pH 7.2.

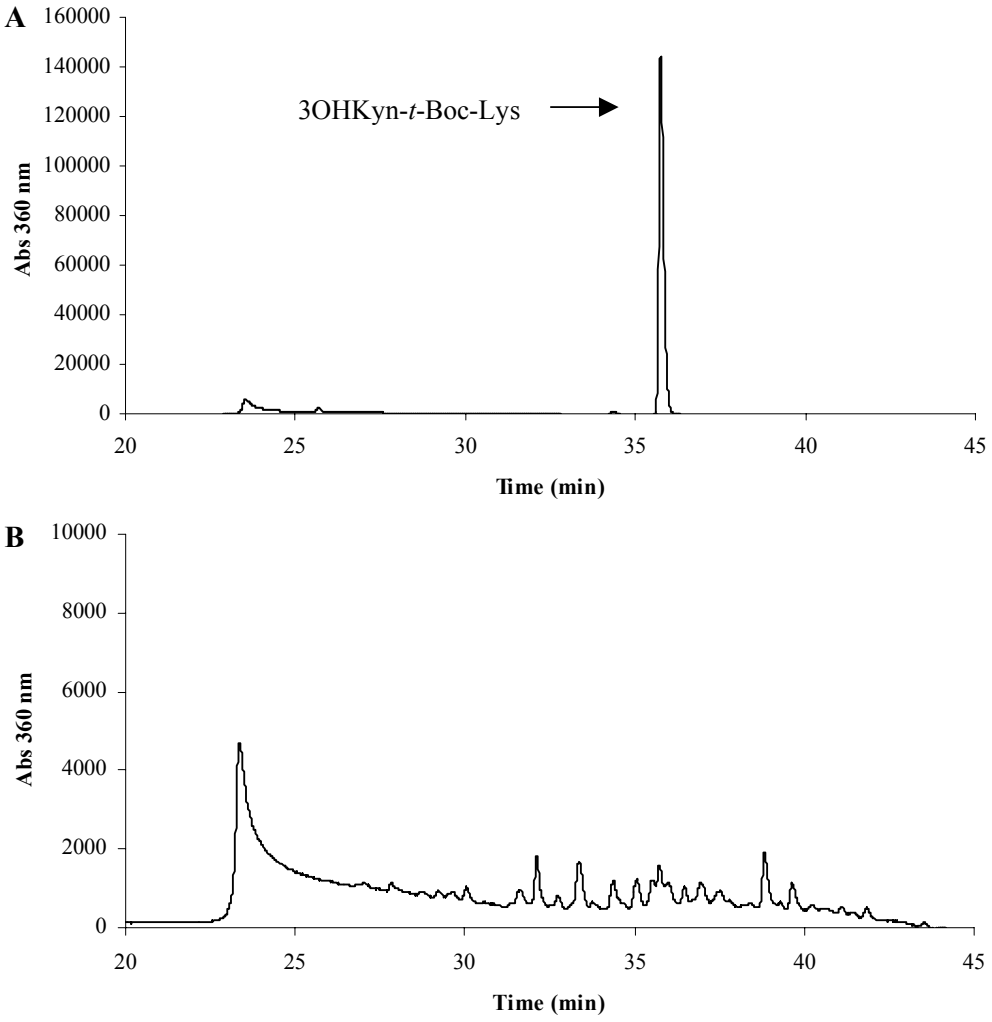


Figure 4.14 Incubation of 3OHKyn-*t*-Boc-Lys at pH 7.2. HPLC chromatograms, *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation.

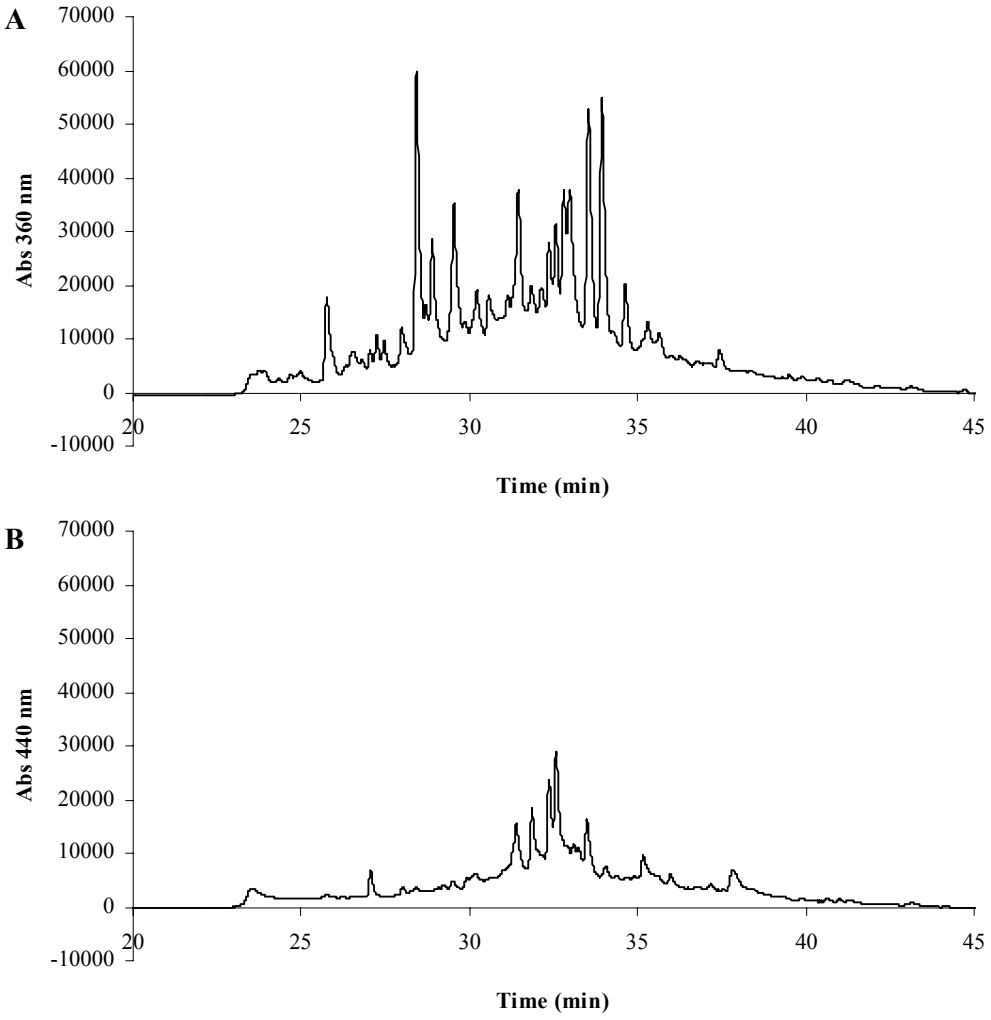


Figure 4.15 HPLC of the acid digest of 3OHKyn-*t*-Boc-Lys following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm; *B*, Absorbance monitored at 440 nm.

Incubation of 3OHKyn-t-Boc-Lys with excess N-α-t-Boc-His

3OHKyn-*t*-Boc-Lys was also incubated with a 20-fold molar excess of N-α-*t*-Boc-His at pH 7.2 for 12 days. Aliquots of the reaction mixture were again taken every 3 days and purified by HPLC. The HPLC chromatogram of the initial reaction mixture is shown in Figure 4.16A. The peak eluting at 36 min is authentic 3OHKyn-*t*-Boc-Lys. Figure 4.16B is the HPLC profile of the reaction mixture after 12 days of incubation. This profile is essentially identical to that of Figure 4.14B.

Following 12 days of incubation, the reaction mixture was applied to a Sep Pak and the 80% ACN eluate was dried and acid hydrolysed with antioxidants. The HPLC separation is shown in Figure 4.17. Numerous peaks eluted at 360 nm (Figure 4.17A), and very few low absorbing compounds eluted at 440 nm (Figure 4.17B).

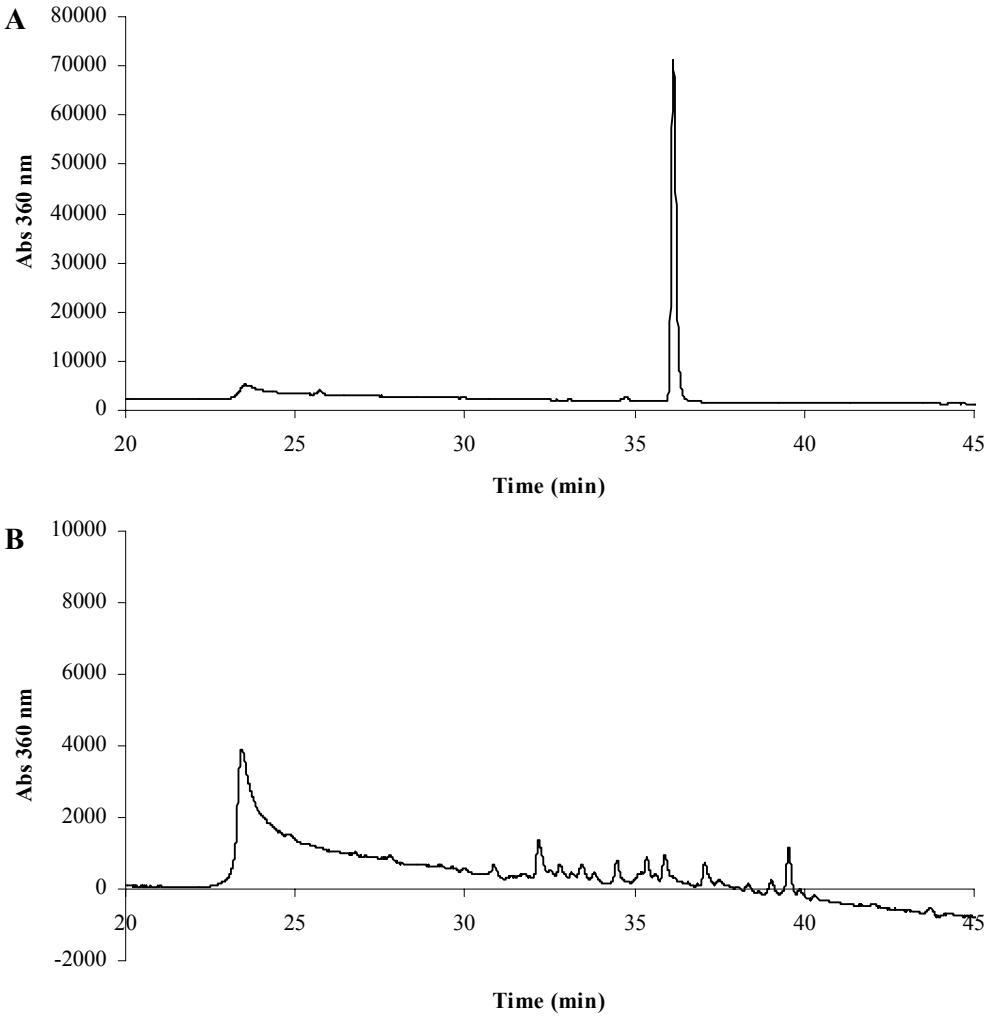


Figure 4.16 Incubation of 3OHKyn-*t*-Boc-Lys with a 20-fold molar excess of N-α-*t*-Boc-His for 12 days. HPLC chromatograms of, *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation.

