INHIBITION OF CONFORMATIONAL CHANGES DUE TO CARBAMYLATION IN LENS CRYSTALLINS BY ASPIRIN

THESIS

Submitted to

The Faculty of Science

University of Karachi

In fulfilment for the degree of

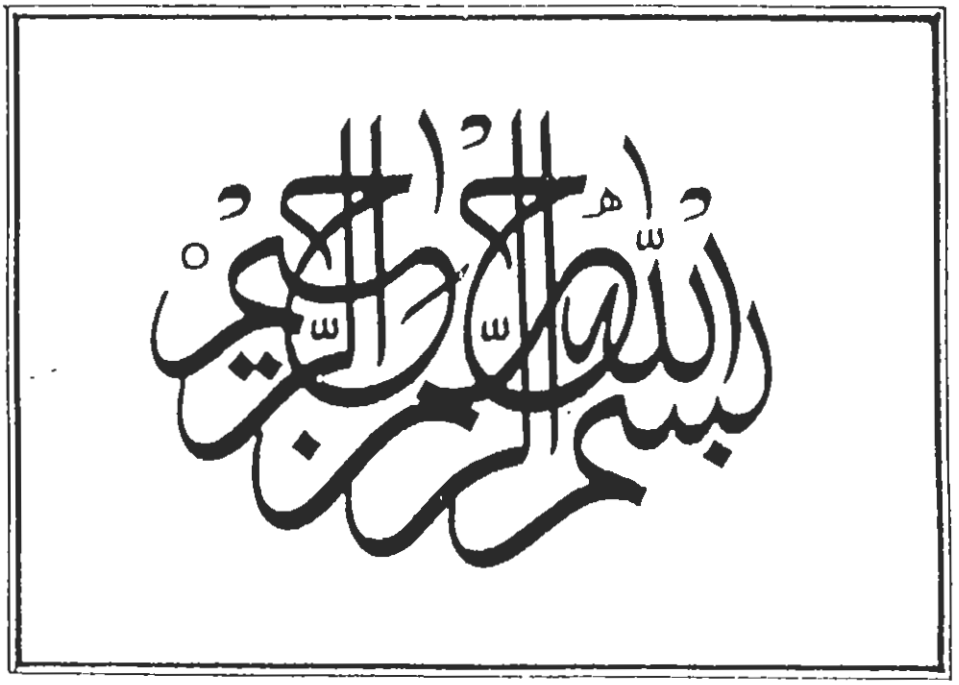
DOCTOR OF PHILOSOPHY

by

SYED AZEEM UL HASAN

H.E.J. RESEARCH INSTITUTE OF CHEMISTRY
UNIVERSITY OF KARACHI
APRIL 1993

Dedicated to
My Parents
Whom I owe everything



In the Name of Allah,
the Beneficent, the Merciful

ACKNOWLEDGMENTS

I take this opportunity to express my deepest gratitude and thanks to my supervisor Prof. Zafar H. Zaidi T.I. for his invaluable concerns, sustained guidance and unstinting support which enabled me to bring this work to completion.

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SUMMARY

The present study describes a new and more accurate technique, based on Fast atom bombardment mass spectrometry, to identify the types and quantitate the extent of modifications which have been produced due to the reactions of αA crystallin with isocyanate and aspirin. These findings would be helpful in understanding the phenomenon of carbamylation of lens crystallins in detail and also to check effectiveness of aspirin as an anticataract agent. The purified αA crystallin was incubated with KNCO and aspirin separately for 24 hours. Modified proteins then subjected to enzymatic digestions. Peptides generated by enzymatic digestions were separated using reverse phase HPLC and analyzed by FAB-MS. For reaction conditions used in this investigation, carbamylated and acetylated lysyl residues were the principal products. For determination of the extent of carbamylation and acetylation, aliquotes of α-crystallin were incubated with isocyanate and aspirin for different time(i.e. 6 hours, 12 hours, 24 hours and 48 hours). After each incubation the modified α-crystallin was separated into αA and αB using RP HPLC. Each modified αA then subjected to enzymatic digestions, peptides generated by enzymatic digestions were recovered on Vydac C18 column and analyzed by FAB-MS. The extent of modifications was quantitated from the intensities of the peaks in the FAB spectra of modified and unmodified peptides. Both isocyanate and aspirin have modified all lysyl residues of αA crystallin but with variable extent. Plots of the extent of carbamylation and acetylation versus time were used to calculate rate constants for reactions at each lysyl residue. Rate constants for carbamylation and acetylation of all lysyl residues except lysines 166 and 11 were found to be similar. The extent of modifications at lysyl residues of αA crystallin were also estimated from concurren. and sequential incubations. Both of these incubations caused a mark reduction in the extent of carbamylation and acetylation as compared to the extent of carbamylation and acetylation of lysyl residues while α-crsytallin was incubated either with isocyanate or

aspirin alone. Similarity in the rate constants and reduction in the extent of modifications due to concurrent and sequential incubations support the notion that "isocynate and aspirin compete for the same residue". But comparison of the yield of acetylated α-crystallin with the yield of carbamylated α-crystallin that might occur due to renal failure indicates that aspirin is not an effective inhibitor of cataract due to carbamylation of lysyl residues.

One letter and three letter symbols for amino acid

Amino acid	Three letter symbol	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Cystine (half)	1/2 Cys	
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

LIST OF ABBREVIATIONS

ACN	Acetonitrile
CSI	Cessium iodide
CM	Carboxymethyl
CNO	Isocyanate
DEAE	Diethylaminoethyl
EDTA	Ethylene diamine tetra acetic acid
FAB-MS	Fast atom bombardment mass spectrometry
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
KNCO	Potassium isocyanate
kDa	Kilodalton
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
R P	Reverse phase
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra violet

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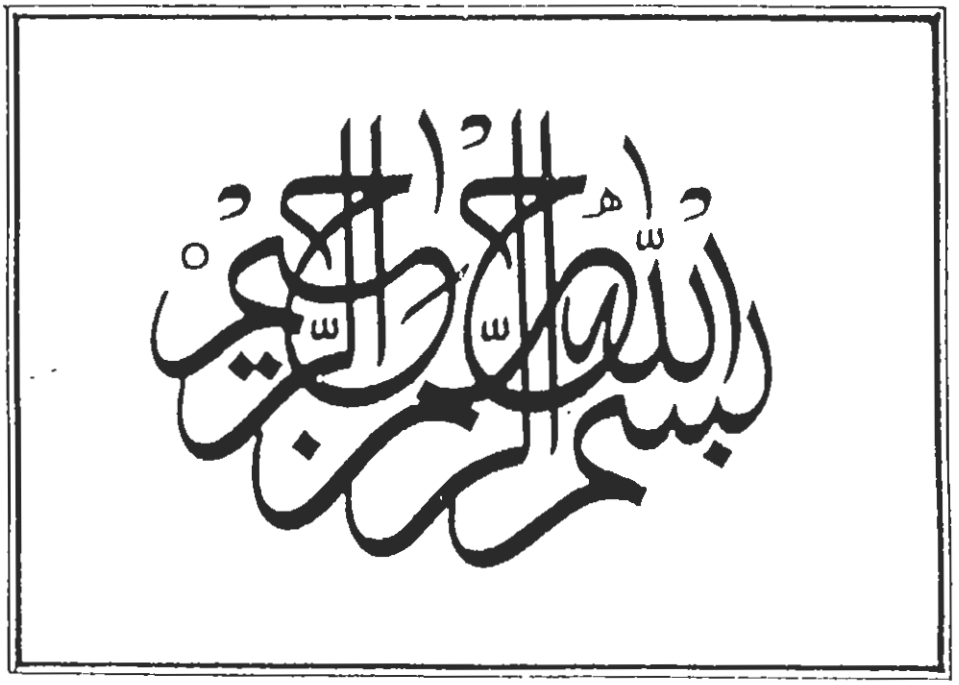

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Aspartic acid	Asp	D
Cysteine	Cys	C
Cystine (half)	1/2 Cys	
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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2.0 EXPERIMENTAL

2. 1. LENS

Bovine lenses from two years old cow were obtained from Purdue University animal science pilot plant, West Lafayette, Indiana, USA. Lenses were either processed immediately or stored at -20°C.

2.2. EXTRACTION OF LENS PROTEINS *(CRYSTALLINS)*

Lenses were decapsulated, homogenized in 30-40 volumes of aqueous buffer (50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.001 M EDTA). Homogenate was centrifuged at 20,000 x g for one hour at 4°C using ultracentrifuge (Hitachi SCP&SH). Sediment was washed twice with the same buffer and supernatant was collected, lyophilized and stored at -20°C.

2.2.1. Isolation of α-Crystallins

Crystallins extracted from bovine were fractionated into α, β and γ crystallins by gel filtration on Sephadex G-200 (Pharmacia). Samples (150 mg/5 ml) was dissolved in 50 mM Tris-HCl (pH 7.6) containing 0.5 M NaCl, 0.001 M EDTA and 0.05% NaN_3 and loaded on column of Sephadex G-200 (90 x 2.5 cm) previously equilibrated with the same buffer. Column was eluted with the same. Flow rate was maintained at 12 ml/hour and the absorbance was recorded at 280 nm. Fraction corresponding to α-crystallins were pooled, lyophilized and stored at -20 °C.

2.2.2. Isolation of αA Crystallins

Crude α-crystallins were separated into αA and αB crystallins by reverse phase HPLC (Ranin instrument Co. Woburn, MA), using a Vydac C4 (4.6 mm x250 mm), cartridge column (Alltech associated

Deerfield IL). Linear gradient of 30-60% acetonitrile in water with 0.1% trifluoroacetic acid over 60 min was used.

2.2.3. Electrophoresis

Electrophoresis of α-crystallins under denatured conditions was carried out according to Laemmli (1970) with slight modifications. Proteins were separated on 12.5% polyacrylamide gels. Samples were diluted with sample diluting buffer (0.0625 M Tris-Glycine pH 8.3 containing 8 M urea) in a ratio of 1:1.

Electrophoresis was carried out at 150 V till tracking dye (bromophenol blue) reached the bottom of the gel. Gel was stained with 0.1% commassie brilliant blue R-250 in methanol (5%) acetic acid (7.5%) mixture for 20-30 minutes and then destained using destaining solution (7 % acetic acid and 5% methanol) over night.

2.2.4. Enzymatic Digestions

α-crystallins (αA) were subjected to enzymatic digestions using enzymes pepsin, chymotrypsin and endoproteinase ASP-N separately.

a) Peptic Digest

10 nmoles (200 μg) of α A crystallin (α was dissolved in 200 μl of 0.2 M formic acid. Pepsin (Sigma Chemical Co., St. Louis, MO) was then added in an enzyme: substrate ratio of 1:50. Mixture was incubated at 37^{o}C for 2 hours.

b) Chymotryptic Digest

10 nmoles (200 μg) αA crystallin dissolved in 200 μl of 0.2 M Tris-HCl buffer (pH 8.2) was incubated with chymotrypsin (Boehringer Mannhein GmbH, West Germany) for 2 hours at 37^{o}C. Enzyme: substrate ratio of 1:50 was maintained.

c) Endoproteinase ASP-N Digest

10 nmoles of protein (αA crystallin) dissolved in 0.2 M Tris-HCl (pH 8.2) was digested with 0.2 μg (2 μg/5 μl) endoproteinase ASP-N (Boehringer Mannhein GmbH, West Germany) solution at 37^{o}C for seven hours.

2.2.5. Separation of Peptides

Peptides generated by enzymatic digestions were separated by reverse phase HPLC using Vydac C18 column with a linear gradient of 5-40% acetonitrile in water containing 0.1% TFA over 60 minutes. Flow rate was maintained 1 ml/min during the whole run and absorbance was recorded at 214 nm.

2.2.6. FAB - MS

FAB - MS analysis were performed on a Kratos MS-50, Mass spectrometer (Kratos Scientific Instruments, Manchester, UK) equipped with DS-90 data acquisition system and a RF magnet. Mass range (m/z 300-4000) was calibrated with CS^{n+1} ions from CSI. Vacuum dried HPLC fractions were dissolved in 22% formic acid and analyzed using 1:1 mixture of glycerol and thioglycerol as the matrix. FAB mass spectra were recorded over the mass range of 300-3500 with a resolution of 3000. Mass spectrometric data were processed using a MACH3 soft ware data system on sun work station.

2.2.7. Confirmation of Peptide

Peptides analyzed by FAB-MS were confirmed using carboxypeptidase Y. Peptide (5 nmoles) was dissolved in 0.2 M ammonium acetate (pH 7.01) 1 μg of carboxypeptides Y was added to the peptide solution and the whole mixture was incubated at 37^{o}C for 20 to 70 minutes. After incubation, mixture was dried under vacuum. Vacuum dried peptide was dissolved in 22% formic acid and analyzed by FAB-MS as described earlier.

2.3. MODIFICATIONS OF α-CRYSTALLINS

α–crystallins were modified with KNCO and aspirin for studying the conformational changes that have occurred due to carbamylation and its possible inhibition with aspirin.

2.3.1. Carbamylation of α-Crystallins

Crude α-crystallin (Peak 1 of Sephadex G- 200) was dissolved in 0.2 M Tris-HCl containing 0.05% NaN_3. To this solution 100 mM of KNCO was added and pH was adjusted to 7.6 with IN NaOH at 37°C and then incubated overnight. After incubation, modified protein was desalted on Sephadex G-25 in order to remove excess of KNCO and other by products. Modified α-crystallins were then separated into αA and αB by reverse phase HPLC on Vydac C4 column. Modified αA crystallins were subjected to enzymatic digestion as described earlier. Peptides generated by enzymatic digestions were first separated on Vydac C18 column and then analyzed by FAB-MS.

2.3.1.1. Determination of Extent of Carbamylation

Aliquots of crude α-crystallins (peak 1 of Sephadex G-200) and αA crystallin (peak 3 of HPLC on C4 column) were incubated with 100 mM KNCO in 0.2 M Tris-HCl (pH 7.6) containing 0.05% NaN_3 at 37°C for different time (i.e. 6 hours, 12, hours, 24 hours and 48 hours). Control samples were prepared for each time by incubating crude α-crystallin (peak 1 of Sephadex G- 200) and αA crystallin (peak 3 of HPLC on Vydac C4 column) in the same buffer but without KNCO. After each incubation solutions were desalted on Sephadex G-25. Each modified crude α-crystallin was then separated into αA crystallin and αB crystallin by reverse phase HPLC on Vydac C4 column.

All modified αA crystallins were subjected to enzymatic digestion individually. Peptides generated by enzymatic digestion were

recovered on Vydac C18 column. Peptides containing modified and unmodified lysine were collected in the same fraction and analyzed by FAB-MS. Quantitation of modification Lys 11, Lys 70, Lys 78 and Lys 88 was carried out using peptic digest whereas quantitation of modification at Lys 145 and Lys 166 was performed from Chymotryptic digest. Lysine 99 was quantified from a peptide in the ASP-N digest. For quantitation, FAB mass spectra were acquired at a scan rate of second per decade over a range covering the molecular mass of the peptide of interest. Intensities of modified and unmodified peptides were averaged over ten scans. Resolution was adjusted to 1500. The modification percentage at each lysyl residue was calculated by comparing intensities of modified and unmodified peptides. Control samples were processed in similar way as for modified proteins.

2.3.2 . Reaction of α-Crystallins with Aspirin

Crude α-crystallins (peak 1 of Sephadex G-200) and αA crystallin (peak iii of HPLC on C4 column) were treated with aspirin, under native and denatured conditions respectively.In case of denatured state, 50 nmoles (1 mg/ml)of αA crystallin was dissolved in 0.2 M Tris-HCl (pH7.6) containing 6M guanidine hydrochloride and 0.05% NaN_3. To the mixture 100 mM aspirin was added and pH was readjusted to 7.6 at 37°C. The solution was incubated for 24 hours at 37°C. In native form, crude α-crystallins (50 nmoles) was dissolved in the same buffer but without 6 M guanidine hydrochloride. The protein solution was incubated with 100 mM aspirin for 24 hours at 37°C and pH was maintained to 7.6. After incubation, each solution was subjected to Sephadex G-25 column removing excess of reagent and other byproducts. Crude modified α-crystallins were then separated into αA and αB using reversed phase HPLC on Vydac C4 column. Aliquots of modified αA crystallin (both from native and denatured states) were digested with enzymes pepsin, chymotrypsin and endoproteinase ASP-N separately as described earlier. Peptides generated by

enzymatic digestions were fractionated on Vydac C18 column. Each peptide was collected individually and analyzed by FAB-MS using similar conditions as described earlier.

2.3.2.1. Determination of Extent of Acetylation

Aliquots of crude α-crystallins (peak 1 of Sephadex G-200) and αA crystallin (peak3 of HPLC) were incubated with 100 mM aspirin in 0.2 M Tris-HCl (pH 7.6) containing 0.05 % NaN_3 at 37^{o}C for different time(i.e, 6 hours, 12 hours, 24 hours and 48 hours). Control samples were prepared for each time by incubating α-crystallins and αA crystallins in the same buffer but without aspirin. After each incubation, solutions were desalted on Sephadex G-25. Each modified crude α-crystallin was separated into αA and αB crystallin by reversed phase HPLC using C4 column. All modified proteins were then subjected to enzymatic digestion individually. Peptides generated by enzymatic digestions were recovered on Vydac C18 column. Lysine containing modified and unmodified peptides were pooled together, vacuum dried and then analyzed by FAB-MS. The percentage modification at each lysyl residue was calculated by comparing intensities of modified and unmodified peptides. Control samples were also subjected in the similar way as for the modified proteins.

2.3.2.2 . On line HPLC Continuous Flow FAB-MS

Two aliquots, each from modified (under denatured state) and unmodified αA crystallin were digested with chymotrypsin using similar conditions as described earlier. Digested materials after desalting on Vydac C18 column, were subjected to on-line HPLC conditions flow FAB-MS. The apparatus used for on-line HPLC continuous flow FAB-MS is shown in Fig. 2.1. It consists of a gradient HPLC system (Ranin Instrument Co, Woburn, MA), two sample injectors,one pre-column injector for loading and other post column injector for tuning and calibrating the mass spectrometer, reversed phase column

(Applied biosystems,aquapore, 50x1mm) and an effluent splitter for controlling the flow rate 3 μl/min to mass spectrometer. The microbore HPLC system was attached to a Kratos MS-50 RF mass spectrometer via a continuous flow probe connected with fused silica (50 υm i.d). The continuous flow probe was designed by David et al., (1990).

5 nmoles of each digest was injected separately and peptides were fractionated using a gradient of 0-50% B in 30 minutes. Solvents A and B contained 10-80% acetonitrile respectively. Both solvents contained 3% glycerol, 3% thioglycerol and 0.1% TFA. The mass spectrometer was operated at a scan rate of 30 sec/decade and 8 KV accelerating voltage. Data were acquired with a Kratos DS-90 data acquisition system and displayed with a Sun work station operating with Kratos MACH 3 software.

2.3.3. Comparative Study of Acetylation and Carbamylation

Effect of acetylation on carbamylation of lens α-crystallin was determined for both concurrent and sequential incubations of α-crystallins in 100 mM aspirin and 100 mM KNCO. 50 nmoles (1mg/ml) of α-crystallin was dissolved in 0.2 M Tris-HCl (pH7.6), containing 0.05 % NaN_3, incubated 100 mM aspirin and 100 mM KNCO together for 24 hours at 37^{o}C. In sequential study, α-crystallin was first incubated with 100 mM aspirin in 0.2 M Tris-HCl (pH 7.6), containing 0.05% NaN_3 at 37^{o}C. After 24 hours the unreacted aspirin was removed using Sephadex G-25 and then acetylated α-crystallin was reincubated in the same reaction buffer containing 100 mM KNCO for 24 hours at 37^{o}C.

Each solution of incubated α-crystallin was desalted after completion of incubation. Modified proteins were separated into αA and αB crystallin by HPLC on Vydac C4 column Each modified αA crystallin was then subjected to enzymatic digestions. Peptides generated by enzymatic

digestions were separated on Vydac C18 column. Modified and unmodified peptides were pooled together, vacuum dried and analyzed by FAB-MS.

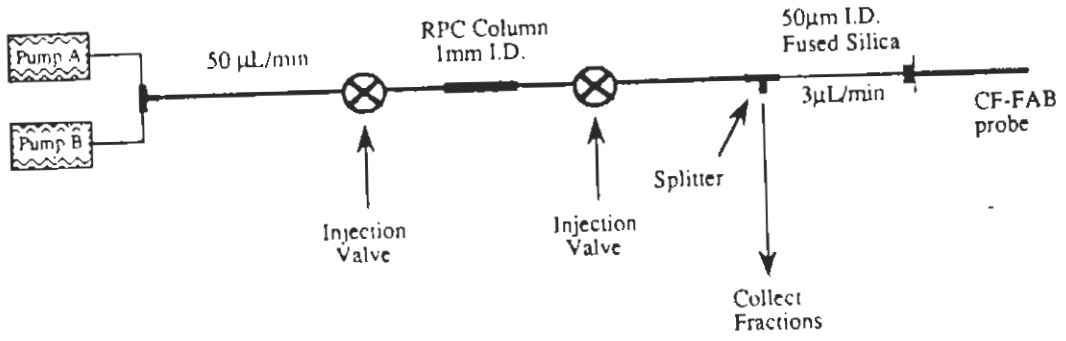


Fig. 2.1. Diagram of on-line HPLC continuous flow FAB-MS system used to analyze chymotryptic digests of αA crystallin.

3.0 RESULTS

3.1. GEL CHROMATOGRAPHY

Separation of water soluble crude lens proteins on Sephadex G-200 resulted in four peaks (Fig. 3.1), peak 1 represents α-crystallins whereas peak 2 and peak 3 represent βH and βL respectively. Peak 4 represents γ-crystallins.

3.2. REVERSE PHASE HPLC

Fractionation of α-crystallins on RP C4 resulted in three major and some minor peaks (Fig. 3.2).

3.3. POLYACRYLAMIDE GEL ELECTROPHORESIS

Figure 3.3 shows electrophoretic pattern of fractions obtained from RP-HPLC.

3.4. ENZYMATIC CLEAVAGE

The purified protein (αA crystallin) was digested with pepsin, chymotrypsin and endoproteinase ASP-N separately. The peptides generated by enzymatic digestions were separated on HPLC using Vydac C18 column. Elution profiles of these peptides are presented in Figs. 3.4 -3.6.

3.5. FAB-MS

Peptides generated by enzymatic digestions were analyzed by FAB-MS as described in section 2.1. A spectrum is shown in Fig. 3.7.

3.6. CONFIRMATION OF PEPTIDES

Amino acid sequence of each peptide was confirmed using carboxypeptidase Y. An example is shown in Fig. 3.8.

3.7. MODIFICATIONS OF α-CRYSTALLINS

α-crystallins were modified with KNCO and aspirin to study the conformational changes that have occurred due to carbamylation and its possible inhibition by aspirin.

3.7.1. Carbamylation

a) Enzymatic Cleavage

The modified αA crystallin was digested with pepsin, chymotrypsin and endoproteinase ASP-N as described in section 2. Peptides generated by enzymatic digestions were separated on Vydac C18 column. Elution profiles of these digests are shown in Figs. 3.9, 3.10 and 3.11 respectively.

b) FAB-MS

Peptides obtained from enzymatic digestions of modified αA crystallin were analyzed by FAB- MS. A spectrum showing modified peptide of αA crystallin is given in Fig. 3.12.

3.7.1.1. Determination of the Extent of Carbamylation

a) Reverse Phase Chromatography

The separation profiles of crude α-crystallin (peak 1 of Sephadex G 200) after carbamylation at various intervals (i.e, 6 hours, 12 hours, 24 hours and 48 hours) on reverse phase C4 column are shown in Figs. 3.13-3.16.

b) Enzymatic Cleavage

Modified αA crystallin was subjected to enzymatic digestions after each incubation as described in section 2. The elution pattern of peptides generated by peptic, chymotryptic and endoproteinase ASP-N digests of carbamylated αA crystallin for 24 hours, showing pooling scheme of

peptides containing unmodified and carbamylated lysines are presented in Figs. 3.17-3.19.

c) FAB-MS

FAB spectra showing carbamylated and uncarbamylated lysine containing peptides are given in Figs. 3.20-3.26.

Graph representing, the percentage of carbamylation at different time (i.e. 6 hours, 12 hours, 24 hours and 48 hours) of lysines 11, 70, 78, 88, 99, 145 and 166 is given in Fig. 3.27. Dotted line indicates decrease in concentration of KNCO determined by negative FAB (Qin et al, 1991).

Graph representing the percentage of carbamylation at different time (i.e. 6 hours, 12 hours, 24 hours and 48 hours) of lysines 11, 70, 78, 88, 99, 145 and 166 from αA crystallin (separated from αB prior to incubation with KNCO) is given in Fig. 3.28. Dotted line indicates decrease in concentration of KNCO determined by negative FAB.

3.7.2. Acetylation

a) Enzymatic Cleavage

Elution profiles of peptides generated by various enzymatic digestions of acetylated αA crystallin on RP C18 column are given in Figs. 3.29-3.31.

b) FAB-MS

A spectrum showing modified peptide(acetylated peptide) of Chymotryptic digest is shown in Fig. 3.32.

3.7.2.1. Determination of the Extent of Acetylation

a) Reverse Phase HPLC

Separation profiles of α-crystallin after acetylation at various time (i.e. 6

hours, 12 hours, 24 hours and 48 hours) on RP C4 column are shown in Figs. 3.33-3.36.

b) Enzymatic Cleavage

Acetylated αA crystallin were digested with enzymes, pepsin, chymotrypsin and endoproteinase ASP-N separately after each incubation as mentioned in section 2.9.The elution patterns of peptides generated by peptic,chymotryptic and endoprotinase ASP-N digests of acetylated αA crystallin are given in Figs. 3.37-3.39. Profile describe the pooling scheme peptides containing unmodified and acetylated lysines in single fraction.

c) FAB-MS

FAB spectra showing acetylation at different lysyl residues alongwith unmodified lysyl residues are shown in Figs. 3.40-3.46.

Graph represents the percentage of acetylation of different lysyl residues at different times is shown in Fig. 3.47.The dotted line indicates decrease in concentration of aspirin with time as determined by RP-HPLC.

3.7.3. Modifications of αA(only) Crystallin

Crude α–crystallins were separated into αA and αB prior to modification by RP-HPLC on C4 column. Aliquots of αA crystallin were then incubated with aspirin for different time as described in section 2.8. After each incubation modified proteins were subjected to enzymatic digestions and analyzed by FAB-MS as described in section 2.9. A spectrum showing ratio of modified and unmodified lysine at 145 position of αA crystallin is shown in Fig. 3.48.

The graph showing effect of time on the percentage of modification at different lysyl residues due to acetylation are given in Figs 3.49.

3.7.4 Competitive Study

The percentage of acetylation and carbamylation of lysyl residue in αA crystallin was compared after incubation of aliquots of crude α-crystallin concurrently and sequentially.

a) Reverse Phase HPLC

Separation profiles of modified crude α-crystallins by concurrent and sequential incubations, on C4 column are shown in Fig. 3.50 and Fig. 3.54.

b) Enzymatic Digestion

Modified αA crystallin (both from concurrent and sequential incubations) were subjected to enzymatic digestions using enzymes pepsin, chymotrypsin and endoproteinase ASP-N separately. Details are given in section 2.10. Peptides generated by enzymatic digestions were separated by RP-HPLC on C18 column. Elution profiles of these peptides are shown in Figs. 3.51, 3.52, 3- 3.53, 3.55 and 3.57.

c) FAB-MS

Each peptide generated by enzymatic digestions was analyzed by FAB-MS as mentioned in section 2.11. Spectra showing ratio of modified and unmodified lysyl residue after concurrent and sequential incubation are given in Figs. 3.58 and 3.59. The percentage of modifications at each lysyl residue calculated from FAB-MS is shown in table 3.1. Comparison of acetylation and carbamylation of lysines in αAcrystallin in 24 hours incubations.

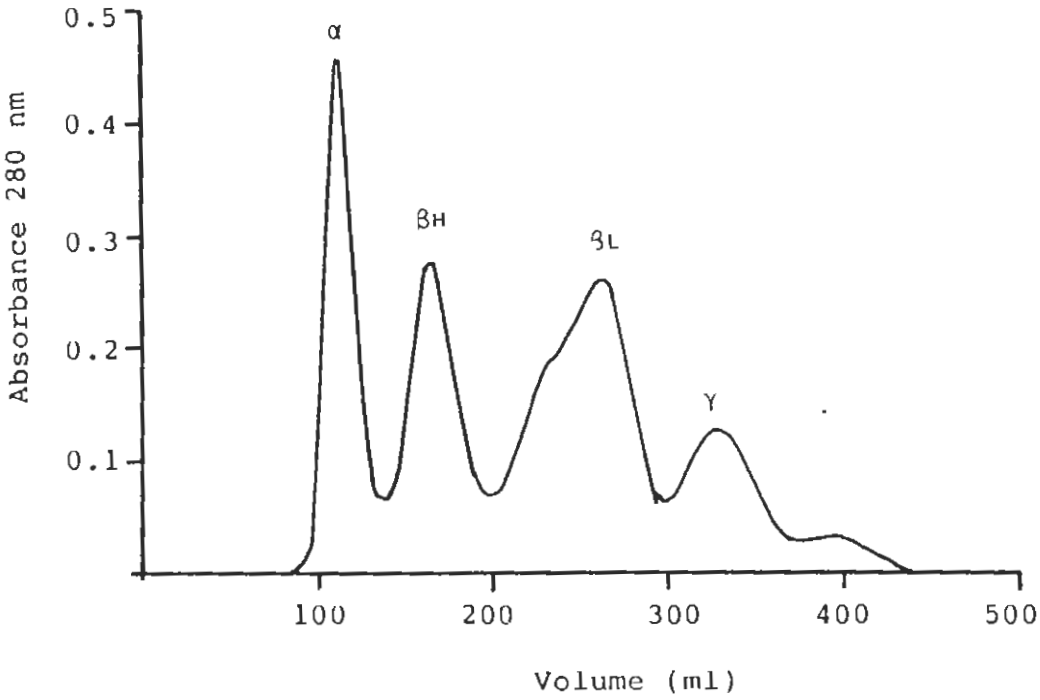


Fig. 3.1. Separation profile of water soluble Proteins (Crystallins) on Sephadex G-200. Column size:90 x 2 cm, eluent:50 mM Tris-HCl (pH = 7.4) containing 0.05% NaN_3, flow rate:12 ml/hr., absorbance:280 nm.

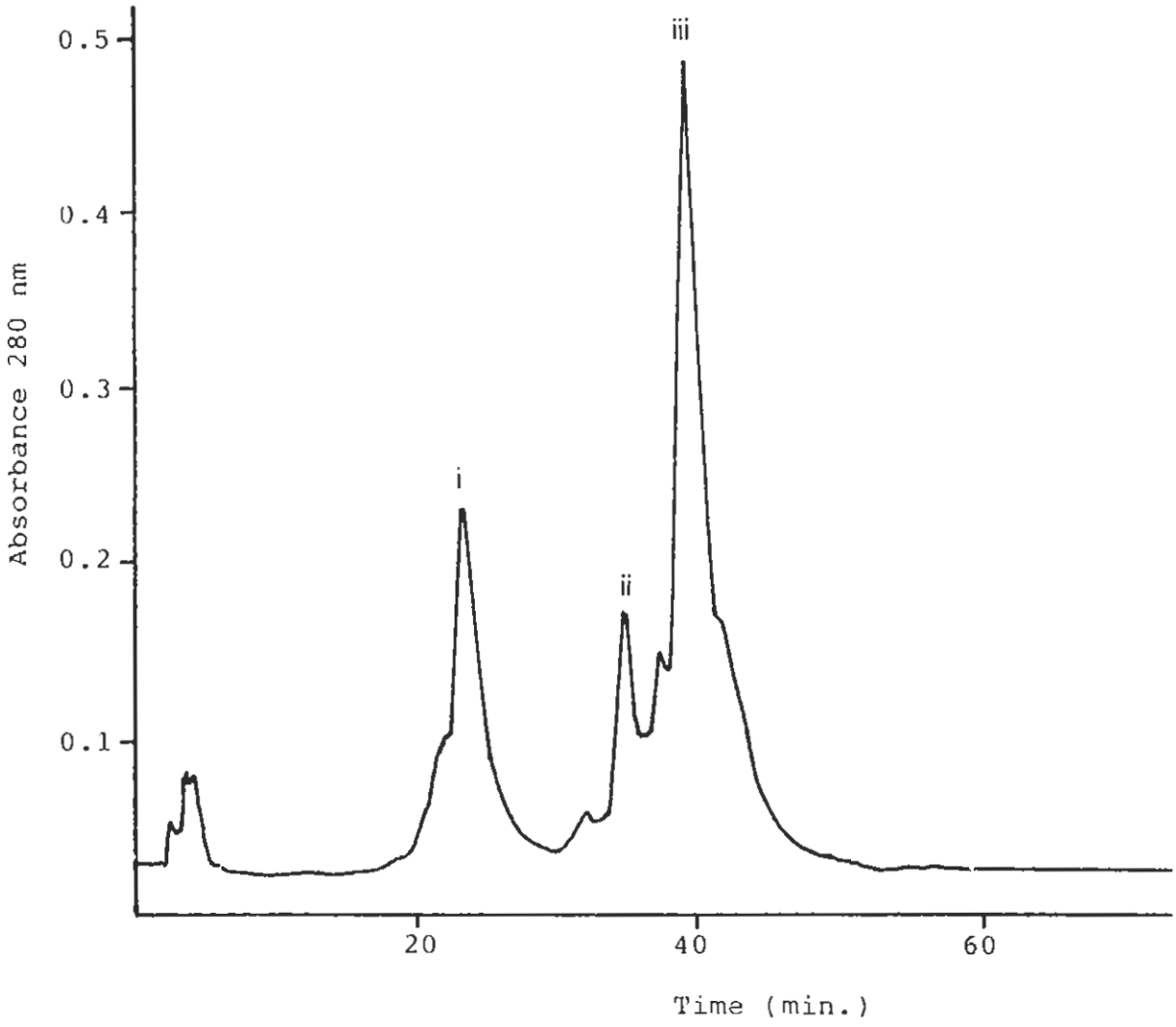


Fig. 3.2. Rechromatography of peak 1 (Fig.3.1.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:280 nm.

