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Post translational modification of crystallins isolated from human lenses

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Abstract

Crystallins, the structural proteins of the lens, have been isolated from human lenses using a combination of gel filtration and reversed phase high performance liquid chromatography (HPLC). The molecular weights of the isolated crystallins have been determined by electrospray ionization mass spectrometry. The isolated crystallins have also been proteolytically digested into peptides, the peptides fractionated by HPLC, and the masses of the peptides determined by fast atom bombardment mass spectrometry. With these techniques, it is possible to confirm and/or correct known protein sequences and identify and locate post translational modifications.

INTRODUCTION

Cataract, which can be defined as an opacity of the eye lens, is the leading cause of blindness worldwide. In countries where surgery to remove a cataractous lens and replace it with a synthetic lens is readily available, cataract may not be a serious impairment; however, in much of the world this surgery is not available, and the development of cataract leads to blindness. The two types of cataract, cortical and nuclear, both involve changes in the lens proteins, called the crystallins. For the lens to be transparent, the crystallins must be tightly and uniformly packed producing a lens with a uniform refractive index. In cortical cataract, the density of the crystallins is altered, causing a non-uniform density with variations in the lens refractive index and consequent light scattering (ref. 1). Nuclear cataract is associated with the formation of insoluble protein aggregates. In both cases, there are modifications to the lens crystallins that either prevent their normal close packing in the cortex or cause their aggregation in the nucleus. The focus of our research is to determine which proteins are modified, where the modifications are located, and the identity of the modifications.

Cataract is most commonly associated with old age. Most people over age 80 have at least early indications of cataract. Cataract occurs earlier in people with diabetes, renal failure, chronic diarrhea or who have experienced prolonged exposure to UV radiation. The mechanisms that have been proposed for the development of cataract vary, depending on the disease with which it is associated. Cataract associated with old age, diabetes, renal failure and chronic diarrhea are all proposed to occur because of modifications to the lysyl residues of the crystallins. In old age, the concentration of glutathione in the lens is lower. This permits an increase in the concentration of dehydroascorbic acid, which is normally reduced by glutathione in younger lenses. Dehydroascorbic acid can react with the amino groups of the lysyl residues, forming a Schiff base which can then rearrange or possibly form cross-links (ref. 2). In diabetes, the elevated concentrations of glucose may lead to formation of a similar Schiff base between the lysyl residues and glucose (ref. 3). Both renal failure and chronic diarrhea are associated with elevated urea, which forms an equilibrium with isocyanate. Isocyanate can also react with lysyl residues forming a carbamylated protein (refs. 4, 5). A different mechanism, oxidation of the tryptophan residues to form kynurenine, has been proposed to explain the development of cataract due to exposure to UV radiation (ref. 6).

RESULTS AND DISCUSSION

Figure 1 is a diagram of our procedure for isolating the lens proteins. The lens is homogenized in a buffer for 1 hour and separated into the water soluble and water insoluble portions. The water soluble portion is further separated by gel filtration (Sephadex G-200) into the α-, β- and γ-crystallins. Reversed phase HPLC further fractionates each crystallin subgroup. Guanidine hydrochloride