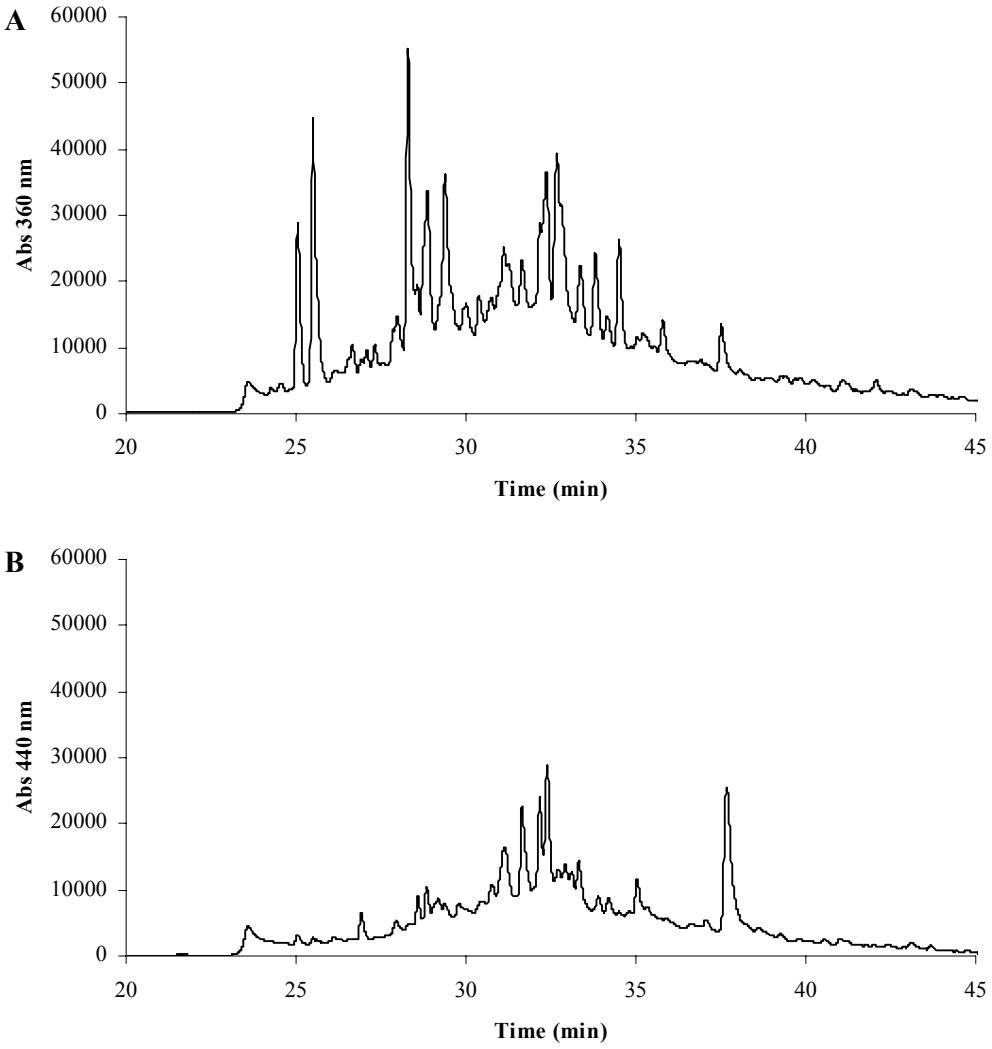


Figure 4.17 HPLC of the acid digest of 3OHKyn-*t*-Boc-Lys with a 20-fold molar excess of N-α-*t*-Boc-His following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm. *B*, Absorbance monitored at 440 nm.

Incubation of 3OHKyn-t-Boc-Lys with excess N-α-t-Boc-Lys

3OHKyn-*t*-Boc-Lys was incubated with excess N-α-*t*-Boc-Lys at pH 7.2 for 12 days. The HPLC chromatogram of the initial reaction mixture is shown in Figure 4.18A. The peak eluting at 37 min is 3OHKyn-*t*-Boc-Lys. Figure 4.18B is the HPLC profile of the reaction mixture after 12 days of incubation. This profile is essentially identical to that of Figure 4.14B and 4.16B.

Following 12 days of incubation, the reaction mixture was applied to a Sep Pak and the 80% ACN eluate was dried and acid hydrolysed with antioxidants. The HPLC separation is shown in Figure 4.19. Numerous peaks again eluted at 360 nm (Figure 4.19A), and very few compounds eluted at 440 nm (Figure 4.19B).

In summary, the HPLC chromatograms (Figures 4.14 to 4.19, inclusive) from incubations of 3OHKyn-*t*-Boc-Lys in the absence and presence of excess amino acids established that it would be a very difficult task to try and structurally identify these numerous minor compounds eluting in each chromatogram. The chromatograms of the hydrolysates were clearly much more complex than those observed for the 3OHKyn-*t*-Boc-His incubations.

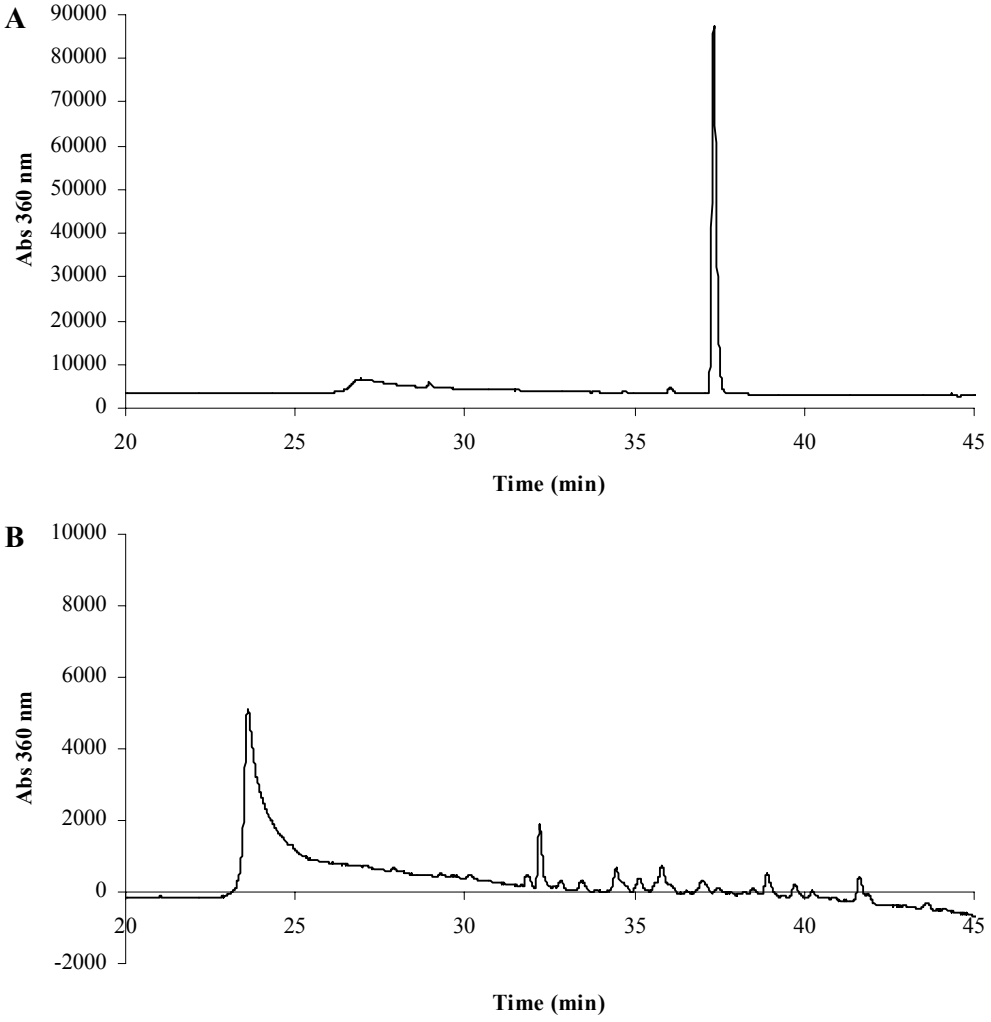


Figure 4.18 Incubation of 3OHKyn-*t*-Boc-Lys with a 20-fold molar excess of N-α-*t*-Boc-Lys for 12 days. HPLC chromatograms of, *A*, Initial reaction mixture; *B*, Reaction mixture after 12 days of incubation.

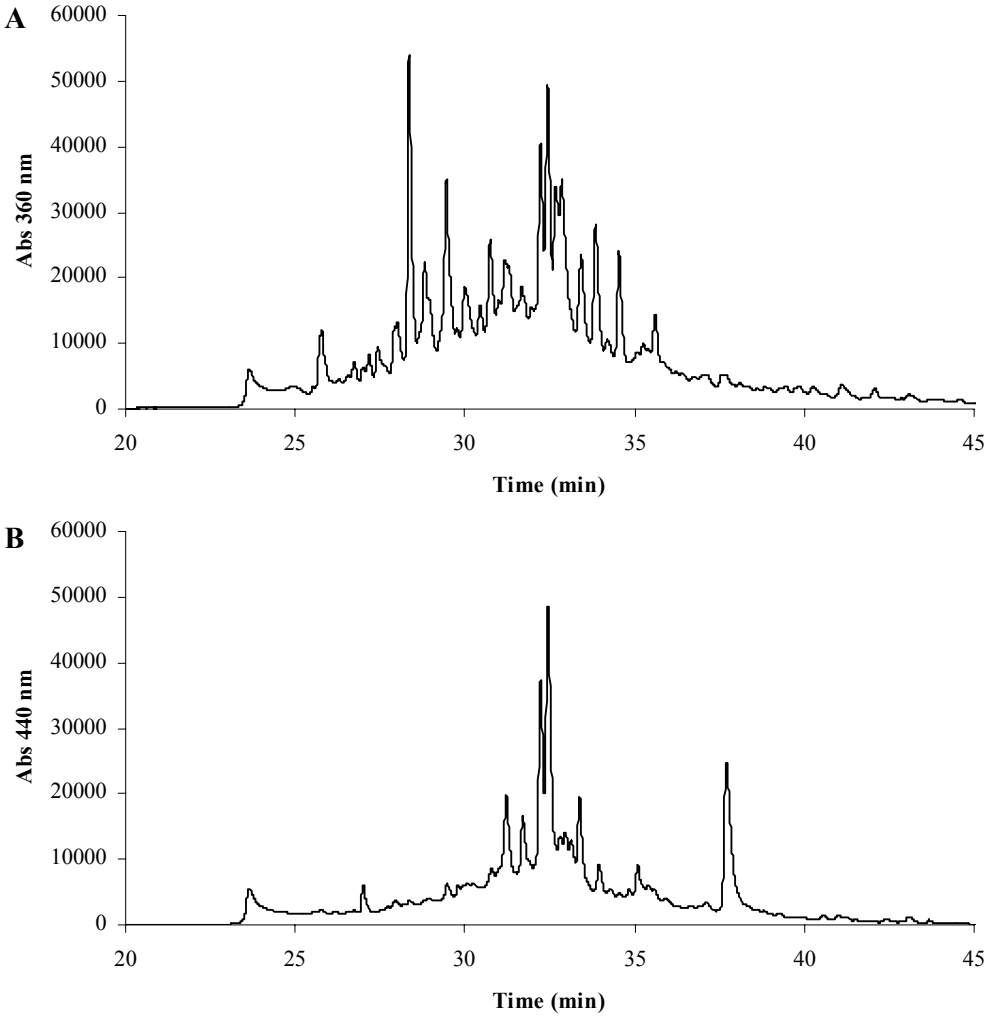


Figure 4.19 HPLC of the acid digest of 3OHKyn-*t*-Boc-Lys with a 20-fold molar excess of N-α-*t*-Boc-Lys following incubation for 12 days. HPLC chromatograms of, *A*, Absorbance monitored at 360 nm. *B*, Absorbance monitored at 440 nm.

4.3.3 Incubation of 3OHKyn-Modified CLP (pH 7.2)

In an attempt to observe the effects of long term exposure of 3OHKyn-modified lens proteins to oxygen, and to hopefully crosslink 3OHKyn in lens proteins, CLP previously modified with 3OHKyn at pH 7.2, (whereby 3OHKyn is only attached to Cys) was incubated for 15 days at pH 7.2. The protein concentration of the solution was 20 mg/mL. Aliquots of the reaction mixture were taken at 0, 1, 2, 3, 6, 9, 12 and at 15 days of incubation, and analysed by, SDS-PAGE, 3-D fluorescence, acid hydrolysis and mass spectrometry.