Fig. 3.3. Polyacrylamide gel electrophoresis of crude(Peak 1 of gel filtration), αA(Peak iii of HPLC) and αB (Peak i of HPLC) Crystallins. From left to right, lane 1: crude α-crystallin, lane 2:αB crystallin, lane 3: αA crystallin.

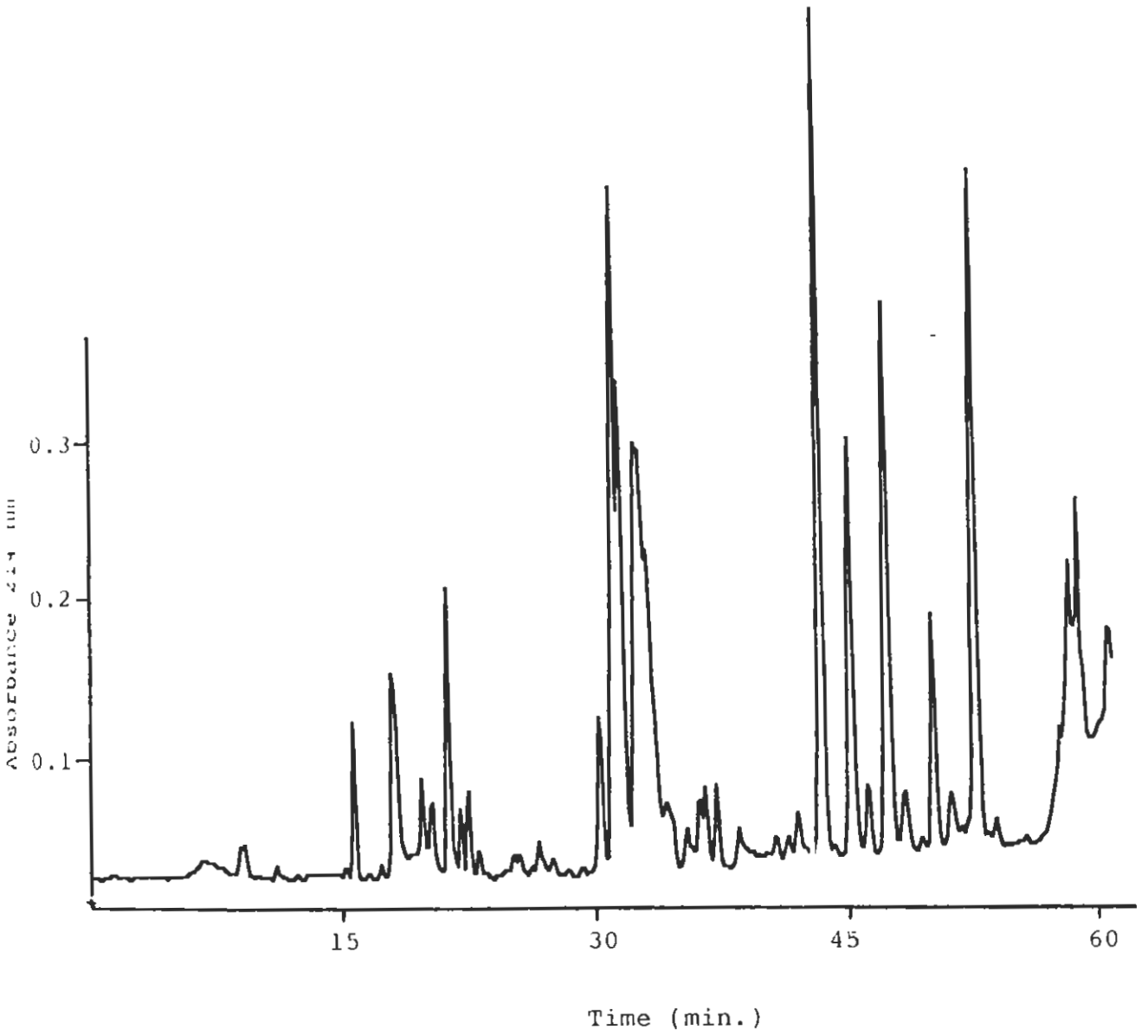


Fig. 3.4. Separation profile of peptic peptides of unmodified αA Crystallin on Vydac C18 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:214 nm.

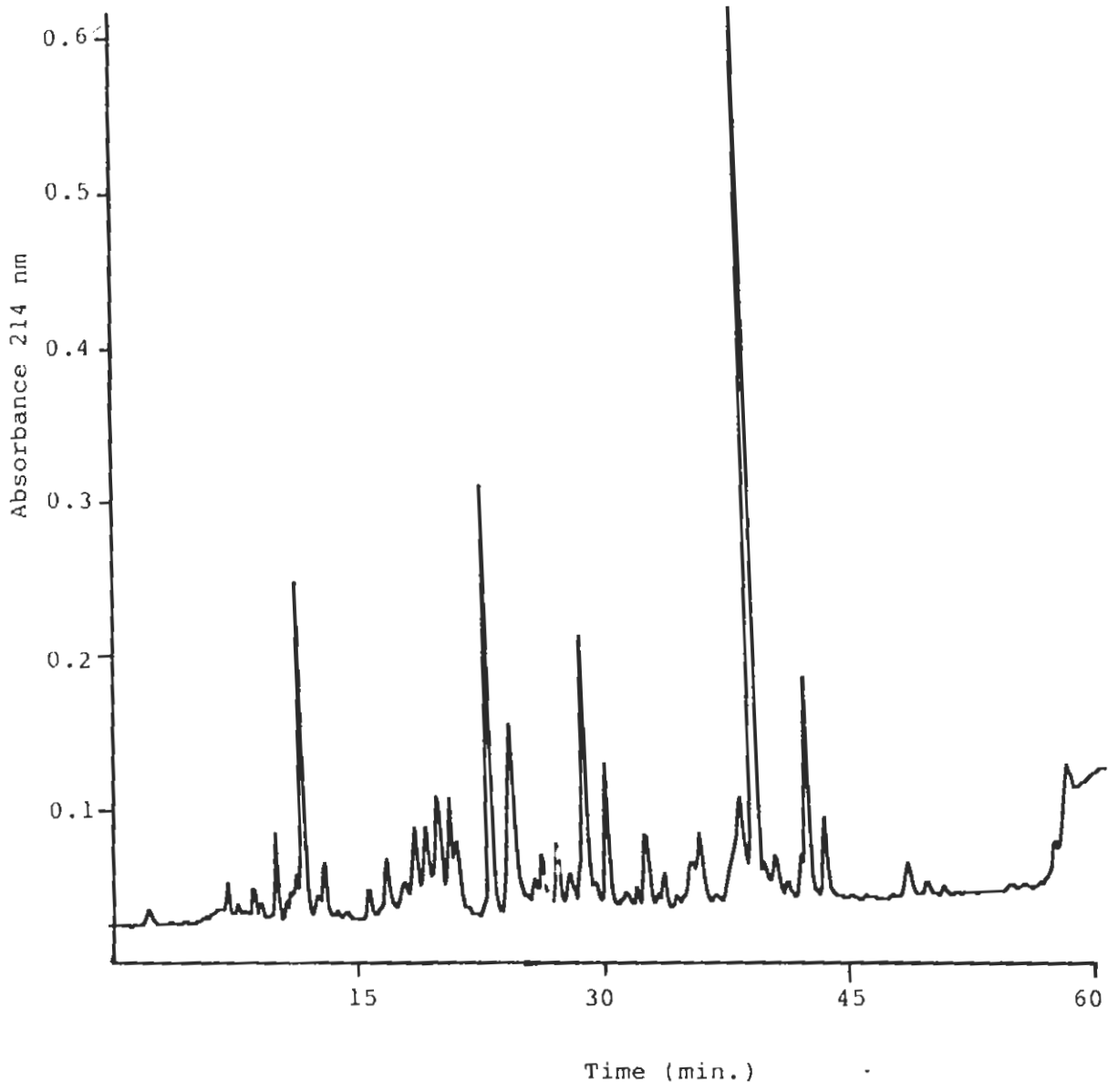


Fig. 3.5. Separation profile of chymotryptic peptides of unmodified αA Crystallin on Vydac C18 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.

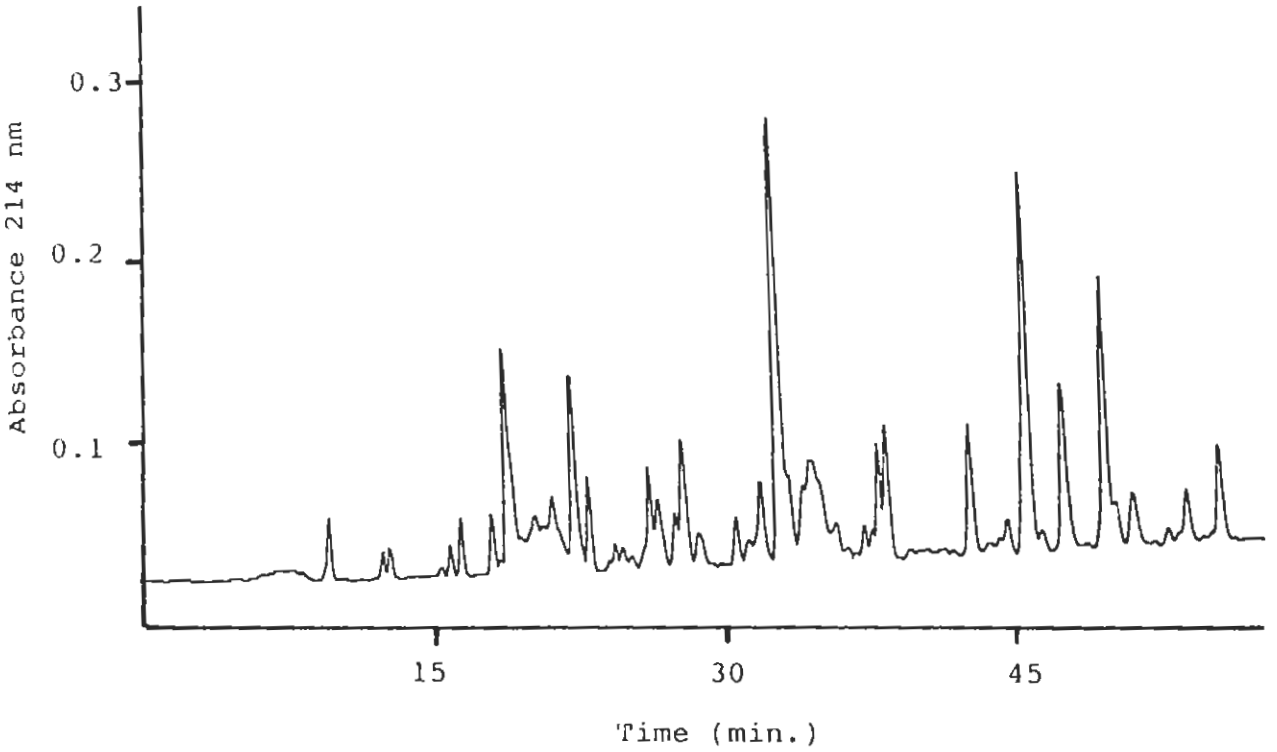


Fig. 3.6. Separation profile of peptides generated by ASP-N digestion of unmodified αA Crystallin. Eluent: 0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:214 nm.

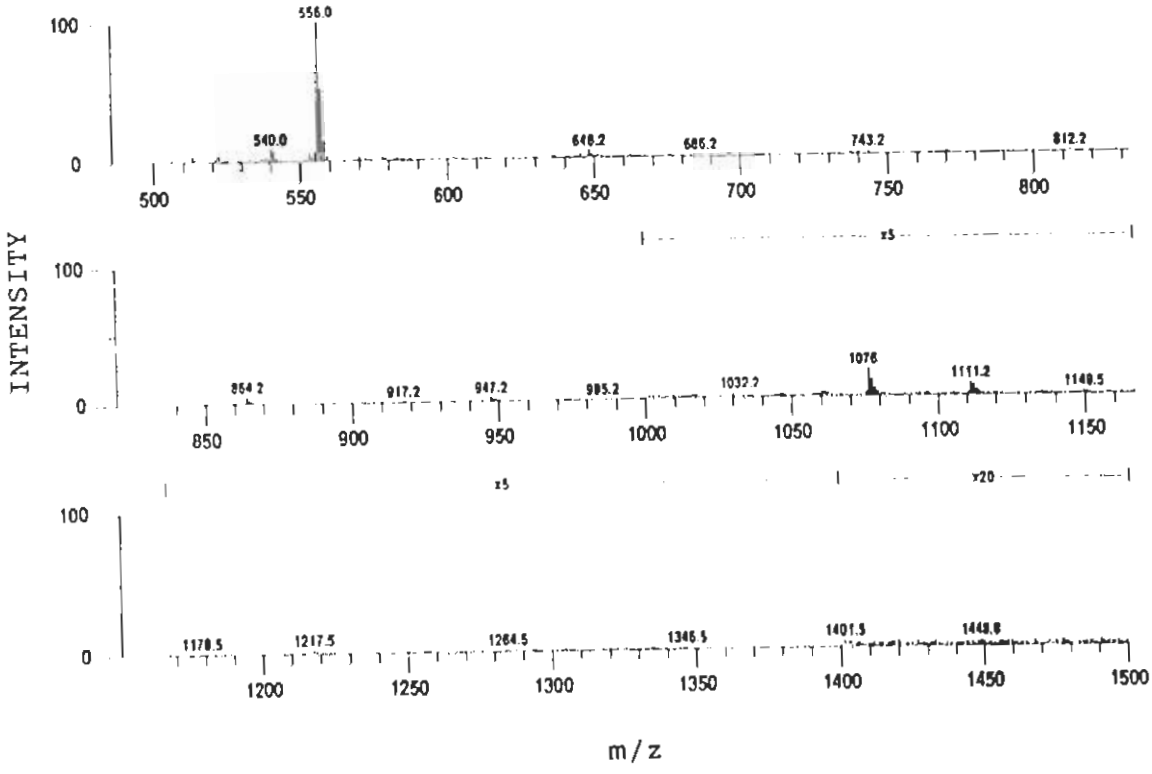


Fig. 3.7. FAB mass spectrum of a fraction from peptic digest of unmodified αA Crystallin. Molecular ions at m/z 556 represent peptide 24-27. Scan rate: 30 sec/decade, resolution: 3000, acceleration voltage: 8kv, calibration range: 300- 4000, matrix: glycerol: thioglycerol(1:1).

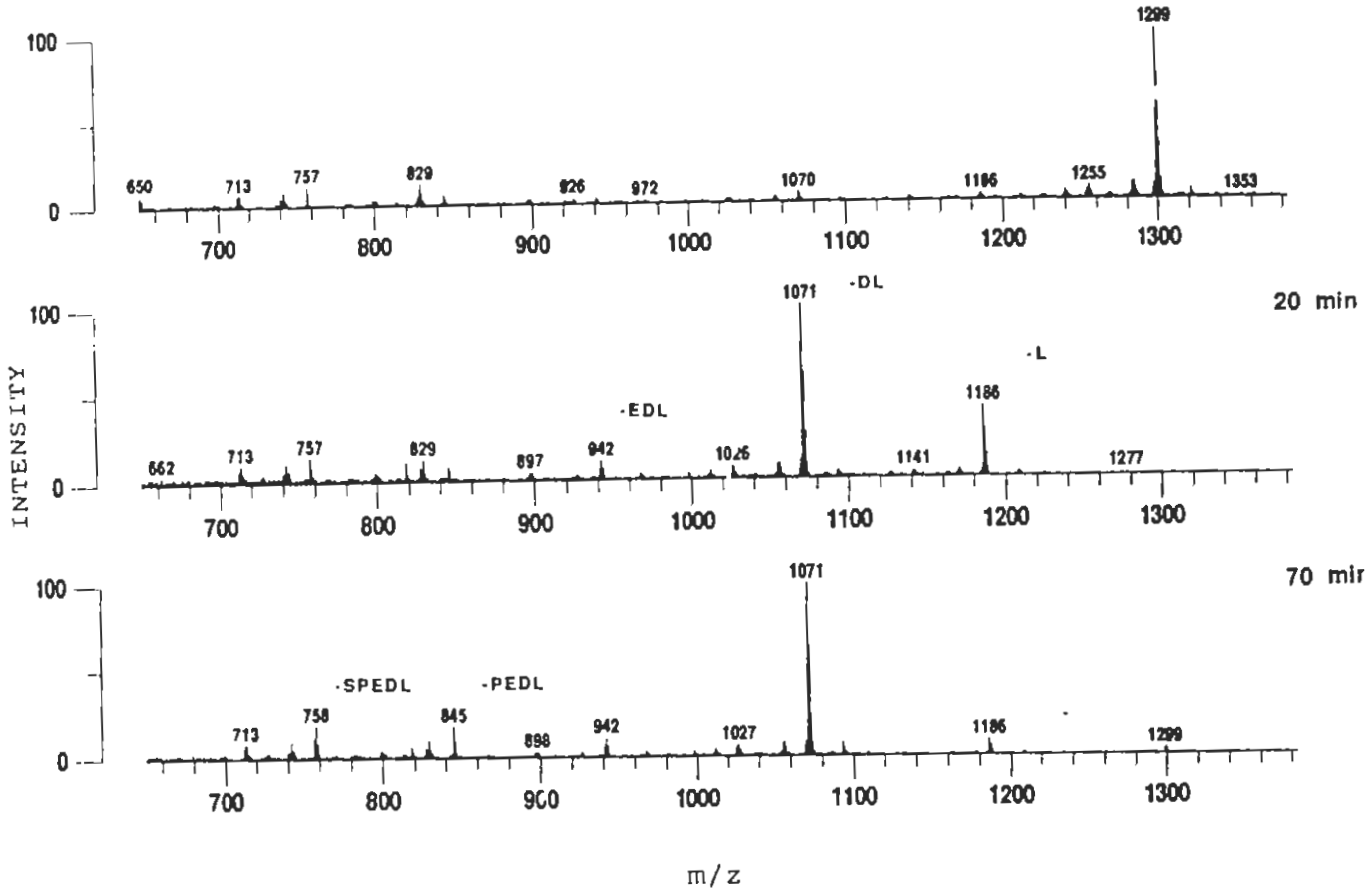


Fig. 3.8. FAB mass spectrum of a peptide(75-85) digested with carboxypeptidase Y for confirmation of the amino acid sequence of peptide. Scan rate: 30 sec/decade, resolution: 3000, acceleration voltage: 8 kv,calibration range: 300-4000, matrix: glycerol: thioglycerol(1:1). The sequence was determined as LDVKHFSPEDL.

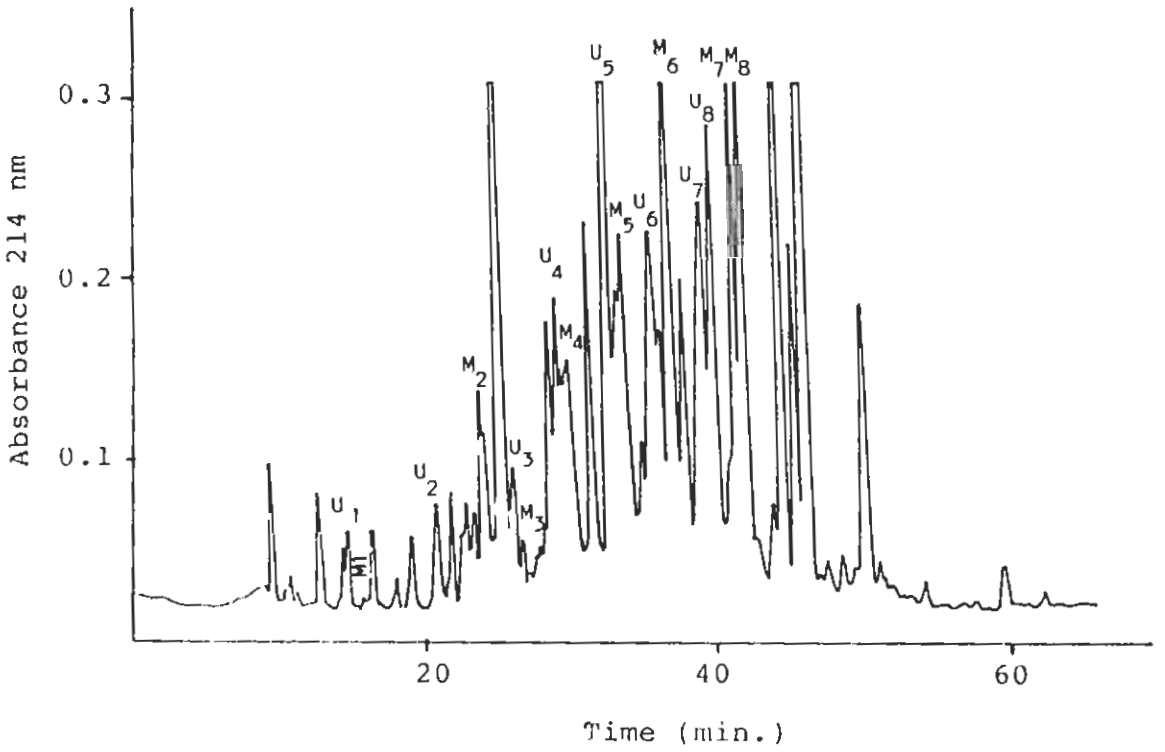


Fig. 3.9. Separation profile of peptic peptides of carbamylated αA Crystallin on Vydac C18 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.U denotes unmodified peptides and M denotes modified peptides.

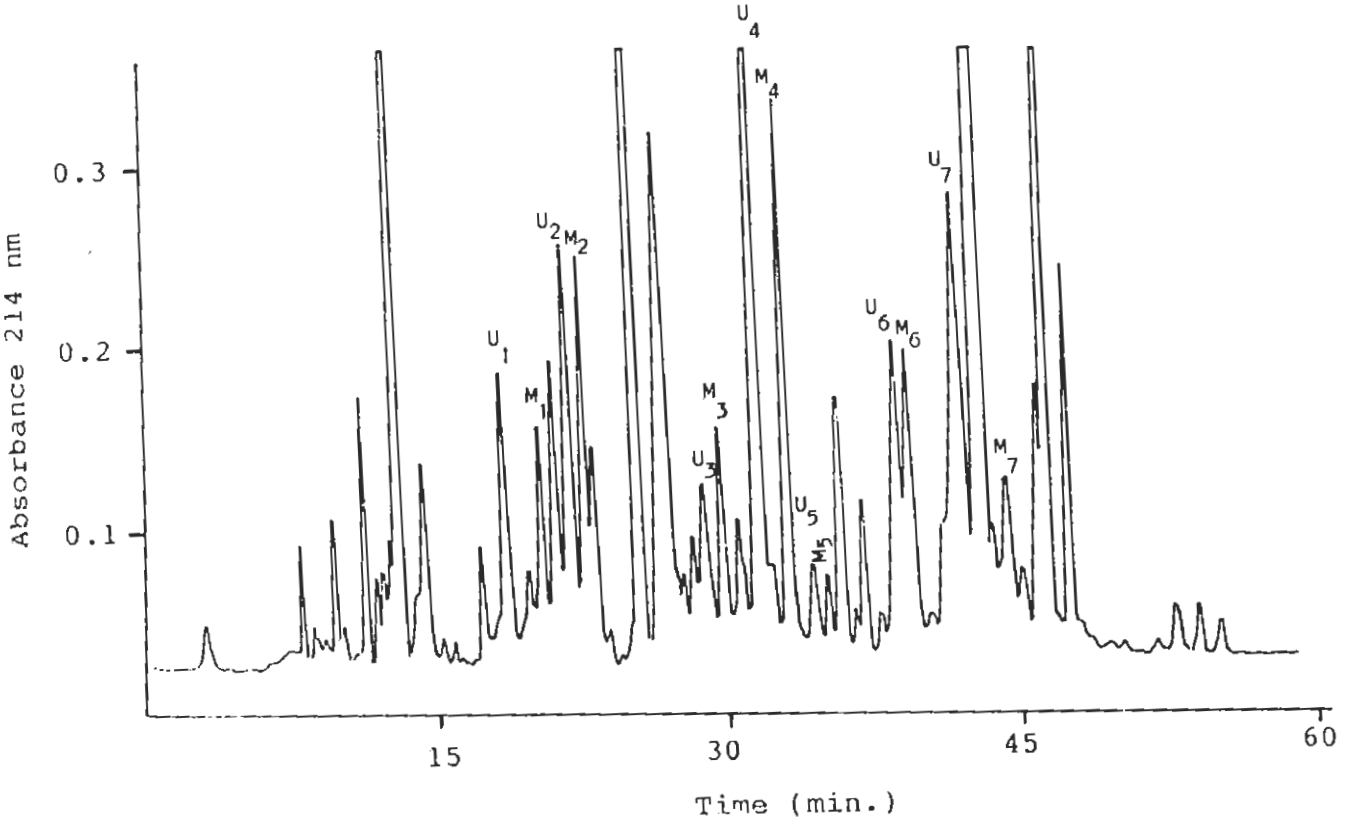


Fig. 3.10 Separation profile of chymotryptic peptides of carbamylated αA Crystallin on Vydac C18 column. Euent:0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:214 nm. U denotes unmodified peptides and M denotes modified peptides.

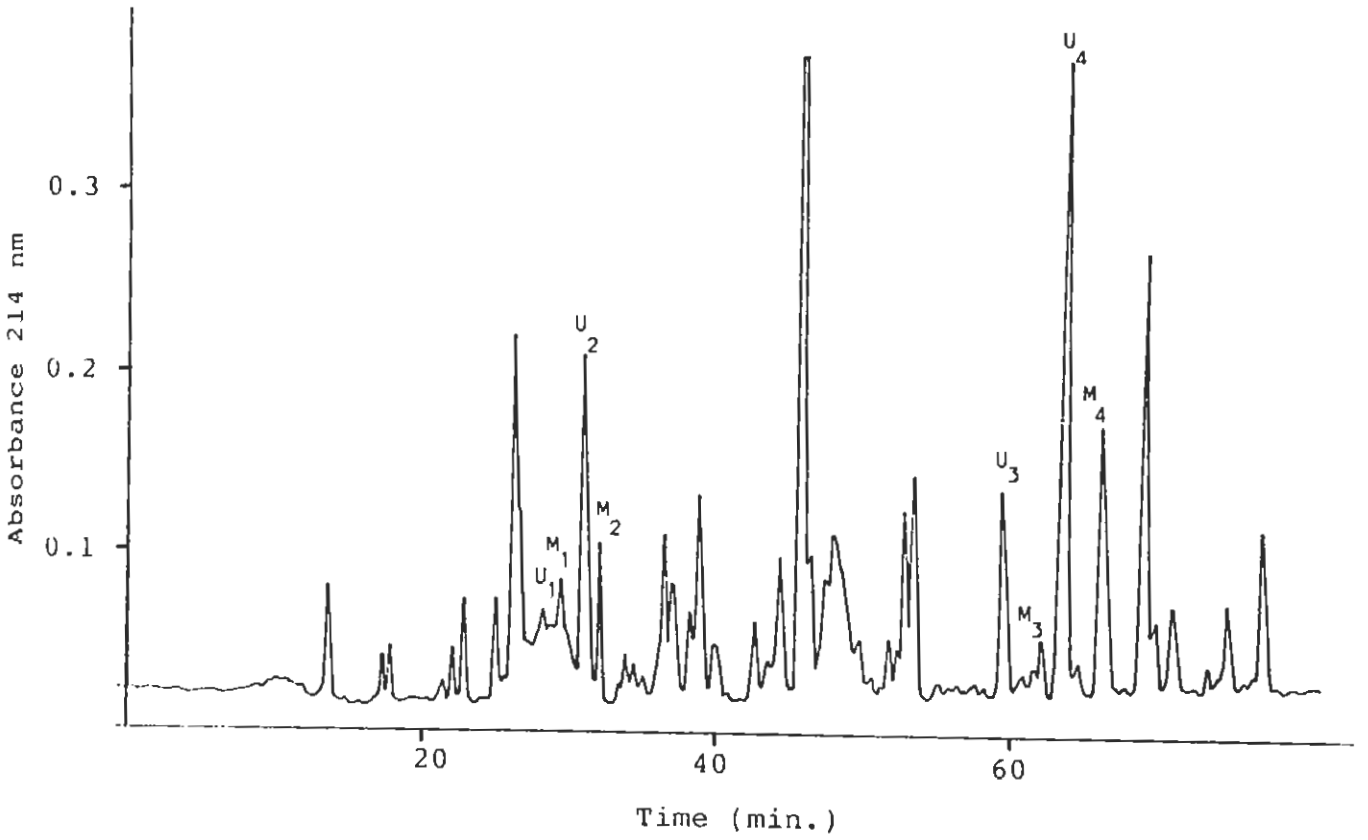


Fig. 3.11 Separation profile of peptides generated by ASP-N digestion of carbamylated αA Crystallin on Vydac C18 column, Eluent:0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:214 nm.U denotes unmodified peptides and M denotes modified peptides.

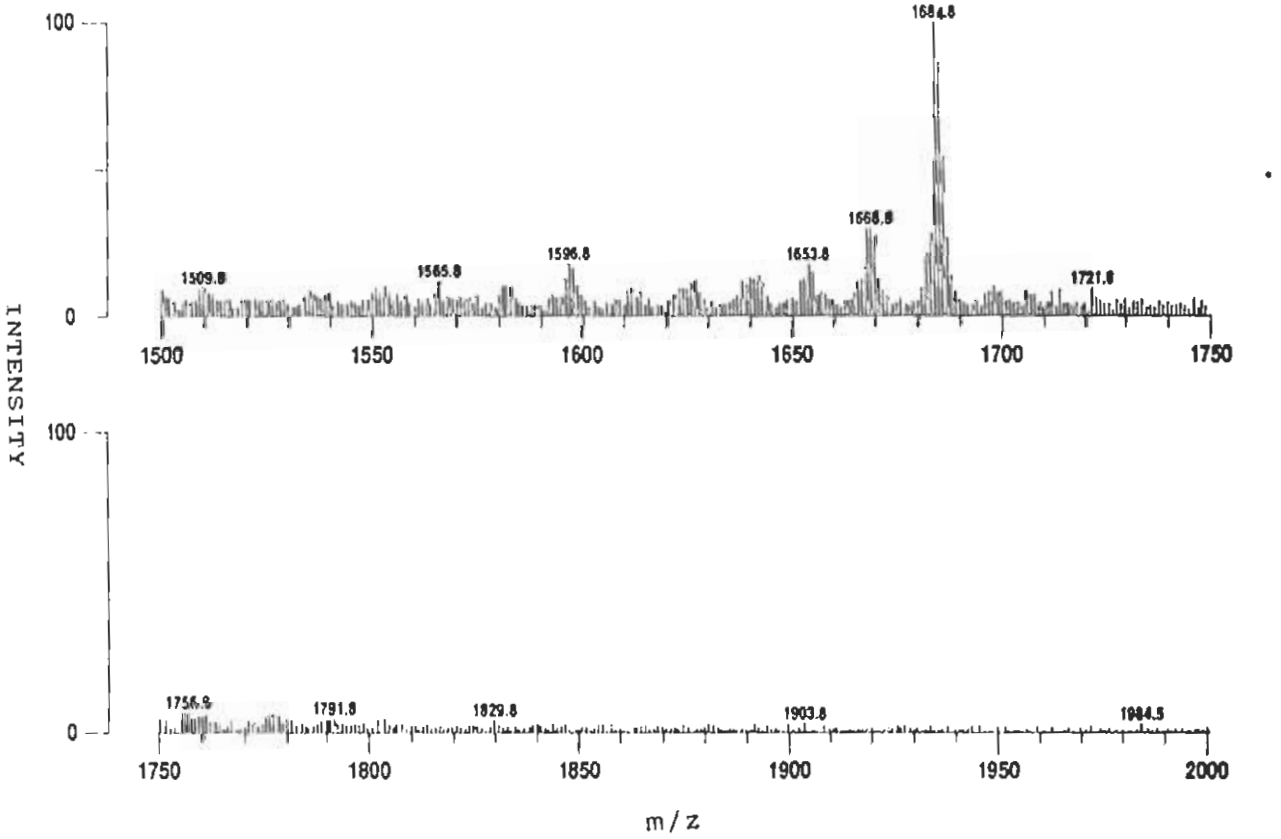


Fig. 3.12 FAB mass spectrum of a fraction from chymotryptic digest of carbamylated αI crystallin, showing carbamylated peptide (m/z = 1684.8).