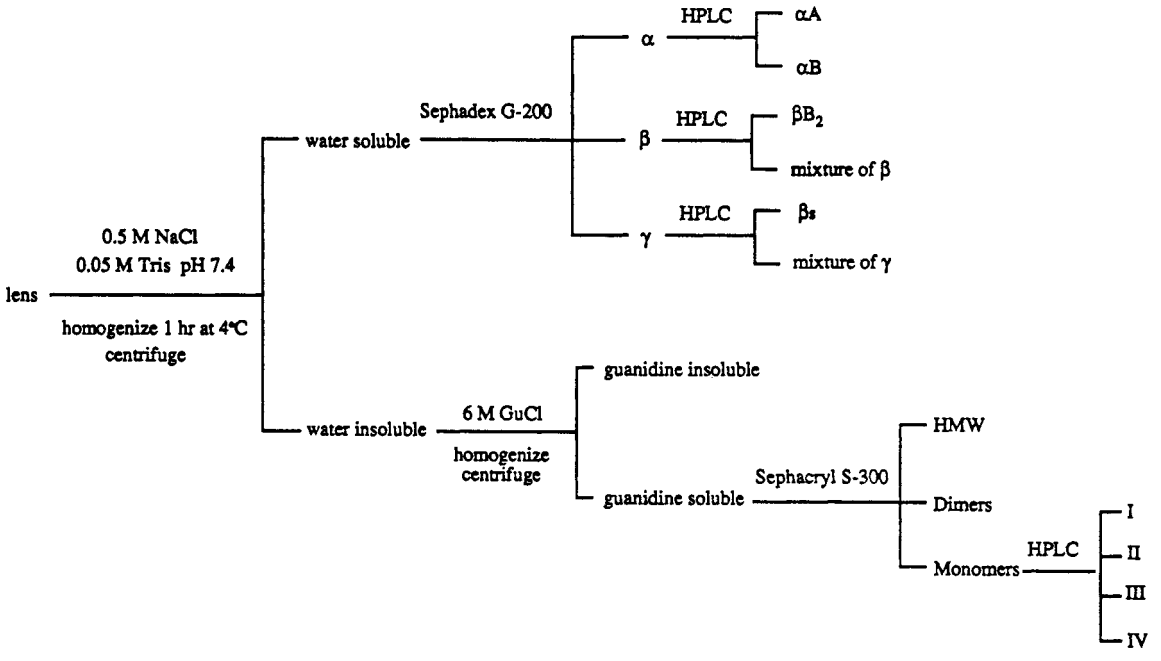

Fig 1. Diagram of the procedure for isolating lens crystallins.

added to the water insoluble portion solubilizes this portion which is then separated into high molecular weight proteins, dimers and monomers by Sephacryl S-300. Again, HPLC is used for further fractionation. Our examination of the isolated proteins includes analysis by two types of mass spectrometry, electrospray ionization mass spectrometry (ESIMS) and fast atom bombardment mass spectrometry (FABMS). The combined use of these techniques to elucidate the primary structure of a protein will be illustrated by our analysis of human αB-crystallin. With ESIMS, it is possible to obtain the molecular weight of the intact protein with an accuracy of 0.01%; i.e., usually to within 2 mu for the monomeric lens crystallins which have molecular weights about 20,000. The sample introduction system of ESIMS causes the protein to become multiply charged, thus making the mass to charge ratio of the whole protein within the range that the mass spectrometer can measure. To calculate the molecular weight of the protein, the m/z from ESIMS is multiplied by the number of charges on the protein. This is referred to as the reconstituted spectrum. Molecular weights of several of the isolated crystallins have been determined by ESIMS. The reconstituted spectrum for αB of the water soluble crystallins is shown in Fig. 2. There is one predominant peak and several weaker ones. The expected molecular weight from the sequence (ref. 7) including acetylation at the N-terminus is 20,201, which is very close to the molecular weight of the principal peak. Assignment of possible identities for some of the other molecular weights are given in Table 1.

Table 1 Assignment of molecular weights for human αB-crystallins from ESIMS data.

ESIMS Molecular Weight	Possible Identity
20,200	αB (1-175)
20,210	
20,070	αB (1-174)
20,280	αB + PO_4
20,130	

