α -Crystallin

THE ISOLATION AND CHARACTERIZATION OF DISTINCT MACROMOLECULAR FRACTIONS

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α -Crystallin was isolated from calf lens periphery by chromatography on DEAE-cellulose and gel filtration. Three distinct populations of macromolecules have been isolated with molecular weights in the ranges approx. 6×10^5 – 9×10^5 , 0.9×10^6 – 4×10^6 and greater than 10×10^6 . The concentration of macromolecules at the molecular-weight limits of a population are very low. The members of the different populations do not appear to be in equilibrium with each other. Further, in those molecular-weight fractions investigated, no equilibrium between members of the same population was observed. The population of lowest molecular weight comprises 65–75% of the total material. The amino acid and subunit composition of the different-sized fractions appear very similar, if not identical. The only chemical difference observed between the fractions is the presence of significant amounts of sugar in the higher-molecular-weight fractions. Subunit molecular weights of approx. 19.5×10^3 and 22.5×10^3 were observed for all α -crystallin fractions.

The lens of the eye contains a higher concentration of protein than most tissues, varying from approx. 30% in the peripheral region to more than 70% in the central area (Philipson, 1969). Almost all of this protein is composed of the structural macromolecules α -, β - and γ -crystallin with the α -crystallin fraction representing approx. 20–25% of the total (Spector, 1964). α -Crystallin has been studied by a number of laboratories and an unusually wide spectrum of molecular weights has been reported (Resnik, 1957; Bloemendal, Bont, Jongkind & Wisse, 1962; Björk, 1963; Spector & Katz, 1965). These observations clearly suggested that the preparations of α -crystallin under investigation were physically heterogeneous. By utilizing sedimentation-equilibrium techniques, this assumption was confirmed (Spector & Katz, 1965). Such results are not too surprising since it has been known for some time that α -crystallin is composed of subunits with mol. wt. approx. 2×10^4 held together by non-covalent forces (Spector & Katz, 1965; Bont, Jongkind, Wisse & Bloemendal, 1962). It has therefore been assumed that there are a number of stable aggregate states containing a variable number of subunits. Since no concentration dependence was observed, the aggregates were thought to be independent of each other

and not in equilibrium. However, no discrete populations of α -crystallin have previously been isolated.

Recently, interest in the question of the size distribution of α -crystallin was renewed by the observation that newly synthesized α -crystallin isolated from calf lenses appeared physically homogeneous (Spector, Wandel & Li, 1968). In the present paper we show that α -crystallin isolated from calf lenses is composed of distinct populations of macromolecules with similar chemical compositions.

A preliminary report of some of this work was presented by Spector (1970).

MATERIALS AND METHODS

Fresh calf lenses (average weight 1.15g) were cored with a no. 3 cork borer. The periphery was homogenized in an equal weight of cold water and then the homogenate was clarified by centrifugation for 10 min at 27600g at 4°C. All subsequent operations were performed at 4°C. The supernatant was dialysed for 18 h against 100 vol. excess of 2mM-phosphate buffer and was either used immediately or frozen. All phosphate buffers were prepared from 0.50M-phosphate (NaH_2PO_4 – K_2HPO_4 , pH 6.80). The soluble lens proteins obtained in this

manner from about 170 lenses were fractionated on a column (9.0 cm × 155 cm) of DEAE-cellulose equilibrated with 2 mM-phosphate buffer. Fractions (250 ml) were collected at a flow rate of 37.5 ml/min (maintained with N₂ at 5 lb/in²) and the fractions were assayed by measuring E_{280} . Elution of the protein was accomplished by stepwise increase in the concentration of phosphate, by using 0.002 M-, 0.01 M-, 0.03 M-, 0.05 M- and 0.4 M-phosphate buffers. Pooled peaks were dialysed against 15–20 vol. of water with four changes over a 27 h period and were then freeze-dried.