Analysis by SDS-PAGE

Aliquots taken during the incubation period were run on an SDS gel to monitor the formation of crosslinks. CLP has a molecular weight of approximately 20 kDa, therefore if a crosslink were to form in the protein during incubation, a band would be visible at approximately 40 kDa. Two gels were performed, one under non-reducing conditions, and the other under reducing condition. Gels are shown in Figure 4.20. The gel in Figure 4.20A was run under non-reducing conditions, and each of the lanes (lanes 2-7) exhibited a visible band between markers 37 and 50 kDa. Aliquots were taken initially, 3, 6, 9, 12 and at 15 days of incubation. A second gel (Figure 4.20B) was undertaken under reducing conditions (*i.e.* DTT was added to reduce disulphide bonds) and, the band between markers 37 and 50 kDa disappeared. This demonstrates that the band present in Figure 4.20A was a result of disulphide bond linkages, and was not a result of any other form of crosslinking in the protein.

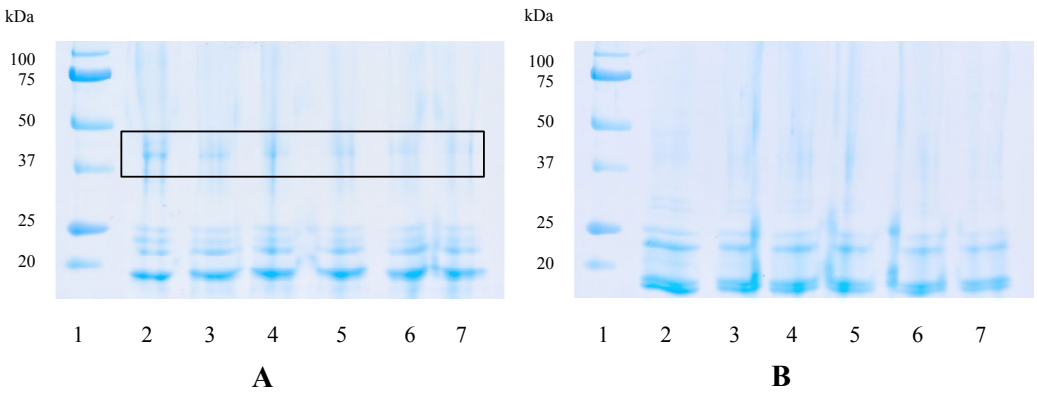


Figure 4.20 SDS-PAGE of proteins from incubation of 3OHKyn modified CLP. Lane 1: Marker; Lane 2: Aliquot time = 0 days; Lane 3: Aliquot time = 3 days; Lane 4: Aliquot time = 6 days; Lane 5: Aliquot time = 9 days; Lane 6: Aliquot time = 12 days; Lane 7: Aliquot time = 15 days. *A*, Non-reducing conditions; *B*, Reducing conditions.

Analysis by 3-D Fluorescence

Aliquots taken during the incubation period were monitored by 3-D fluorescence. Each of the aliquots was ultra-filtered with a 10 kDa cut-off membrane, to remove any non-covalently bound material that may have formed during the incubation period. A change in fluorescence was an indication that the protein had altered with respect to its 3OHKyn attachment. Initially the 3OHKyn-modified protein exhibited maximal fluorescence intensity at Ex 370 nm/Em 485 nm (Figure 4.21A). Aliquots taken after 2 days of incubation revealed that the emission of the protein had changed 10 nm from the initial aliquot (see Table 4.2). The fluorescence intensities for all of the protein aliquots are listed in Table 4.2. At the end of the incubation period the maximal fluorescence intensity was observed at Ex 365 nm/Em 470 nm (Figure 4.21B).

Table 4.2 List of the 3-D fluorescence intensities for each aliquot from the incubation.

Time (Days)	Ex (nm)/Em (nm)
0	370/485
1	370/480
2	370/475
3	370/470
6	370/470
9	365/470
12	365/470
15	365/470

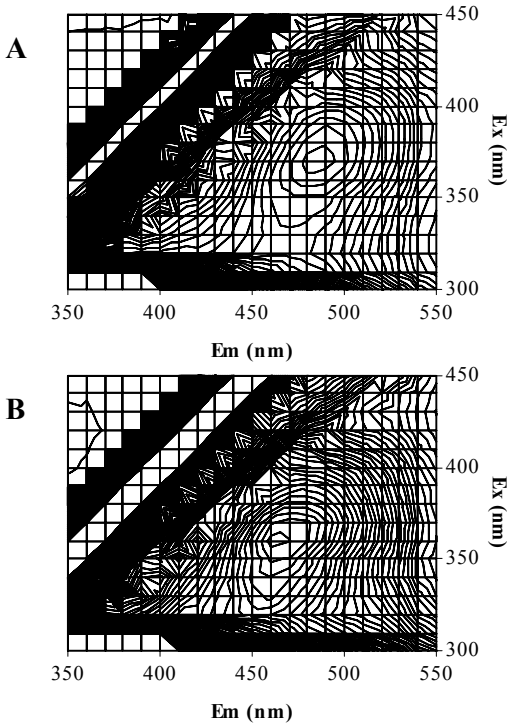


Figure 4.21 3-D Fluorescence spectra of aliquots from the protein mixture. *A*, Initial aliquot; *B*, Aliquot after 15 days of incubation.

Analysis of Filtrate

Aliquots of the protein at the various time intervals were taken for acid hydrolysis. Each protein aliquot was ultra-filtered to remove non-covalently bound material from the protein. Each filtrate was analysed by HPLC and peaks that eluted were examined by mass spectrometry. Figure 4.22 shows the HPLC chromatogram of the filtrate from the aliquot taken after 1 day of incubation of 3OHKyn-modified protein at pH 7.2.

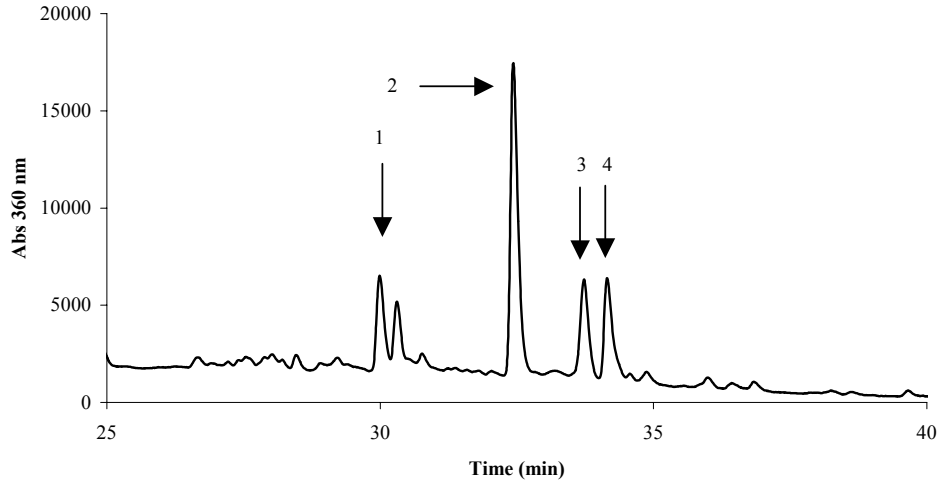


Figure 4.22 HPLC chromatogram of the filtrate from the protein aliquot after 1 day of incubation at pH 7.2.

Figure 4.22 exhibited 5 peaks eluting between 30 and 35 min, and the arrowed peaks were collected. Peak 1 eluted at 30 min and the ESI mass spectrum (Figure 4.23A) of this peak showed a molecular ion of *m/z* 206. MS/MS of this ion (spectrum not shown) yielded fragment ions *m/z* 188 and 160. This compound was identified as xanthurenic acid, since standard xanthurenic acid also yields the same fragment ions (spectrum not shown) and co-eluted with authentic xanthurenic acid. Xanthurenic acid is formed from oxidation of 3OHKyn.[109,191] Peak 2 eluted at 32.4 min on the HPLC chromatogram. The mass spectrum of this peak (Figure 4.23B) showed an ion at *m/z* 395, MS/MS of this ion (spectrum not shown) resulted in fragment ions *m/z* 377, 349 and 297. This compound could not be identified, since none of these ions are 3OHKyn fragment ions. Peak 3 eluted at 33.7 min on the HPLC chromatogram. The mass spectrum (Figure 4.23C) showed a prominent ion at *m/z* 300, and MS/MS of this ion (spectrum not shown) yielded fragment ions *m/z* 282, 208 and 110. The molecular ion of 3OHKyn-yellow is

m/z 208, therefore MS/MS was done on this ion (spectrum not shown) and fragment ions *m/z* 190, 162 and 110, confirmed that this peak contains 3OHKyn-yellow, which is also attached to a small compound of mass 92 Da. Finally, peak 4 eluted at 34.2 min, and the mass spectrum was identical to that for peak 3 (Figure 4.23C) with a prominent ion at *m/z* 300. MS/MS (spectrum not shown) yielded the same fragment ions as peak 3. It appears that peak 3 and peak 4 are diastereoisomers.

These findings show that some of the 3OHKyn attached to the CLP has been released from the protein as deaminated 3OHKyn, and due to the instability at pH 7.2 (Chapter 2, stability study at pH 7.2) it has oxidised, to form novel compounds.

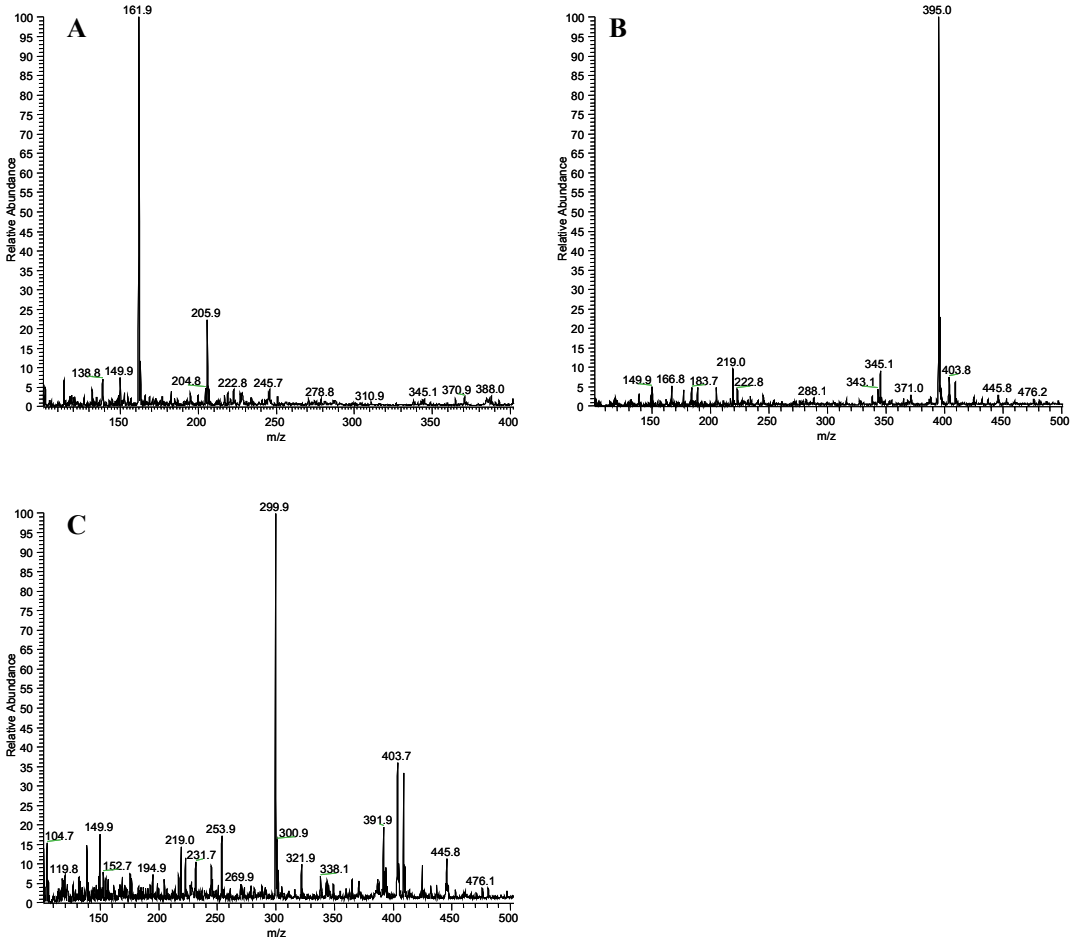


Figure 4.23 ESI mass spectra of the peaks eluting in Figure 4.22. *A*, Peak 1 eluting at 30 min; *B*, Peak 2 eluting at 32.4 min; *C*, Peak 3 eluting at 33.7 min.