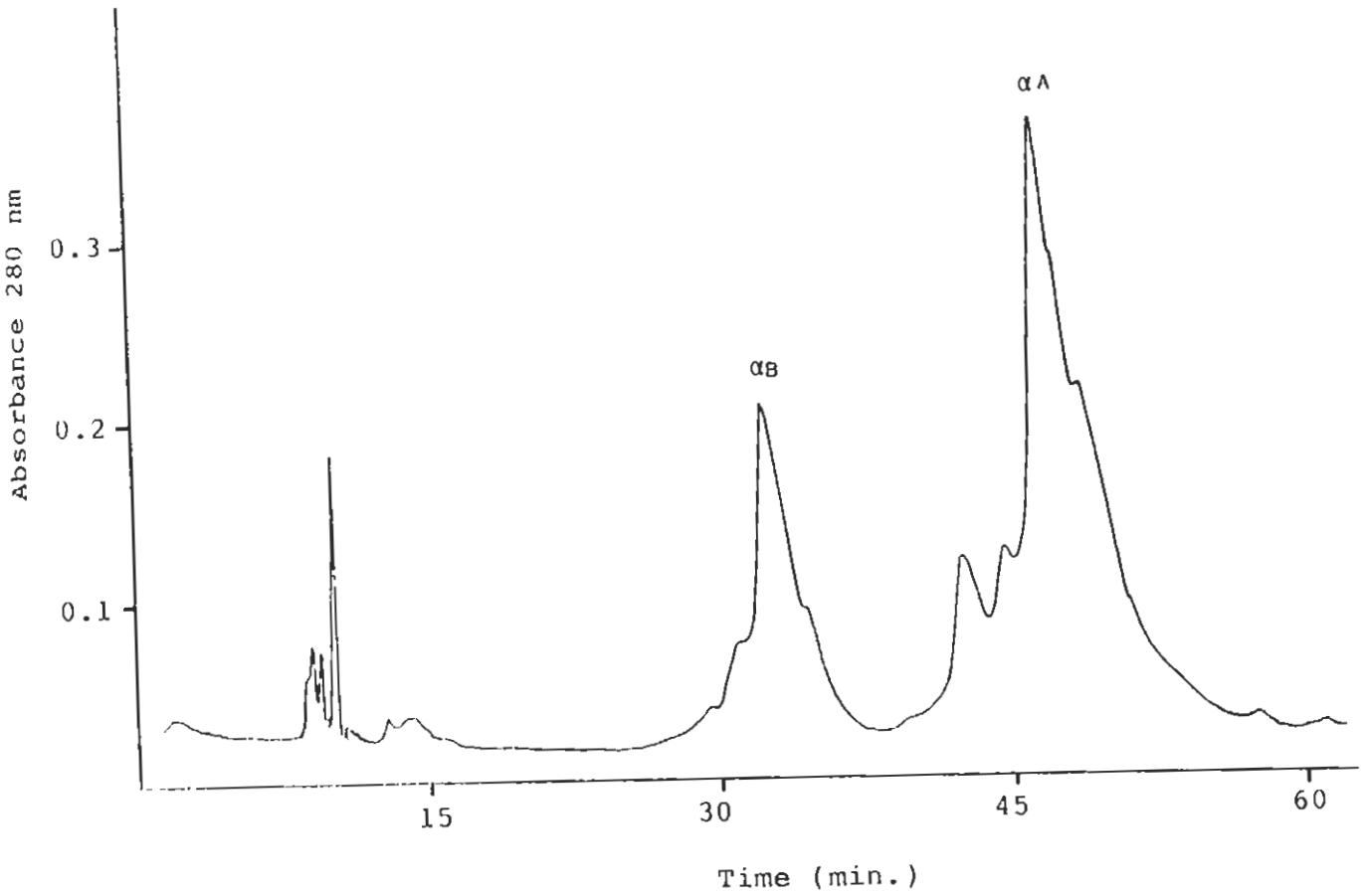


Fig. 3.13 Separation profile of crude α-crystallin (incubated with 100 mM KNCO for 6 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:280 nm.

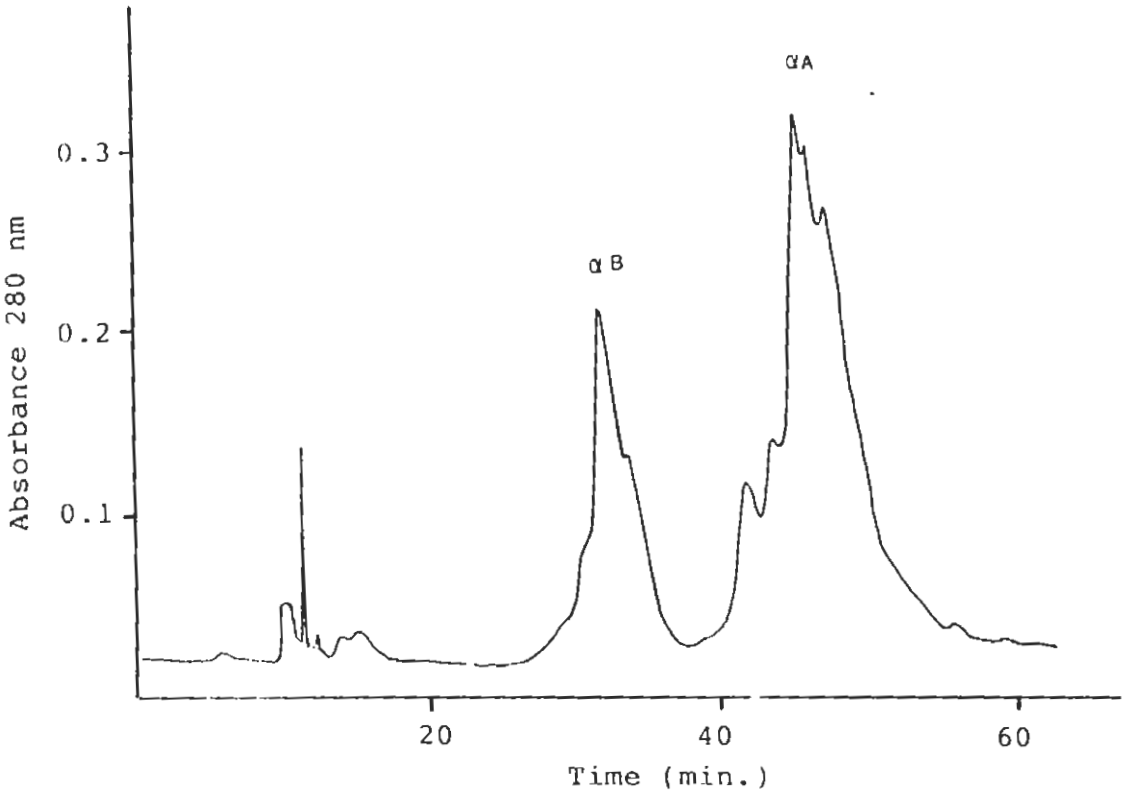


Fig. 3.14 Separation profile of crude α-crystallin (incubated with 100 mM KNCO for 12 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance: 280 nm.

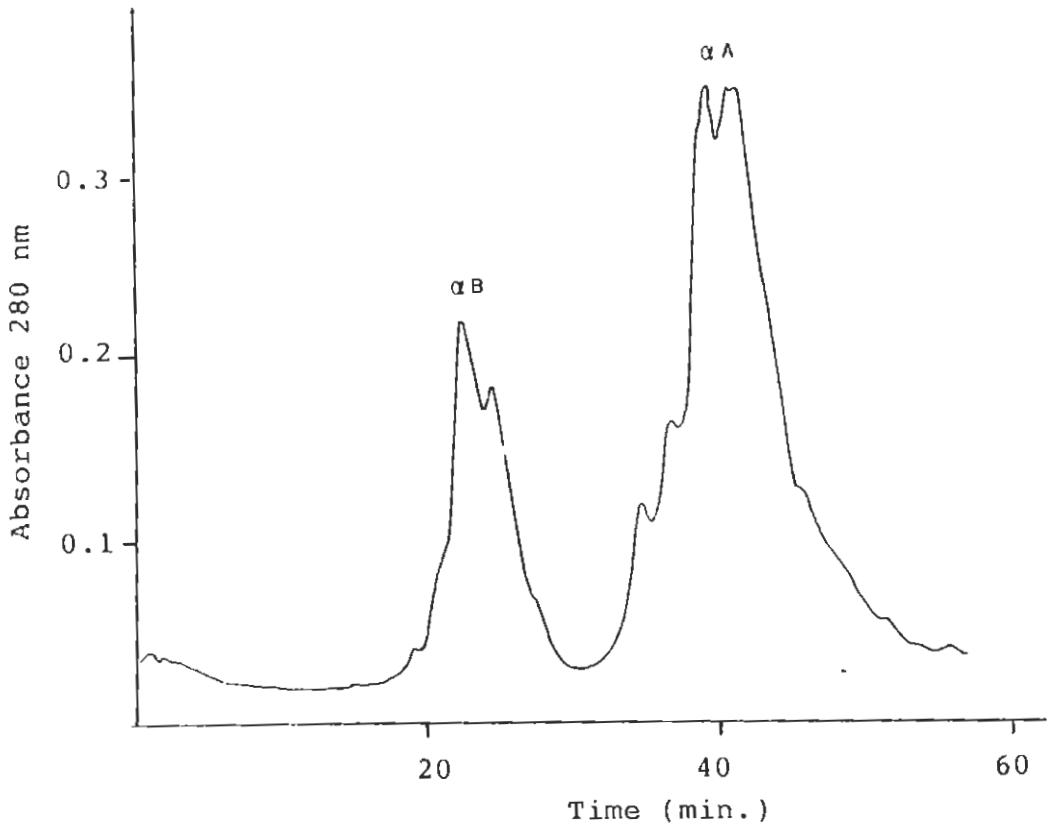


Fig. 3.15 Separation profile of Crude α-crystallin (incubated with 100 mM KNCO for 24 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance: 280 nm.

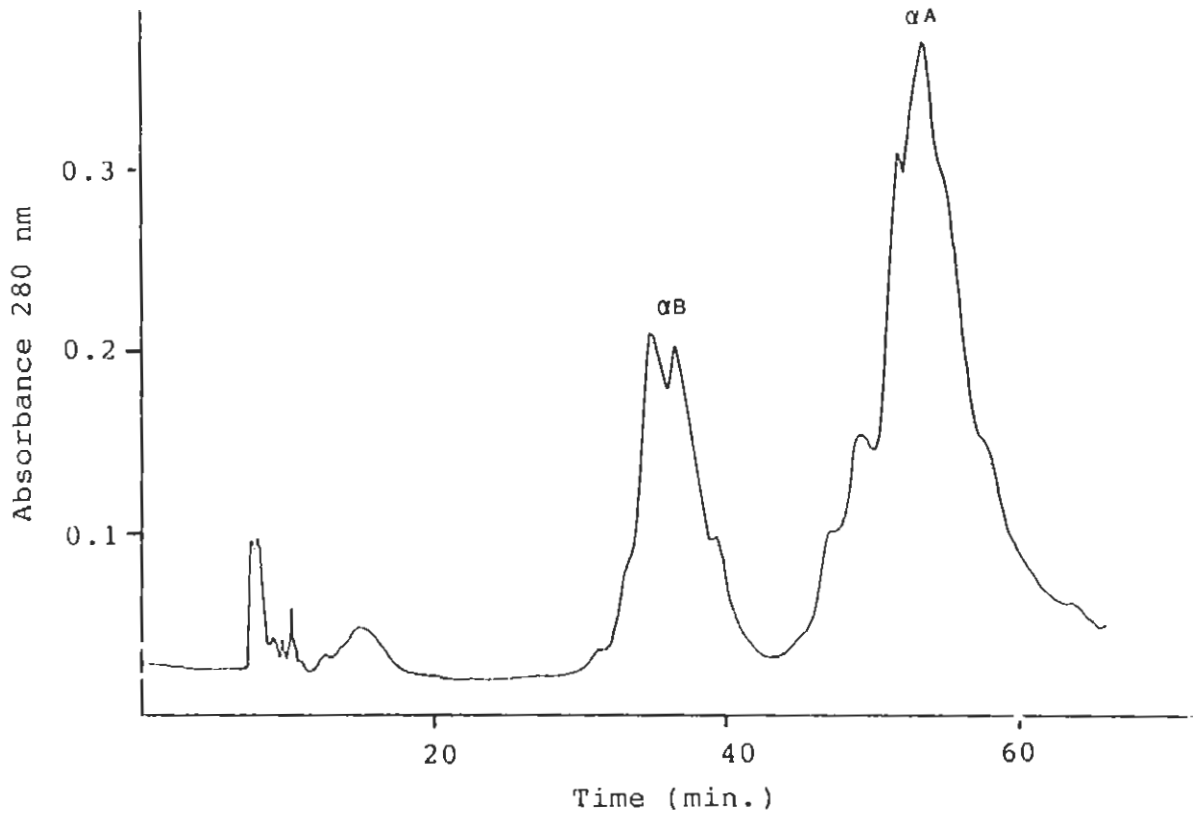


Fig. 3.16 Separation profile of crude α-Crystallin (incubated with 100 mM KNCO for 48 hours) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance: 280 nm.

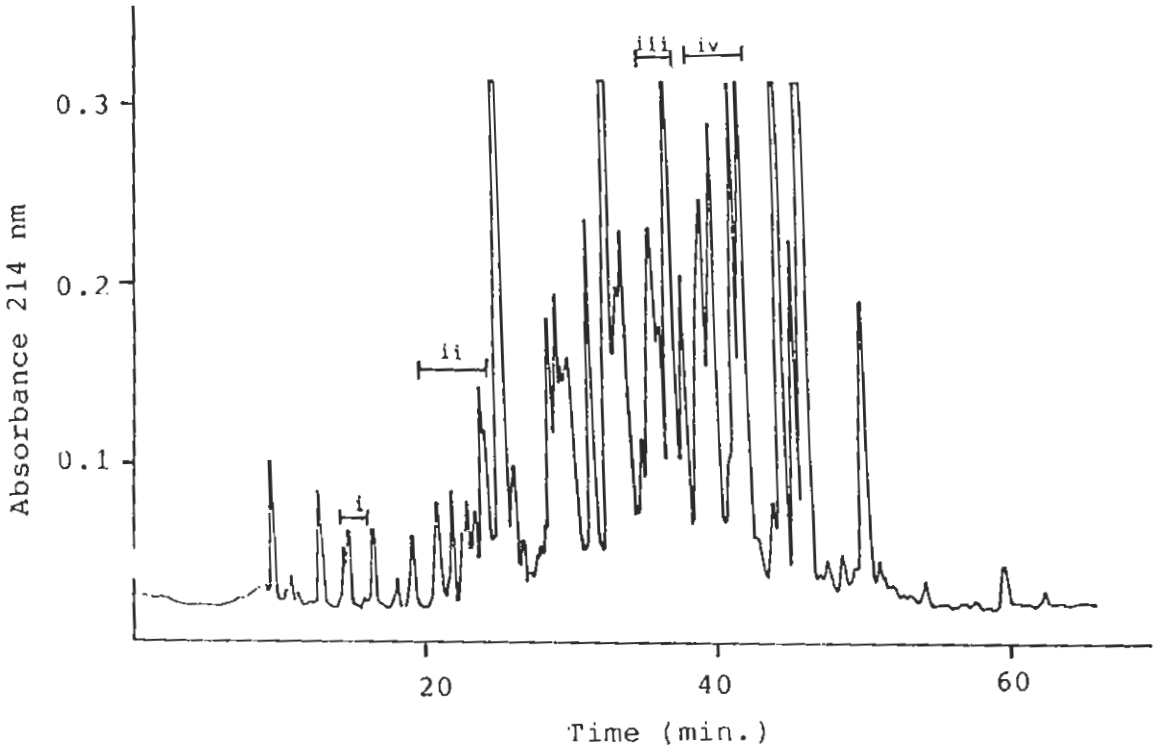


Fig. 3.17 Separation profile of peptic peptides of αA Crystallin (carbamylated for 24 hours) on Vydac C18. Eluent:0.1% TFA-ACN, flow rate: 60 ml/hr., absorbance: 214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i,ii,iii and iv represent peptides containing unmodified and carbamylated lysines 11,70, 78 and 88 respectively.

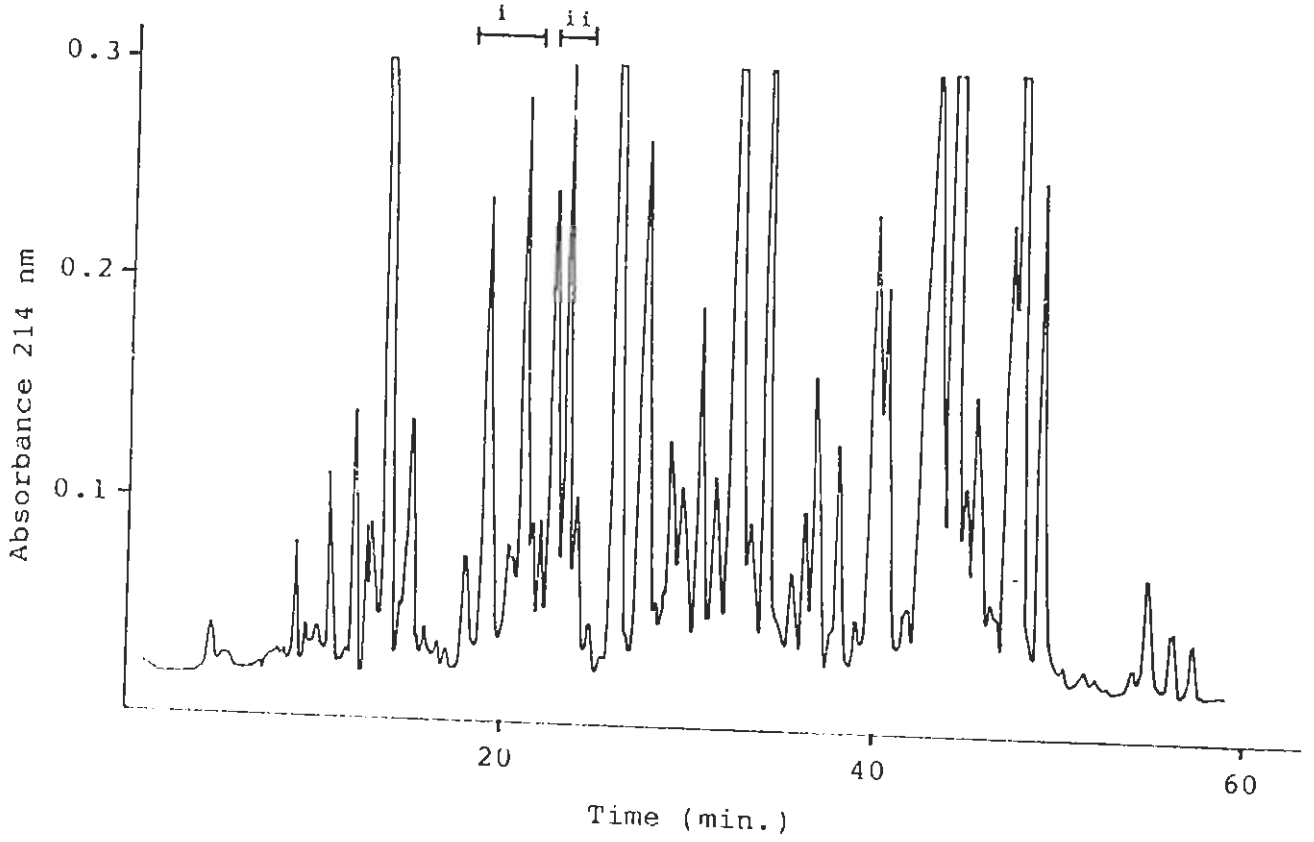


Fig. 3.18 Separation profile of chymotryptic peptides of αA Crystallin (carbamylated for 24 hours) on Vydac C18. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i and ii represent peptides containing unmodified and carbamylated lysines 166 and 145 respectively.

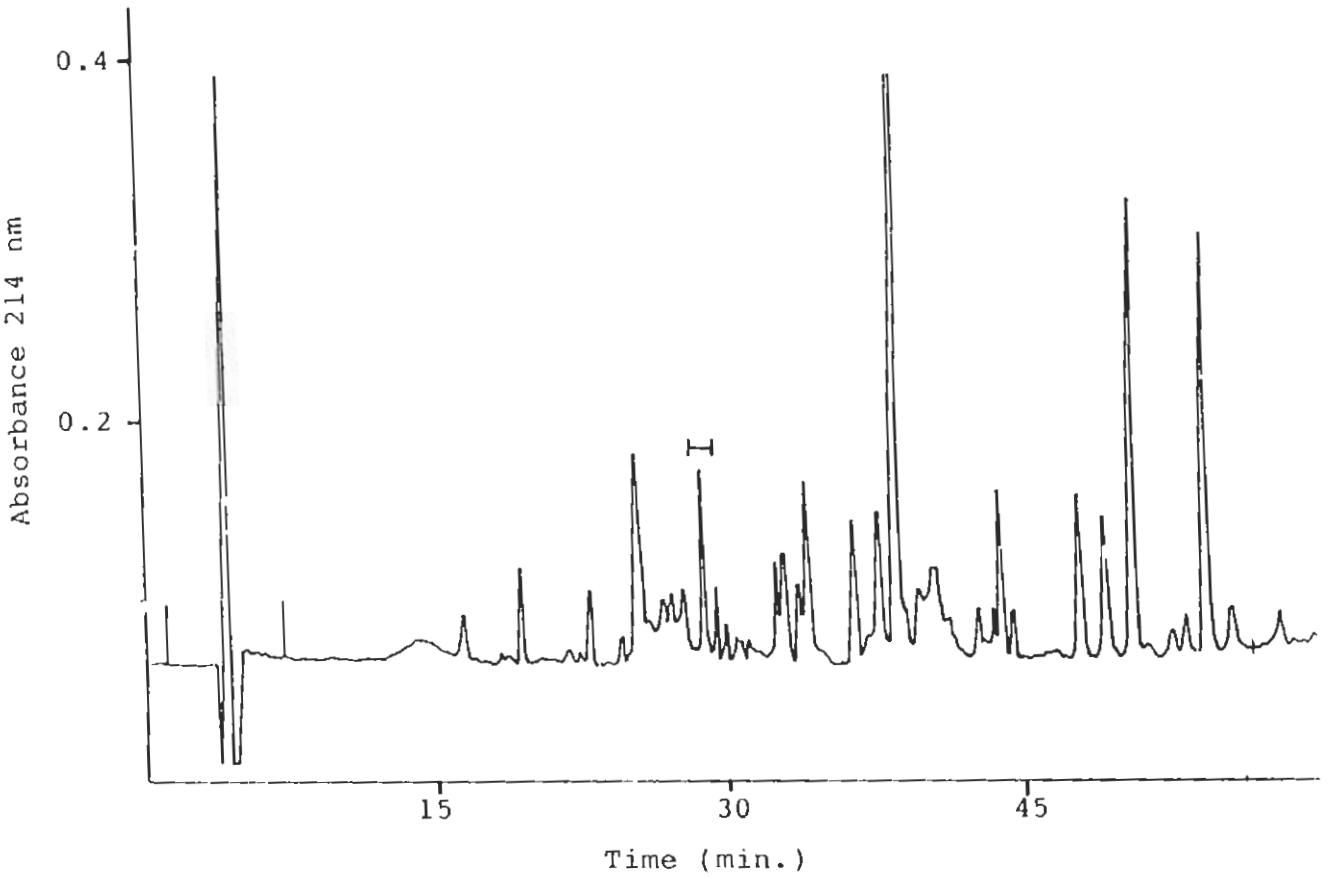


Fig. 3.19 Separation profile of peptides generated by ASP-N digestion of carbamylated αA crystallin (for 24 hours) on Vydac C18. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr :., absorbance: 214 nm. Horizontal bar indicates the peptides. containing unmodified and carbamylated lysine 99.

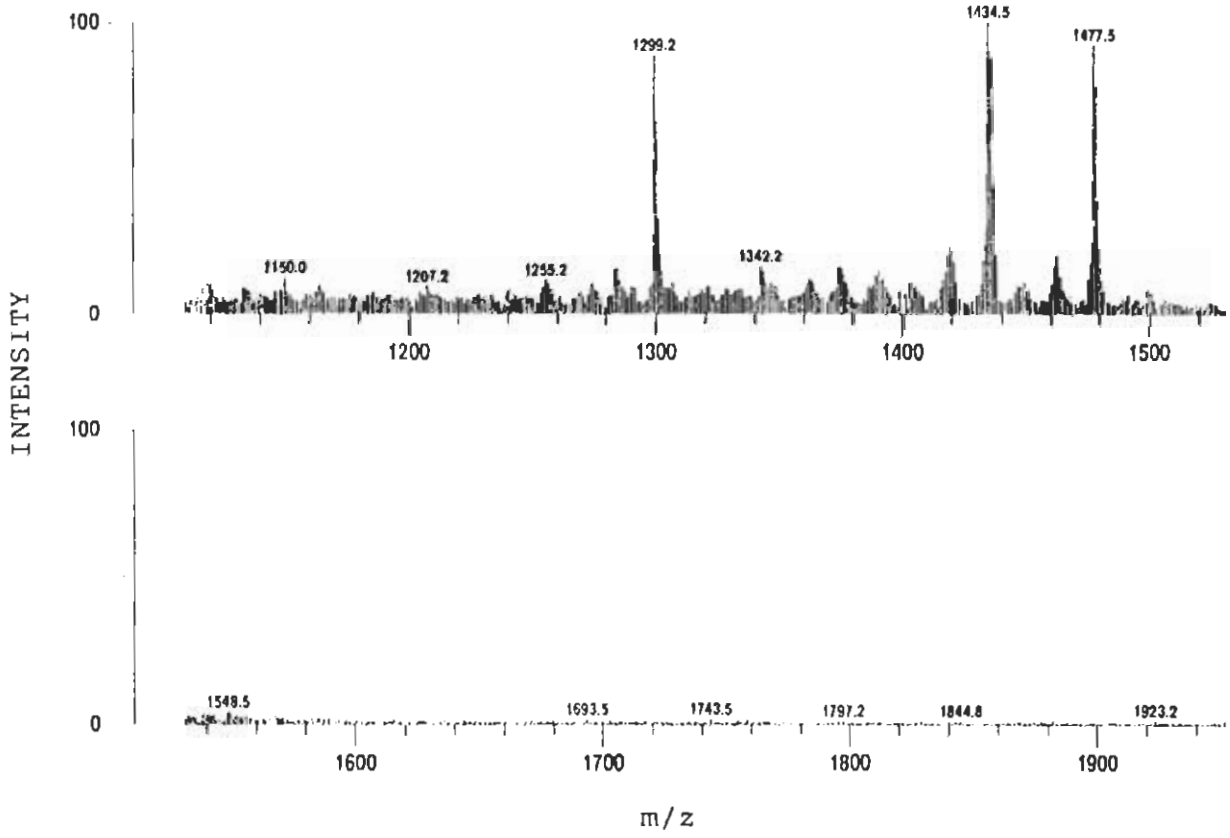


Fig. 3.20 FAB mass spectrum of fraction iv from peptic digest of carbamylated αA Crystallin(Fig.3.17), showing unmodified (m/z = 1434.5) and carbamylated(m/z = 1477.5) Peptides containing lysine11. Scan rate: 12 sec/decade, resolution: 1500,acceleration voltage:8 kv,calibration range:300- 4000, matrix: glycerol:thioglycerol(1:1).

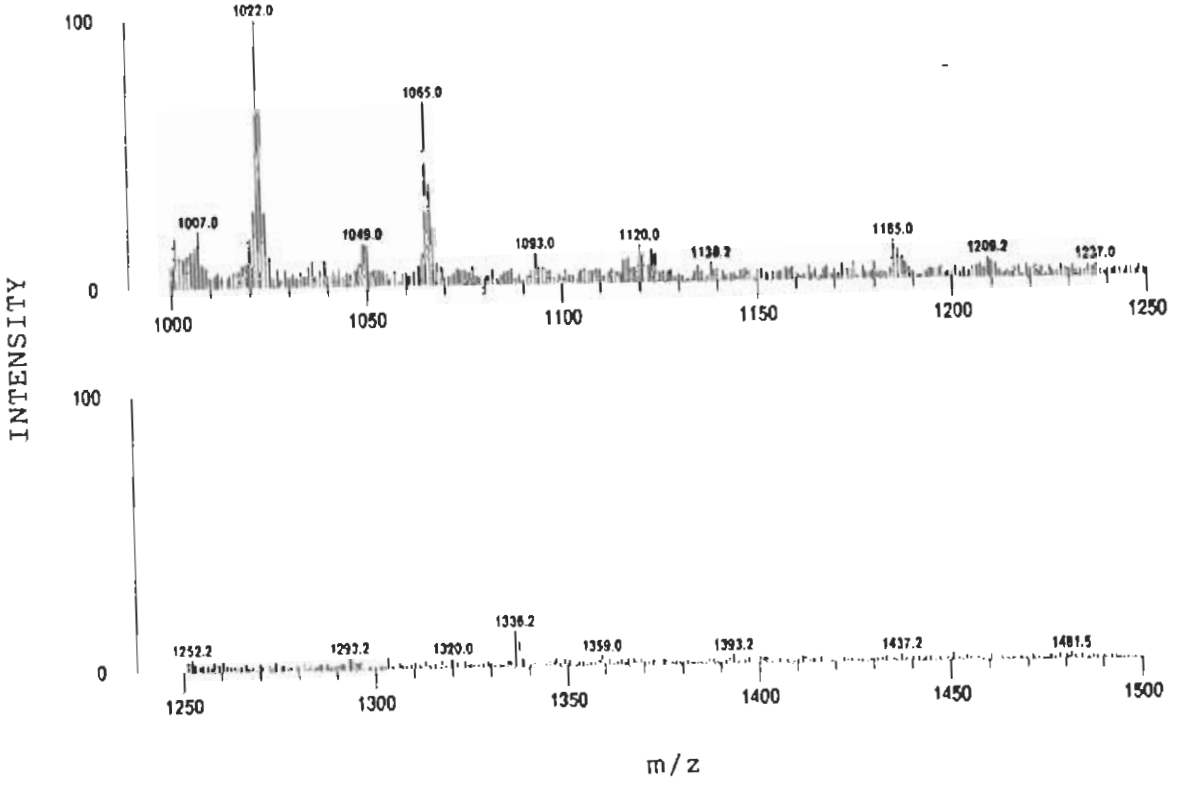


Fig. 3.21 FAB mass spectrum of fraction ii from peptic digest of carbamylated αA Crystallin(Fig.3.17), showing unmodified (m/z = 1022) and carbamylated(m/z = 1065) peptides containing lysine 70. Scan rate: 15 sec/decade, resolution: 1500,acceleration voltage:8 kv,calibration range:300-4000, matrix: glycerol:thioglycerol(1:1).

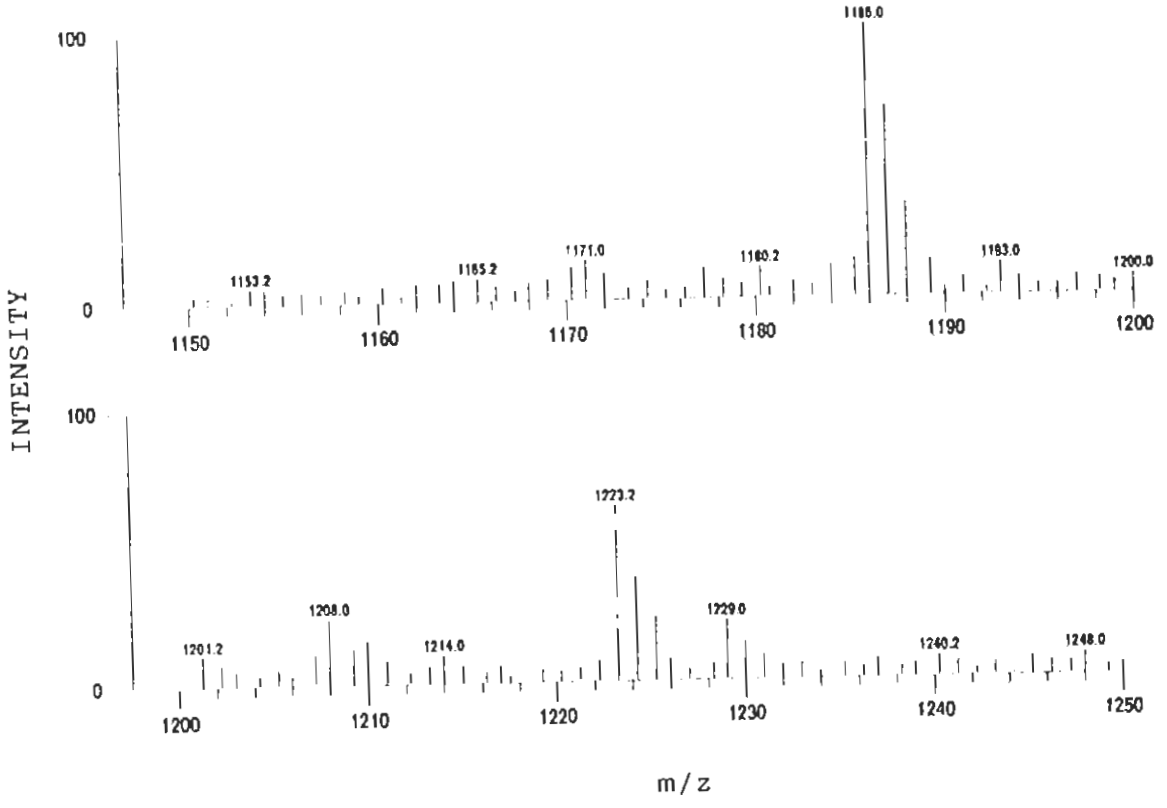


Fig. 3.22 FAB mass spectrum of fraction iii from peptic digest of carbamylated αA Crystallin (Fig.3.17), showing unmodified (m/z = 1186) and carbamylated (1229) peptides containing lysine 78. Scan rate: 15 sec/decade, resolution: 1500, acceleration voltage:8 kv, calibration range: 300-4000, matrix: glycerol:thioglycerol(1:1).