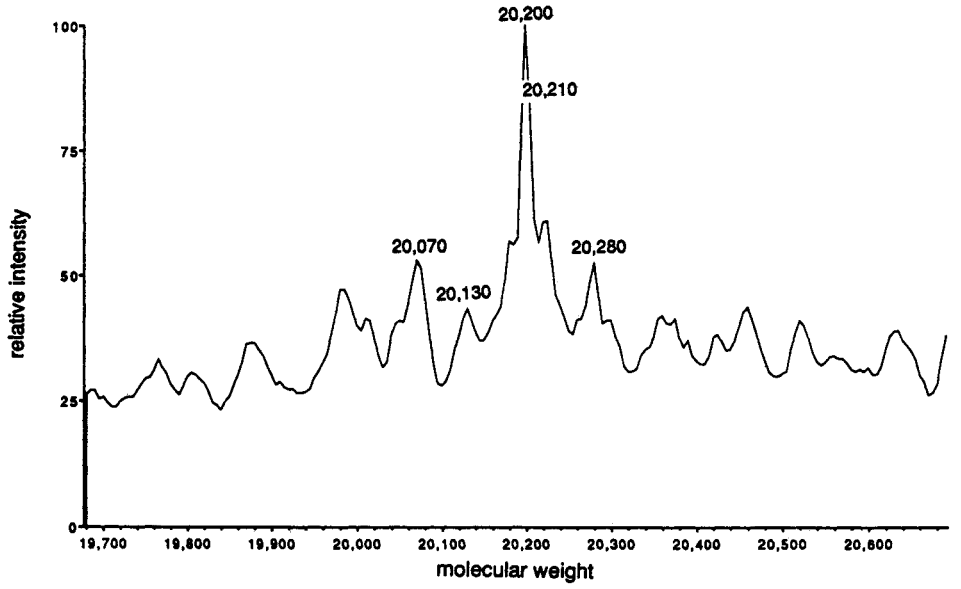

Fig. 2 Electrospray ionization mass spectrum of human αB-crystallins.

The protein with a molecular weight of 20,280 indicates the presence of a post-translational phosphorylation, a modification proposed for bovine αB-crystallin by Chiesa et al. (ref. 8) and Voorter et al.(ref. 9). The molecular weight of 20,070 is close to the weight of αB-crystallin minus the C-terminal Lys, similar to the loss of the C-terminal Ser reported for the bovine αA-crystallin (ref. 10). The other two molecular weights do not fit previously reported modifications.

To locate a site of modification, the protein is proteolytically digested into peptides, the peptides are fractionated by reversed phase HPLC, and the molecular weights of the peptides are determined by FABMS. These molecular weights can be compared with the expected molecular weights calculated from the known protein sequence taking into consideration the specificity of the enzyme. Our laboratory has computer programs which assist in these calculations. Enzymes commonly used for digestion include trypsin, Asp-N, Glu-C, chymotrypsin and pepsin. For example, trypsin is expected to cleave C-terminal to Lys and Arg except before Pro. The HPLC chromatogram of a tryptic digest is shown in Fig. 3 with some of the fraction numbers indicated.

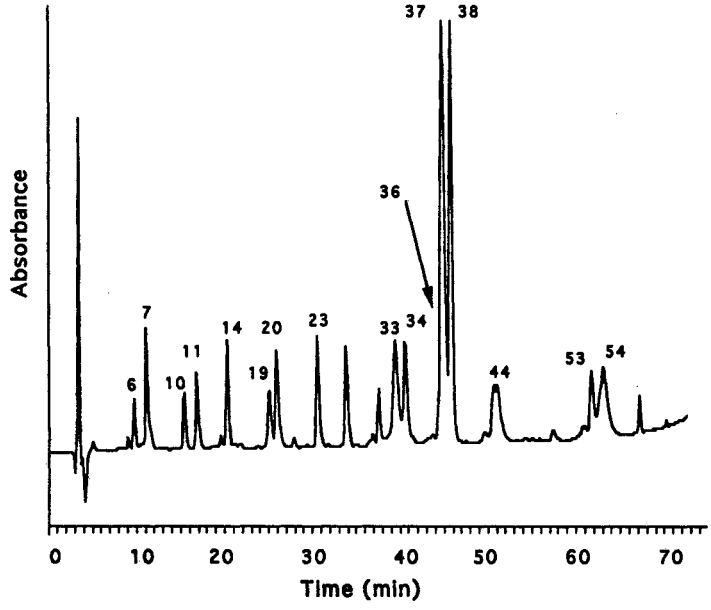

Fig. 3 Chromatogram of an HPLC fractionation of a tryptic digest of αB-crystallin.

The peptides expected in this tryptic digest are compared with the masses found in Table 2. Also the fraction numbers in which they were found are listed in the last column. Note that the masses found are one mu higher than the mass of the peptide because the protonated form is seen in FABMS. A fraction may contain two or more peptides.

In addition to the peptides listed in Table 2, peptides with MH + of 1454, 4084, and 1576 were also found, 80 mu higher than the expected masses for peptides 12-22, 23-56, and 57-59, respectively. These peptides indicate a likely phosphorylation in each of these peptides. The mass spectrum showing peptide 12-22 and its phosphorylated counterpart is given in Fig. 4. The other two phosphorylated peptides eluted in a fraction just prior to their unphosphorylated peptides.