Acid Hydrolysis of Protein

Protein aliquots from the reaction mixture were acid hydrolysed after ultra-filtration to remove non-covalently bound compounds. The HPLC chromatogram of the hydrolysate of the initial aliquot is shown in Figure 4.24A. The doublet compound eluting at 28 min was identified as 3OHKyn-Cys. This result was expected since this protein was initially prepared by incubating CLP with 3OHKyn at pH 7.2 for 48 hours (see Chapter 3). The HPLC chromatogram of the hydrolysate of the protein after 15 days of incubation at pH 7.2 is shown in Figure 4.24B. As a result of this incubation period, the chromatogram indicates that the protein had changed in a major way from the initial aliquot. The numbered peaks indicated with an arrow, in Figure 4.24B, were collected and analysed by mass spectrometry for 'marker' ions, which were later used to compare with peaks obtained from cataract lens proteins.

In Figure 4.24B, peak 1 eluted at 25 min. The ESI mass spectrum (spectrum not shown) of this peak showed a molecular ion of *m/z* 363. MS/MS of this ion (spectrum not shown) yielded fragment ions *m/z* 317, 208, 156 and 110, which are the fragment ions for 3OHKyn-His. Therefore this peak was identified as 3OHKyn-His. Peak 2 eluted at 26.6 min, and the mass spectrum of this peak showed an ion at *m/z* 518 (Figure 4.25A). MS/MS of this ion (spectrum not shown) resulted in fragment ions *m/z* 499, 475, 429, 387 and 279. This compound could not be identified. Peak 3 eluted at 28.2 min. The mass spectrum showed many ions (Figure 4.25B) however none could be identified as the molecular ion. Peak 4 eluted at 28.5 min. The mass spectrum exhibited many ions centred between *m/z* 400 and *m/z* 600 (Figure 4.25C), MS/MS of *m/z* 684, exhibited fragment ions *m/z* 667, 641, 591, 505, 429 and 299 (spectrum not shown). This compound could not be identified. Peak 5 eluted at 29 min. The mass spectrum showed an ion at *m/z* 528 (Figure 4.25D), MS/MS resulted in fragment ions *m/z* 511, 484, 423 and 250 (spectrum not shown). Peak 6 eluted at 32.5 min. The mass spectrum exhibited many ions above *m/z* 400 (Figure 4.25E), however it was difficult to determine the molecular ion for this peak. Peak 7 eluted at 33.5 min. The mass spectrum also showed numerous ions eluting above *m/z* 400 (Figure 4.25F), making it difficult to determine the molecular ion.

Peak 8 eluted at 33.9 min. The mass spectrum is shown in Figure 4.26A. Ions in this spectrum could not be identified. Peak 9 eluted at 35.3 min and the mass spectrum (Figure 4.26B) failed to show any familiar ions. Peak 10 eluted at 37.8 min, the mass spectrum is shown in Figure 4.26C, ions in the spectrum were not familiar. Peak 11 eluted at 41.2 min and the mass spectrum showed an abundant ion at *m/z* 542 (Figure 4.26D), MS/MS of this ion yielded fragment ions *m/z* 497 and 375 (spectrum not shown), this data alone makes it difficult to conclude if this is indeed the molecular ion of this peak. Peak 12 eluted at 42.2 min and the mass spectrum is shown in Figure 4.26E. Finally peak 13 eluted at 46 min and the mass spectrum is shown in Figure 4.26F.

The structures of the majority of these compounds could not be determined from the mass spectrometry data alone and were not pursued further.

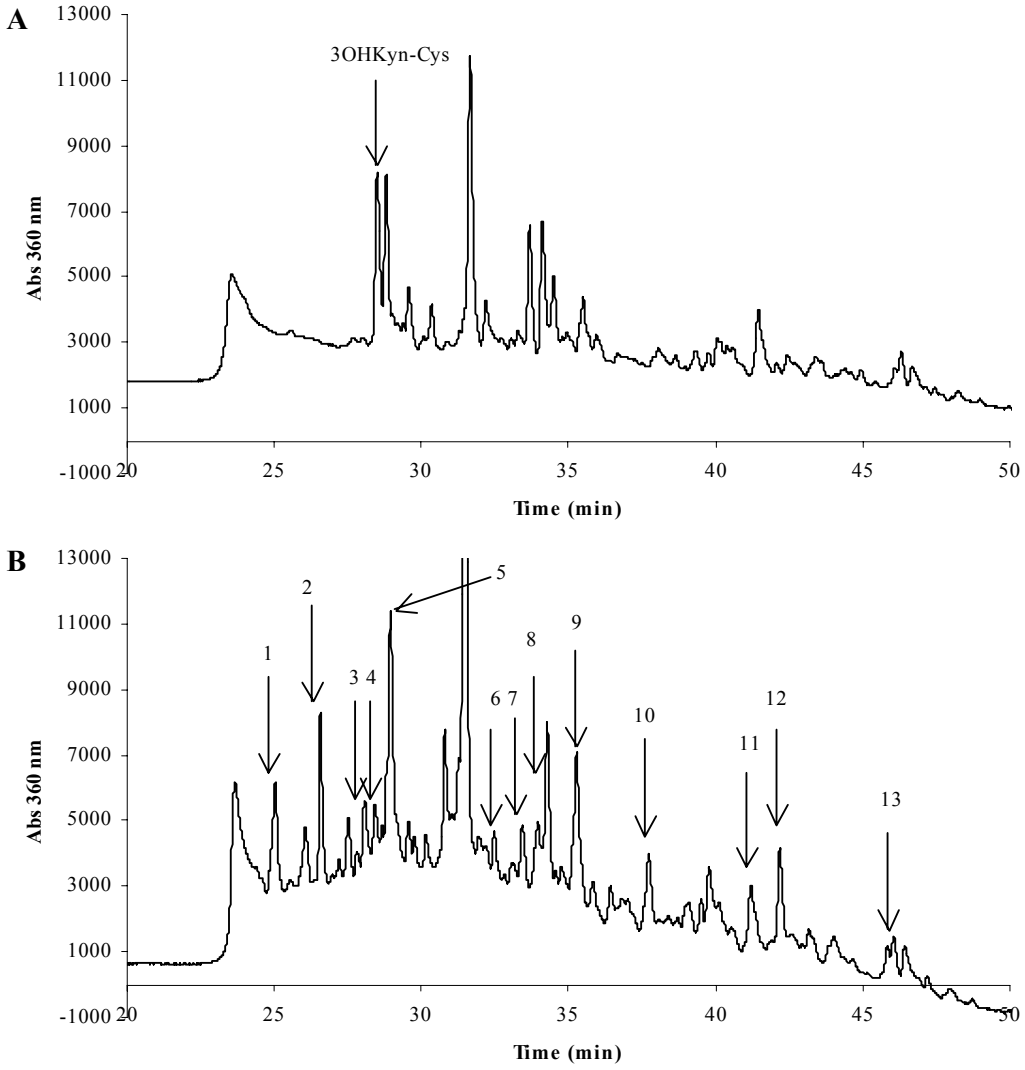


Figure 4.24 HPLC chromatograms of acid hydrolysed proteins. *A*, Initial aliquot; *B*, Aliquot after 15 days of incubation. Arrowed peaks were collected for mass spectrometry.

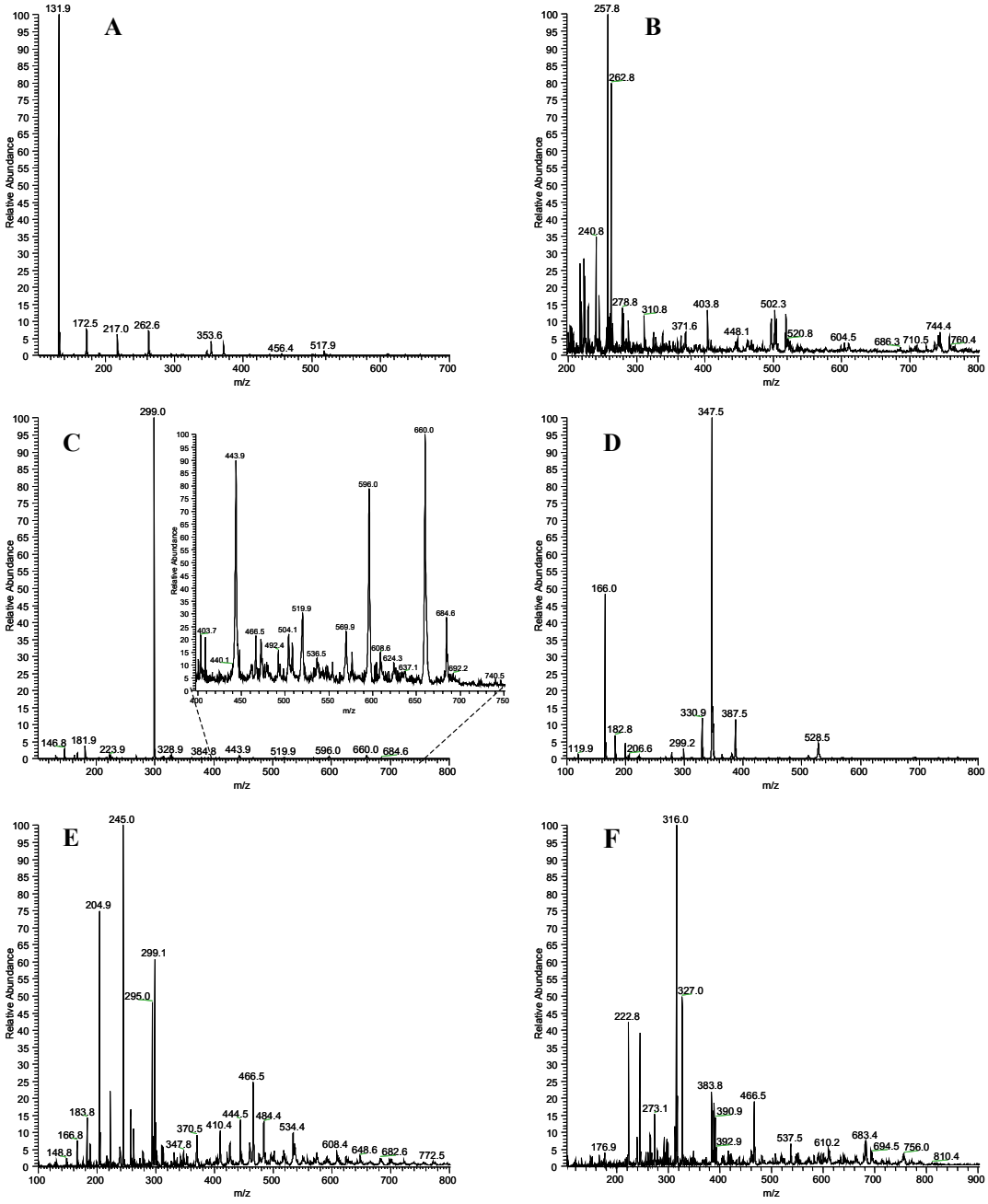


Figure 4.25 ESI mass spectra of peaks eluting in Figure 4.24B. *A*, Peak 2; *B*, Peak 3; *C*, Peak 4; *D*, Peak 5; *E*, Peak 6; *F*, Peak 7.