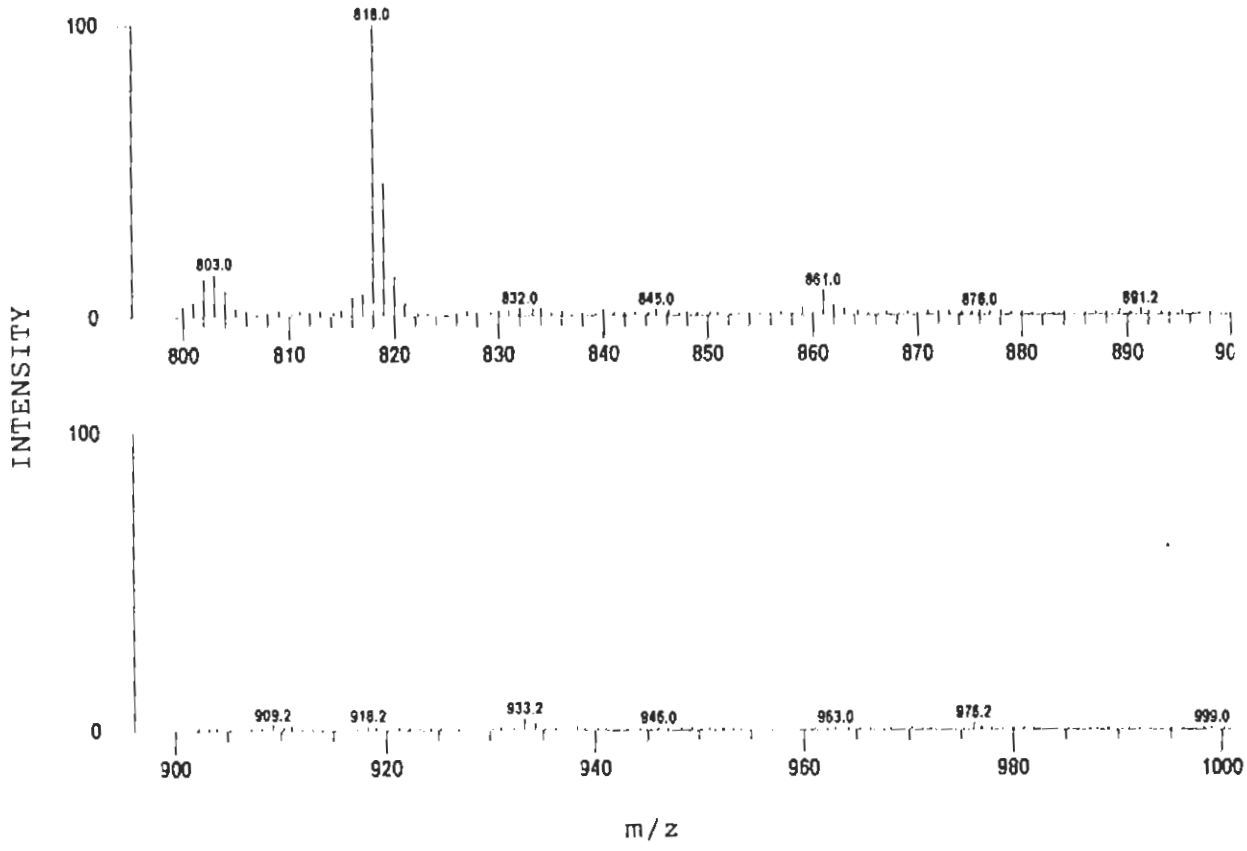


Fig. 3.23 FAB mass spectrum of fraction i from peptic digest of carbamylated αA Crystallin (Fig.3.17), showing unmodified (m/z = 818) and carbamylated (m/z = 861) peptides containing lysine 88. Scan rate: 15 sec/decade, resolution: 1500,acceleration voltage:8 kv,calibration range:300-4000, matrix: glycerol:thioglycerol(1:1).

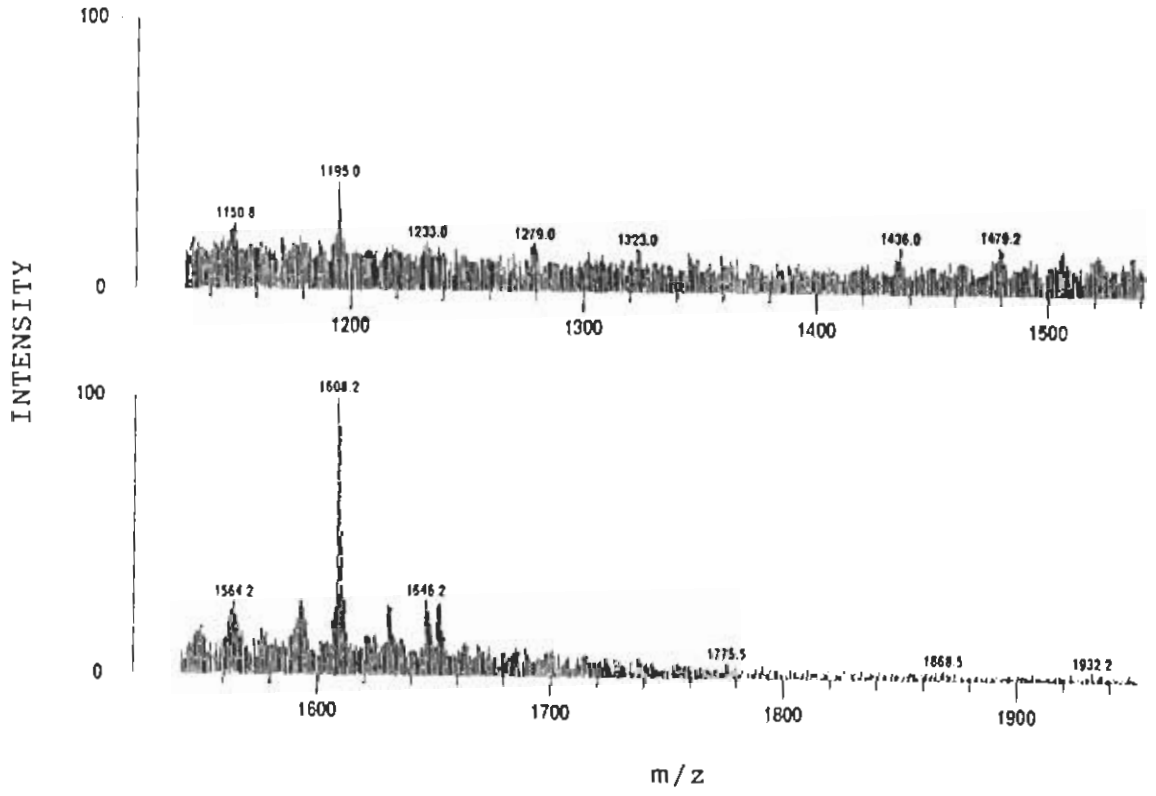


Fig. 3.24 FAB mass spectrum of a fraction (indicated by horizontal bar in Fig.3.19) from ASP-N digest of carbamylated αA Crystallin, showing unmodified (m/z = 1608.2) and carbamylated (m/z = 1651.2) peptides containing lysine 99.Scan rate:8 sec/decade, resolution:1500,acceleration voltage:8 kv,calibration range:300- 4000, matrix: glycerol: thioglycerol (1:1).

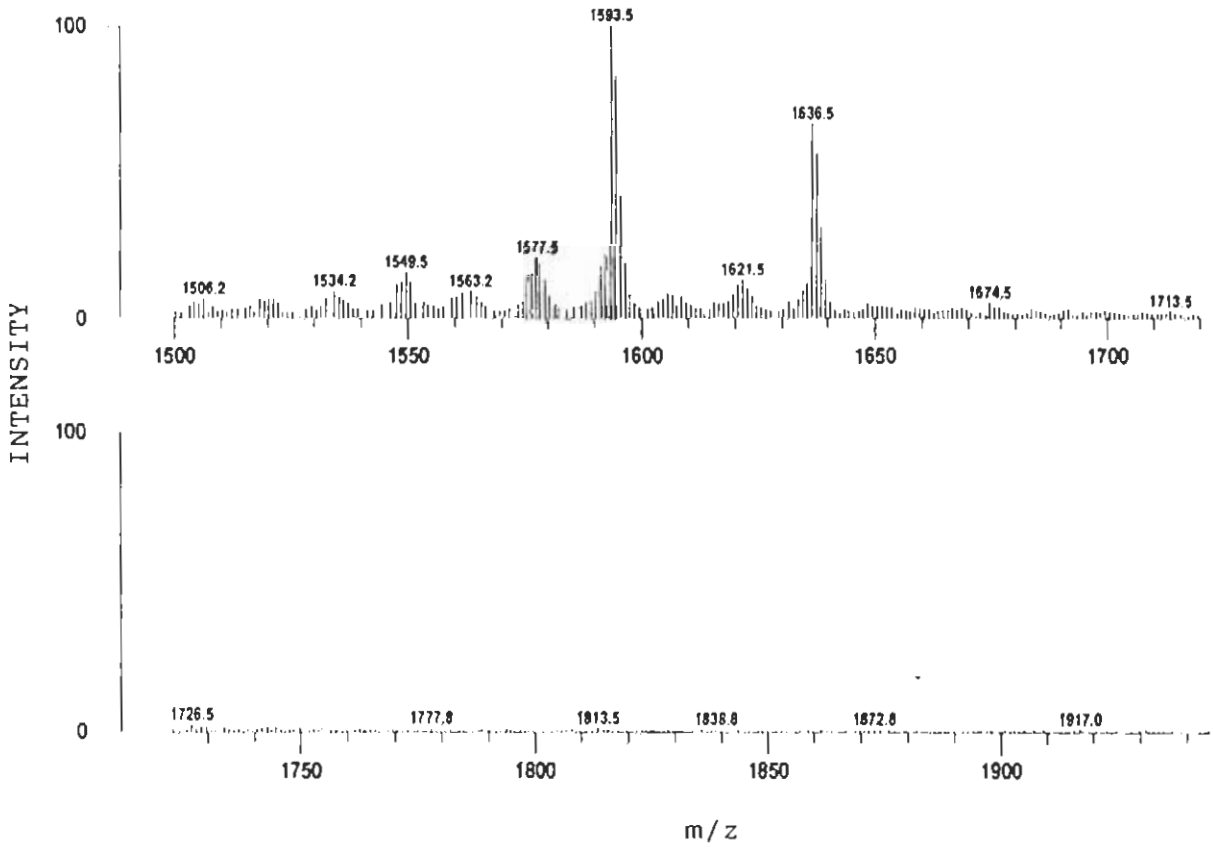


Fig. 3.25 FAB mass spectrum of fraction ii from chymotryptic digest of carbamylated αA Crystallin (Fig.3.18), showing unmodified (m/z = 1593.5) and carbamylated(m/z = 1636.5) peptides containing lysine 145. Scan rate: 8 sec/decade, resolution: 1500, acceleration voltage:8 kv,calibration range:300- 4000, matrix: glycerol:thioglycerol(1:1).

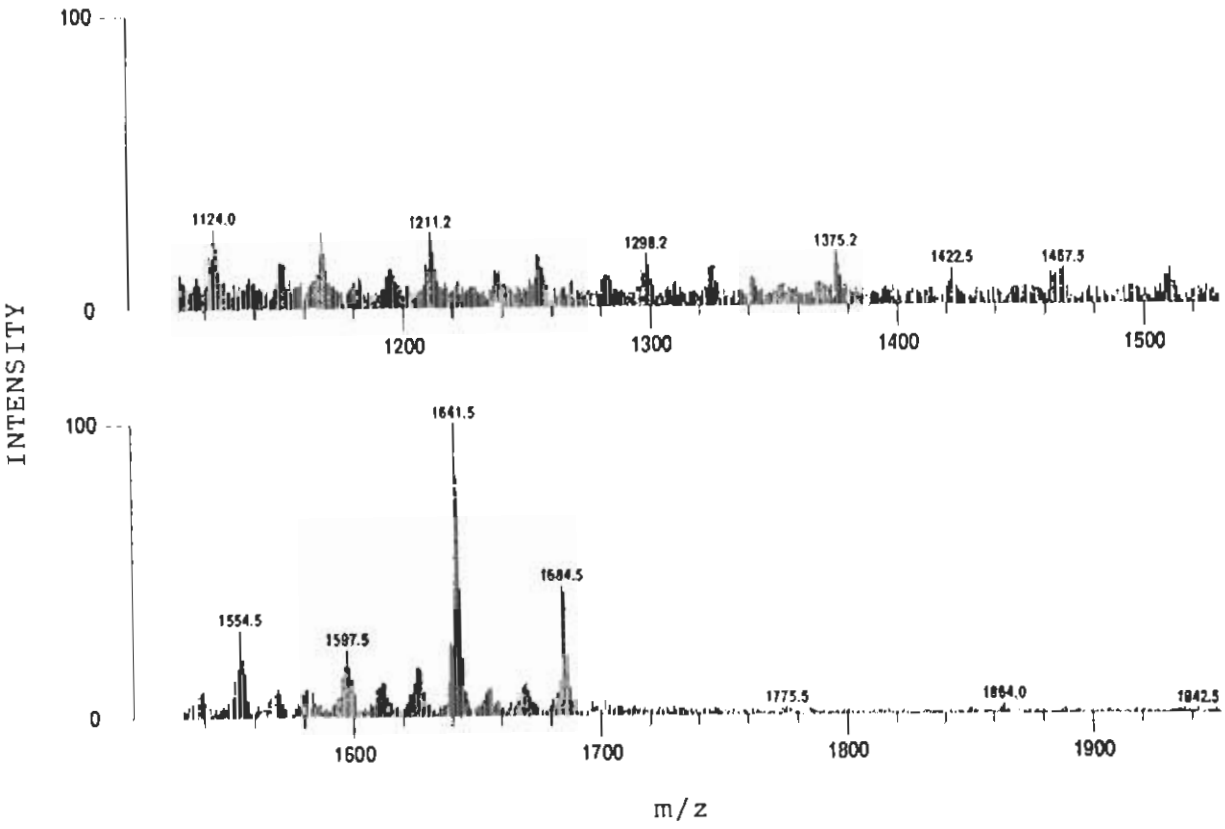


Fig. 3.26 FAB mass spectrum of fraction i from chymotryptic digest of carbamylated αA Crystallin (Fig.3.18), showing unmodified (m/z = 1641.5) and carbamylated(1684.5) peptides containing lysine 166. Scan rate: 8 sec/decade, resolution: 1500, acceleration voltage:8 kv,calibration range:300- 4000, matrix: glycerol:thioglycerol(1:1).

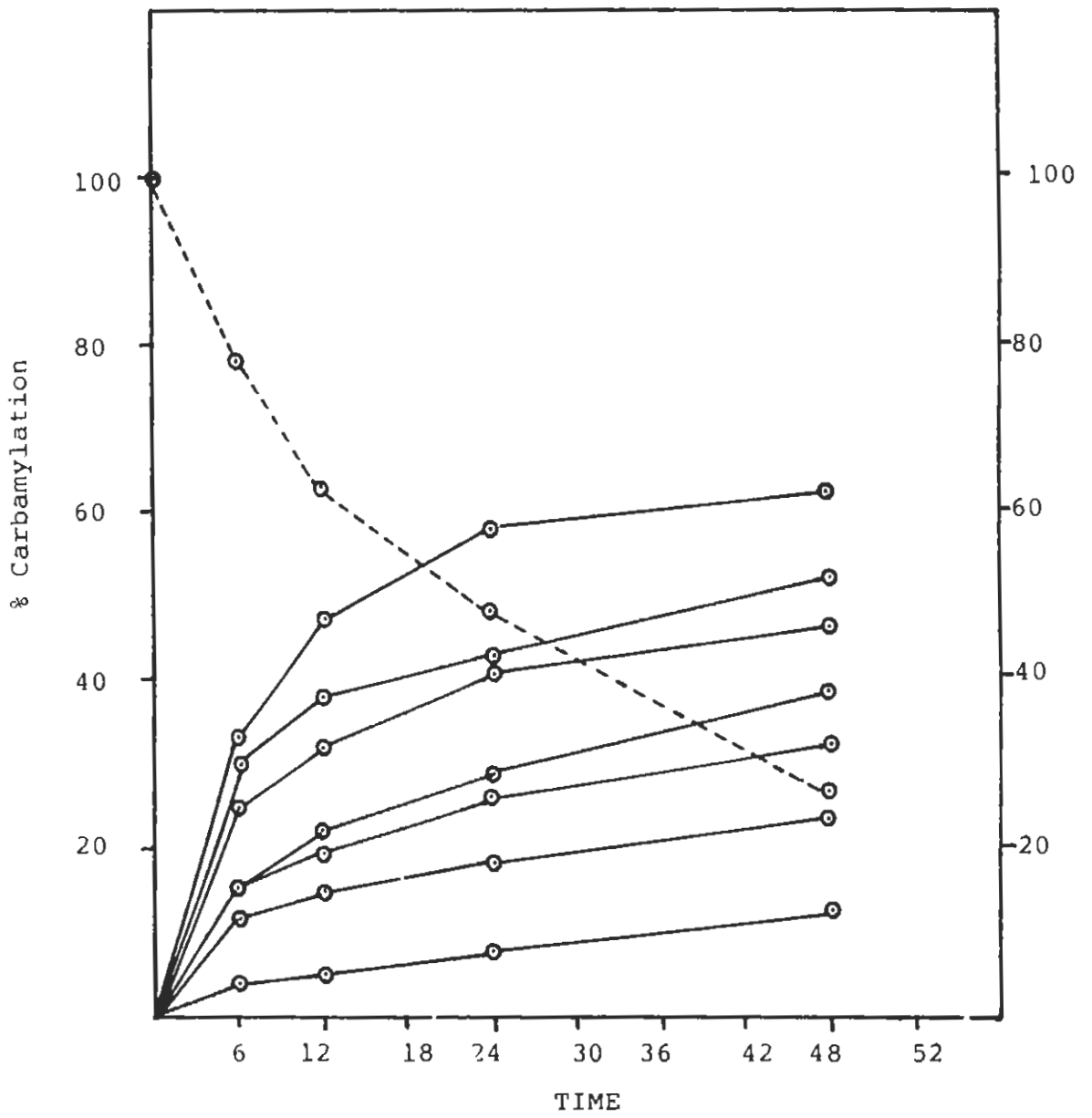


Fig. 3.27 Modification of each lysyl residue of αA crystallin as a function of incubation time. Dotted line indicates the concentration of KNCO with time.

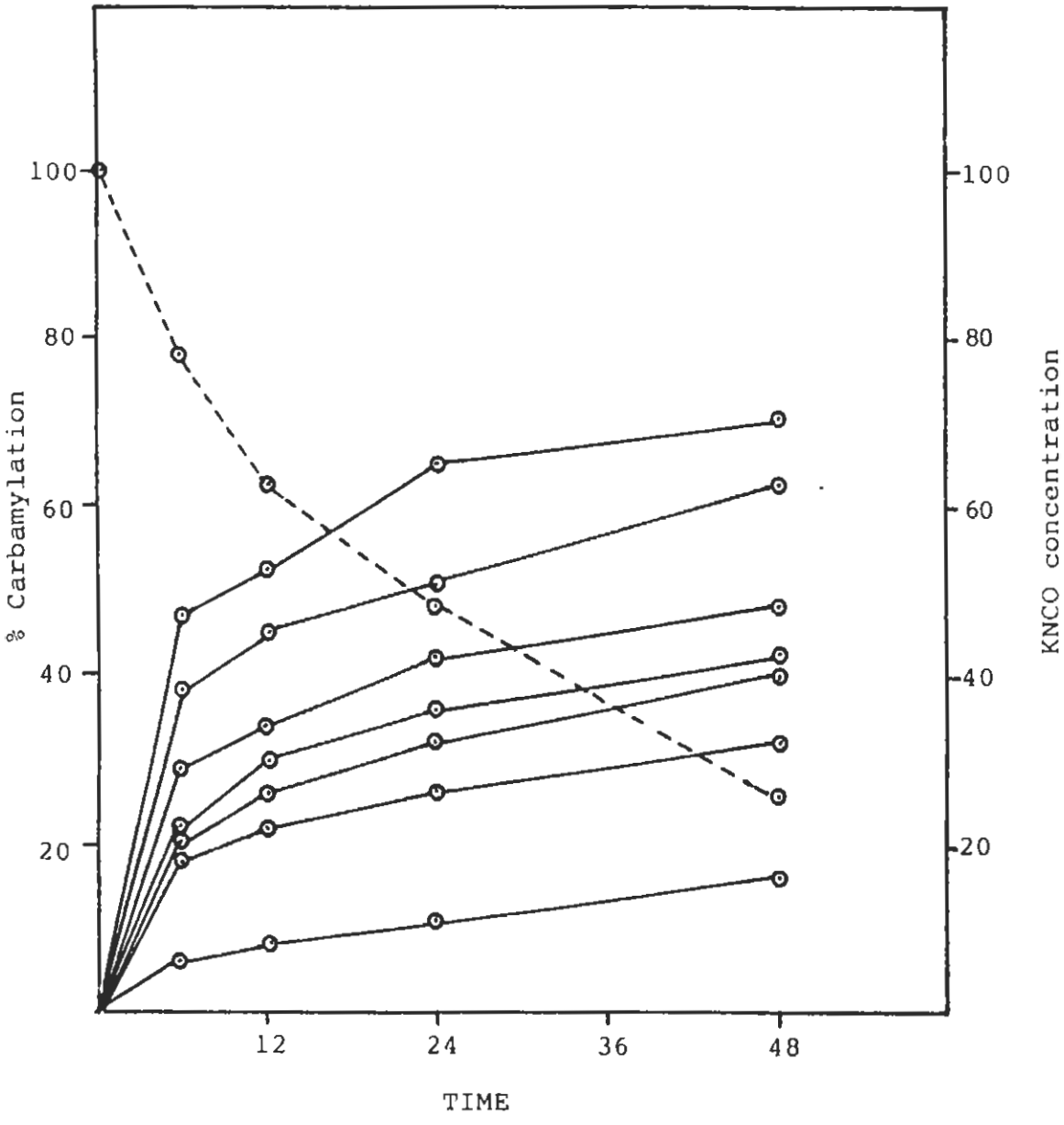


Fig. 3.28 Modification of each lysyl residue of αA crystallin (separated from αB prior to incubation with KNCO) as a function of incubation time. Dotted line denotes the concentration of KNCO with time.

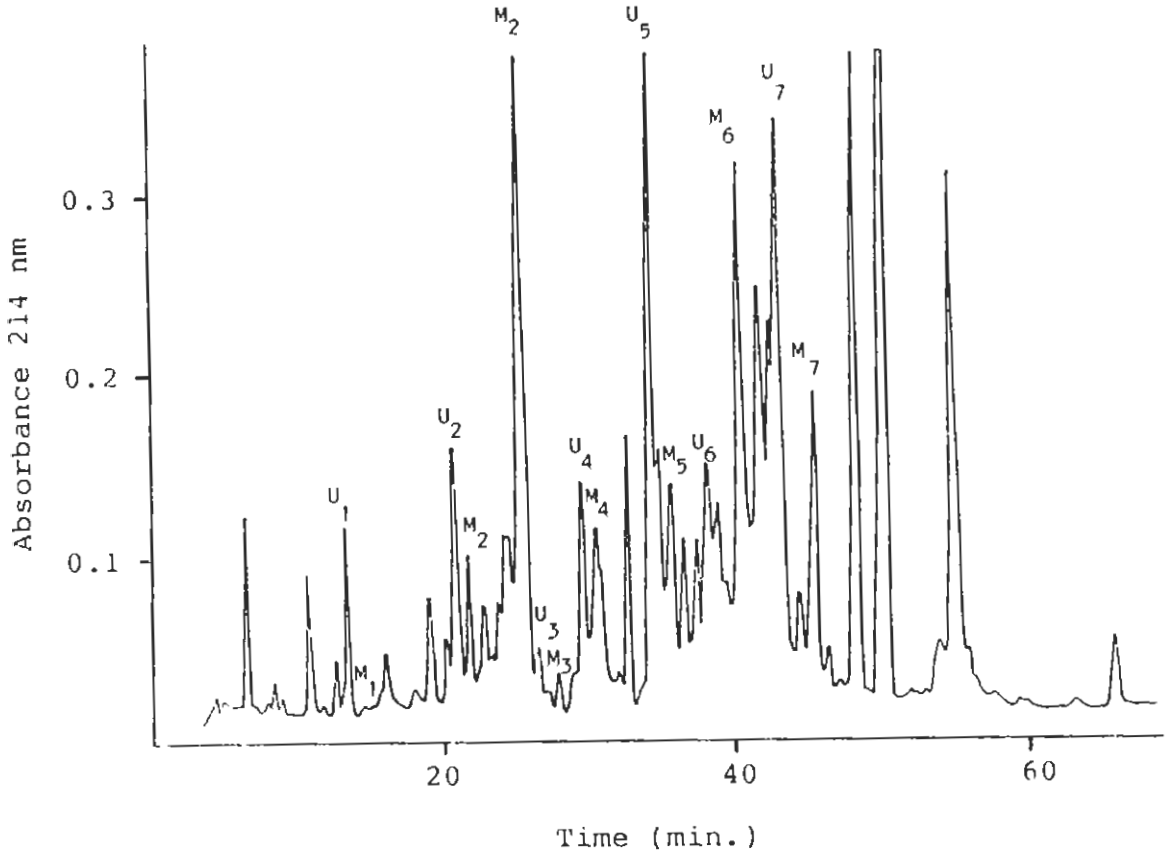


Fig. 3.29 Separation profile of peptic peptides of acetylated αA Crystallin on Vydac C18 column. Eluent: 0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.U denotes unmodified peptides and M denotes modified peptides.

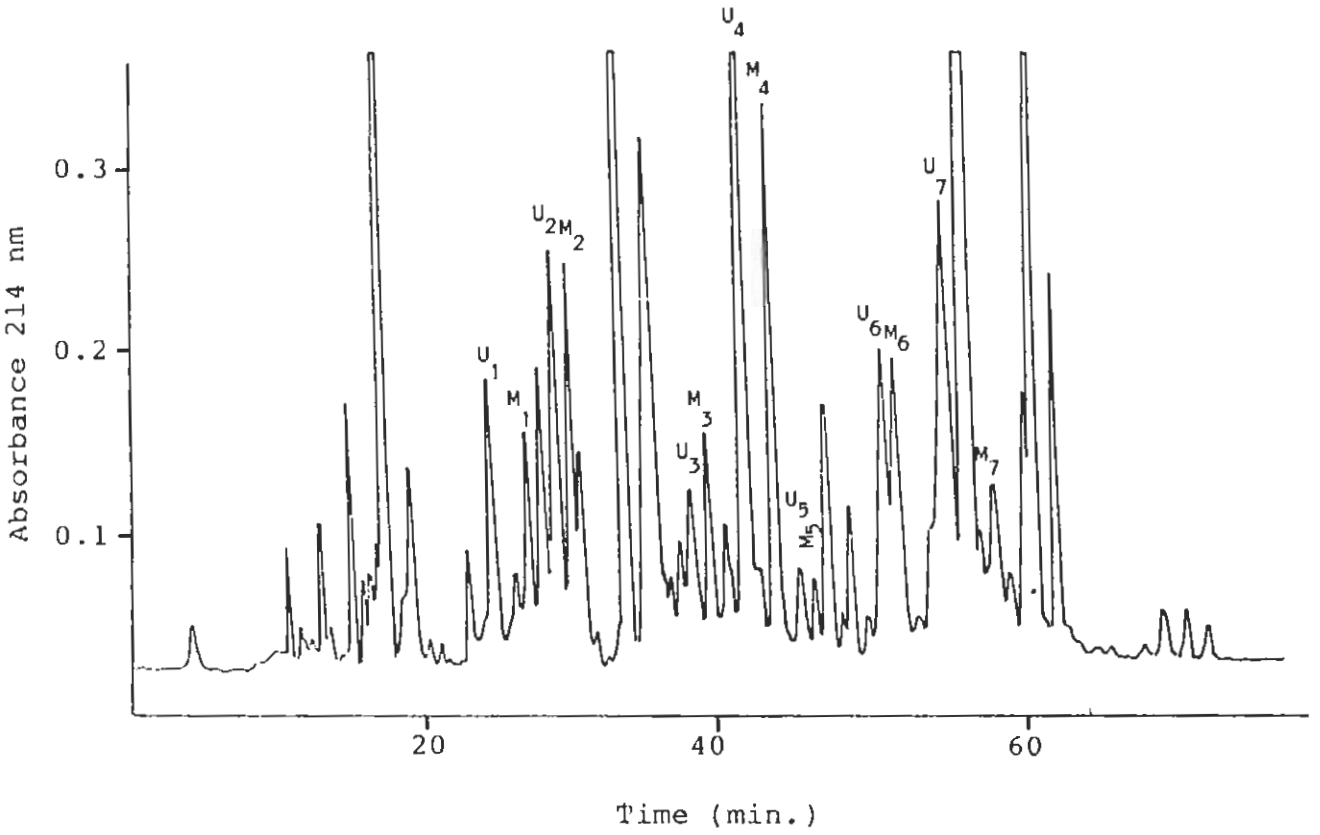


Fig. 3.30 Separation profile of chymotryptic peptides of acetylated αA Crystallin on Vydac C18 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.U denotes unmodified peptides and M denotes modified peptides.

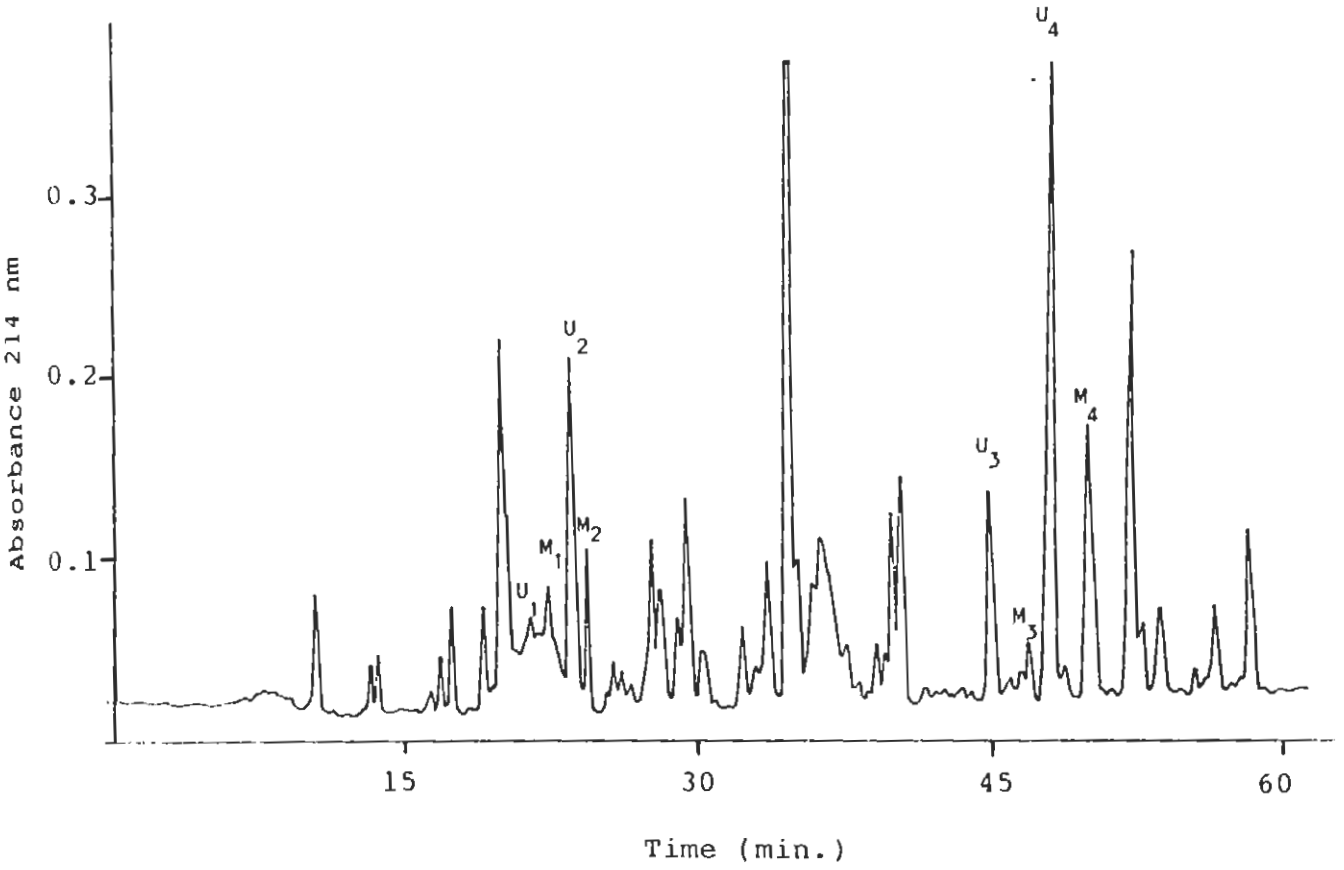


Fig. 3.31 Separation profile of peptides generated by ASP-N digestion of acetylatd αA Crystallin on Vydac C4 column, Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.U denotes unmodified peptides and M denotes modified peptides.

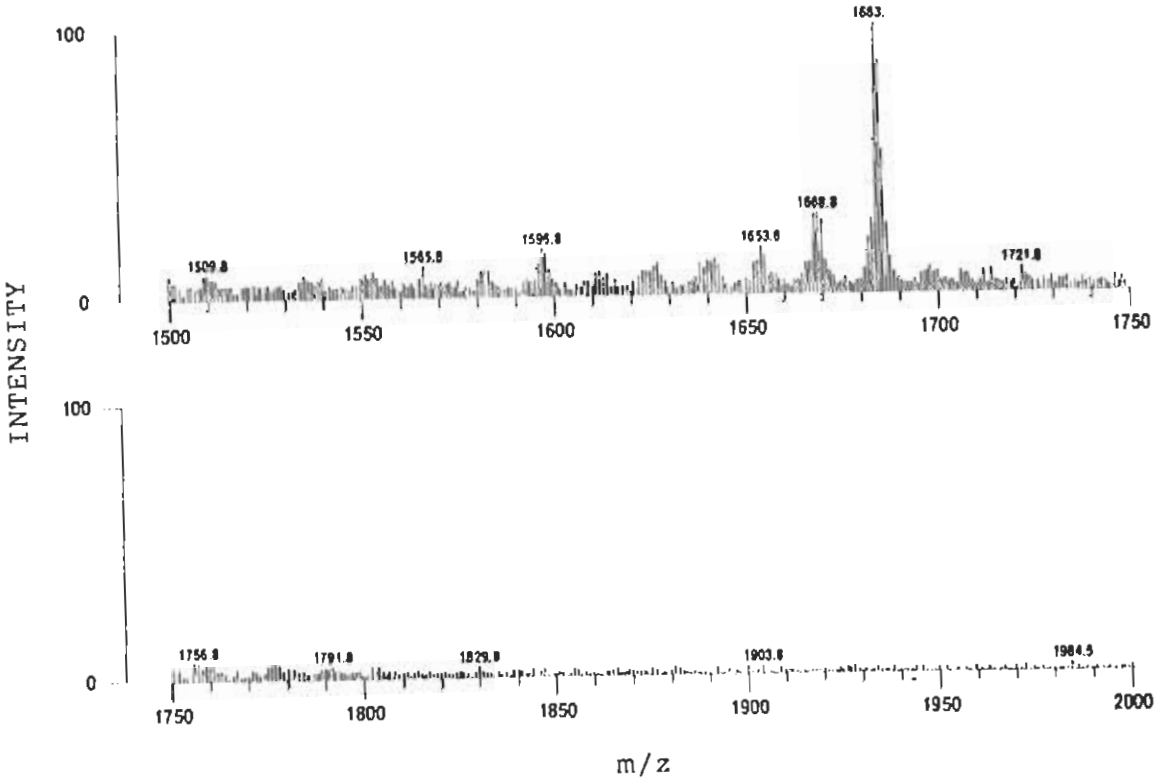


Fig. 3.32 FAB Mass spectrum , showing acetylated peptide 158-173-(m/z = 1683).Scan rate:30 sec/decade, resolution:3000, acceleration voltage: 8 kv,calibration range: 300-4000, matrix: glycerol:thioglycerol(1:1).