If the identity of a peptide is ambiguous, it can be further digested with another enzyme and the products again analyzed by FABMS. For example, another peak in Fig. 4, m/z 1148, fits peptide 48-56, which could occur only if there had been a cut after Phe 47, a cleavage not expected, but possible, in a tryptic digest. The other peptide that would be produced by cleavage at Phe 47, peptide 23-47 (MW 2873) was in fraction 55 of the HPLC chromatogram (Fig. 3). The FAB mass spectrum of this peptide is shown in Fig. 5A. To confirm the identity of this peptide, it was treated with carboxypeptidase which removes residues from the C-terminus (Fig. 5B) and with pepsin which cleaves rather non-specifically (Fig. 5C). The carboxypeptidase digestion shows peptides corresponding to removal of 10 residues from the C-terminus, clearly confirming its identity. The peptic digest is equally satisfactory for confirmation with the production of 5 peptides that fit parts of the sequence of peptide 23-47. In this case both methods of confirmation were conclusive. Because any one method does not always yield useful information, the protein chemist needs several confirmatory methods at his disposal.

Table 2 Peptides in a tryptic digest of human αB-crystallin

Co-ordinates	Sequence	Expected MW	Found MH^+	Fractin #
1-11	CH3CO-MDIAIHHPWIR	1429	1430	37
12-22	RPFFPFHSPSR	1373	1374	31
23-56	LFDQFFGEHLLES DLFPTSTSLSPFYLR PPSFLR	4003	4004	54
57-69	APSWFDTGLSEMR	1495	1496	38
70-72	LEK	388	389	6
			660	7
73-74	DR	289		
			1191	28
75-82	FSVNLDVK	920	921	29
			1888	35
83-90	HFSPEELK	985	986	23
92-92	VK	245		
93-103	VLGDVIEVHGK	1164	1165	26
104-107	HEER	569		
108-116	QDEHGFISR	1087	1088	20
117-120	EFHR	587	588	11
121	K	146		
122-123	YR	337		44
			2943	
124-149	IPADVDPLTITSSLSS DGVLTVNGPR	2623	2624	43
150	K	146		10
			900	
151-157	QVSGPER	771	772	10
158-163	TIPITR	699	700	19
164-174	EEKPAVTAAPK	1139	1140	14
			1268	
175	K	146		13,

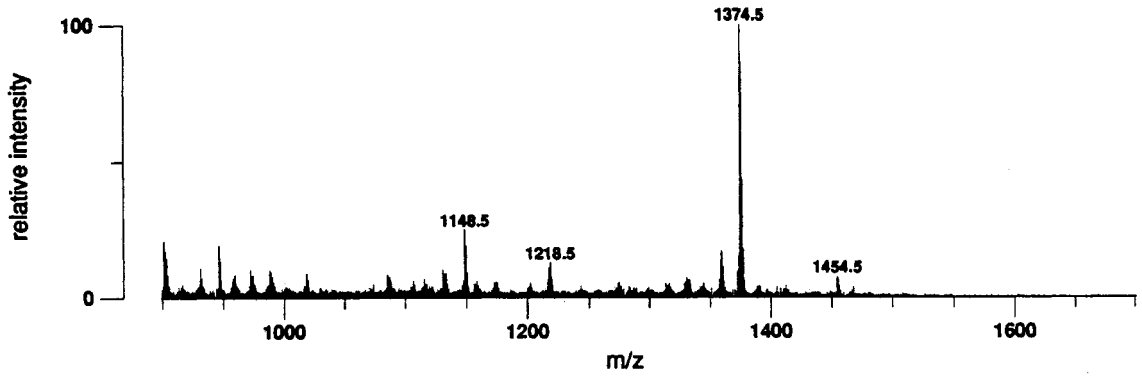

Fig. 4 FAB mass spectrum showing peptide 12-22 of αB-crystallin (m/z 1374) and its phosphorylated counterpart (m/z 1454).