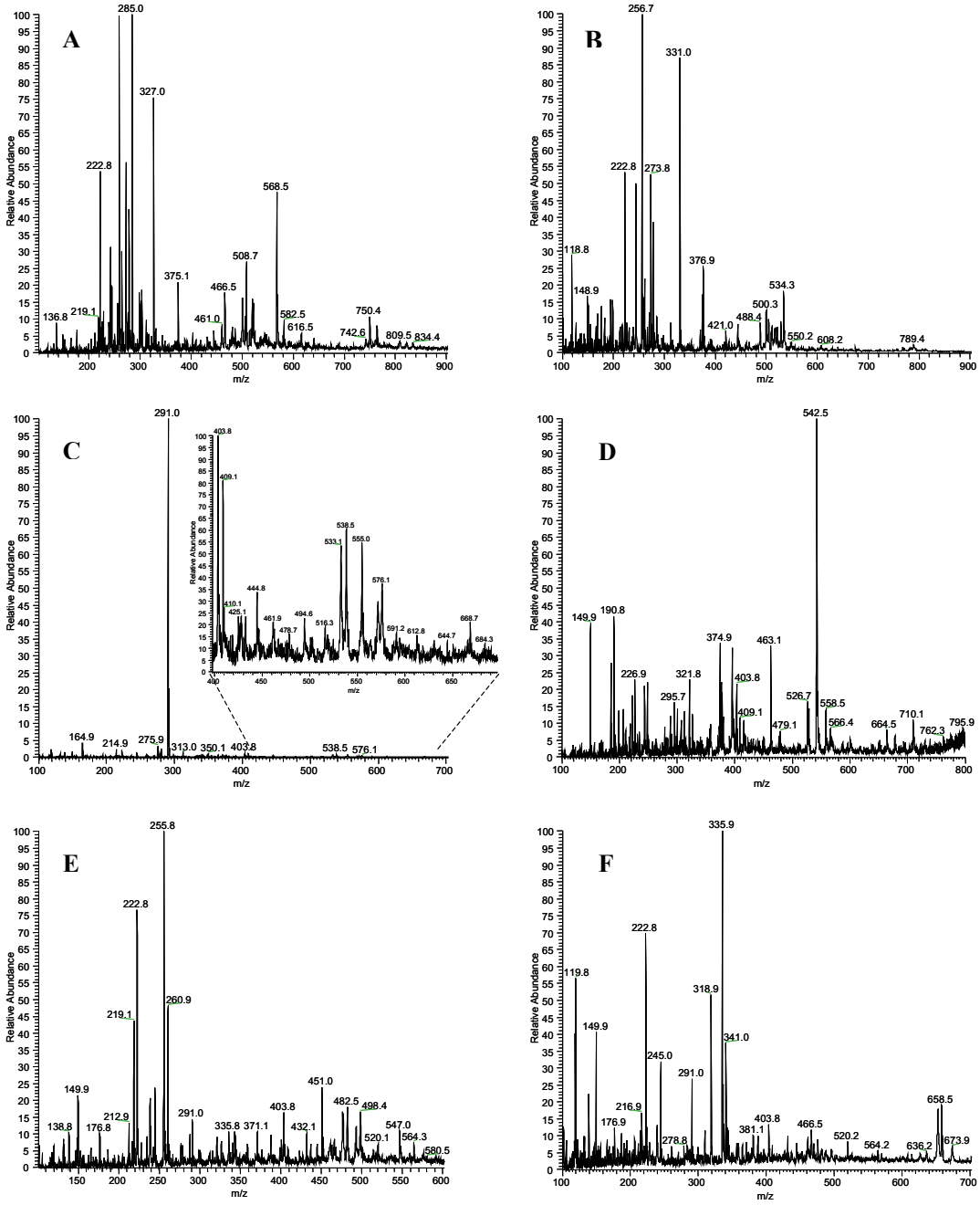


Figure 4.26 ESI mass spectra of peaks eluting in Figure 4.24B. *A*, Peak 8; *B*, Peak 9; *C*, Peak 10; *D*, Peak 11; *E*, Peak 12; *F*, Peak 13.

The modified CLP used in this study was known to contain modification primarily at Cys. The concentration of 3OHKyn-Cys in the protein aliquots during the incubation at pH 7.2 was quantified and is shown in Figure 4.27. The concentration of 3OHKyn-Cys decreased by 63% after 2 days of incubation and could not be detected after 3 days of incubation. Analysis of the HPLC hydrolysates and mass spectrometry confirmed that 3OHKyn-His was formed after 6 days of incubation This adduct presumably formed as a result of decomposition of 3OHKyn-Cys with release of deaminated 3OHKyn, which then bound to His residues (see Figure 4.24A and Figure 4.24B).

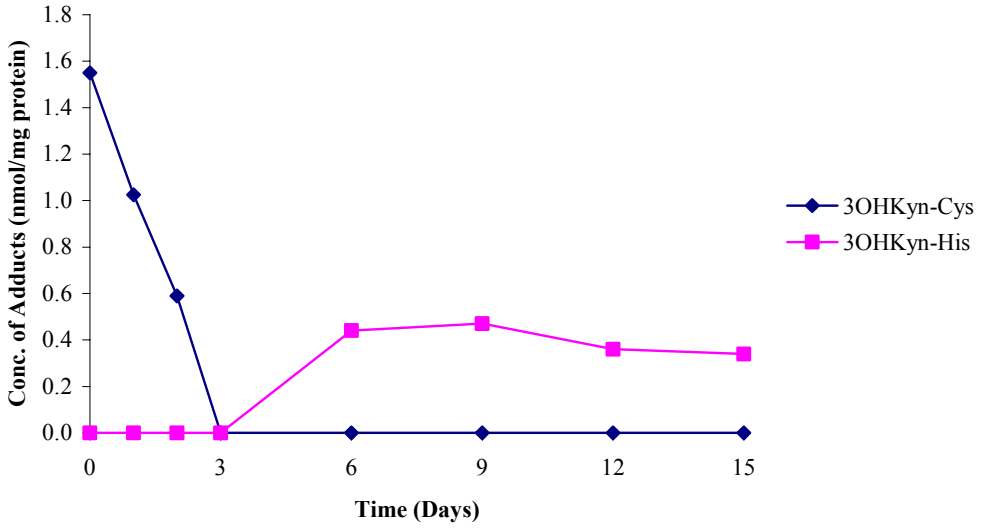


Figure 4.27 Concentration of 3OHKyn-Cys and 3OHKyn-His in protein samples during incubation of CLP originally modified by 3OHKyn at pH 7.2.

4.3.4 Incubation of 3OHKyn-Modified CLP (pH 9.5)

In this section, another attempt was made to crosslink 3OHKyn-modified lens proteins. The modified protein used in this incubation was CLP modified by 3OHKyn at pH 9.5 which contains modifications at Cys, His and Lys, and a greater degree of modification by 3OHKyn. The protein was incubated for 10 days at pH 7.2 and aliquots were taken at 0, 1, 2, 3, 4, 6, 8 and 10 days. Since this protein was originally modified at a high pH (pH 9.5), the protein reaction mixture at the start of the incubation was a dark brown/black colour (Chapter 3), and this colour was possibly indicative of a high amount of 3OHKyn oxidation in the protein. Each of the aliquots was again analysed by SDS-PAGE, 3-D fluorescence acid hydrolysis and mass spectrometry.

Analysis by SDS-PAGE

Aliquots of the reaction mixture were again monitored by SDS gels for the formation of crosslinks (*i.e.* band at approximately 40 kDa). Two gels were performed, one under non-reducing conditions and the other under reducing condition. Gels are shown in Figure 4.28. The gel in Figure 4.28A was run under non-reducing conditions, and each of the aliquots exhibited a visible band between markers 37 and 50 kDa. The second gel (Figure 4.28B) was undertaken under reducing conditions (*i.e.* DTT was added to reduce disulphide bonds) and, the band between markers 37 and 50 kDa disappeared. This demonstrates that the band present in Figure 4.28A was a direct result of disulphide bond linkages, and was not the result of any other form of crosslinking in the protein.

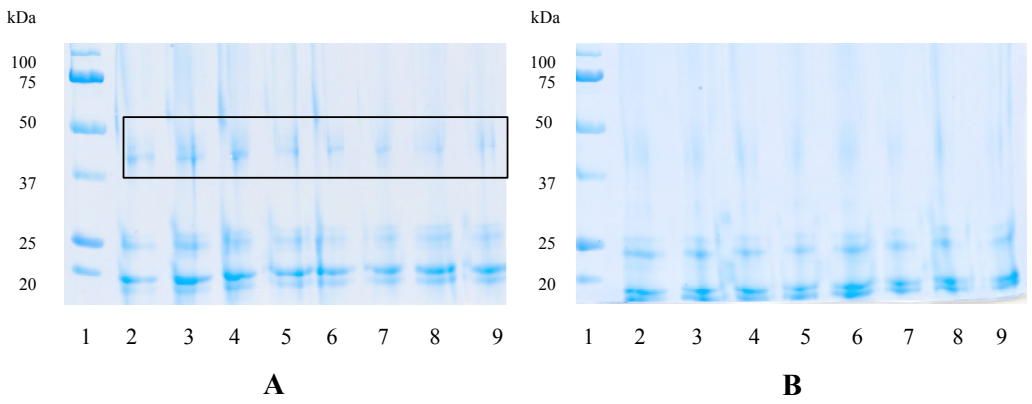


Figure 4.28 SDS-PAGE of proteins from incubation of 3OHKyn modified CLP. Lane 1: Marker; Lane 2: Aliquot time = 0 days; Lane 3: Aliquot time = 1 days; Lane 4: Aliquot time = 2 days; Lane 5: Aliquot time = 3 days; Lane 6: Aliquot time = 4 days; Lane 7: Aliquot time = 6 days; Lane 8: Aliquot time = 8 days; Lane 9: Aliquot time = 10 days. *A*, Non-reducing conditions; *B*, Reducing conditions.

Analysis by 3-D Fluorescence

Aliquots of the protein mixture were monitored for modifications by 3-D fluorescence. Each aliquot was first ultra-filtered to remove non-covalently bound compounds. Initially the 3OHKyn-modified protein exhibited maximal fluorescence intensity at Ex 360 nm/Em 480 nm (Figure 4.29A). The fluorescence intensities for all the protein aliquots are listed in Table 4.3. The maximum excitation wavelength for all aliquots remained constant, however the emission gradually decreased by 15 nm over the 10 day incubation period. At the end of the incubation, the protein exhibited maximal fluorescence intensity at Ex 360 nm/Em 465 nm (Figure 4.29B).

Table 4.3 List of the 3-D fluorescence intensities for each aliquot from the incubation.

Time (Days)	Ex (nm)/Em (nm)
0	360/480
1	360/475
2	360/470
3	360/470
4	360/470
6	360/470
8	360/465
10	360/465

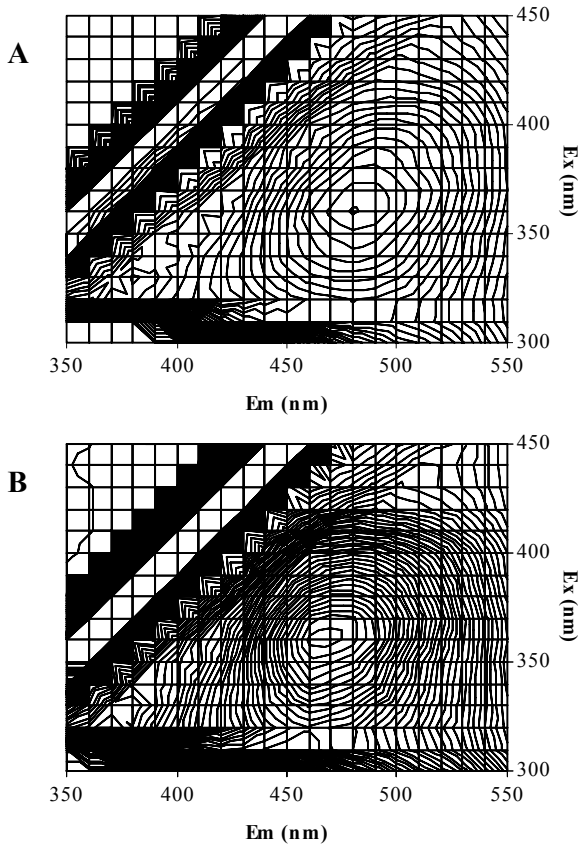


Figure 4.29 3-D Fluorescence spectra of the aliquots from the protein mixture. *A*, Initial aliquot; *B*, Aliquot after 10 days of incubation.