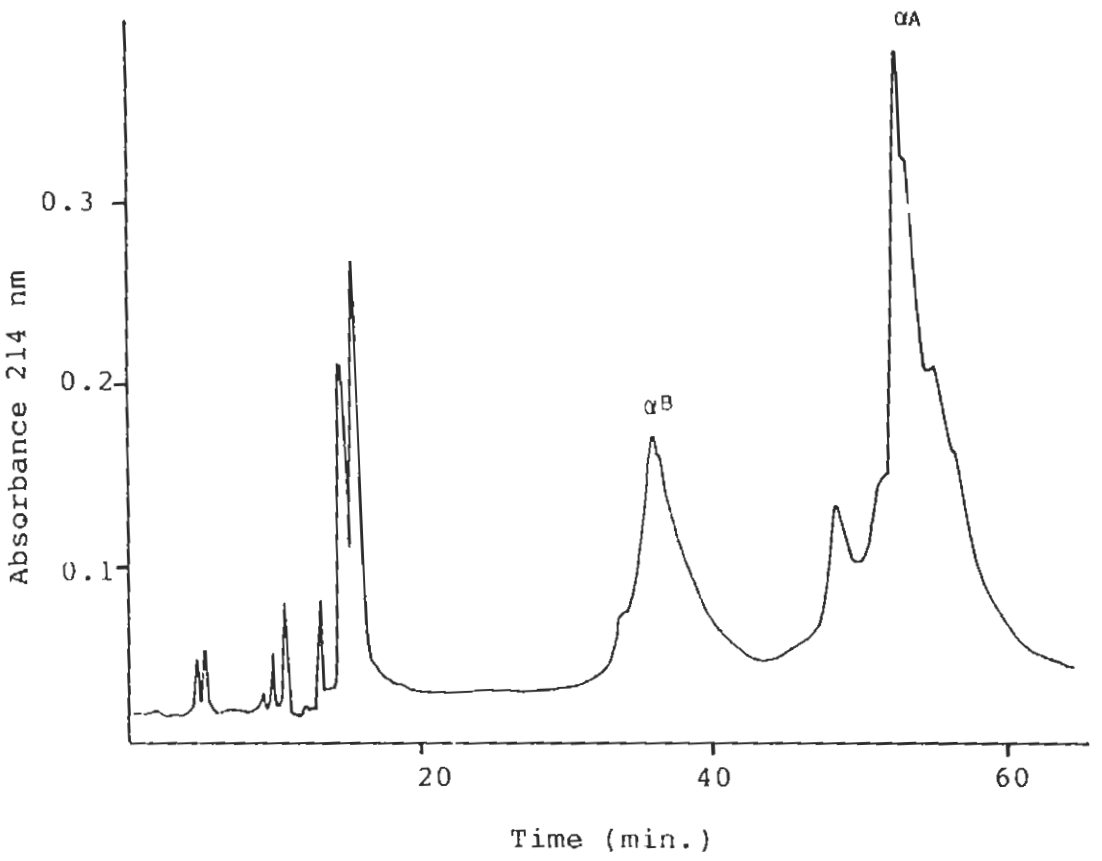


Fig. 3.33 Separation profile of crude α-Crystallin (incubated with aspirin for 6 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr , absorbance:280 nm.

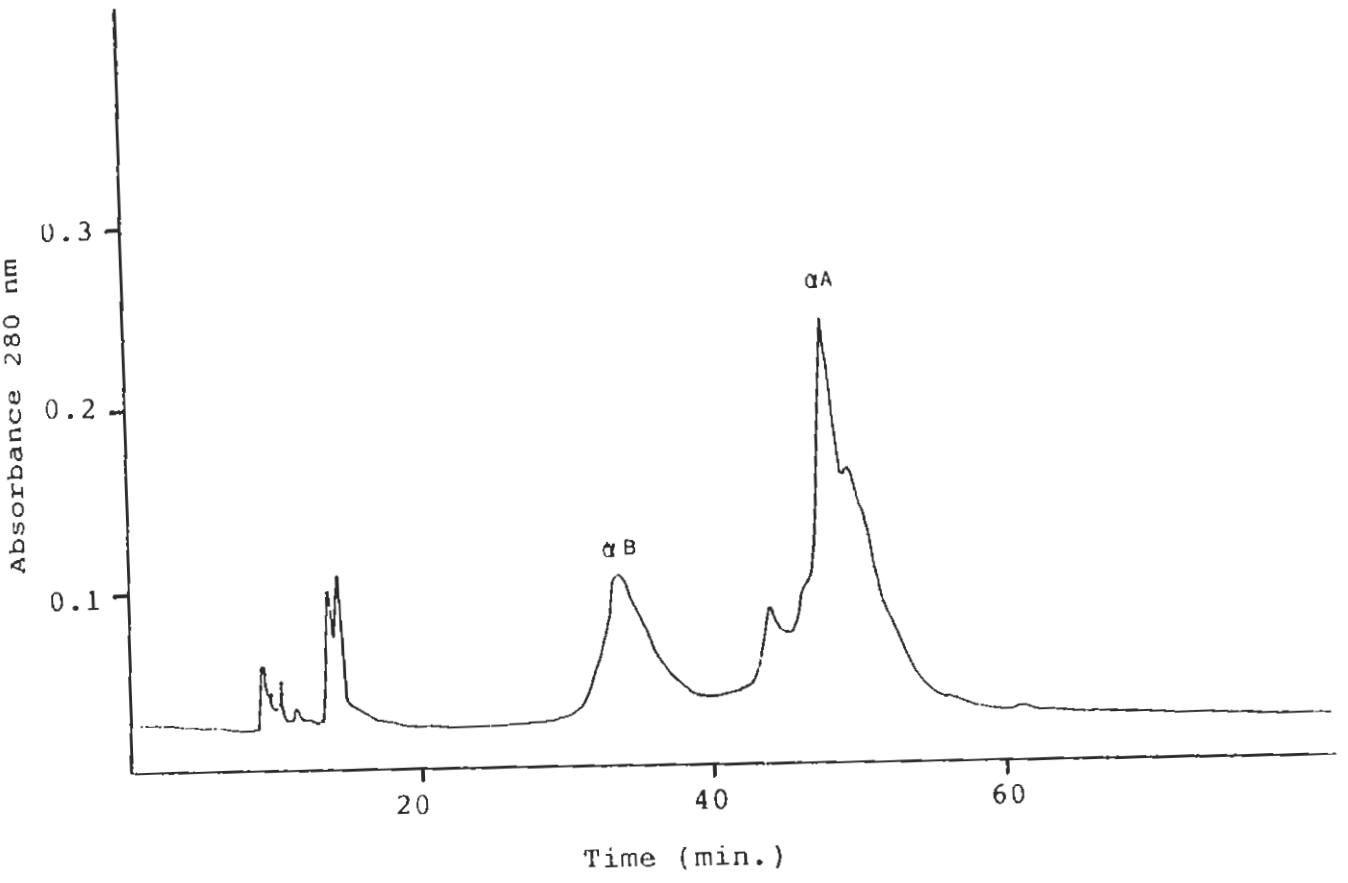


Fig. 3.34 Separation profile of crude α-crystallin (incubated with aspirin for 12 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:280 nm.

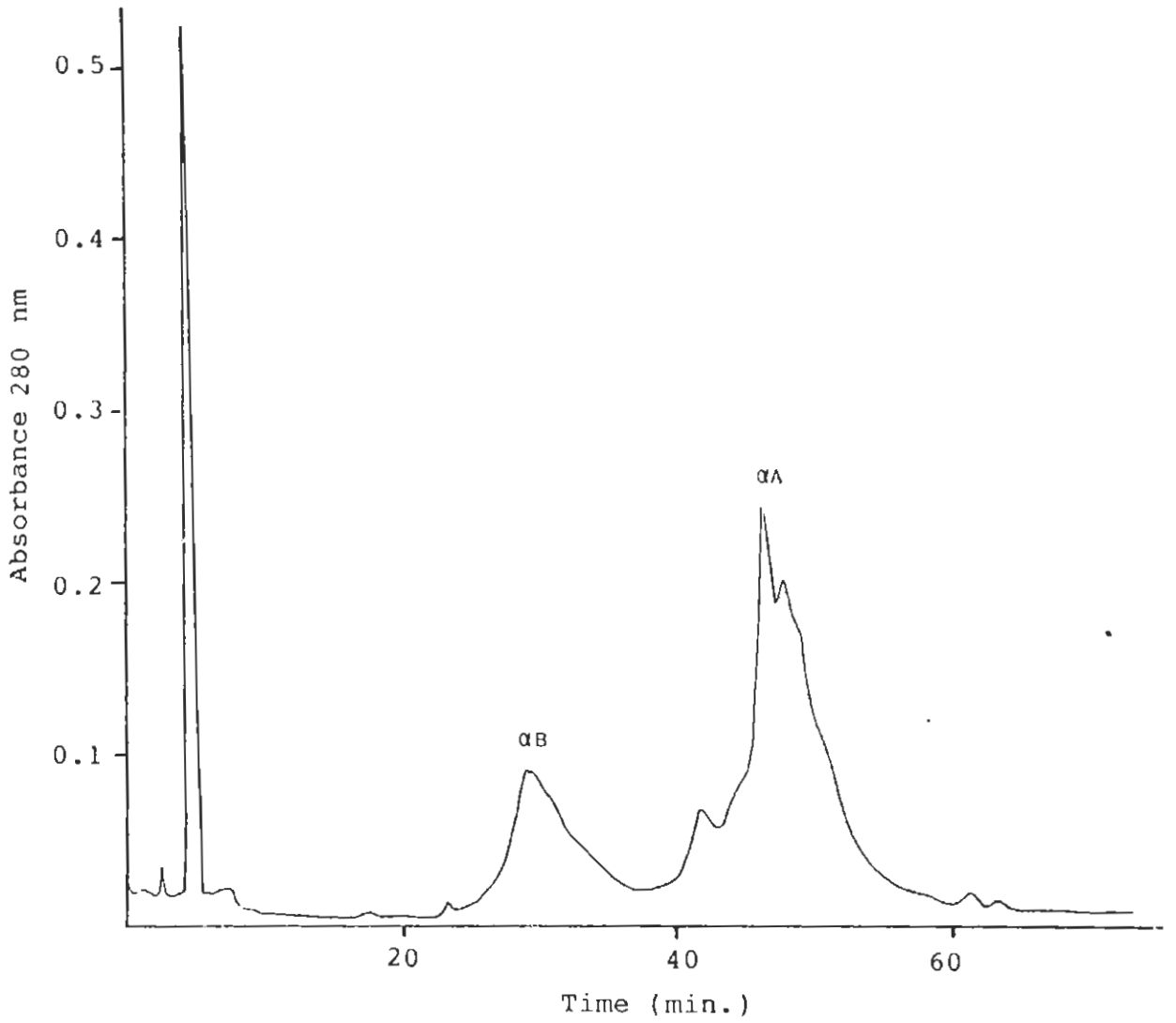


Fig. 3.35 Separation profile of crude α-Crystallin (incubated with aspirin for 24 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr .. absorbance:280 nm.

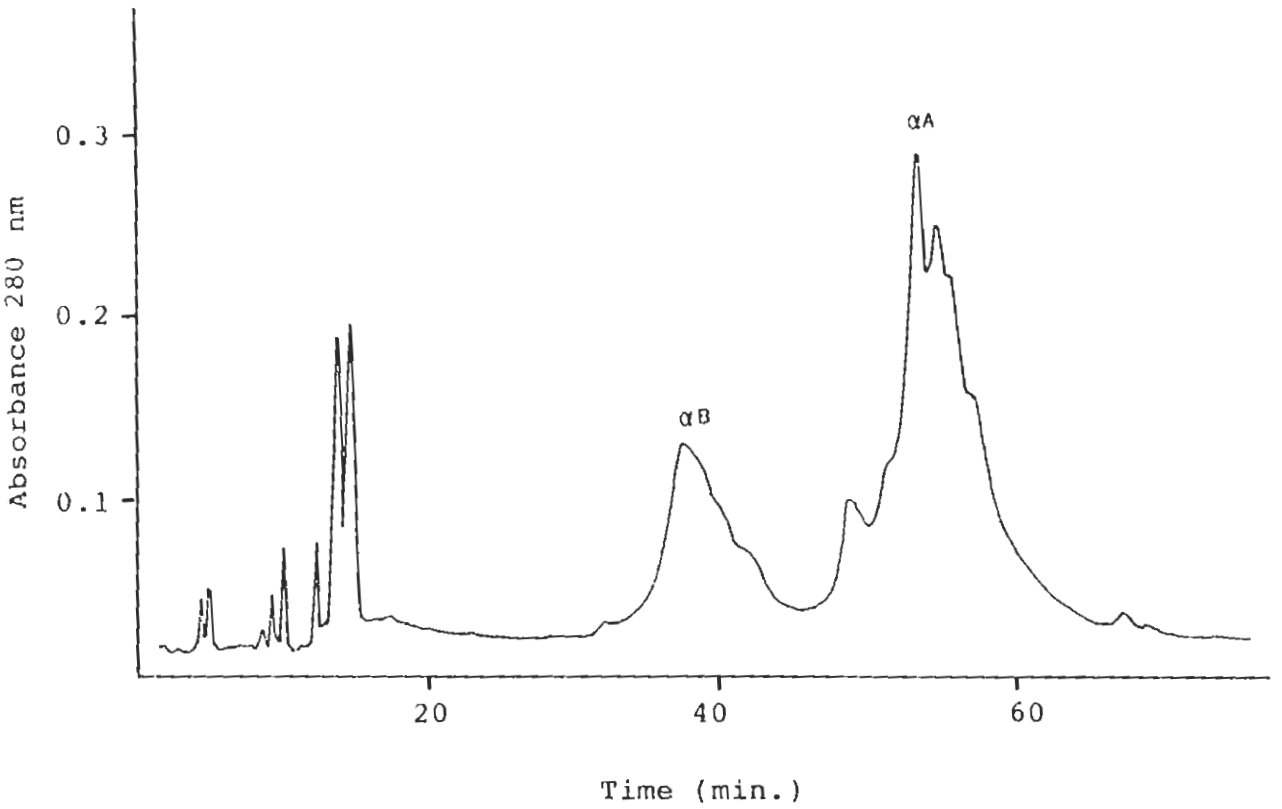


Fig. 3.36 Separation profile of crude α-Crystallin (incubated with aspirin for 48 hours.) on Vydac C4 column. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr., absorbance:280 nm.

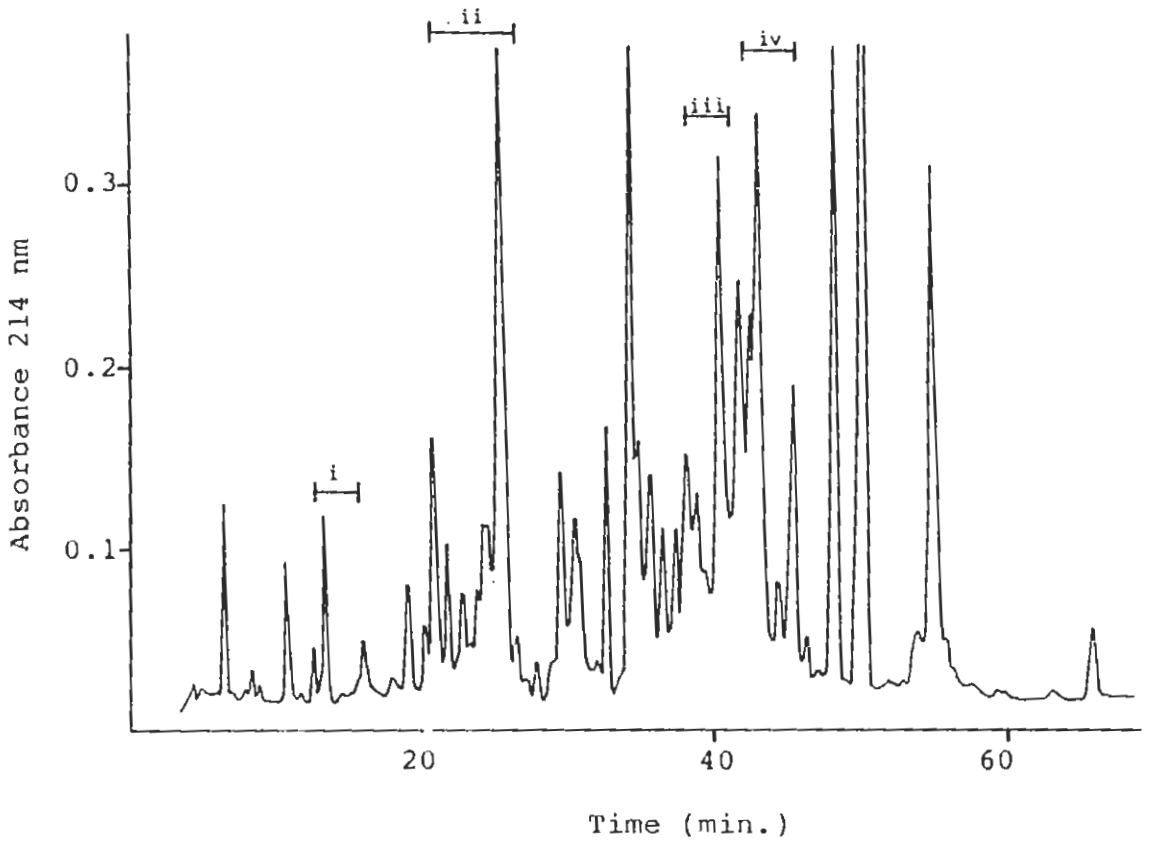


Fig. 3.37 Separation profile of peptic peptides of αA Crystallin (acetylated for 24 hours) on Vydac C18 column. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance: 214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i,ii,iii and iv represent peptides containing unmodified and acetylated lysines 11,70, 78 and 88 respectively.

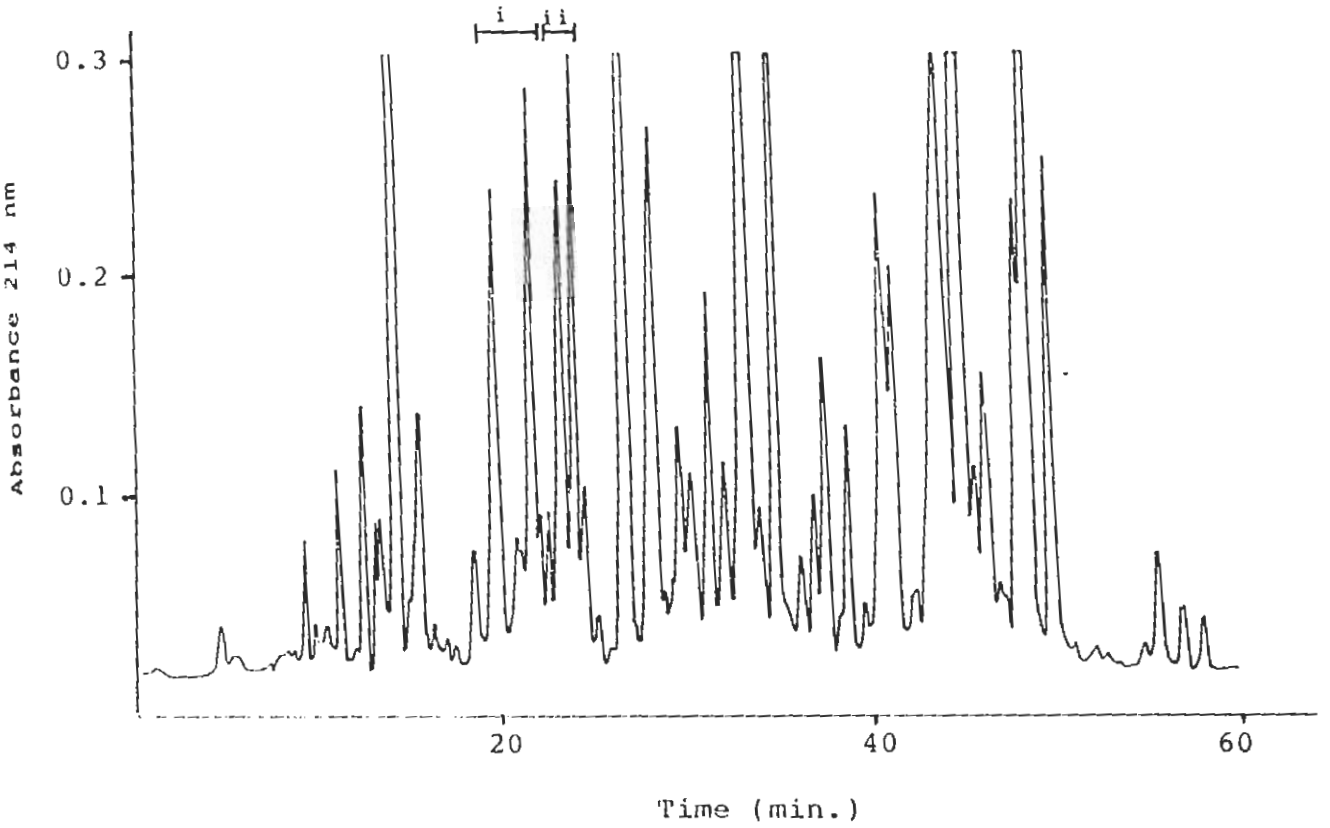


Fig. 3.38 Separation profile of chymotrypsin peptides of αA Crystallin(acetylated for 24 hours) on Vydac C18 column. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr., absorbance: 214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i and ii represent peptides containing unmodified and acetylated lysines 166 and 145 respectively.

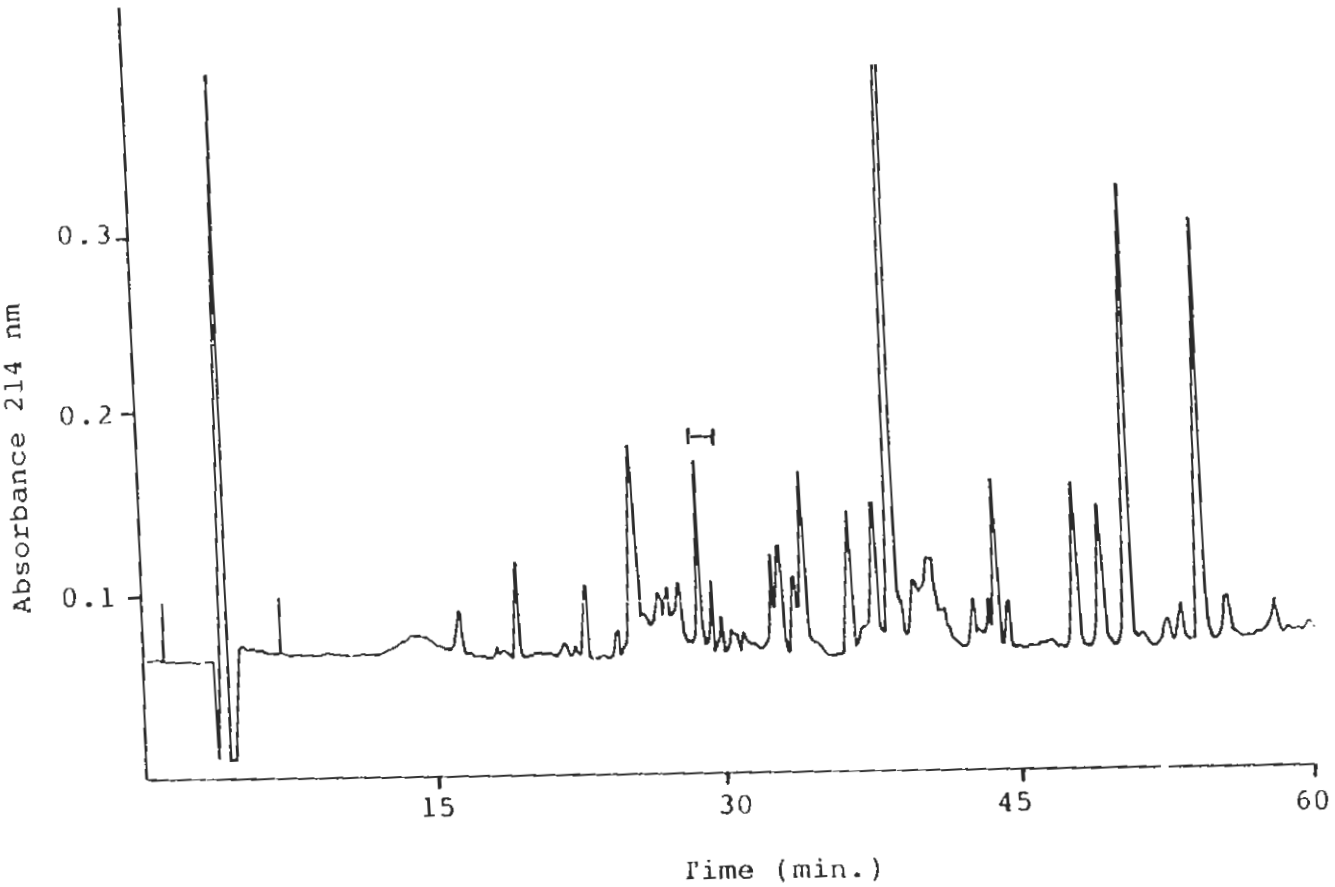


Fig. 3.39 Separation profile of peptides generated by ASP-N digestion αA Crystallin(acetylated for 24 hours) on Vydac C18 Column. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr .., absorbance: 214 nm. Horizontal bar indicates the pooling scheme of peptides containing unmodified and acetylated lysine 99.

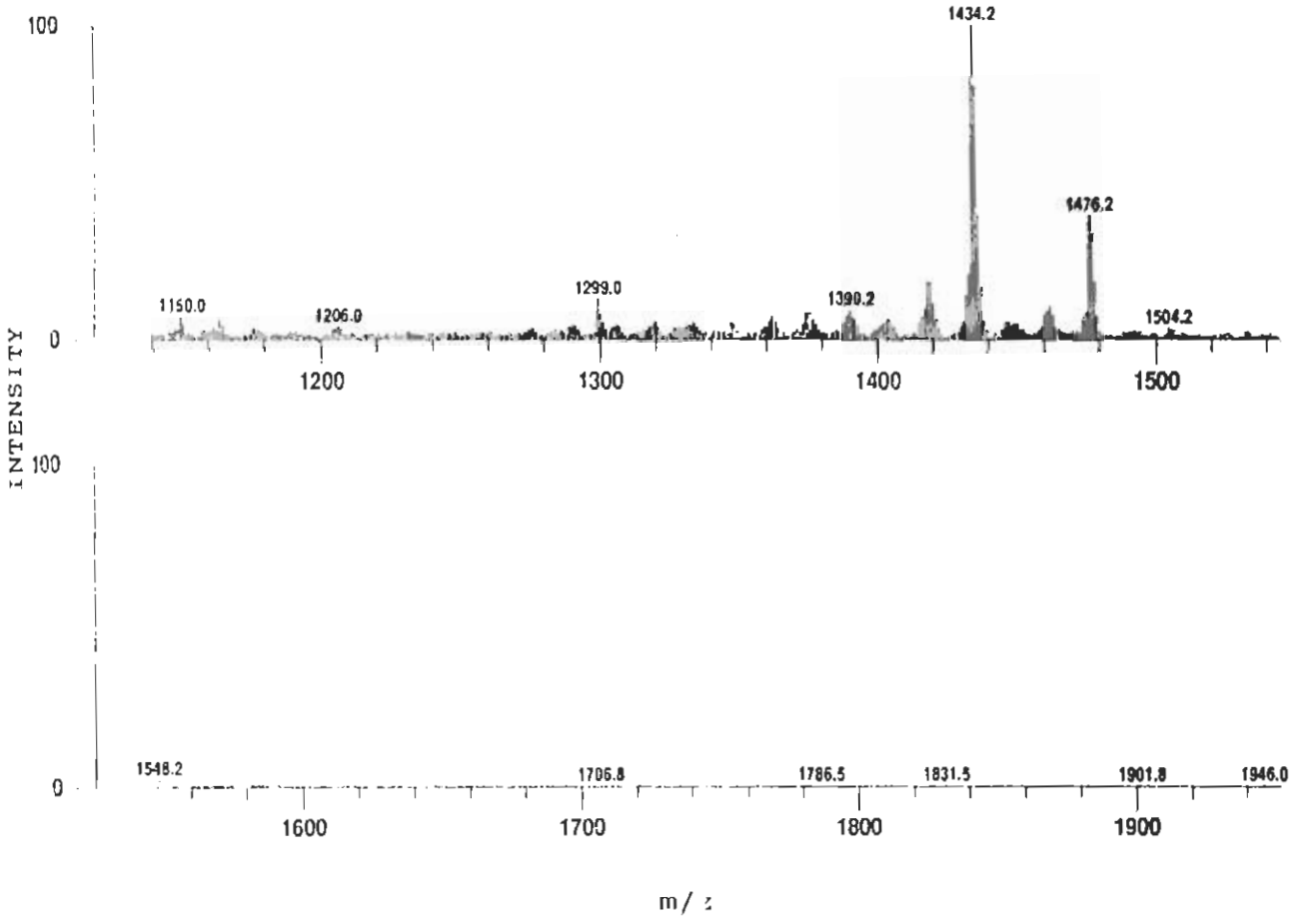


Fig. 3.40 FAB mass spectrum of fraction iv from peptic digest of acetylated αA Crystallin (Fig.3.36), showing unmodified (m/z = 1434.2) and acetylated (m/z = 1476.2) Peptides containing lysine11. Scan rate:12 sec/decade, resolution: 1500, acceleration voltage: 8 kv, calibration range: 300-4000, matrix: glycerol: thioglycerol (1:1).

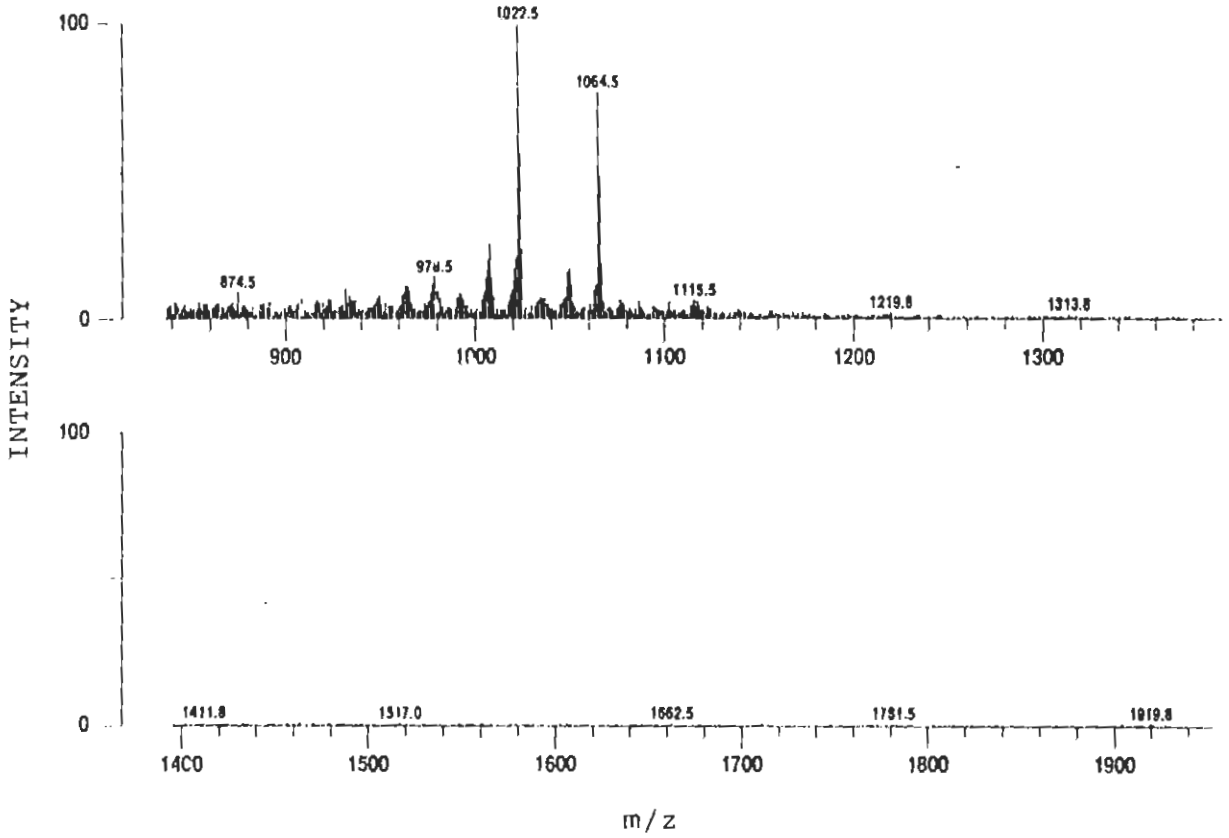


Fig. 3.41 FAB mass spectrum of fraction ii from peptic digest of acetylated αA Crystallin(Fig.3.36), showing unmodified (m/z = 1022.5) and acetylated(m/z = 1064.5) peptides containing lysine 70. Scan rate: 15 sec/decade, resolution: 1500, acceleration voltage: 8 kv, calibration range: 300-4000, matrix: glycerol: thioglycerol (1:1).