Approx. 4 g of the α -crystallin fraction eluted from the DEAE-cellulose column by 0.4 M-phosphate was dissolved in 50 ml of 0.01 M-tris buffer, pH 7.8, containing 0.1 M-KCl and 0.1 mM-EDTA and applied to an Agarose column (9 cm × 155 cm) of Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). In some experiments smaller columns of Bio-Gel A-5m as well as of Bio-Gel A-15m were used. Fractions (20 ml) were collected at a flow rate of 4.1 ml/min. The fractions were examined by measuring their E_{280} and the peaks were pooled, dialysed and freeze-dried as described above. Pooled peaks from several experiments were rechromatographed on the same column to eliminate cross-contamination. The α -crystallin fractions were then dialysed and freeze-dried.

α -Crystallin samples of approx. 3 mg were hydrolysed with 6 M-HCl at 110°C for 20, 40 and 70 h after sealing under vacuum (0.05 mmHg or less). Amino acid analyses were performed in duplicate on a Beckman model 121 Amino Acid Analyser or on a Technicon AutoAnalyzer by the method of Spackman, Stein & Moore (1958). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967) and by oxidation with *N*-bromosuccinimide (Spande & Witkop, 1967). Cysteine was determined as carboxymethylcysteine after reaction of the protein with iodoacetic acid by a modification of the method of Crestfield, Moore & Stein (1963).

N-Terminal residues were determined by the dansylation method of Gray & Hartley (1963). DNS-amino acids were identified by t.l.c. (Morse & Horecker, 1966). Sugar was determined as described by Dische & Danilichenko (1967). Gel electrophoresis was performed in sodium dodecyl sulphate by the method of Shapiro, Vinuela & Maizel (1967) as modified by Weber & Osborn (1969).

Equilibrium centrifugation was conducted in a filled Epon double-sector cell equipped with sapphire windows. A Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics was used. The temperature was maintained at 4°C. The high-speed equilibrium method of Yphantis (1964) and a modification of LaBar's (1965) technique applied to low-speed equilibrium was used (Li, 1971). In the latter method C_0 , the initial concentration of the solution, is determined after overspeeding by the expression:

$$C_0 = \int_{r_m}^{r_b} f_r dr^2 / (r_b^2 - r_m^2)$$

where r = distance from the centre of rotation, f_r = fringe displacement where $f_m = 0$, b = base of the solution column and m = meniscus of the solution column. An An-J rotor was used for all experiments. Photographs were taken with Kodak spectrographic plates and

were examined with a Nikon Shadowgraph model 6C. A partial specific volume of 0.73 was determined from the amino acid composition (McMeeekin & Marshall, 1952). Samples were dialysed against the appropriate buffer before analyses. A small fraction of the peak 1 material isolated from Bio-Gel A-5m columns was insoluble and was therefore removed by centrifugation before sedimentation-equilibrium analyses.

RESULTS

A method has now been developed that will allow fractionation of as much as 11 g of α -crystallin in a 24 h period. A typical elution profile obtained when approx. 41 g of calf lens periphery was fractionated on DEAE-cellulose is shown in Fig. 1. The bars under the curves indicate the section of each peak that was recovered for further analysis. The use of a stepwise elution procedure resulted in a clean separation of the different fractions. γ -Crystallin has previously been shown to be the first material eluted from the column, followed by the β -crystallins and finally, with 0.4 M-phosphate buffer, α -crystallin. Investigation of these fractions gave results similar to that formerly found with much smaller columns (Spector, 1964). The results obtained are summarized in Table 1. Essentially all of the protein applied was recovered. The quantities of protein isolated in the different peaks fall within the limits previously expected for the relative amounts of the crystallins (Spector, 1964). Peak 3 is eluted from the column with 0.03 M-phosphate buffer. However, in these experiments elution with 0.05 M-phosphate was started before peak 3 came through the column. Approx. 25% was regularly recovered as α -crystallin.