Analysis of Filtrate

Aliquots of the incubation mixture were taken for acid hydrolysis of the protein, and were ultra-filtered to remove non-covalently bound material from the protein. Each filtrate was examined by HPLC and peaks that eluted were analysed by mass spectrometry. Figure 4.30 is the HPLC chromatogram of the filtrate from the protein aliquot after 1 day of incubation at pH 7.2.

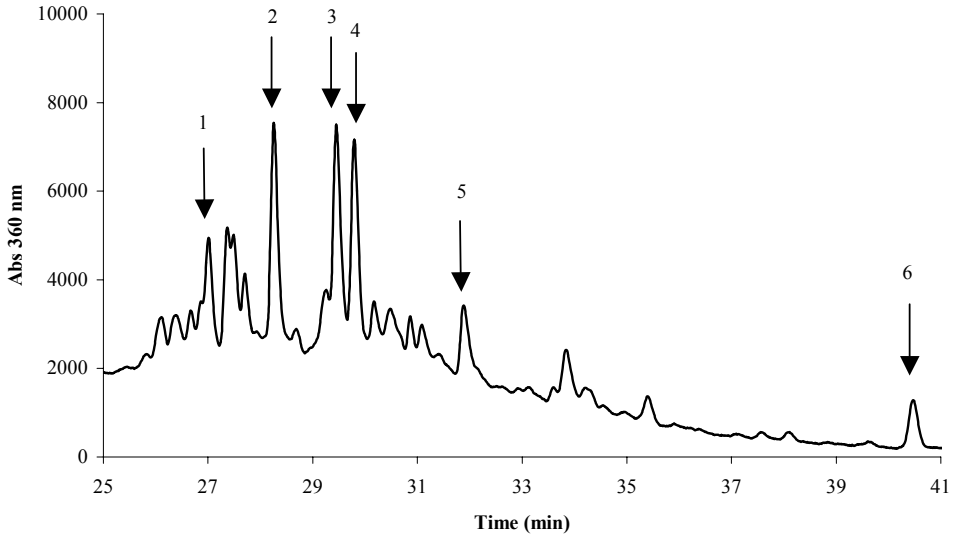


Figure 4.30 HPLC chromatogram of the filtrate from the protein aliquot after 1 day of incubation.

The HPLC chromatogram exhibited numerous peaks eluting between 26 and 41 min. Six peaks in the HPLC chromatogram were collected and analysed by mass spectrometry (Figure 4.30). Peak 1 eluted at 27 min, and the mass spectrum showed numerous ions (Figure 4.31A). MS/MS of ion *m/z* 371 (spectrum not shown) yielded fragment ion *m/z* 268, and MS/MS of ion *m/z* 343 (spectrum not shown), yielded fragment ion *m/z* 240. The identity of this peak could not be determined. Peak 2 eluted at 28.3 min on the HPLC chromatogram. The mass spectrum of this peak showed an abundant ion at *m/z* 305 (Figure 4.31B). MS/MS of this ion yielded fragment ions *m/z* 286, 261, 212 and 194. This peak was not identified. Peak 3 eluted at 29.4 min on the HPLC chromatogram, and the mass spectrum showed an abundant ion at *m/z* 206

(Figure 4.31C). MS/MS of this ion resulted in fragment ions *m/z* 188 and 160 (spectrum not shown), confirming that this peak was indeed xanthurenic acid, since authentic xanthurenic acid co-eluted with this sample. Peak 4 eluted at 29.8 min on the HPLC chromatogram. The mass spectrum of this peak is shown in Figure 4.31D. The mass spectrum shows that there is a small ion at *m/z* 206, indicating a small amount of xanthurenic acid was present, since this peak eluted so close to peak 3. However this peak did contain an abundant ion at *m/z* 162, and MS/MS of this ion yielded fragment ions *m/z* 143, 134, 120 and 105 (spectrum not shown). The ion *m/z* 162 is a known fragment ion of 3OHKyn (Chapter 2), however the fragment ions (*m/z* 143, 134, 120 and 105) are not characteristic fragment ions of 3OHKyn. Peak 5 eluted at 31.9 min on the HPLC chromatogram. The mass spectrum showed an abundant ion at *m/z* 395 (Figure 4.31E). MS/MS of this ion yielded fragment ions *m/z* 377, 349 and 297. This compound could not be identified, however this compound together with these fragment ions was also seen in the filtrate of Section 4.3.3. Peak 6 eluted at 40.5 min on the HPLC chromatogram, the mass spectrum (Figure 4.31F) showed many ions, however none could be identified. These findings again show that 3OHKyn has deaminated from the protein and formed numerous oxidised compounds which were not structurally elucidated.

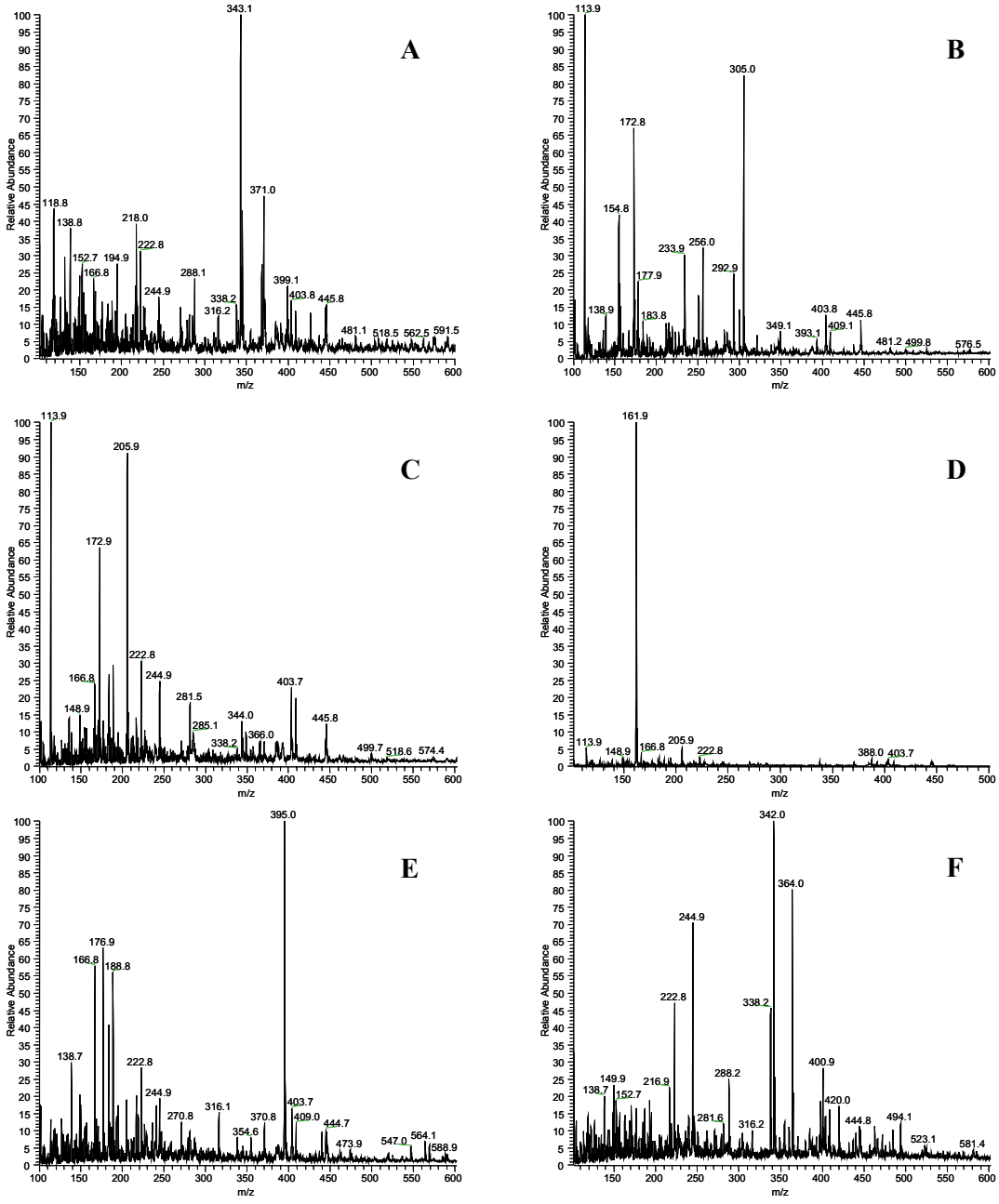


Figure 4.31 ESI mass spectra of the peaks eluting in Figure 4.30. *A*, Peak 1 eluting at 27 min; *B*, Peak 2 eluting at 28.3 min; *C*, Peak 3 eluting at 29.4 min; *D*, Peak 4 eluting at 29.8 min; *E*, Peak 5 eluting at 31.9 min; *F*, Peak 6 eluting at 40.5 min.

Acid Hydrolysis of Protein

Each protein aliquot from the incubation at pH 7.2 was ultra-filtered prior to acid hydrolysis. The HPLC chromatogram of the hydrolysate of the initial aliquot is shown in Figure 4.32A. The peak eluting at 25.3 min was identified as 3OHKyn-Lys, the peak at 26.1 min was identified as 3OHKyn-His and the doublet eluting at 29 min was identified as 3OHKyn-Cys, from MS/MS analysis. These 3OHKyn adducts have been previously observed in Chapter 3, since CLP modified by 3OHKyn at a high pH yields all three adducts.

The protein (modified by 3OHKyn at pH 9.5) was incubated at pH 7.2 for 10 days. Figure 4.32B is the HPLC chromatogram of the hydrolysate of that aliquot. Numerous peaks differed from those in Figure 4.32A, however only a few were analysed by mass spectrometry (Figure 4.32B). Peak 1 eluted at 24.9 min, the mass spectrum (spectrum not shown) of this peak showed a molecular ion of *m/z* 363, MS/MS of this (spectrum not shown) confirmed that this was indeed 3OHKyn-His. Peak 2 eluted at 28 min as what appeared to be a doublet, analysis of this peak by mass spectrometry showed few ions, but none greater than *m/z* 600 (Figure 4.33A). MS/MS of *m/z* 589 (spectrum not shown) resulted in fragment ions *m/z* 571, 460, 442 and 424, of all these ions, *m/z* 424 is the only familiar ion, since it is also the molecular ion of xanthommatin, however this peak cannot be identified with such little information. Peak 3 eluted at 29.2 min, the mass spectrum (Figure 4.33B) showed numerous ions. MS/MS of *m/*z 162 (spectrum not shown) showed that it was not a fragment ion of 3OHKyn, since the fragment ions were not characteristic fragment ions of 3OHKyn. Peak 4 eluted at 32.2 min on the HPLC chromatogram. The mass spectrum (Figure 4.33C) exhibited numerous ions, however none were familiar. Peak 5 eluted at 34.2 min on the HPLC chromatogram. The mass of this peak (Figure 4.33D) showed many ions of varying abundance, and thus it was difficult to determine the molecular ion for this peak.

Peak 6 eluted at 35.5 min on the HPLC chromatogram. The mass spectrum of this peak (Figure 4.34A) showed many ions but none could be identified as the molecular ion. Similarly peak 7 eluted at 36.9 min and analysis of the mass spectrum (Figure 4.34B) again showed many ions. MS/MS of *m/z* 919 resulted in fragment ions *m/z* 901, 883,

829, 769, 737, 681, 589, 531, 499, 443 and 351. Since many of these fragment ions do appear in the original mass spectrum (Figure 4.34B), it is assumed that *m/z* 919 is the molecular ion for this peak. Peak 8 eluted at 38 min, the mass spectrum is shown in Figure 4.34C. This peak exhibited very few ions and they were all below *m/z* 600. Finally peak 9 eluted at 47 min on the HPLC chromatogram. The mass spectrum is shown in Figure 4.34D, and there are numerous ions present, however analysis failed to identify any characteristic ions. Although the HPLC chromatogram of the hydrolysate changed during the incubation of the protein, many peaks could not be identified after mass spectrometry, and the molecular ions in each peak again could not be easily identified.