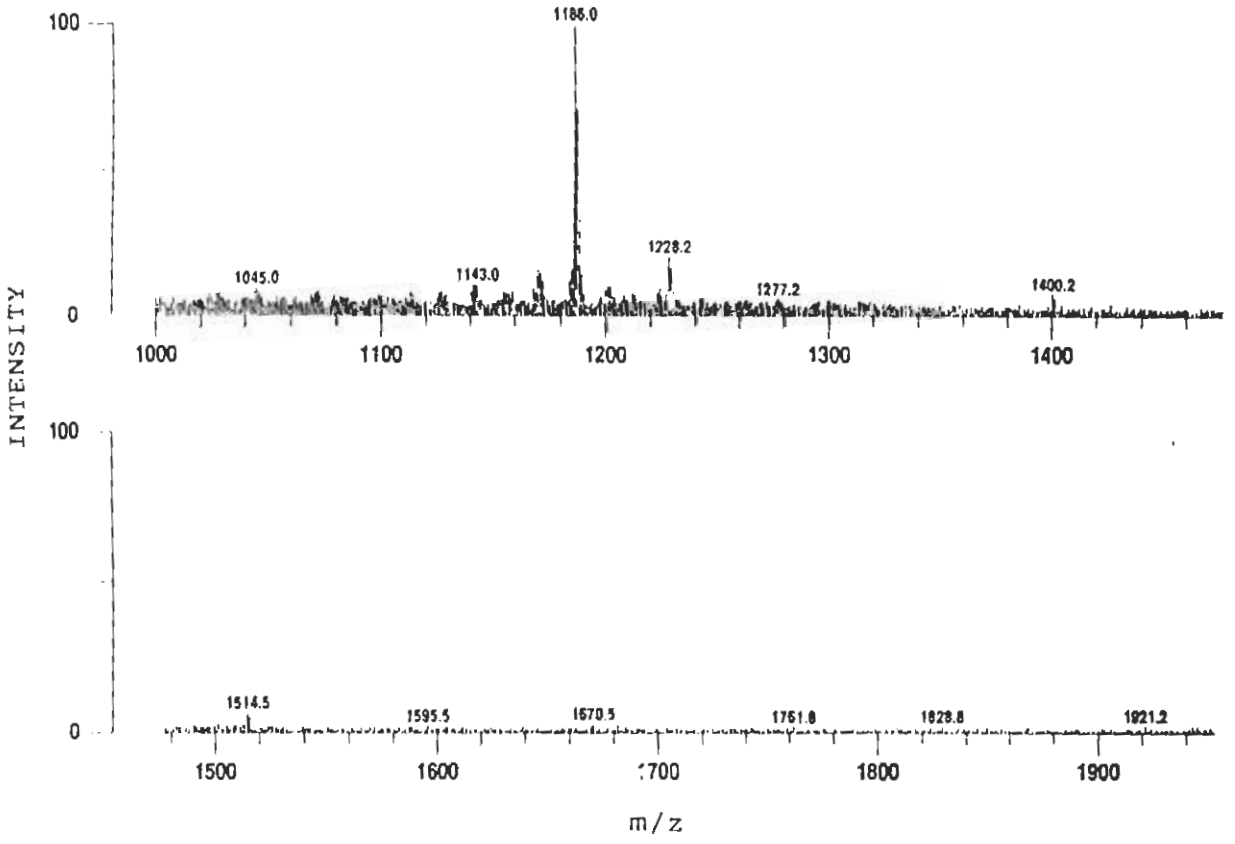


Fig. 3.42 FAB mass spectrum of fraction iii from peptic digest of acetylated αA Crystallin (Fig.3.36), showing unmodified (m/z = 1186) and acetylated (m/z = 1228) peptides containing lysine 78. Scan rate: 15 sec/decade, resolution: 1500, acceleration voltage: 8 kv, calibration range: 300-4000, matrix: glycerol: thioglycerol(1:1).

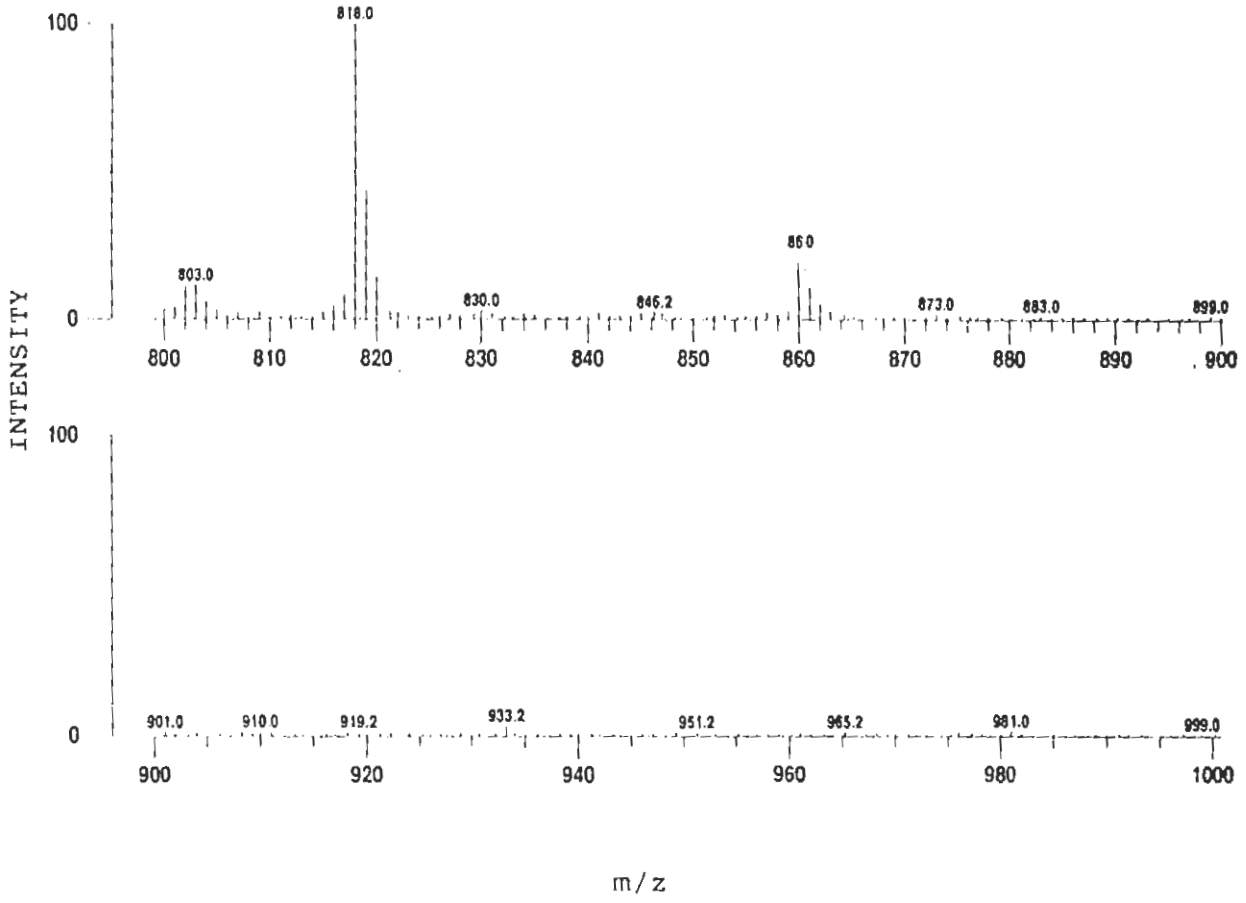


Fig. 3.43 FAB mass spectrum of fraction i from peptic digest of acetylated αA Crystallin (Fig.3.36), showing unmodified (m/z = 818) and acetylated (m/z = 860) peptides containing lysine 88. Scan rate: 15 sec/decade, resolution: 1500, acceleration voltage: 8 kv,calibration range:300-4000, matrix: glycerol:thioglycerol(1:1).

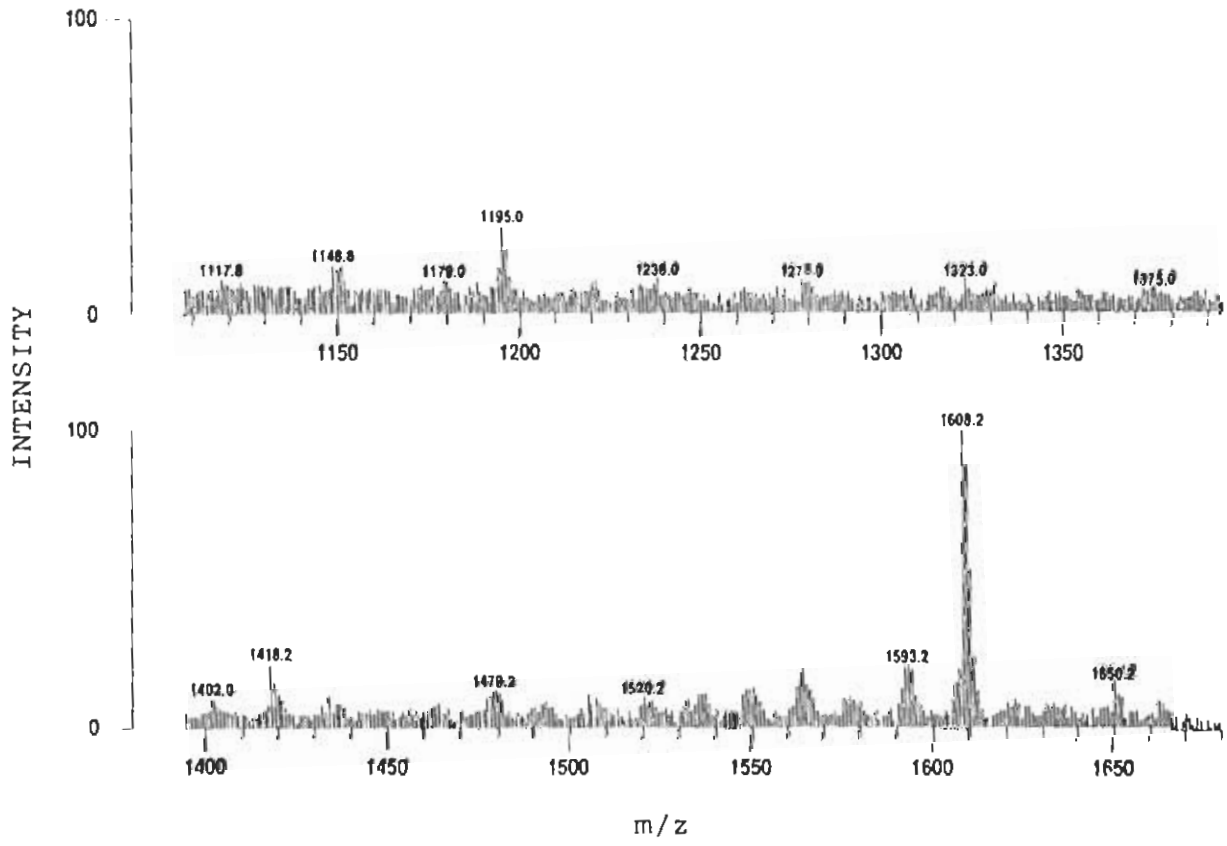


Fig. 3.44 FAB mass spectrum of a fraction (indicated by horizontal bar in Fig. 3.38) from ASP-N digest of acetylated αA Crystallin, showing unmodified (m/z = 1608.2) and acetylated (m/z = 1650.2) peptides containing lysine 99. Scan rate: 8 sec/decade, resolution: 1500, acceleration voltage:8 kv,calibration range: 300- 4000, matrix: glycerol: thioglycerol (1:1).

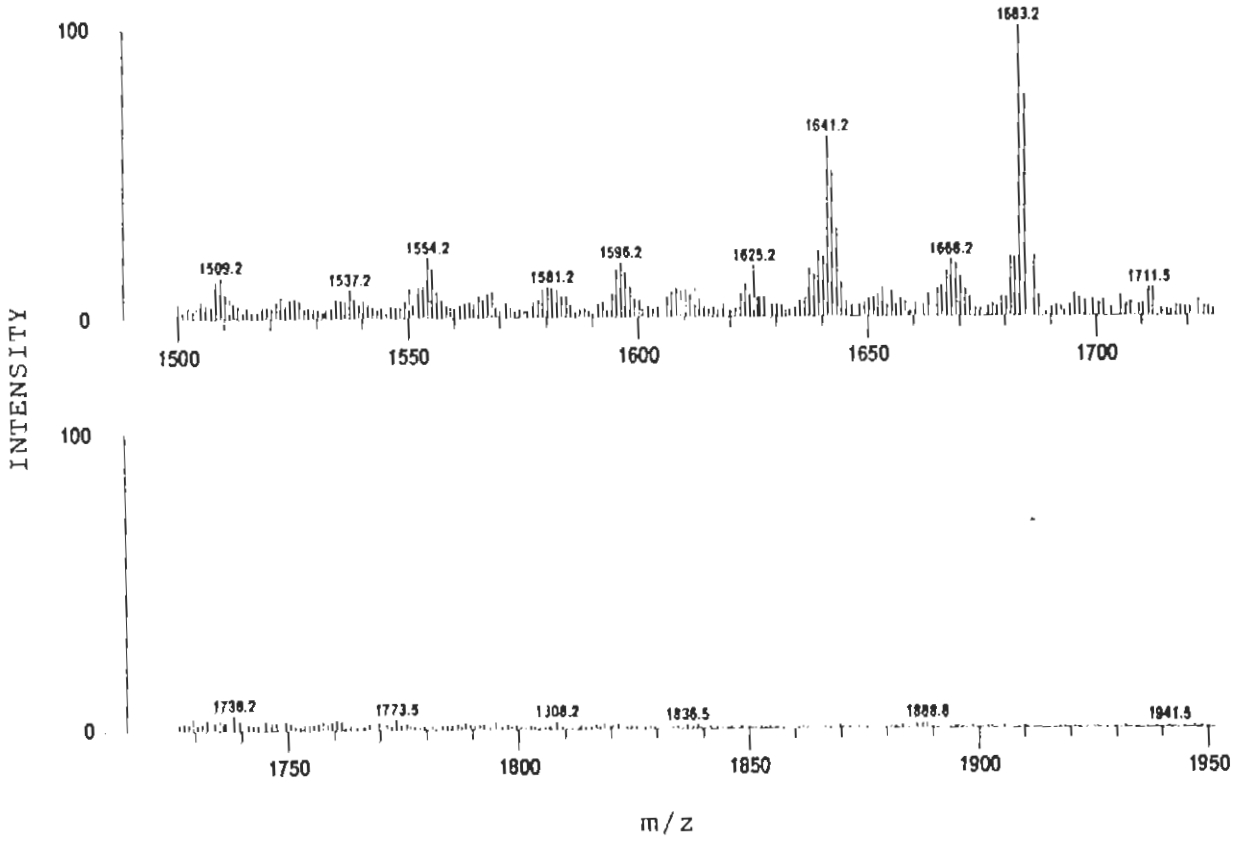


Fig. 3.45 FAB spectrum of fraction i from chymotryptic digest of acetylated αA Crystallin (Fig.3.37), showing unmodified (m/z = 1641.2) and acetylated (1683.2) peptides containing lysine 166. Scan rate: 8 sec/decade, resolution:1500, acceleration voltage: 8 kv,calibration range:300-4000, matrix: glycerol: thioglycerol (1:1).

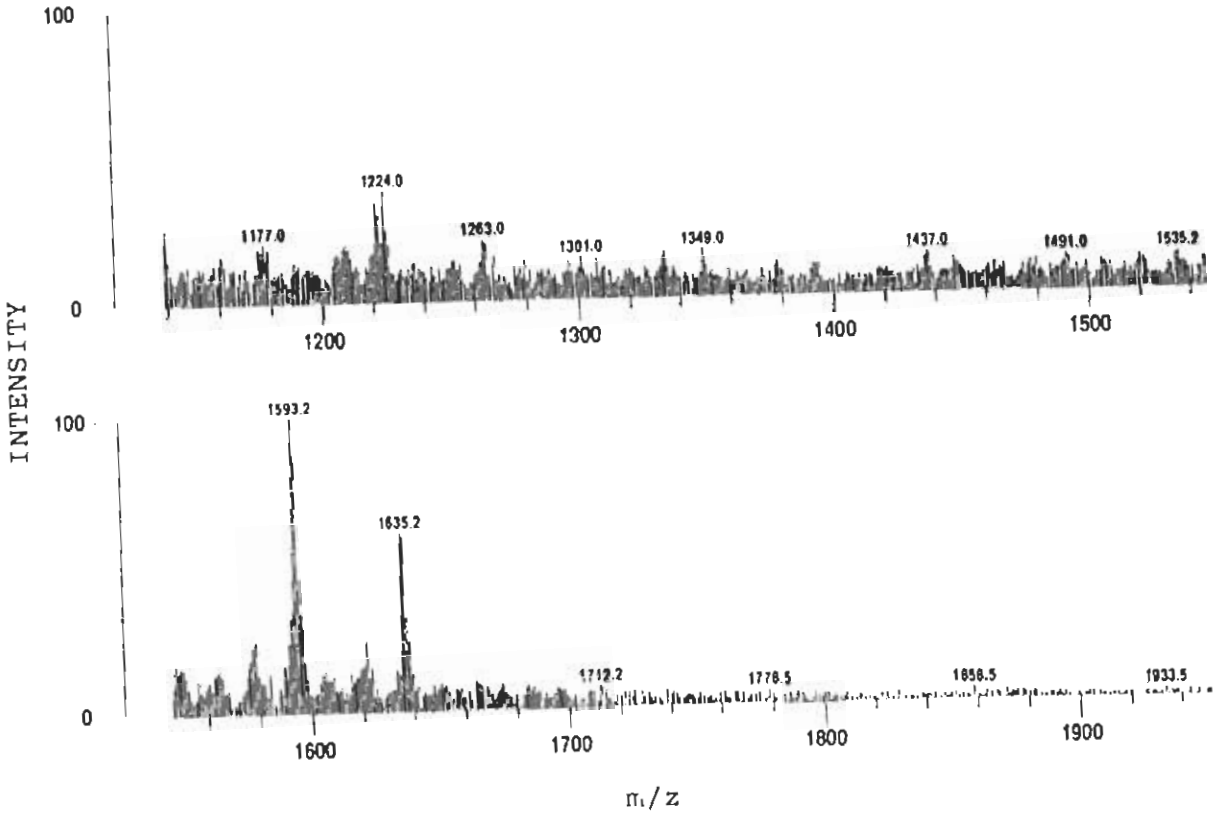


Fig. 3.46 FAB mass spectrum of fraction ii from chymotryptic digest of acetylated αA Crystallin (Fig.3.37), showing unmodified (m/z = 1593.2) and acetylated (m/z = 1635.2) peptides containing lysine 145. Scan rate: 8 sec/decade, resolution: 1500, acceleration voltage: 8 kv,calibration range: 300-4000, matrix: glycerol:thioglycerol (1:1).

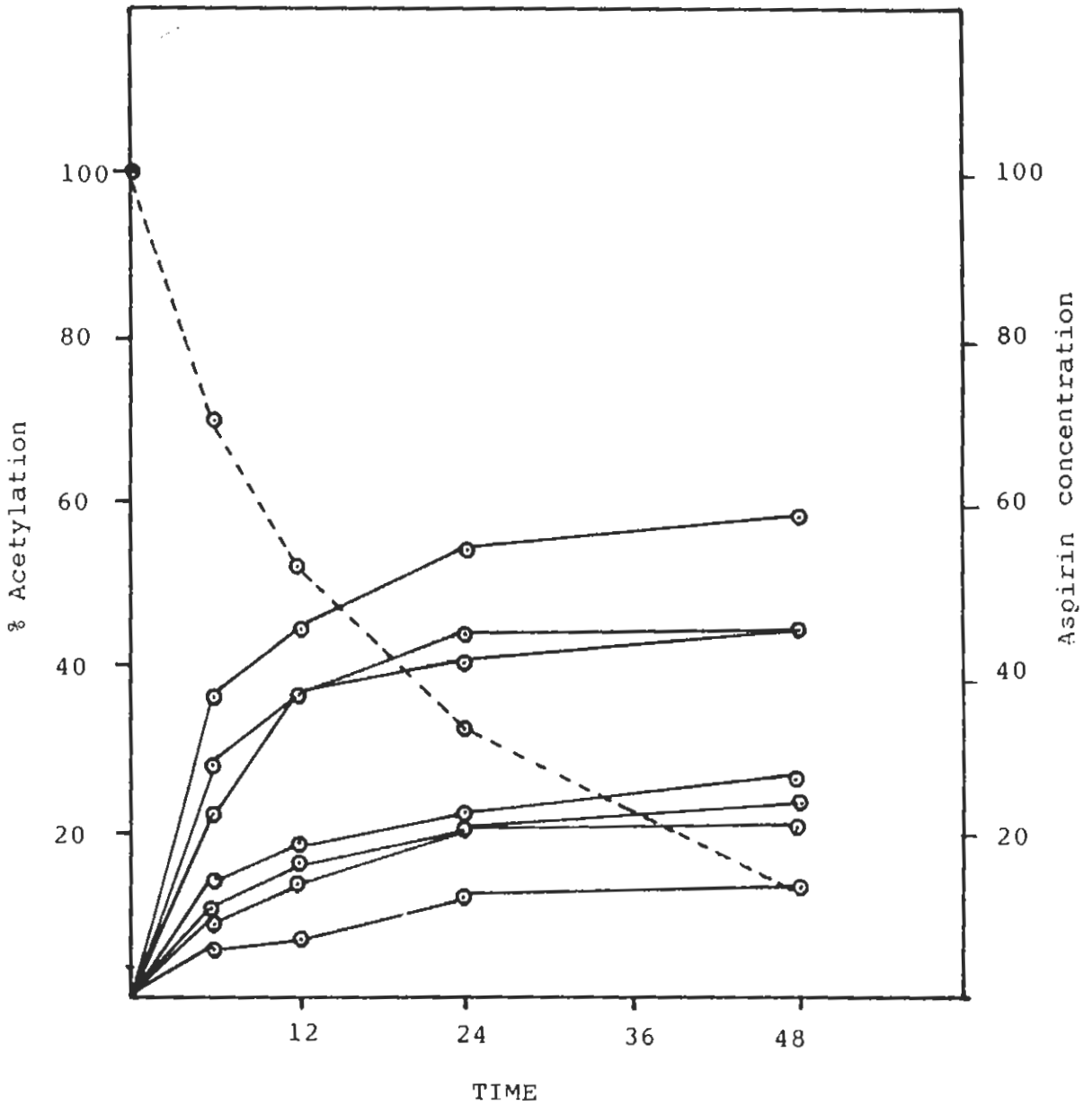


Fig. 3.47 Modification of each lysyl residue of αA crystallin as a function of incubation time. Dotted line denotes the concentration of unhydrolyzed aspirin with time.

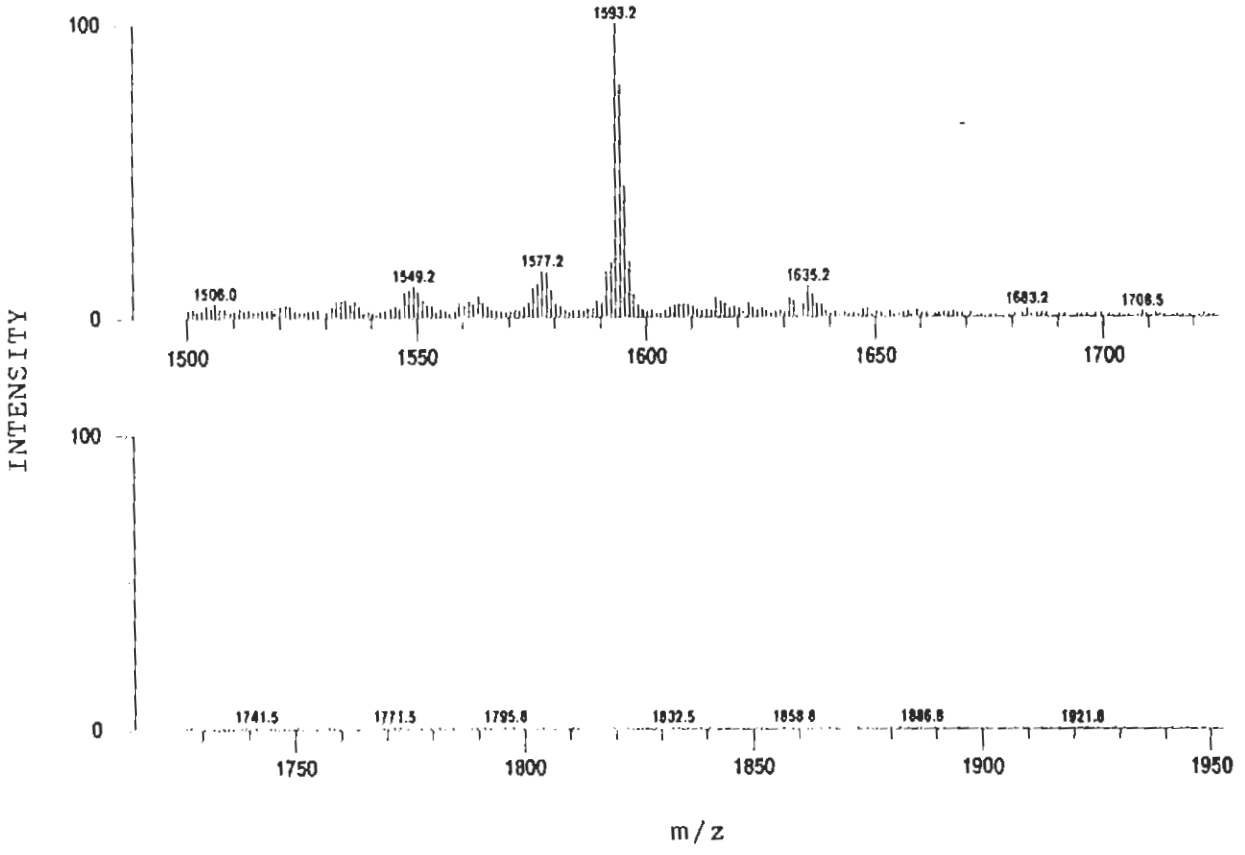


Fig. 3.48 FAB mass spectrum of a fraction from chymotryptic digest of αA (only) crystallin, showing peptides containing unmodified and acetylated lysine 145.

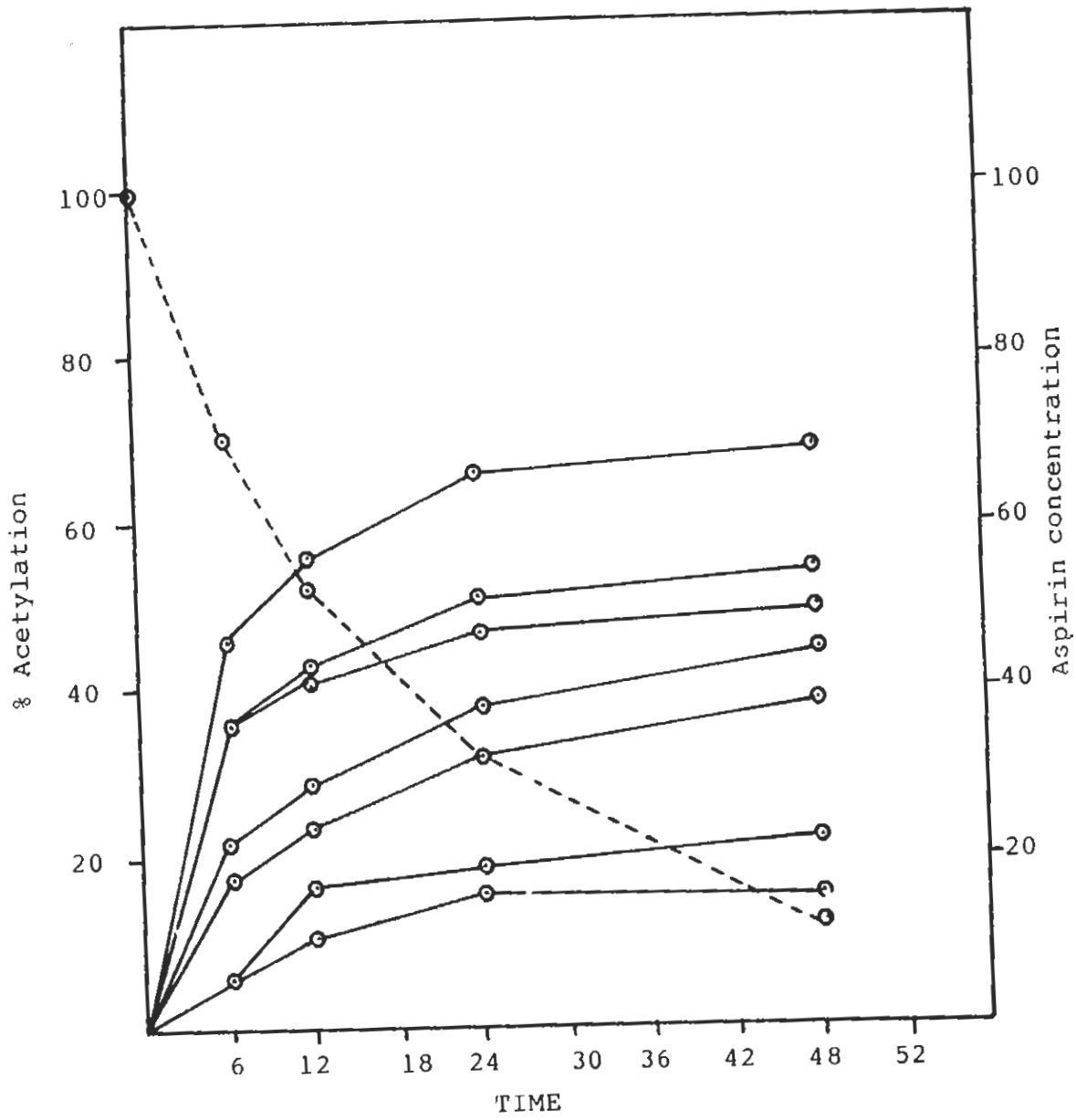


Fig. 3.49 Modification of each lysyl residue of αA crystallin (separated from αB crystallin prior to incubation with aspirin) as a function of incubation time. Dotted line denotes the concentration of unhydrolyzed aspirin with time.

Table 3.1. Rate constants for acetylation and carbamylation of αA-crystallin lysyl residues (10^3 x k, M^{-1} hr^{-1})

Lysine	Acetylation	Carbamylation
11	32.2	54.4
70	42.1	31.4
78	15.8	10.3
88	10.0	5.1
99	16.1	20.4
145	20.9	23.2
166	69.9	20.4

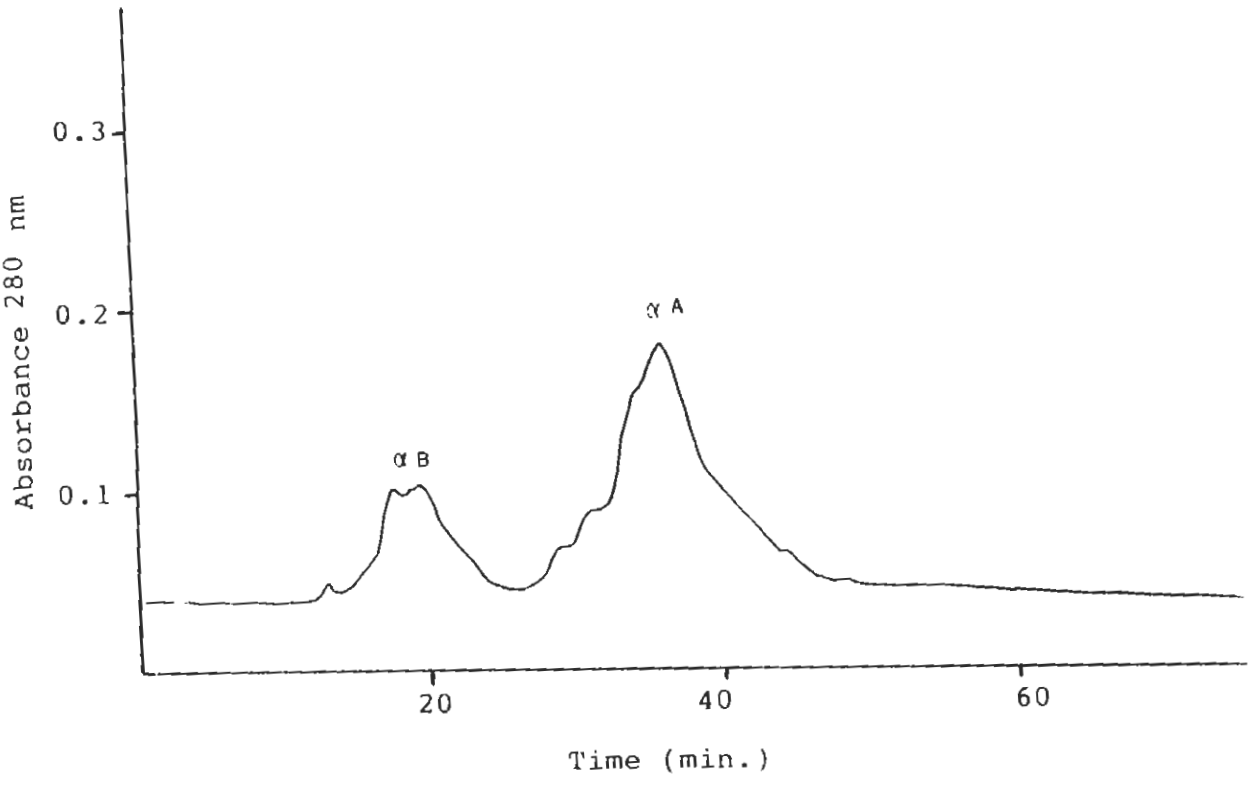


Fig. 3.50 Separation profile of crude a-Crytallin on Vydac C4 column after concurrent incubation. Eluent: 0.1% TFA-ACN, flow rate:60 ml/hrs., absorbance: 280 nm.