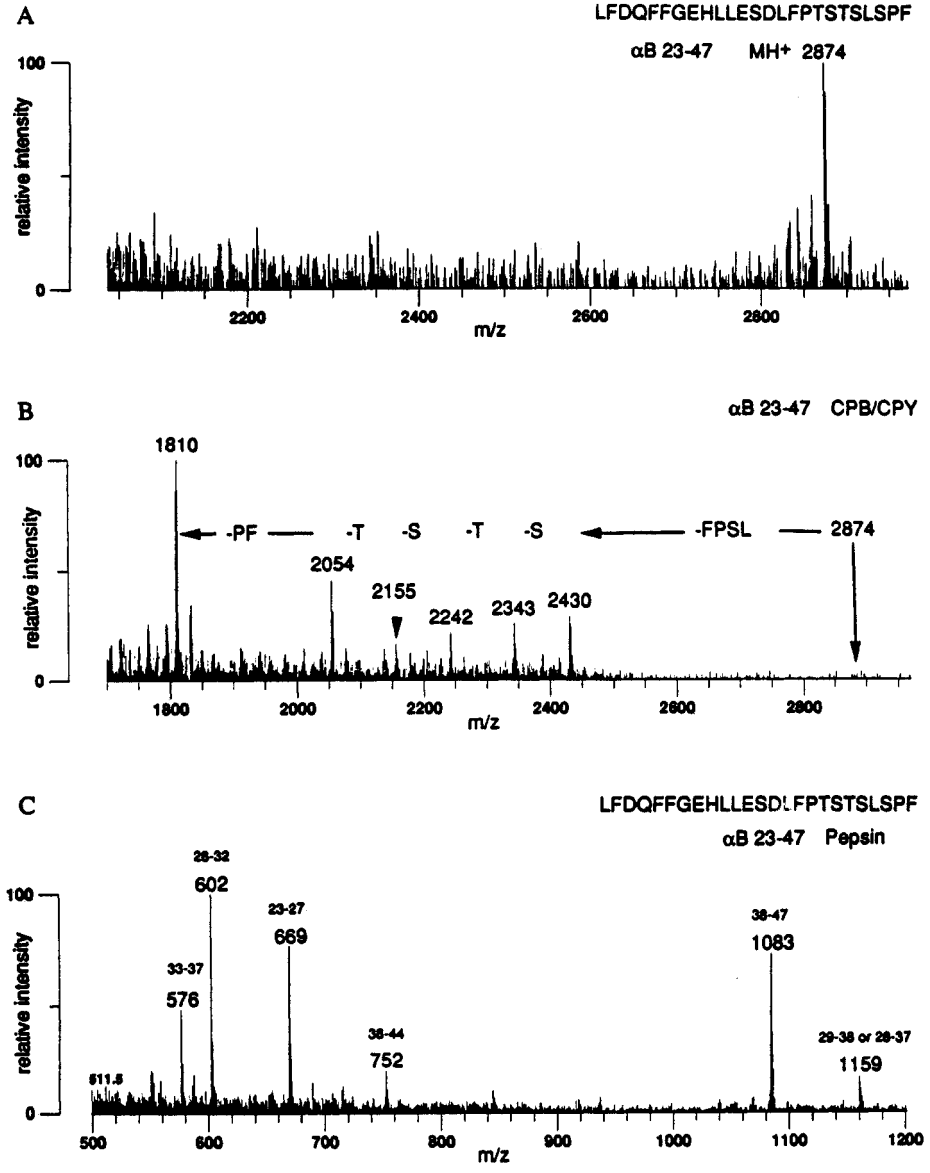

Fig. 5 FAB mass spectra showing (A) peptide 23-47 of αB-crystallin in a tryptic digest, (B) the same peptide after further digestion with carboxypeptidase and (C) the same peptide after further digestion with pepsin.

The procedure described here for determining the primary structure of αB-crystallin, i.e; a combination of ESIMS analysis to obtain the mass of the intact protein and FABMS of peptides formed in enzymatic digests to locate modifications to the protein, has facilitated our examination of the crystallins of the human lens. At present, we have confirmed and/or corrected the sequences of the αA-, αB-, βB2-crystallins (ref. 11) and parts of βs and the γ-crystallins.

Acknowledgements

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