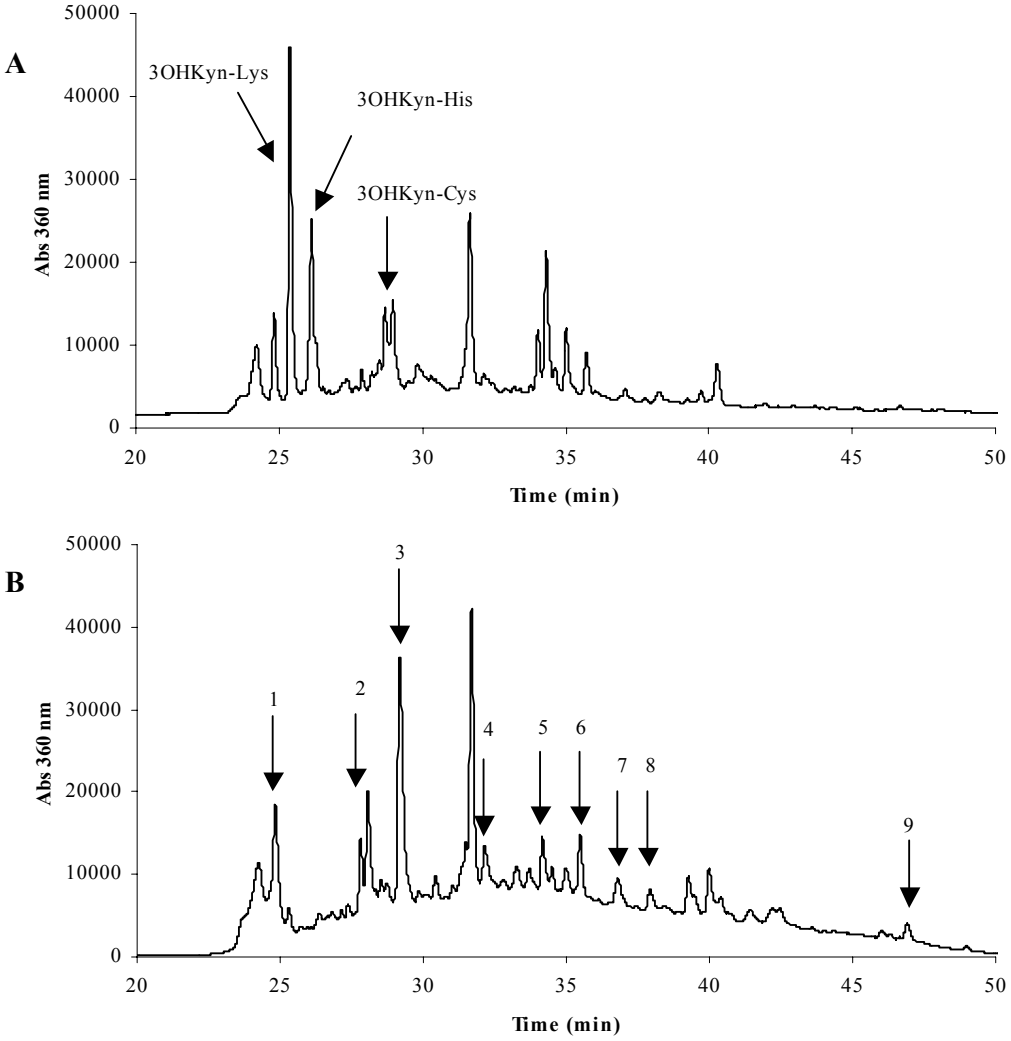


Figure 4.32 HPLC chromatograms of acid hydrolysed proteins. *A*, Initial aliquot; *B*, Aliquot after 10 days of incubation. Arrowed peaks were collected for mass spectrometry.

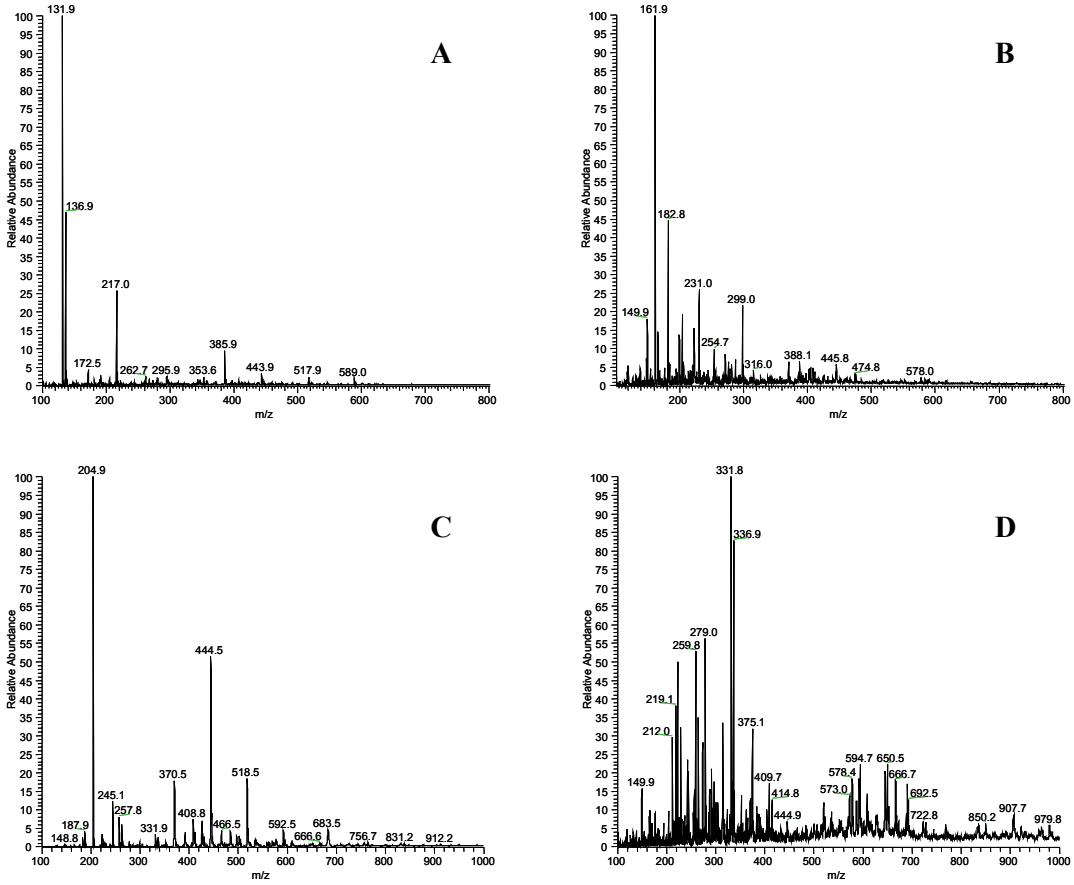


Figure 4.33 ESI mass spectra of peaks eluting in Figure 4.32B. *A*, Peak 2; *B*, Peak 3; *C*, Peak 4; *D*, Peak 5.

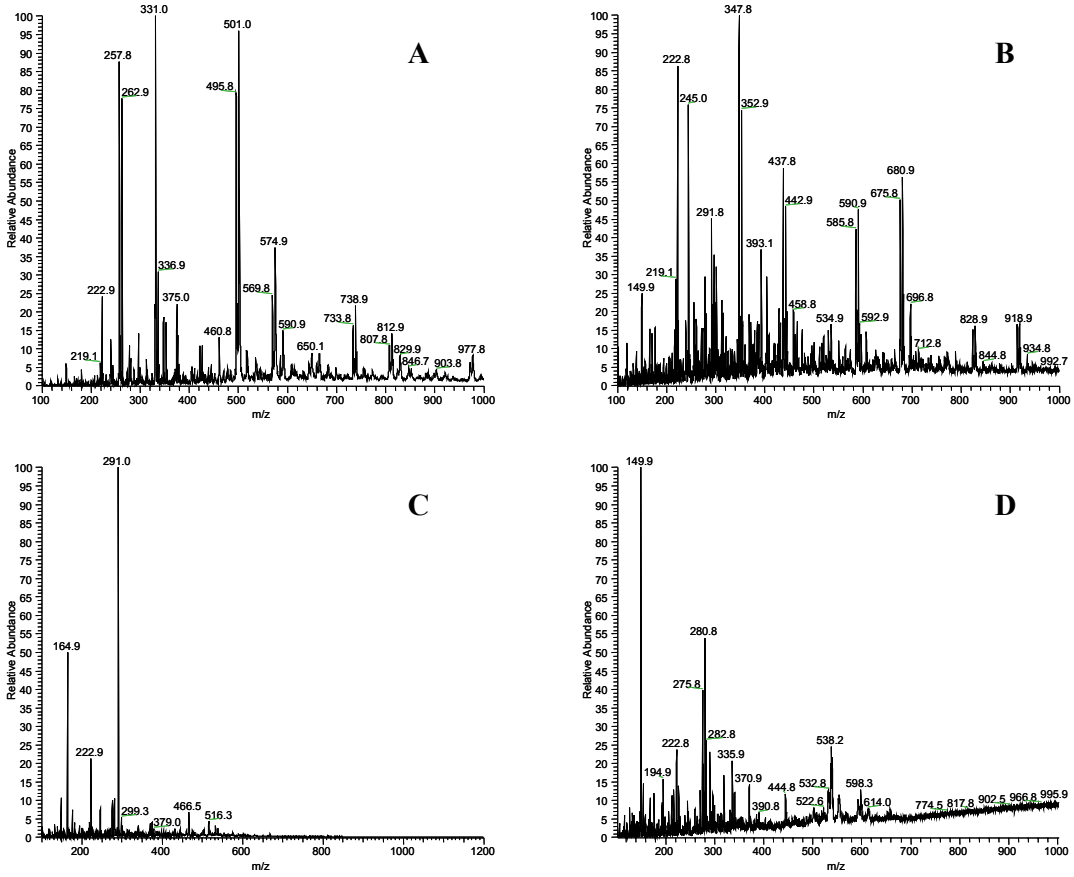


Figure 4.34 ESI mass spectra of peaks eluting in Figure 4.32B. *A*, Peak 6; *B*, Peak 7; *C*, Peak 8; *D*, Peak 9.

The modified CLP used in this study was known to originally contain modifications at Cys, His and Lys. The concentration of each adduct was quantified and are shown in Figure 4.35. 3OHKyn-Cys was not recovered after 3 days of incubation, and similarly 3OHKyn-Lys was not recovered after 3 days of incubation. 3OHKyn-His was recovered during the whole incubation period. MS/MS was used to identify the adducts in each protein aliquot.