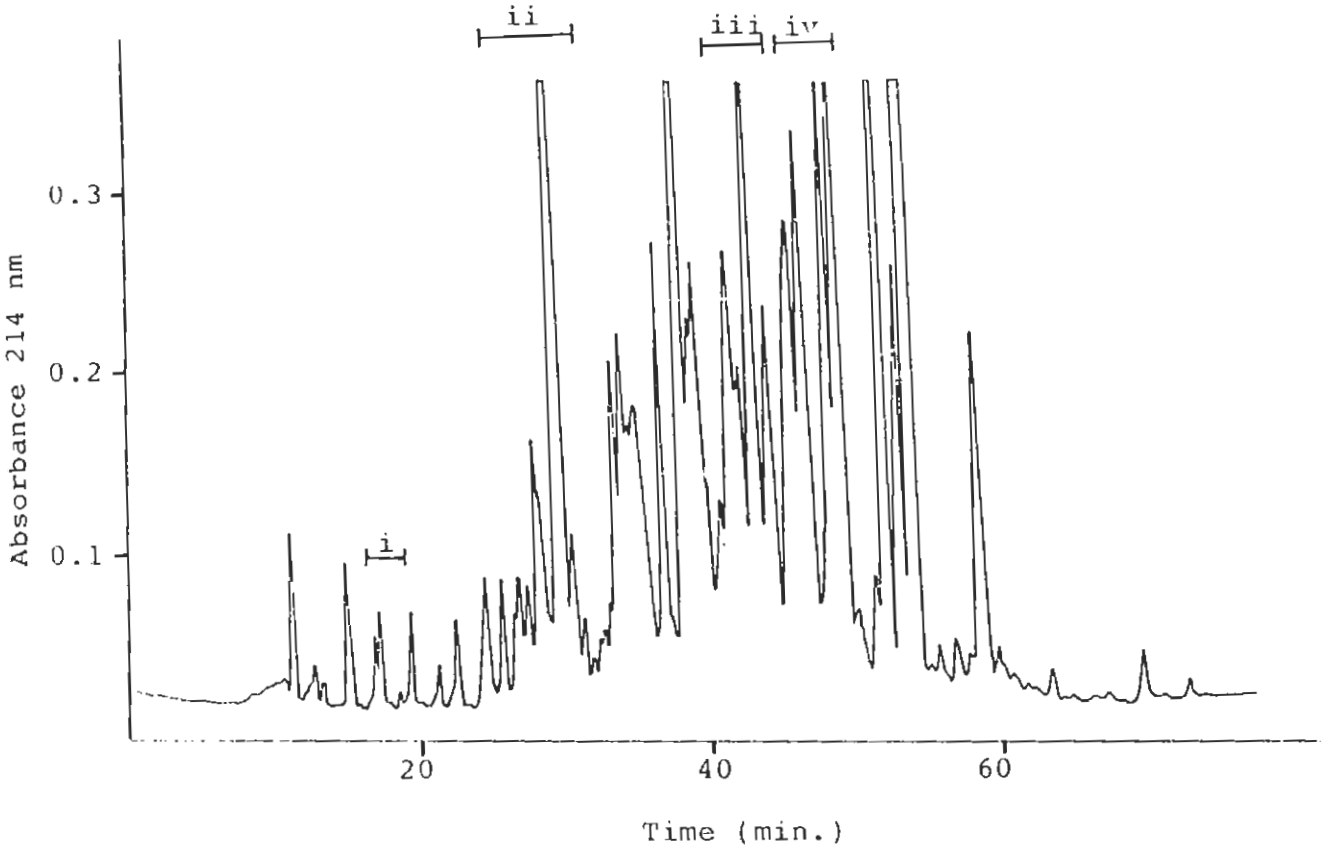


Fig. 3.51 Separation profile of peptic peptides of modified αA Crystallin on Vydac C18 column after concurrent incubation. Eluent: 0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance: 214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i,ii,iii and iv represent peptides containing unmodified and carbamylated lysines 11,70, 78 and 88 respectively.

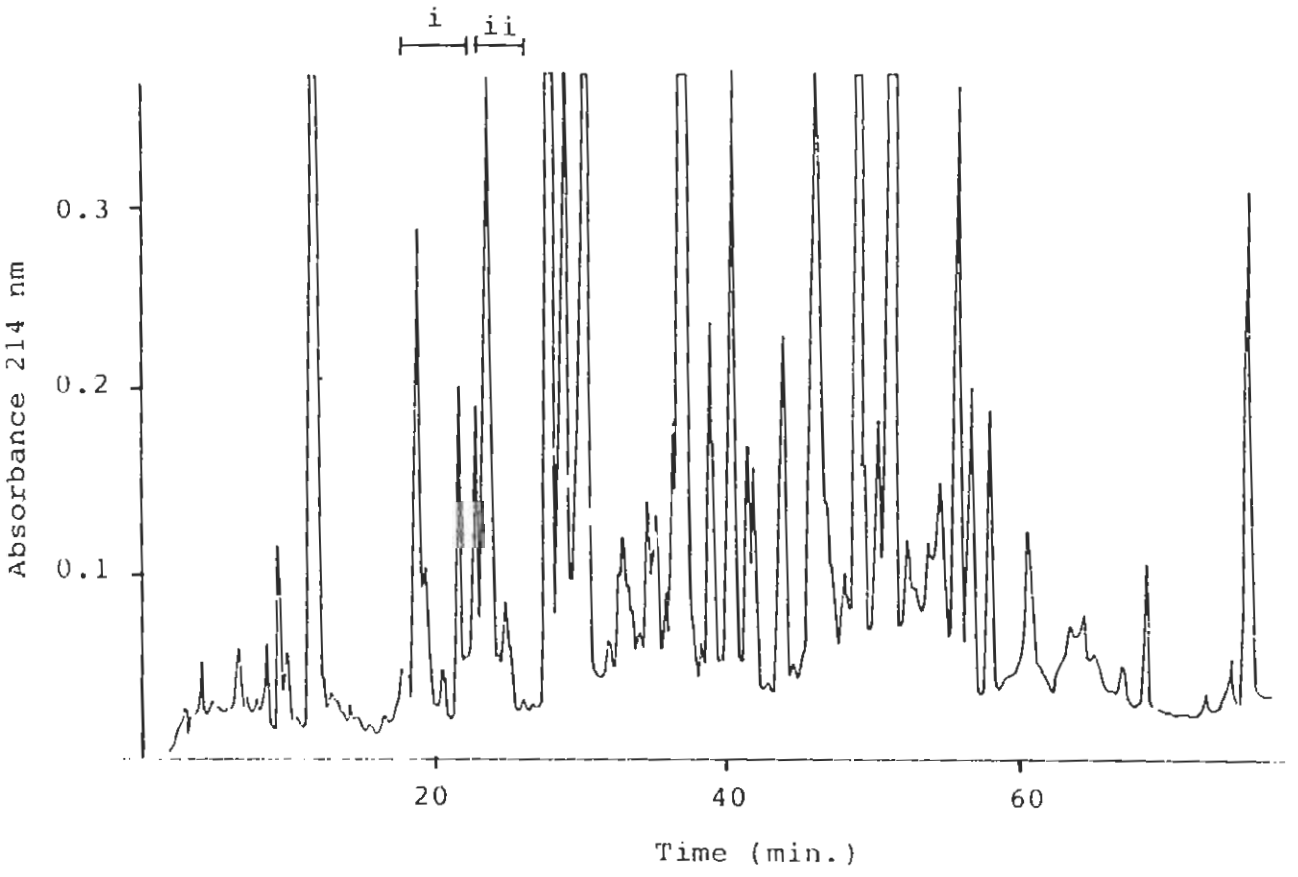


Fig. 3.52 Separation profile of chymotryptic peptides of modified αA Crystallin on Vydac C18 column after concurrent incubation. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance: 214 nm.Horizontal bars indicate the pooling scheme of peptides. Fractions i and ii represent peptides containing unmodified and carbamylated lysines 166 and 145 respectively.

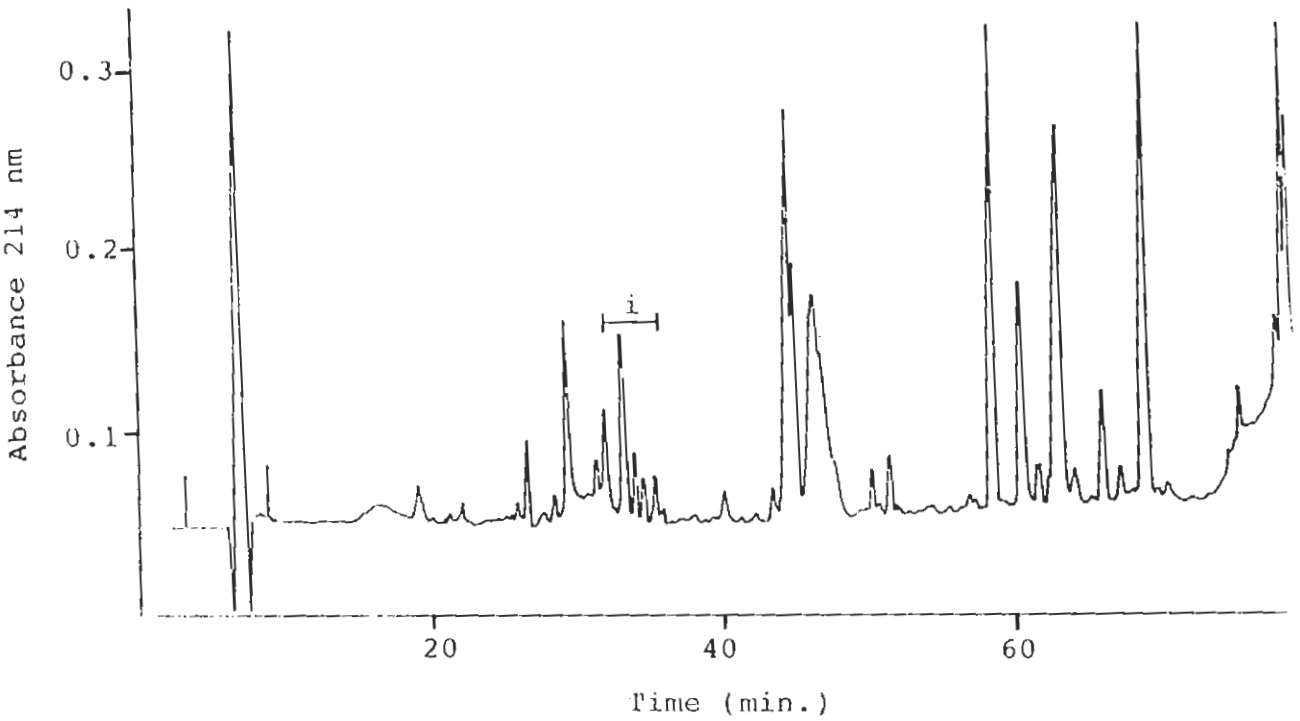


Fig. 3.53 Separation profile of ASP-N peptides of modified αA Crystallin on Vydac C18 column after concurrent incubation. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance: 214 nm.Horizontal bar indicates the pooling scheme of peptides containing unmodified and acetylated lysine 99.

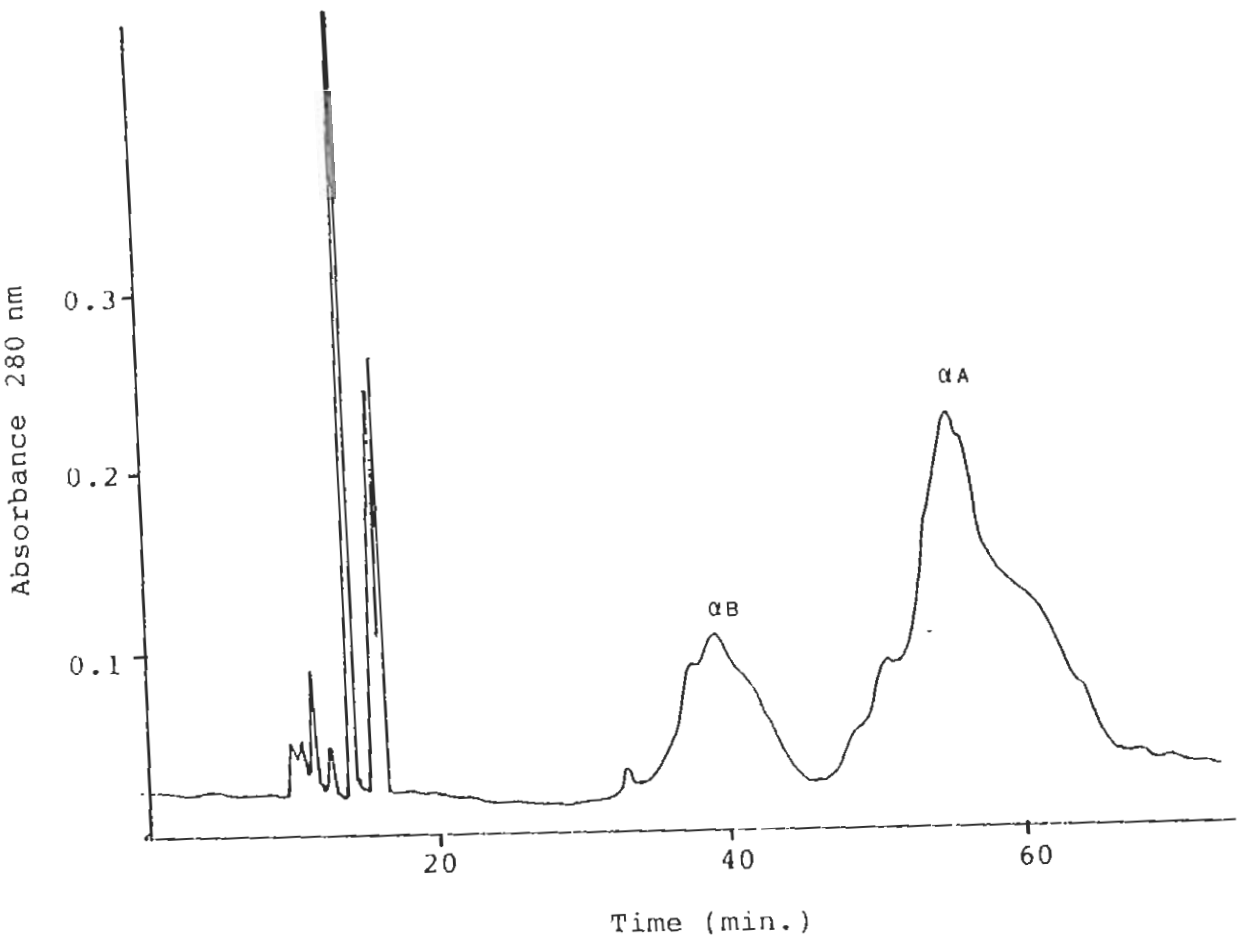


Fig. 3.54 Separation profile of crude α -Crystallin on Vydac C4 column after sequential incubation. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance: 280 nm.

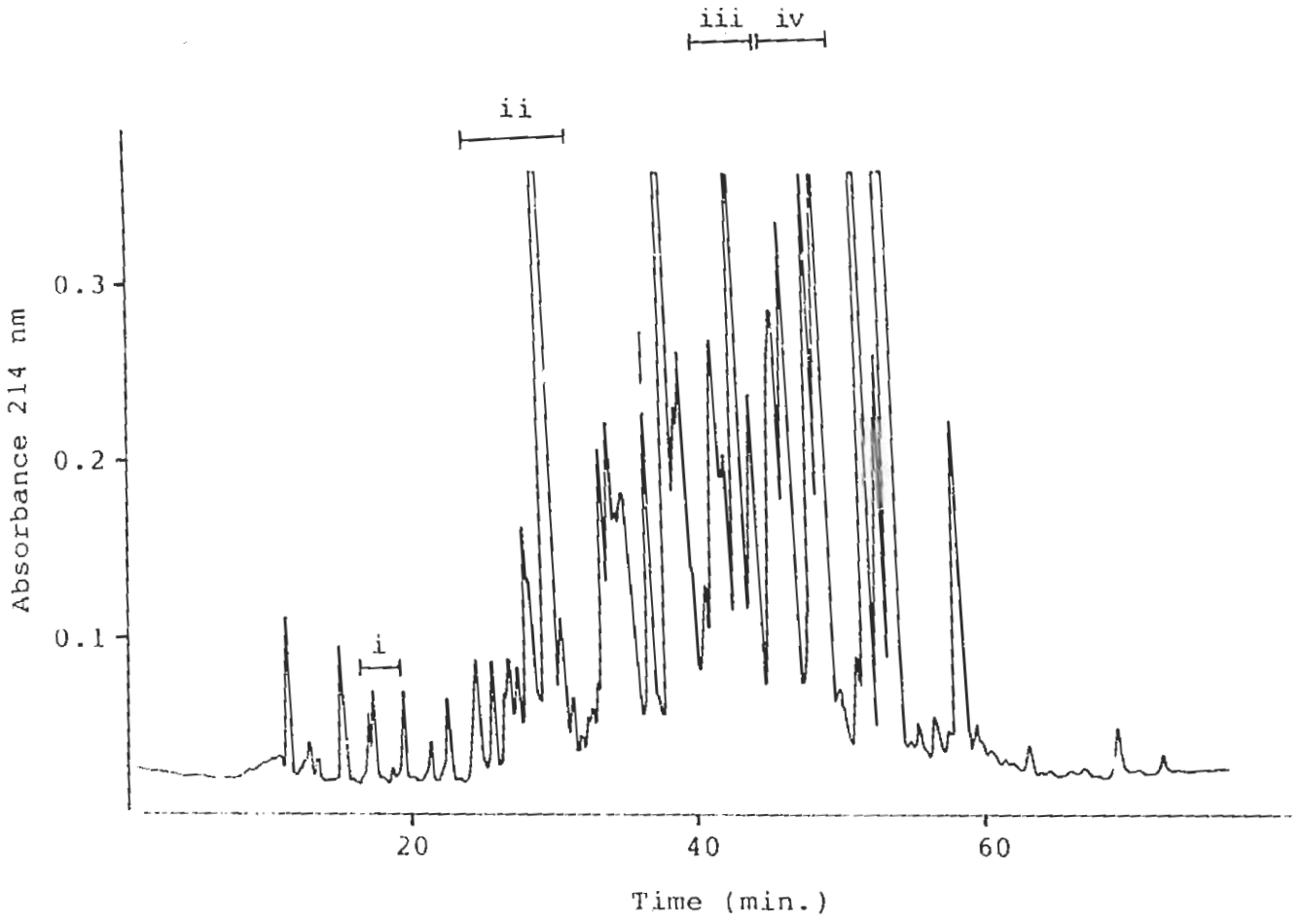


Fig. 3.55 Separation profile of peptic peptides of modified αA Crystallin on Vydac C18 column after sequential incubation. Eluent: 0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance:214 nm. Horizontal bars indicate the pooling scheme of peptides. Fractions i,ii,iii and iv represent peptides containing unmodified and carbamylated lysines 11,70, 78 and 88 respectively.

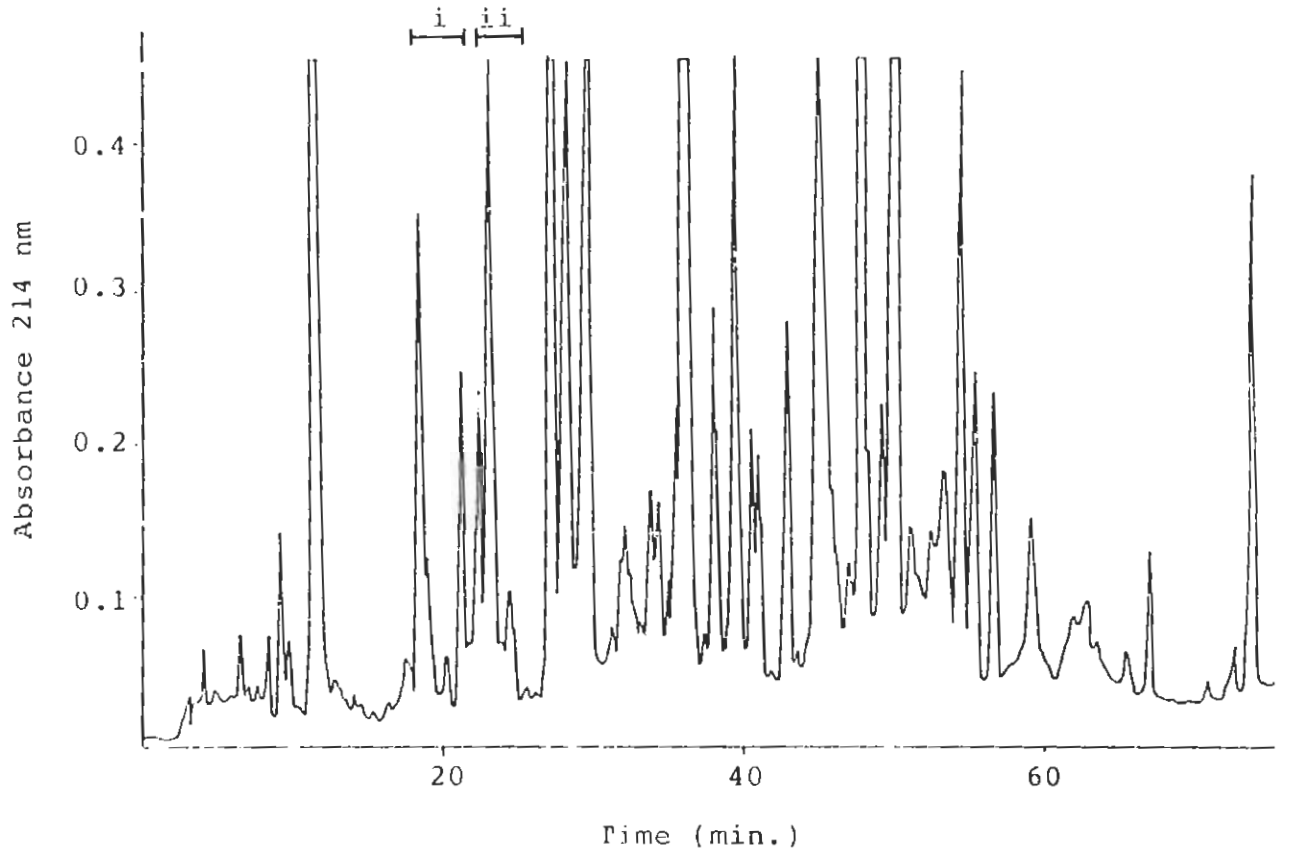


Fig. 3.56 Separation profile of chymotryptic peptides of modified αA Crystallin on Vydac C18 column after sequential incubation Eluent:0.1% TFA-ACN, flow rate: 60 ml/hr ., absorbance:214 nm.Horizontal bars indicate the pooling scheme of peptides. Fractions i and ii represent peptides containing unmodified and carbamylated lysines 166 and 145 respectively.

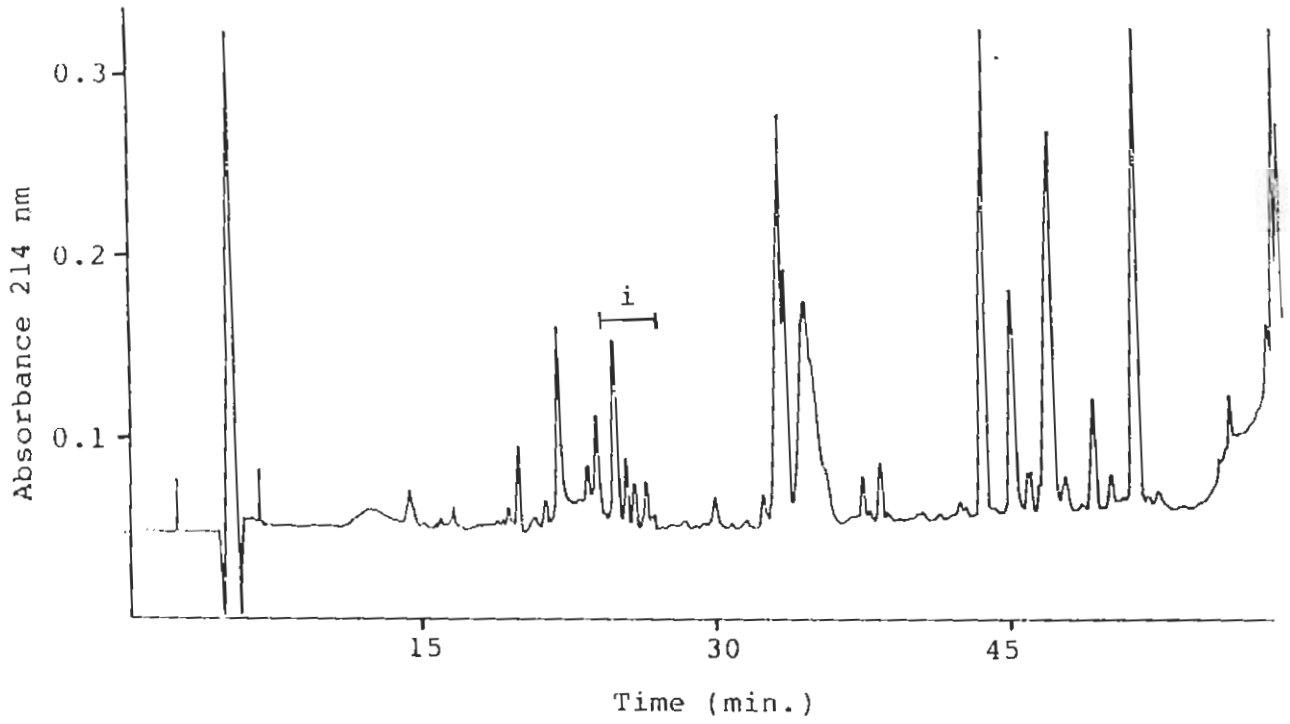


Fig. 3.57 Separation profile of ASP-N peptides of modified αA Crystallin on Vydac C18 column after sequential incubation. Eluent:0.1% TFA-ACN, flow rate:60 ml/hr ., absorbance:214 nm.Horizontal bar indicates the pooling scheme of peptides containing unmodified and acetylated lysine 99.

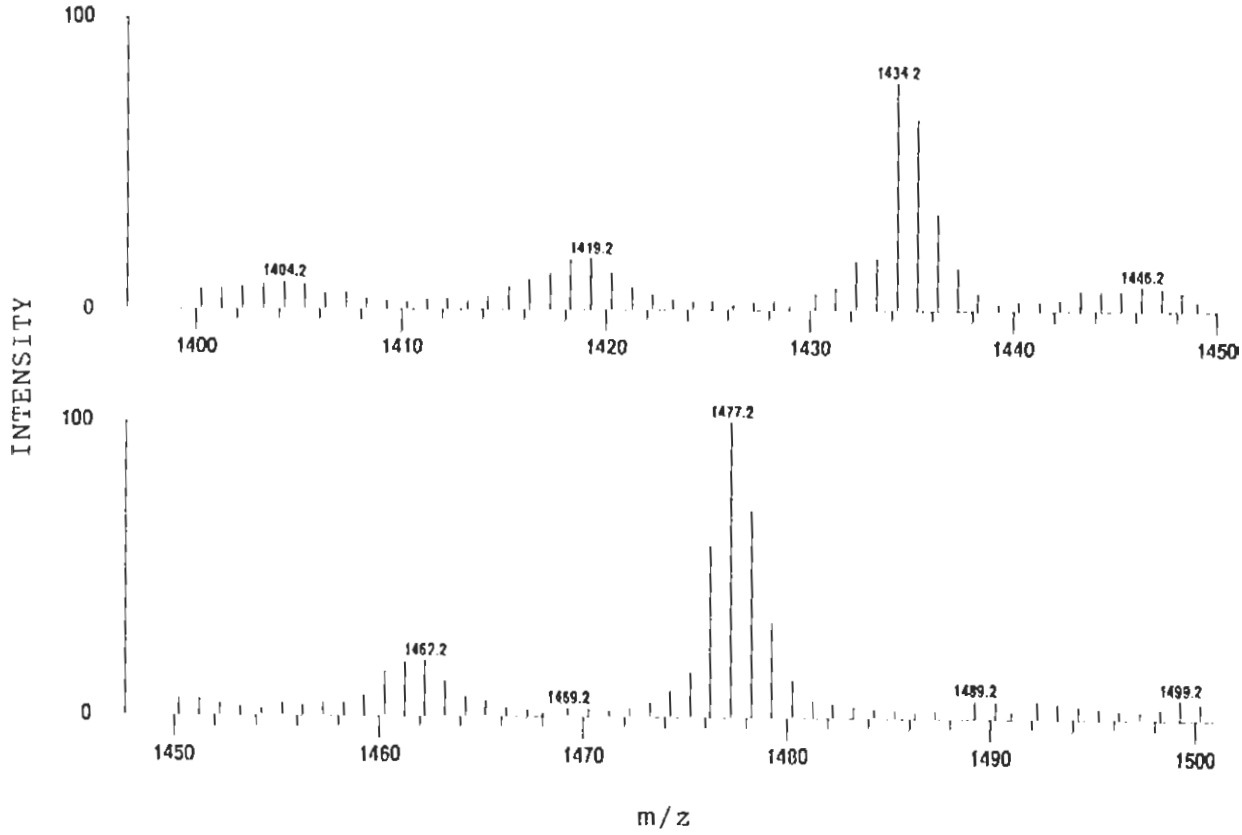


Fig. 3.58 FAB mass spectrum of a fraction from peptic digest of modified αA Crystallin after concurrent incubation, showing peptides containing unmodified and modified lysine 11.Scan rate:8 sec/decade, resolution:1500,acceleration voltage:8 kv,calibration range:300-4000, matrix: glycerol:thioglycerol (1:1).

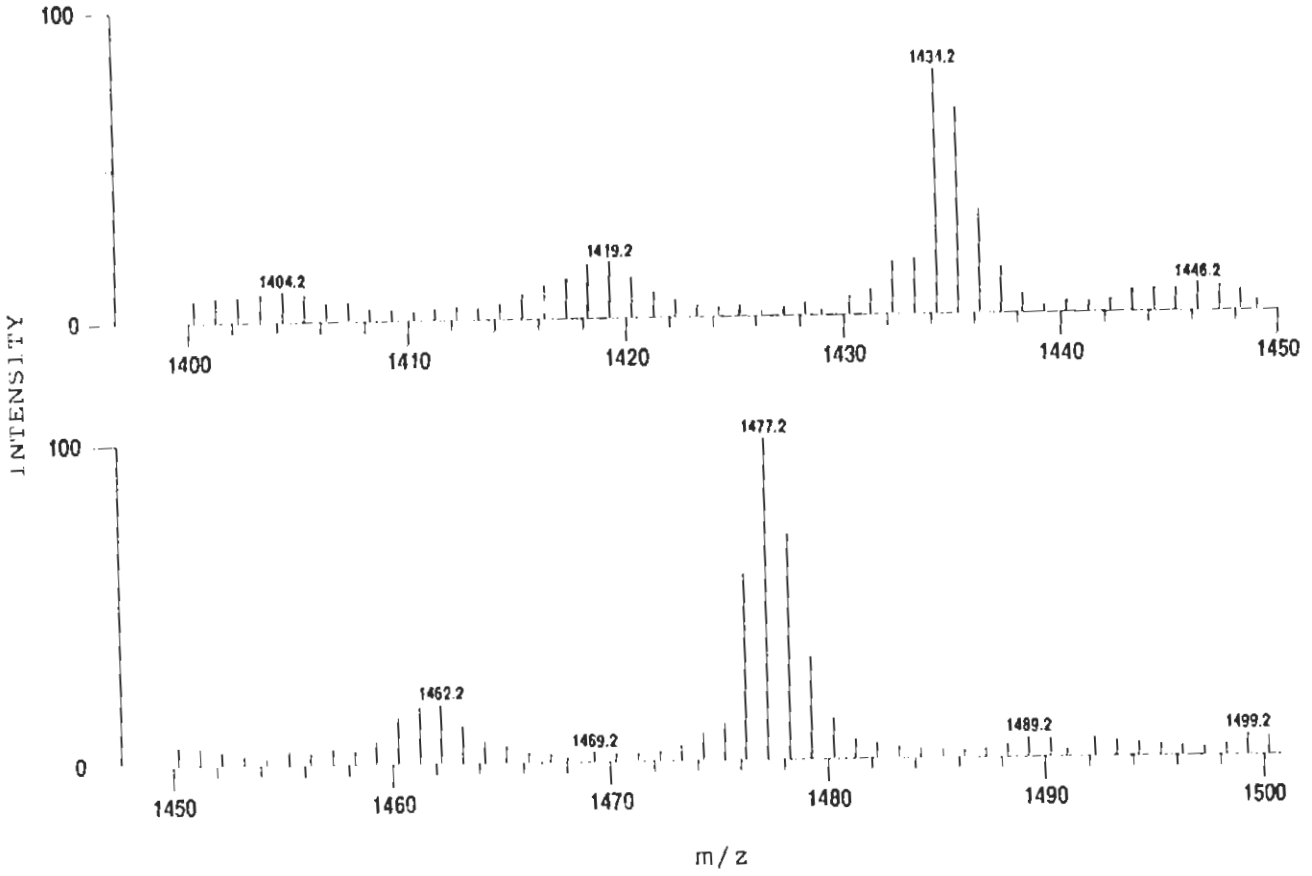


Fig. 3.59 FAB mass spectrum of a fraction from peptic digest of modified αA Crystallin after sequential incubation, showing peptides containing unmodified and modified lysine 11.

Table 3.2 **Comparison of acetylation ard carbamylation of lysines in αA crystallin 24 hours incubations.**

Lysine Number	Carbamylation Only	Acetylation only	Concurrent		Sequential	
			Acetylation	Carbamylation	Acetylation	Carbamylatio
11	58	40	27	32	32	26
70	42	43	36	25	36	25
78	18	20	17	14	17	11
88	08	12	04	08	04	06
99	26	22	21	16	22	10
145	34	20	22	31	21	26
166	29	54	44	19	46	20

4.0 DISCUSSION

In the present studies,we have employed a new strategy to determine the type and sites of chemical modification and have exploited the complex,formed of bovine lens crystallin with isocyanate. Earlier, incorporation of radioactivity into lens crystallins was used for monitoring the effect of chemical agents such as isocyanate, sugars e.t.c. on lens crystallins but these studies were incomplete and inconclusive because,these described only the extent of reaction with the whole molecule and did not provide any information about the reaction sites.Similarly amino acid analyses and sequencing methods did not furnish implicit information about the reaction sites (Jabusch,1985) as these techniques require peptides with high purity. Fast atom bombardment mass spectrometry does not require purity of peptides of that order and several peptides in a fraction can be identified by their molecular masses. FAB-MS can also be utilized in calculating the ratio of modified to unmodified peptide which in turn would be useful in determining the extent of modification in a reaction at known sites.