Since preparations of α -crystallin frequently contain small amounts of lower-molecular-weight impurities, the fraction eluted with 0.4 M-phosphate buffer was further purified on a Bio-Gel A-5m Agarose column. A profile similar to that shown in Fig. 2 was obtained when 3–4 g of α -crystallin was chromatographed on the column. Two major peaks representing the bulk of the material were isolated. The lower-molecular-weight components account for approx. 6% of the material applied to the column. Their amino acid compositions indicate that they are truly contaminants and not α -crystallin components. The results of a number of runs indicate that peak 1 material accounted for 12–16% of the initial load and peak 2 material for 50–57%. Between 77 and 92% of the protein was recovered from the column. The isolated peak 1 and peak 2 materials were re-run to remove the overlapping contamination that resulted from the close proximity of the peaks. When purified protein from peak 1 or peak 2 was re-run on the Agarose column, each gave a single

Fig. 1. Stepwise elution of soluble lens proteins from a column (9cm $\times$ 155cm) of DEAE-cellulose. The bars indicate the fraction of each peak pooled for further analyses. Concentrations of phosphate buffer prepared by dilution of 0.50M-phosphate buffer, pH 6.80, are marked on the figure by arrows.

Table 1. Summary of DEAE-cellulose chromatography of crystallins

Soluble lens protein was fractionated on DEAE-cellulose as indicated in the text. The buffers were prepared from 0.50M-phosphate, pH 6.80.

Peak	Concn. of elution buffer	E_{280} units in peak	Recovery (%)
1	0.002M	9161	21.8
2	0.03M	15899	37.9
3		2874	6.9
4	0.05M	3004	7.2
5	0.4M	10478	25.0
Total recovery		41416	98.8

Fig. 2. Elution profile of α -crystallin from Bio-Gel A-5m column. The bars indicate the fractions of each peak that were pooled. The elution buffer was 0.01M-tris-HCl buffer, pH 7.8, containing 0.1M-KCl and 1mM-EDTA. See the text for additional information.

peak. Assuming equal percentage recovery of the protein in each peak of the elution profile, peak 2 represents approx. 75% of the starting material.

Since the basis for separation on Agarose columns is primarily molecular size, the fractionation suggested that there are two distinct populations of α -crystallin. Sedimentation-equilibrium experiments were therefore undertaken to characterize the peaks from the Agarose columns. When the peak 2 material was investigated with the Yphantis (1964) high-speed procedure at 4000–6800 rev./min, a $\bar{M}_w$ of $7.2 \times 10^5 \pm 4\%$ was obtained (Fig. 3). A linear relationship between $\ln f$ and r^2 was always observed. No concentration dependence was found. However, when peak 2 material was allowed to reach equilibrium at low speed (1500 rev./min as described by Li, 1971) $\bar{M}_w$ approx. 9×10^5 was obtained. Such an observation suggests that Agarose peak 2 material is polydisperse even though the $\log f$ versus r^2 relationship is linear under all conditions investigated.

To examine the nature of the polydispersity more critically, the peak 2 material was re-run on a Bio-Gel A-5m column. A single peak with the expected profile and elution volume was obtained. Single fractions α_{2-I} and α_{2-II} as shown in Fig. 4

were taken from the ascending and descending sides of the elution profile. These fractions were then subjected to equilibrium sedimentation at high speed. The results are shown in Fig. 4. Both fractions appeared homogeneous, with fraction α_{2-I} having $\bar{M}_w$ 7.95×10^5 and fraction α_{2-II} $\bar{M}_w$ 6.19×10^5 . These experiments show that the peak 2 material represents a polydisperse system not involving chemical equilibrium. A major proportion of the macromolecules in this population appear to have molecular weights in the range 6×10^5 – 8×10^5 . The results obtained with peak 1 from the Agarose 5m column differed markedly from those obtained with peak 2 (Fig. 3). Molecular heterogeneity was apparent in this material from the individual sedimentation-equilibrium experiments. At 4800 rev./min, a $\bar{M}_w$ range of 1.1×10^6 – 1.4×10^6 was observed. At 6800 rev./min the $\bar{M}_w$ obtained at the meniscus was 9×10^5 and the bottom value

was 1.2×10^6 . By using low-speed equilibrium conditions (1500 rev./min) a $\bar{M}_w$ of 1.11×10^6 was observed at the meniscus and a maximum $\bar{M}_w$ of 3.9×10^6 at the bottom (Fig. 5). These experiments suggest that the higher-molecular-weight components present in peak 1 may not have been detected under these conditions.