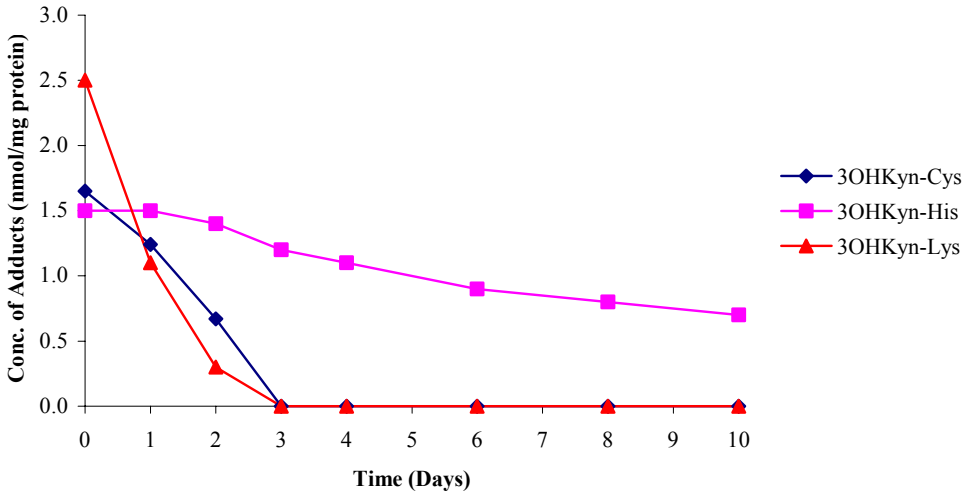
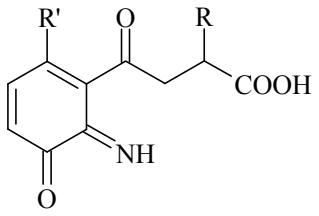


Figure 4.35 Concentration of 3OHKyn-Cys, 3OHKyn-His and 3OHKyn-Lys in protein samples during incubation of CLP originally modified by 3OHKyn at pH 9.5.

4.4 Discussion

This chapter is a preliminary investigation, examining the potential for 3OHKyn to modify and crosslink lens proteins and to compare the modified proteins with authentic human cataract lens proteins. Crosslinking is characteristic of ARN cataract.[166] Scheme 4.1 showed the expected products from this study. Table 4.4 shows the molecular ions of the expected 3OHKyn crosslink compounds of which none were observed in this study.

Table 4.4 Expected molecular ions of the 3OHKyn crosslink compounds.



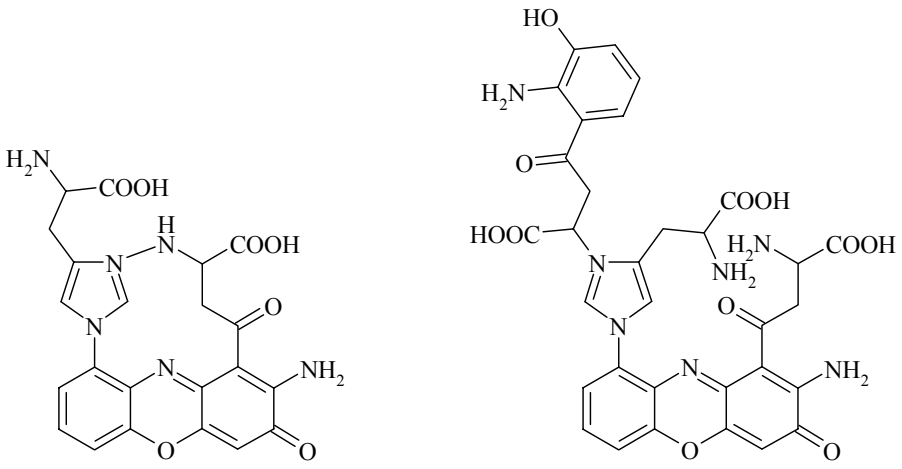
R'	R	Molecular Weight	Molecular Ion
His	His	513.5 Da	514.5 Da
Lys	His	504.5 Da	505.5 Da
Lys	Lys	495.5 Da	496.5 Da
Cys	Cys	445.5 Da	446.5 Da
His	Cys	479.5 Da	480.5 Da
Lys	Cys	470.5 Da	471.5 Da

3OHKyn-t-Boc-His

Firstly, the stability of the various 3OHKyn amino acid adducts were examined and the products formed were analysed. The study in Section 4.3.1 demonstrated that the products following incubation of 3OHKyn-*t*-Boc-His in the absence or presence of excess amino acids were essentially identical. In particular one major product (compound with an ion *m/z* 550) with a high absorbance at both 360 and 440 nm was observed in the acid digest of all reactions. Attempts were made to characterise this compound. Large scale reactions were undertaken with the 3OHKyn-*t*-Boc-His adduct however following HPLC purification, only small yields were observed. Therefore although a relatively large amount of adduct was used, only a small amount of product

was formed for NMR analysis. NMR was performed with the product that was available. NMR could not elucidate the structure of this product. Although the starting material in this experiment was 3OHKyn-*t*-Boc-His, the NMR of 3OHKyn-*t*-Boc-His was distinctly different from that in Figure 4.13. It is likely that the unknown compound contains an impurity that made interpretation of the NMR spectrum difficult. In addition, there was not enough product available to run additional NMR experiments and gain sufficient data for structural elucidation. The HPLC profiles of the acid digests monitored at 440 nm suggested that this product absorbed highly at this wavelength, however the UV-visible spectrum (Figure 4.12) showed that the wavelength maximum of the chromophore was ~ 408 nm, and therefore this data shows that it is unlikely that this compound contains a simple phenoxazone moiety. 3OHKyn yields a phenoxazone when it is oxidised.[184]

The UV-visible spectrum (Figure 4.12) also showed that this unknown product is stable to acid, since Figures 4.12A and 4.12B are identical, and the molecular ion in the mass spectra of Peak 2 and Peak 2' differed by 100 Da, which is equivalent to a *t*-Boc group. The MS/MS spectrum (Figure 4.10) suggest that the imidazole ring of His is linked twice, since there were very few His-related fragment ions resulting from MS/MS of *m/z* 550. Some possible structures of this unknown compound are shown in Figure 4.36, however the molecular ions of these structures do not equal the mass of the unknown (*m/z* 550).



m/z 481 (M+1) *m/z* 690 (M+1)

Figure 4.36 Possible structures of the unknown compound formed from the 3OHKyn-*t*-Boc-His incubations, whereby the imidazole ring is linked twice.

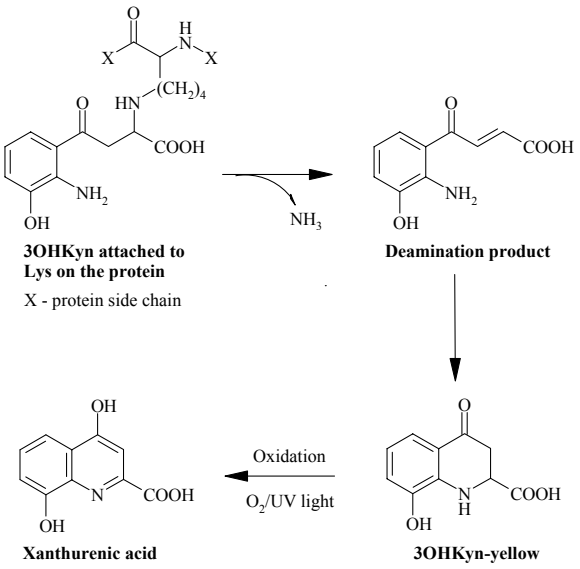
3OHKyn-t-Boc-Lys

The incubations with 3OHKyn-*t*-Boc-Lys further demonstrated instability of this 3OHKyn amino acid adduct at pH 7.2. Numerous products were formed as a result of each incubation. However the HPLC profiles of the acid digests were not the same for all three reactions, and therefore, as a result of the inconsistency, it was decided that the products from these incubations would not be further investigated at this time.

Protein

A study of proteins modified by 3OHKyn further demonstrated the reactivity of 3OHKyn. For example, protein originally modified at pH 7.2 and then incubated for 15 days, showed that 3OHKyn 'transferred' during the incubation period *i.e.* 3OHKyn initially modified Cys on the protein however, after six days of incubation 3OHKyn had become covalently attached to His residues. This further demonstrates the intrinsic instability of the Cys adduct, but it also suggests how bound UV filters may react *in vivo*. The filtrate showed the presence of xanthurenic acid, an oxidation product of 3OHKyn, which is formed when 3OHKyn deaminates at neutral pH.[109] The reactive intermediate cyclises to form 3OHKyn-yellow[191] and is then oxidised in the presence of

UV light or oxygen forming xanthurenic acid (Scheme 4.2). This also shows that in an older lens when 3OHKyn is attached to lens protein at pH 7.2, it may ultimately result in the production of xanthurenic acid if the environment is oxidising (as is the case for cataract lenses).[91,159,163] Xanthurenic acid 8-*O*-β-D-glucoside is a compound, which has been reported in brunescent cataracts. It is derived from deamination of 3OHKynG, followed by cyclisation to form 3OHKynG-yellow and then oxidation[224] (similar to Scheme 4.2 for 3OHKyn). Additional studies by Shirao, *et al*. showed that xanthurenic acid 8-*O*-β-D-glucoside was not derived artificially through sample preparation since sample preparation conditions were done under acidic conditions at 4^0C.[224] Similarly, the sample preparation conditions in this thesis were also done at 4^0C, and at acidic pH, therefore the xanthurenic acid identified in the filtrates was not derived from sample preparation. Rather xanthurenic acid was derived from the intrinsic instability of 3OHKyn at pH 7.2 during the incubation. Bova, *et al*. have shown that 3OHKynG does not deaminate significantly at pH 5.[85]



Scheme 4.2 Route of formation of xanthurenic acid from autoxidation of protein-bound 3OHKyn.

Similarly the protein originally modified at pH 9.5 and further incubated for 10 days also showed the presence of xanthurenic acid in the filtrate. These modified proteins also showed that 3OHKyn-Cys and 3OHKyn-Lys are both unstable at pH 7.2, but 3OHKyn-His was relatively stable over the incubation period (10 days) (Figure 4.35).

The SDS-PAGE of the protein incubations at 15 days and 10 days did not show evidence of non-disulfide crosslinks forming (*i.e.* a band at ~40 kDa was not observed) in protein modified by 3OHKyn. The fluorescence data did however show that the fluorophore had changed, or that the environment of the fluorophore had changed. Fluorescence measurements were done at pH 5.5 (protein aliquots were added to 6 M guanidine HCl solution). At this pH the protein in Section 4.3.3 had an initial fluorescence at Ex 370 nm/Em 485 nm. These wavelengths are not identical to those of the 3OHKyn amino acid adducts at that pH, but they are close (e.g. 3OHKyn-Cys Ex 340 nm/Em 510 nm, Ex 430 nm/Em 510 nm; 3OHKyn-*t*-Boc-His Ex 350 nm/Em 520 nm; 3OHKyn-*t*-Boc-Lys Ex 350 nm/Em 520 nm, Ex 410 nm/Em 520 nm). The fluorescence at the end of the incubation was measured at Ex 365 nm/Em 470 nm (Table 4.2). Similarly the fluorescence data in Section 4.3.4 was similar to that of Section 4.3.3. Initially the fluorescence was measured at Ex 360 nm/Em 480 nm, and at the end of the incubation the fluorescence of the protein was Ex 360 nm/Em 465 nm (Table 4.3), which is again comparable to the 3OHKyn amino acid adducts. The modified protein may contain other species (*i.e.* oxidised compounds attached to protein), which may be responsible for the observed fluorescence intensities.

The HPLC chromatogram of the hydrolysate of 3OHKyn-modified protein showed observable changes (*i.e.* there were additional peaks eluting in the HPLC chromatograms) at the end of the incubation period compared to the original HPLC profile. Many of the peaks that eluted were collected and examined by mass spectrometry, however the expected crosslink compounds that are listed in Table 4.4 were not observed. The aim of this study was to identify crosslink compounds in proteins modified by 3OHKyn. Chapter 5 is a study of the novel compounds, which are observed in the hydrolysate of cataract lens proteins. The HPLC profiles of cataract lens

proteins will be analysed and compared to those of oxidised 3OHKyn-modified protein and conclusions will be drawn at the end of Chapter 5.

In summary, the findings from this preliminary investigation show that 3OHKyn does not appear to crosslink lens proteins (SDS-PAGE data). However the proteins did undergo changes during the incubation periods, since the fluorescence intensities shifted significantly during the incubation, and the HPLC profiles of the acid digests at the end of the incubation were distinctly different to the initial HPLC profiles. One reason for a lack of observable crosslinking may be that the incubations were done at a protein concentration of 20 mg/mL. The protein concentration in the human lens has been reported as ~450 mg/mL,[21] therefore it would be ideal in the future to attempt incubations at a higher protein concentration.