We, therefore, selected FAB-MS in combination with HPLC for determining the effect of isocyanate on lens crystallin.

Aspirin is capable of acetylation of a variety of proteins including lens crystallins (Pincard, 1966., Cotlier, 1981., Raoetal., 1985., Crompton, 1988). However,there is contraversy about the effectiveness of aspirin as anti cataract agent. Therefore, FAB-MS has been used to check the effectiveness of aspirin as anticataract agent.

We have selected αA crystallin as it is the major protein of α-crystallins constituting one third part of the total lens crystallins (Harding, 1976). Its primary structure has been elucidated by Ouderaa, (1974). It contains seven lysyl residues at positions 11, 70, 78, 88, 99, 145 and 166 and one cysteine at position 131. The earlier investigations have indicated that lysyl residues have more affinity for chemical modification

than any other residue (Beswick,1975). It is also known that disulfide bonding plays a major role in the aggregation of crystallins that leads to cataract formation. In view of these observations αA crystallin serves as a good model for determining the extent of modifications at lysyl residues and effect of chemicals such as isocyanate and aspirin on disulfide bonding.

4.1. ISOLATION OF α-CRYSTALLINS

We employed a two steps procedure for the isolation of αA crystallin i.e. gel filtration and reversed phase HPLC. Gel filtration was carried out on Sephadex G- 200.The crude extract was fractionated into four peaks as reported earlier (Asselbergs, 1979) (Fig. 3.1). Peak 1 represents high molecular mass α-crystallins, while peaks 2 and 3 contain heavy β crystallin (βH) and light βcrystallin (βL) respectively. Peak 4 represents low molecular mass γ-crystallins. Peak 1 of gel filtration was further purified by reversed phase HPLC. We obtained better separation of α-crystallins by HPLC method as compared to conventional chromatographic techniques such as ion exchange chromatography on CM Cellulose (dejong, 1975) and sulfoethyl Sephadex (Schoenmakers, 1969) . Rechromatography of α-crystallins (peak 1 of gel filtration) on reversed phase column revealed three major and some minor peaks (Fig. 3. 2). Peak 1 was identified as αB crystallin whereas peak 2 and peak 3 were identified as αA crystallin on alkaline polyacrylamide gel electrophoresis (Fig. 3.3). We, therefore, collected whole region covering peak 2 and peak 3 as αA crystallin. Our identification has been further confirmed by electrospray analysis (Smith, 1991).

4.2. ENZYMATIC DIGESTION AND PEPTIDE MAPPING

Purified αA crystallin was subjected to enzymatic digestions by pepsin,chymotrypsin and endoproteinase ASP-N to generate peptides

and construct molecular mass maps of these peptides after FAB-MS analysis. We used more than one enzyme for digestion of αA crystallin as it is known that some peptides in a digest may not be recorded in FAB-MS either due to their hydrophobic nature or their molecular masses did not fall within the required range (i.e. 300- 400) (Smith, 1991). αA crystallin cleaved into 40 fractions by pepsin. Peptides obtained by pepsin were first analyzed by FAB-MS and then identified from a list showing all possible peptide coordinates and their molecular masses produced by pepsin (Table 4.1). Identification of each peptic peptide was confirmed by further digestion of peptide with carboxypeptidase. An example is shown in Fig. 3.8. For the peak at m/z 1299, the computer assisted mass search listed four peptides (Table 4.1). As the carboxypeptidase removed residues from C-terminus, new peaks appeared after 20 and 70 minutes incubations. Mass spectra indicating that residue were SPEDL. This confirms the identification of peptide as segment 75- 85. About 70% sequence of αA crystallin was detected from the peptic digest. Purified αA crystallin was also cleaved with chymotrypsin to get rest of the sequence of αA crystallin. More than 40 peptides were generated by chymotrypsin. These peptides were analyzed by FAB-MS and identified from list containing molecular masses of all peptides generated by chymotrypsin (Table 4.2). Sequence of each chymotryptic peptide was confirmed using carboxypeptidase. 82% sequence of αA crystallin was detected in chymotryptic digest. Combined molecular weight maps of pepsin and chymotrypsin digests covered 90% sequence of αA crystallin (Fig. 4.1). But still some peptides were missing, particularly peptides containing lysine 99 was not obtained in both the digests. Although there were some peaks determined by FAB-MS whose molecular masses corresponded to the peptides containing lysine 99, but their intensities were too low for adequate confirmation. Several enzymes were tried to isolate the peptide containing lysine 99. These include, elastase, thermolysin, endoproteinase V8 and endoproteinase

ASP-N. We successfully isolated the desired peptide using endoproteinase ASP-N. 30 peptides were isolated from endoproteinase ASP-N digestion of αA crystallin. These peptides were analyzed by FAB-MS and identified from list (Table. 4.3). Confirmation of peptide sequence was carried out using carboxypeptidase. About 99% sequence of αA crystallin was observed when molecular mass maps of three digests were combined (Fig. 4.1).

4.3. MODIFICATIONS OF α-CRYSTALLINS

Molecular mass maps obtained from enzymatic digestions have been utilized for locating the site as well as the type of modification occurred due to incubation with isocyanate and aspirin.

4.3.1. Carbamylation of αA Crystallin

Incubation of αA crystallin with KNCO created a 43 mass unit increase in the molecular masses of all lysine containing peptides. Complete FAB-MS analysis of peptides from all three enzymatic digestions indicated the carbamylation of lysyl residues and there was no sign of carbamylation at any other residue.

4.3.1.1. Quantitative Determination of Carbamylation

The extent of carbamylation at each lysyl residue of αA crystallin was determined by incubating α-crystallin with 100 mM KNCO for different time (i.e. 6 hours,12 hours,24 hours and 48 hours). Reversed phase HPLC chromatograms of α-crystallins after incubation at different time showed the effect of carbamylation on the elution profiles of αA and αB. In case of unmodified α-crystallin αB was eluted between 39-41% acetonitrile whereas fractions corresponding to αA were eluted between 44-46% acetonitrile. After 6 hours incubation of α-crystallin with isocyanate, peaks corresponding to αB and αA became broader and eluted later indicating the presence of more than one components. A shoulder peak was detected with αA fraction. After 12 hours

incubation the shoulder peak became prominent and two separate peaks were observed after 24 hours of incubation. After 48 hours incubation the modified peak (carbamylated) became dominant indicating the completion of carbamylation. Although HPLC profiles gave some idea about the extent of carbamylation of α-crystallins but this information is not conclusive. These profiles describe the extent of carbamylation of whole αA crystallin but they do not provide information about the extent of carbamylation at each lysyl residue. A combination of enzymatic digestion, HPLC separation, pooling of modified and unmodified peptides in same fraction and quantitation of percentage of modification by FAB-MS would serve as a better strategy for determining the extent of carbamylation at each lysyl residue.

Peptic digest was selected for the quantitation of the percentage of modification at lysines 11,70,78 and 88. Although we also observed some peaks whose molecular masses corresponded to the peptide containing lysines 99,145 and 166 but their intensities were too low for adequate confirmation. Chymotryptic digest produced peptides containing all seven lysines but this digest was used for the quantitation of the percentage of modification at lysines 145 and 166 only, because in addition to chymotryptic peptides, we also found evidence of some peptides formed from cleavage after lysines 78,88 and 99. Therefore chymotryptic digest was not appropriate for quantitation of percentage of modification at lysine 78,88 and 99 due to unequal production of these peptides in modified and unmodified proteins. Percentage of carbamylation at lysine 99 was determined using endoproteinase ASP-N. In peptic digest several segments were identified as lysine containing peptides for lysines 11, 70,78 and 88, but we studied only those segments which are listed in table 4.4 because the enzymatic digestion gave adequate yield of these peptides and mass spectral responses of these peptides were good.

Unmodified and carbamylated peptides were collected in the same fraction to facilitate the comparison of the relative intensities of their FAB-MS responses. Under HPLC conditions used in present study, only peptide 86-92 and its carbamylated form eluted between 12.6-14.2% (Fig. 3.17) was collected without contamination of coeluting components. Fraction ii containspeptides 64-71 and 85- 92, fraction iii contains peptides 75-84 and 86-93 whereas fraction iv contains peptides 75-85 and 11-22. All these fractions also contained the corresponding carbamylated peptides. Use of a slower gradient produced only a slightly better separation with considerable peak broadening. Although rechromatography could be employed for removing unwanted coeluting peptides, but it was time consuming and also introduced the possibility of errors arising from unequal recovery of modified and unmodified peptides. For quantitative purpose, mixtures of modified and unmodified peptides were examined over several scans to assure that the surface activity of other components in the matrix did not affect the ratio of modified to unmodified peptides during analysis. We observed that the intensities of the peaks changed with different scans, but the ratio of modified to unmodified peptides remained the same. An example is shown in Fig. 4.2.

Different lysyl residues of αA crystallin was found to be carbamylated at different rates. Lysine 11 was the most preferred site of modification whereas Lysine 88 was the least preferred. The percentage of carbamylation at each lysyl residue was increased with the progress of time and a plateau was observed after 24 hours. This might be due to the decomposition of isocyanate rather due to the saturation of binding sites as suggested previously (Cromptom, 1985).

Negative FAB-MS was used to determine the rate of decomposition of isocyanate at different time (Qin et al., 1992). Carbamylation of lysyl residue at different rate probably due to the availability of lysine residue

which in turn depends on the conformation of the αA crystallin in the reaction mixture.

To date no direct information is available concerning the three dimensional structure of α-crystallins. Techniques such as measurement of absorbance, fluorescence, circular dichroism and immunoassay, provide partial information about the conformation of α-crystallins. The intrinsic fluorescence measurement of the carbamylated α-crystallins indicated that the only tryptophan at position 9 in αA crystallin is located in a relatively hydrophobic environment (Beswick and Harding, 1984).

A limited tryptic digestion showed that there are two nicking sites at both C and N termini of αA crystallin (Siezen and Hoenders, 1977). First nick by trypsin occurred at Arg 157 resulting in the production of small peptides that remained attached with the α-crystallin aggregates and could not be removed even after gel filtration and extensive dialysis. These observations suggest that nick occurred at the surface loop consisting of residues 146 to 158 while the other portions at C terminal including lysines 145 and 166 were not exposed. Siezen and Hoenders (1977) also reported that the second nick occurred at Arg 12 thus the N terminus become exposed due to the dissociation of α crystallin aggregate at the point. Our results of FAB MS analysis supported these findings. Lysine 11 was modified most readily and of the two C terminal lysines, lysine 166 was slightly less reactive than lysine 145. This may be due to the presence of two glutamic acid residues nearby lysine 166 which could repel the negatively charged CNO^-. Bovine αA crystallin shows approximately 90% sequence homology with bovine αB crystallin (Siezen, 1981) and it has been claimed that a domain, consisting of residue 72-148 in bovine αB crystallin is responsible for aggregate formation (Ingolia, 1982). Thus a decreased reactivity of lysines 78 and 88 could be due to the presence of these residues in the shielding region (residue, 72- 148).

Similar pattern of the carbamylation of lysyl residues were observed for αA crystallin that had previously been separated by reversed phase HPLC from αB crystallin (Fig. 3.2) and incubated at different time (i.e. 6 hours, 12 hours, 24 hours and 48 hour) with 100 mM KNCO. These results suggested that after HPLC separation the native conformation of αA crystallin is recovered.

4.3.2. Reaction Of α-Crystallin With Aspirin

Incubation of α-crystallin with aspirin for 24 hours showed an increase of 42 mass unit in the molecular masses of lysyl containing peptides. Under denatured state, we observed, serines residues were also acetylated. Acetylation of serine residue has also been reported previously by Roth (1983). We also found signal of minor product which could probably be due to partial acetylation of cystenyl residue, because we used highly acidic conditions during isolation, analyses and storage of peptides. It was possible that acetyl group might have been detached from cystein residue under these conditions.Therefore, we used an on-line continuous FAB-MS for confirmation of partial acetylation at cystein residue of αA crystallin but did not find any sign of acetylation at cysteine residue. The observed signal could be an artifact as this was observed only in one experiment.

4.3.2.1. Quantitative Determination of the Acetylation

The extent of acetylation at each lysyl residue of αA crystallin was determined by incubating α-crystallin with aspirin for different time (6 hrs, 12 hrs, 24 hrs and 48 hrs). Acetylation altered the HPLC profiles of α-crystallins in a similar way as was observed in case of carbamylation but the progress of acetylation was slower as compared to carbamylation and modified peak remained as minor peak even after 48 hours of incubation (Fig. 3.36). Peptiedes listed in table 4.5 were selected for quantitation of percentage of acetylation at lysyl residues because of their high yield and good response to FAB MS.

A mean value of 30% acetylation was measured at lysyl residues of α A crystallin. This value was similar to the decrease in number of ε groups of acetylated crystallins (Rao and Cotlier, 1988), indicating possible involvement of ε group of lysine in the reaction. Aspirin acetylated all the seven lysyl residues of αA crystallin in a similar way as observed in case of acetylation of hemoglobin (Shemsuddin,1974), but it differ from acetylation of other proteins such as human serum albumin and prostaglandin synthetase, where only one residue i.e, serine of prostaglandin synthetase and lysine of human serum albumin were acetylated (Walker,1976; Roth,1983). We observed approximately seven fold range of reactivity from lysine 88, (the least reactive) to lysine 166, (the most reactive). This is because of the accessibility of the lysyl residues which depends on the adjacent residues and the conformation of the α-crystallins in the incubation solution.

Acetylation of lysyl residues follows a pattern similar to the carbamylation. But some lysyl residues showed more affinity for acetylation than carbamylation or vice versa. This can be explained in detailed by comparing the rate constants for acetylation and carbamylation of each lysyl residue of αA crystallin.

We assume, the rate constant of acetylation is of pseudo first order, varying only with the concentration of unmodified lysine. Aspirin concentration would not be expected to change significantly due to acetylation of protein because the concentration of aspirin was much higher than concentration of lysine. Since aspirin was hydrolyzed during incubation. We therefore corrected the extent of acetylation by determining the rate of hydrolysis of aspirin by RP-HPLC to express the concentration of product (acetylated lysine) that would has been formed if decrease in aspirin concentration had not occurred. The rate constants for acetylation of each lysine were calculated using following equation.

$$\ln [\text{unmodified lysine}] = K [100\ \text{mM aspirin}] \times t$$

Rate constants (K) determined from plots of ln [unmodified lysine] against time for each lysine are given in table 3.1 (column ii). Similar approach has been used to calculate the rate constants for carbamylation of each lysine. Rate constants for carbamylation of each lysine residue are given in table 3.1 (column iii). The acetylation rate constants were greater than the carbamylation rate constants for lysines 166 and 70 but less for lysines 145, 11 and 99. The larger rate constants for acetylation VS carbamylation at lysines 166 and 70 may due to the presence of two glutamic acid adjacent to lysine 166 and an aspartic acid adjacent to lysine 70, which could repel the negatively charged NCO^-. Hydrophobic valine residues nearby lysines 78 and 88 may encourage reaction with the more hydrophobic aspirin moiety. Higher rate constants for carbamylation VS acetylation at lysine 145,99 and 11 were observed probably due to some steric hinderance produced by the residues adjacent to these lysines. These include proline, histidine and phenylalanine. An increased rate of acetylation has been observed, when αA crystallin, after separation from αB by RP-HPLC was incubated with aspirin. This suggests that in aggregate form lysine 145 of αA crystallin is surrounded by a moiety such as proline that causes some resistance for aspirin molecule to react with lysine 145. This view is supported by the observation that there is no difference in the rate of carbamylation of lysine 145 in both states i.e. aggregated and non-aggregated forms of αA crystallins.

The extent of modifications were also calculated from concurrent and sequential incubations of α-crystallins with isocyanate and aspirin. HPLC patterns of modified crude α-crystallin were identical for both incubations i.e. concurrent and sequential. Similarly no major difference has been observed in the separation profiles of peptides generated

from enzymatic digestions of modified α-crystallin after concurrent and sequential incubations. Both incubations have modified all lysyl residues but with lesser extent as compared to the extent that has been measured while α- crystallin was incubated either with isocyanate or aspirin alone.

The similarity in the rate constants for acetylation and carbamylation of several lysyl residues of αA crystallin and the reduction in the extent of modifications after concurrent and sequential incubations support the in vitro studies of Crompton et al (1985) in which they concluded that aspirin and isocyanate were competing for the same sites

The similarity in the rate constants raises question about the potential effectiveness of aspirin in inhibiting the carbamylation in vivo.

In vivo, ratio of acetylated lysine to the carbamylated lysine will depend not only on the rate constants but also on the concentration of aspirin and NCO^- in the lens. Physiological concentrations of isocyanate and aspirin can be predicted. The concentration of urea in the lens has been reported about to be 60% of the plasma concentration (Paterson and Green, 1979). Reed (1972) have estimated the concentration of urea in the plasma about 20 mM. For this value the concentration of urea in the lens should be about 12 mM. In lens, at equilibrium 0.8% of the urea (plasma urea) exists as NCO^- (Dirnhuber and Schutz, 1948) hence the concentration of NCO^- in normal lens would be about 0.17 mM. It is known that the level of urea in renal failure patient is elevated so the concentration of NCO^- would be much higher than the calculated value. The concentration of un-hydrolyzed aspirin in the lens can also be calculated for comparison with concentration of NCO^-. It has been reported that 30 minutes after ingestion of 1300 mg (4 tablets) of aspirin, 0.05 mM remained as un-hydrolyzed aspirin in human plasma (O'Kruk., et al 1948). Experiments on rabbit lens have shown that the concentration of aspirin in the lens is about one tenth of

the plasma concentration (Valerie et al, 1988). If this distribution is similar for human, then a dose of 1300 mg aspirin will provide 0.005 mM un-hydrolyzed aspirin inside the lens after 30 minutes of ingestion. This value is less then 1/30 of the concentration of NCO^- in a uremic patients.

We can therefore conclude, that concentration of aspirin in the lens as well as the rate constants for acetylation are not sufficient to inhibit the carbamylation of lysine residues in uremic patients.

4.4 CONCLUSIONS

The present study leads to the following conclusions.

1) Isocyanate carbamylates all the lysyl residues of αA crystallin.

2) The extent of carbamylation vary from one lysyl residue to another and lysine 11 was found to be most reactive site whereas lysine 88 was least reactive.

3) Acetylated lysyl residues were the principal products, formed after the reaction of α-crystallin with aspirin.

4) Approximately five folds of range of reactivity from lysine 88 (the least reactive) to lysine 166 (the most reactive) was measured.

5) Concurrent and sequential incubations of α-crystallin with isocyanate and aspirin have reduced the extent of carbamylation and acetylation of lysyl residues.

6) Similarity in the rate constants for acetylation and carbamylation of the lysyl residues (except for lysine 166 and 11) of α-crystallin and decreased in the extent of modifications of lysyl residues of α-crystallin after concurrent and sequential incubations support the notion that "aspirin and isocyanate compete for the same residue".

7) Comparison of the yield of acetylated α-crystallin with the yield of carbamylated α-crystallin that might occure due to renal failure indicates that aspirin is not an effective inhibitor of cataract due to carbamylation of lysyl residues.

Table 4.1 List of all possible peptides generated by digestion of αA2 crystallin with pepsin.

Coordinates	Sequence	Weight	Hydrophobicity
(1-10)	MDIAIQHPWF	1256.59	-357
(11-14)	KRTL	516.34	-53
(11-14)	KRTL	516.34	-53
(15-17)	GPF	319.15	-293
(18-18)	Y	181.07	-1430
(19-22)	PSRL	471.28	-178
(23-23)	F	165.08	-1520
(24-26)	DQF	408.16	20
(27-27)	F	165.08	-1520
(28-29)	GE	204.07	660
(30-31)	GL	188.12	-420
(32-32)	F	165.08	-1520
(33-33)	E	147.05	510
(34-34)	Y	181.07	-1430
(35-36)	DL	246.12	-520
(37-37)	L	131.09	-1650
(38-39)	PF	262.13	-845
(40-40)	L	131.09	-1650
(41-47)	SSTISPY	753.35	-214
(48-48)	Y	181.07	-1430
(49-52)	RQSL	502.28	108
(53-53)	F	165.08	-1520
(54-57)	RTVL	487.31	-355
(58-63)	DSGISE	6026.24	220
(64-71)	VRSDRDKF	1021.52	151
(72-74)	VIF	337.23	-1240
(75-75)	L	131.09	-1650
(76-80)	DVHHF	644.32	-102
(81-83)	SPE	331.14	253
(86-91)	TVKVQE	702.39	122
(92-93)	DF	280.11	-455
(94-95)	V	3 246.12	-120
(96-101)	IHGKHNE	833.41	371
(103-109)	RQDDHGY	889.36	421
(110-113)	ISRE	503.27	43
(114-114)	F	165.08	-1520
(115-118)	HRRY	630.33	160
(119-120)	RL	287.19	-480
(121-129)	PSNVDQSAL	929.44	150

(130-133)	SCSL	408.17	-113
(134-139)	SADGML	592.25	23
(140-143)	TF	266.3	-615
(142-156)	HEPKIPSQVDAGHSE	1436.68	269
(157-164)	RAIPVSRE	926.5	2 69
(165-165)	E	147.05	510
(166-173)	KPSSAPSS	759.37	301

Cleavage points, C-terminal to: L,E,F, Y

Table 4.2 List of all possible peptides generated by digestion of αA2 crystallin with chymotrypsin.

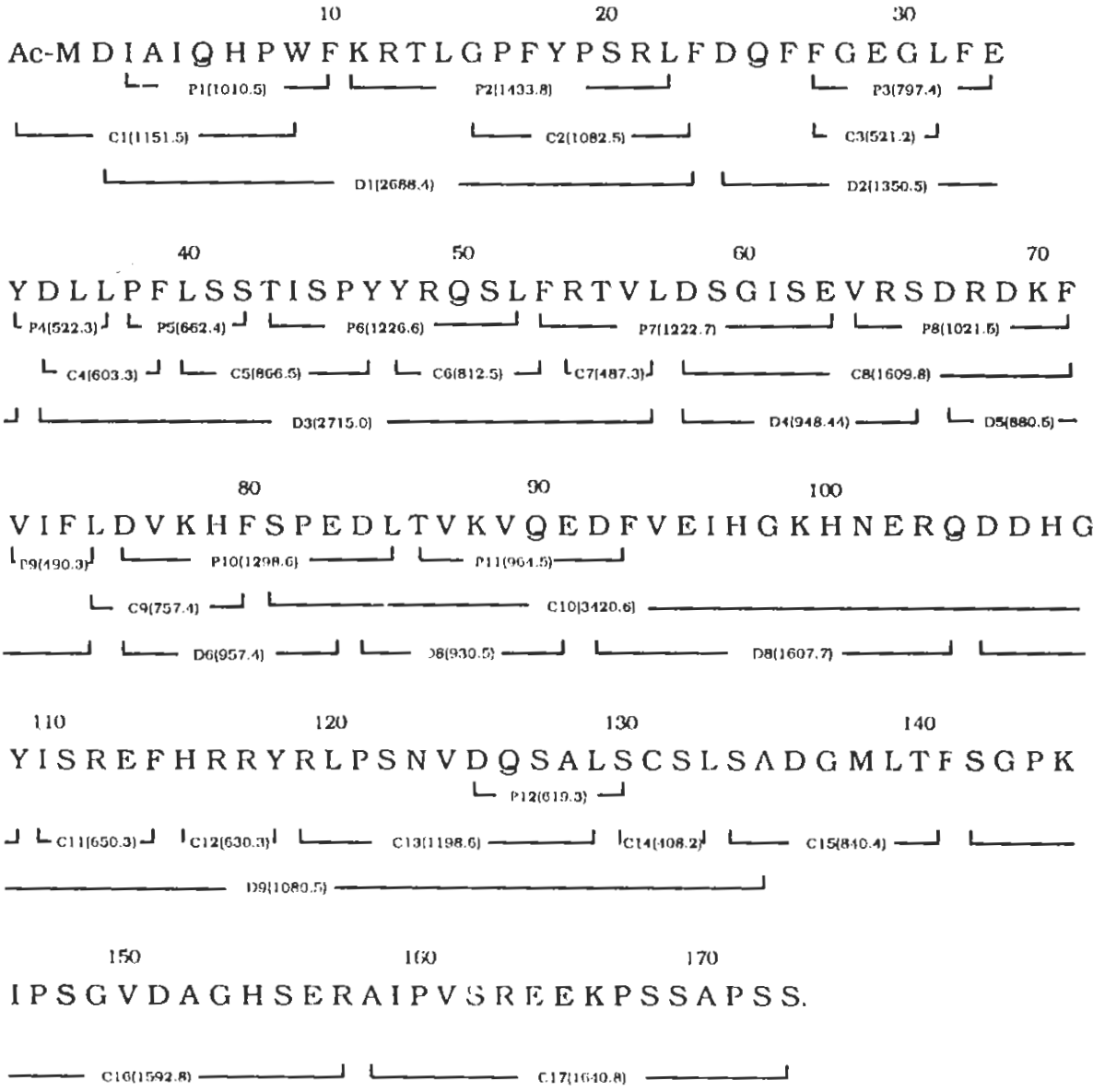
Coordinates	Sequence	Weight	Hydrophobicity
(1-9)	MDIAIQHPW	1109.53	-228
(10-10)	F	165.08	-1520
(11-14)	KRTL	516.34	-53
(15-17)	GPF	319.15	-293
(18-18)	Y	181.07	-1430
(19-22)	PSRL	471.28	-178
(23-23)	F	165.08	-1520
(24-26)	DQF	408.16	20
(27-27)	F	165.08	-1520
(28-31)	GEGL	374.18	120
(32-32)	F	165.08	-1520
(33-34)	EL	310.12	-460
(35-36)	DL	246.12	-520
(37-37)	L	131.09	-1650
(39-39)	PF	262.13	-845
(40-40)	L	131.09	-1650
(41-47)	SSTISPY	753.35	-214
(48-48)	Y	181.07	-1430
(49-52)	RQSL	502.28	108
(53-53)	F	165.08	-1520
(54-57)	RTVL	487.31	-355
(58-71)	DSGISEVRSDRDKF	1609.76	181
(72-74)	VIF	377.23	-1240
(75-75)	L	131.09	-1650
(76-80)	DVKHF	644.32	-102
(81-85)	SPEDL	559.25	-56
(86-93)	TVKVQEDF	964.48	-23
(94-109)	VEIHGKHNERQDDHGY	1932.87	332
(110-114)	ISREF	650.34	-270
(115-118)	HRRY	630.33	160
(119-120)	RL	287.19	-480
(121-129)	PSNVDQSAL	929.44	150
(132-133)	SCSL	408.17	-113
(134-139)	SADGNL	592.25	23
(140-141)	TF	266.13	-615
(142-157)	SGPKIPSGVDAGHSER	1593.7	234
(158-173)	AIPVSREEKPSSAPSS	1640.7	234

Cleavage points, C-terminal to: L,F,W,Y

Table 4.3 List of all possible peptides generated by digestion of αA2 crystallin with endoproteinase ASP-N.

Coordinates	Sequence	Weight	Hydrophobicity
(1-1)	M	166.97	-25
(2-23)	DIAIQHPWFKRTLGPFYPSRLF	2785.03	-348
(24-34)	QFFGESGLFEY	1252.97	-311
(35-57)	DLLPFLSSTISPYYRQSLFRTVL	2715.44	-499
(58-66)	DSGISEVRS	948.44	187
(67-68)	DR	289.14	650
(69-75)	DKFVIFL	880.50	-831
(76-83)	DVKHFSPE	957.45	31
(84-91)	DLTVKVQE	930.50	-39
(92-104)	DFVEIHGKHNERQ	1607.70	239
(105-105)	D	133.08	610
(106-124)	DHGYISREFHRRYRLPSNV	2401.20	-32
(125-135)	DQSALSCSLLSA	1080.47	140
(136-150)	DGMLTFSGPKIPSGV	1504.75	-116
(151-173)	DAGHSERAIPVSREEKPSSAPSS	2392.15	296

Cleavage point, N-terminal to: D



P denotes peptides found in peptic digest.
C denotes peptides found in chymotryptic digest.
D denotes peptides found in endoproteinase ASP-N digest.

Figure 4.1: Sequence of α A_2 crystallin.

Table 4.4 List of Lysine Containing Peptides found in Enzymatic Digestions used for the Quantitation of percentage of Carbamylation.

Fragment	Sequence	m/z[a]	m/z[b]	Lys No.
11-22	KRTLGPFYPSRL[p]	1434.5	1477.5	11
64-71	VRSDRDKF[p]	1022.3	1065.3	70
75-84	LDVKHFSPED[p]	1186.5	1229.3	78
86-92	TVKVQED[p]	818.3	861.3	88
92-104	DFVEIHGKHNERQ[d]	1608.5	1651.5	99
142-157	SGPKIPSGVDAGHSER[c]	1593.7	1536.7	145
158-173	AIPVSREEKPSSAPSS[c]	1641.8	1684.8	166

a) before acetylation
b) after acetylation
c) chymotryptic peptides
d) ASP-N peptides
p) Peptic peptides.

Table 4.5 List of Lysine Containing Peptides found in Enzymatic Digestions used for the Quantitation of percentage of Acetylation.

Fragment	Sequence	m/z[a]	m/z[b]	Lys No.
11-22	KRTLGPFYPSRL[p]	1434.5	1476.5	11
64-71	VRSDRDKF[p]	1022.3	1064.3	70
75-84	LDVKHFSPED[p]	1186.5	1228.3	78
86-92	TVKVQED[p]	818.3	860.3	88
92-104	DFVEIHGKHNERQ[d]	1608.5	1650.5	99
142-157	SGPKIPSGVDAGHSER[c]	1593.7	1535.7	145
158-173	AIPVSREEKPSSAPSS[c]	1641.8	1683.8	166

a) before acetylation
b) after acetylation
c) chymotryptic peptides
d) ASP-N peptides
p) Peptic peptides.

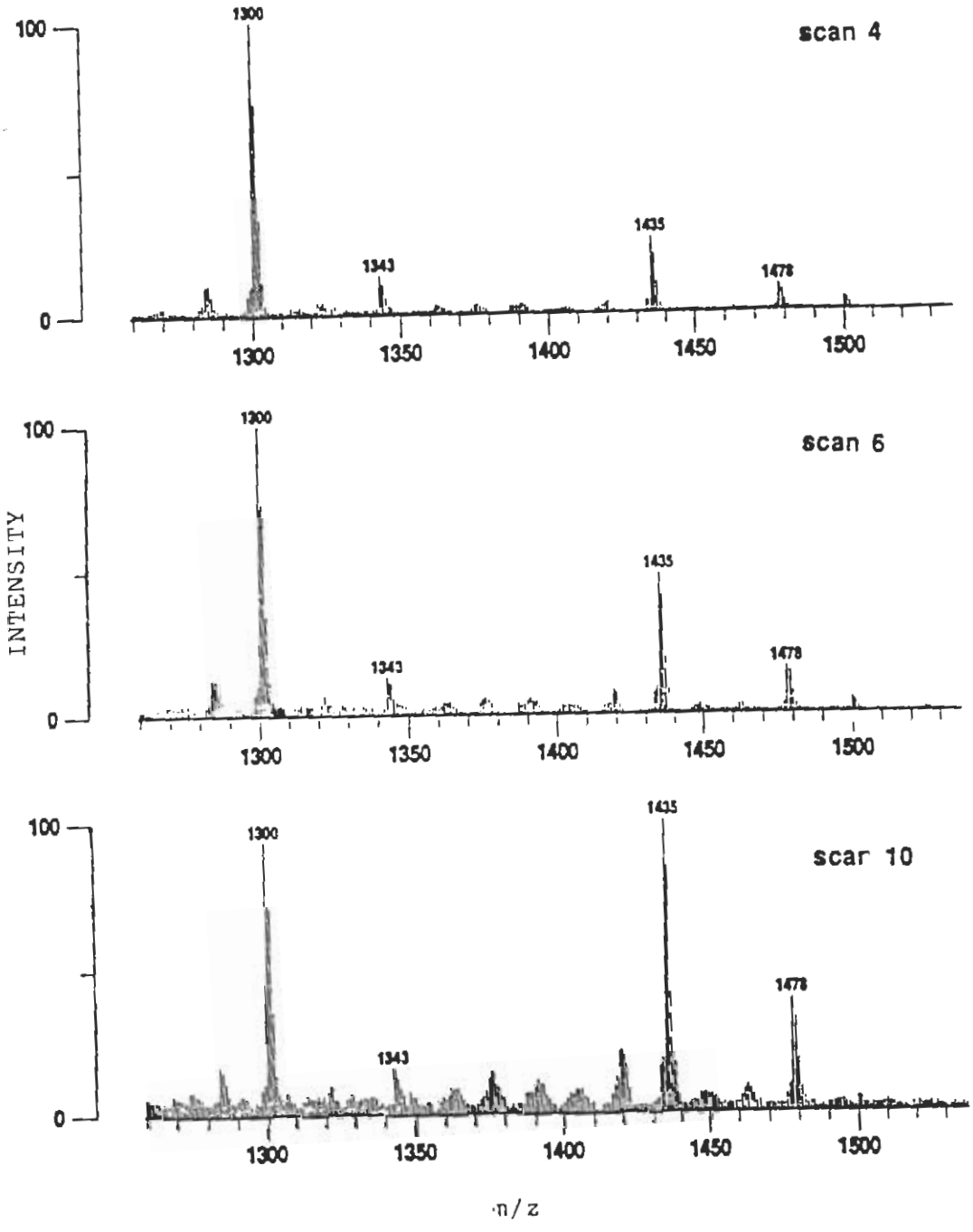


Fig. 4.2 FAB mass spectra, showing ratio of modified to unmodified peptides in different scans.

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