Fig. 3. Sedimentation equilibrium of peaks 1 and 2 from a Bio-Gel A-5m column. The solutions contained 0.1M-tris-HCl buffer, pH 7.0, 0.1M-KCl and 0.1mM-dithiothreitol. The peak 1 material was run in the analytical ultracentrifuge at 4800 rev./min. The peak 2 material was examined at 6000 rev./min. Log f refers to the log of the fringe displacement (μm) and % to the concentration in g/100ml. M_m refers to the molecular weight at the meniscus and M_b at the base. $\circ$, α_1 ($M_m = 1.1 \times 10^6$; $M_b = 1.4 \times 10^6$); $\bullet$, α_2 ($M_w = 7.3 \times 10^5$).

Amino acid analyses of the Agarose peaks were carried out to characterize further the materials (Table 2). The reported values were obtained by extrapolation of 20, 40 and 70h hydrolysis results to zero time. The 'residues per subunit' values are based on an average molecular weight of 20500. The results suggest that peak 1 and peak 2 materials are essentially identical and correspond well to previous reports of the amino acid composition of α -crystallin. Dansylation indicated no free N -terminal group in either peak 1 or peak 2 material.

Although subunits of α -crystallin can easily be demonstrated in the ultracentrifuge in the presence of high concentrations of disaggregating reagents such as urea and guanidinium chloride (Spector & Katz, 1965), difficulty has previously been encountered in delineating species that varied in molecular weight by only a few thousand. The method of determining molecular weight by polyacrylamide-gel electrophoresis in the presence of 0.1M-sodium dodecyl sulphate was therefore adopted (Shapiro *et al.* 1967; Weber & Osborn, 1969). This method appears to be particularly effective for resolving subunits that have only small differences in their molecular weights. When a group of polypeptide chains with molecular weights from 35000 (pepsin) to 11000 (chymotrypsin subunit) were treated in this manner, a linear relationship between the logarithm of the molecular weight and the mobility was obtained (Fig. 6). These results are similar to those reported by

Fig. 4. Sedimentation equilibrium of fractions from a re-run of peak 2 from a Bio-Gel A-5m column. The peak 2 elution profile and positions of the α_{2-I} and α_{2-II} fractions are shown in the insert. The equilibrium experiment was conducted at 6800 rev./min in 0.1M-tris-HCl buffer, pH 7.8 containing 0.1M-KCl and 0.001M-EDTA. $\circ$, α_{2-I} ($\bar{M}_w$ 795000); $\bullet$, α_{2-II} ($\bar{M}_w$ 619000).

Fig. 5. Sedimentation equilibrium of peak 1 from the Bio-Gel A-5m column. The experiment was conducted in the buffer described in Fig. 3 at a speed of 1500 rev./min for 90 h at 4°C. Equilibrium was reached between 48 and 64 h. Arrows indicate the base and the meniscus of the liquid column. $\bar{M}_{\min.} = 1.11 \times 10^6$; $\bar{M}_{\max.} = 3.90 \times 10^6$.

Weber & Osborn (1969). The results obtained when the Agarose fractions were analysed are shown in Fig. 7. Two major components with molecular weights of 22500 and 19500 were found. A number of runs gave variations of approx. ± 500 . No differences could be detected between the two Agarose peaks (*a* and *b*, Fig. 7). The lower-molecular-weight species appear to represent approximately twice as much material as the heavier species based on the intensity of the Coomassie Blue staining. Only when the gels were loaded with large quantities of protein was it possible to detect small amounts of a number of additional components with different molecular weights.

The broad heterogeneity of peak 1 and its elution from the Agarose 5m column at approximately the breakthrough volume of the column prompted us to examine this material with a larger-pore gel, (Bio-Gel A-15m). Such experiments indicate that a significant fraction of the peak 1 material from the calf lens periphery is represented by a component which is much larger than the population of molecules detected above in peak 1. Estimates based on gel filtration suggest that the molecular weight of this material may be above 10×10^6 . Gel electrophoresis (*c*, Fig. 6) gave the same subunit profile as was previously found for the Bio-Gel A-5m peaks and amino acid analyses indicate the same composition as observed above for Bio-Gel A-5m peak 1 and peak 2.

In an effort to determine a chemical difference between these fractions, examination for non-protein components was undertaken. All assays were negative except for hexose. By the method of

Table 2. Comparison of amino acid composition of Bio-Gel A-5m peaks 1 and 2

	Composition			
	(residues/ 1000 residues)		(residues/ subunit*)	
	Peak 1	Peak 2	Peak 1	Peak 2
Aspartic acid	86	87	15.0	15.1
Threonine	34	33	5.9	5.7
Serine	103	107	17.9	18.6
Glutamic acid	105	101	18.1	17.6
Proline	81	81	14.1	14.1
Glycine	60	58	10.4	10.1
Alanine	44	41	7.6	7.1
Cysteine†	5	5	0.9	0.9
Valine	58	58	10.1	10.1
Methionine	12	12	2.0	2.0
Isoleucine	47	50	8.2	8.7
Leucine	87	83	15.1	14.5
Tyrosine	31	31	5.4	5.4
Phenylalanine	76	78	13.2	13.6
Lysine	48	46	8.4	8.0
Histidine	39	42	6.8	7.3
Arginine	73	76	12.7	13.2
Tryptophan‡	12	12	2.0	2.0

* Based on average subunit mol.wt. 20500.

† Cysteine was determined as carboxymethylcysteine (Crestfield *et al.* 1963).

‡ Tryptophan was determined by the spectrophotometric method of Edelhoch (1967) and by oxidation with *N*-bromosuccinimide (Spande & Witkop, 1967).

Dische & Danilchenko (1967), we demonstrated the presence of a significant amount of hexose in the higher-molecular-weight fractions. Although the fraction of lowest molecular weight contained little hexose, as much as 3% hexose was observed in the high-molecular-weight material. The hexose could not be removed by exhaustive dialysis.

DISCUSSION

The need to fractionate gram quantities of lens proteins efficiently has led to the development of procedures for handling up to 41g of protein with a column (9cm $\times$ 155cm) of DEAE-cellulose. The procedure utilizing stepwise elution takes approx. 24h and a second batch of protein can be run through without column regeneration. Small DEAE-cellulose columns have also been used effectively with as little as 5mg of material, making this procedure applicable for the fractionation of lens protein obtained from small animals such as the rat. The method provides a gentle isolation procedure that does not utilize isoelectric precipitation or the addition of heavy metal.

Fig. 6. Relationship of mobility to molecular weight obtained from gel electrophoresis in sodium dodecyl sulphate. The 10% gels were run in phosphate buffer at pH 7.0 in the presence of 0.3% sodium dodecyl sulphate. The preparations were first incubated at 37°C for 2 h in 1% sodium dodecyl sulphate and 1% mercaptoethanol. Electrophoresis was run for 4.5 h at 8 mA/gel. Bromphenol Blue was used as a tracking dye. All proteins were run on duplicate gels. The chymotrypsin subunit molecular weights of 11000 and 13000 were calculated from the amino acid sequences reported by Dayhoff & Eck (1968). Similar results were reported by Weber & Osborn (1969).

The present work indicates that all populations of α -crystallin contain two major subunit species with molecular weights of 19500 and 22500 in a ratio of approx. 2:1. In α -crystallin isolated from rabbit a component with a molecular weight of 18500 has also been reported (Shapiro, 1968).

In spite of the fact that all α -crystallin macromolecules are composed of low-molecular-weight subunits held together by non-covalent forces, only populations of macromolecules of certain sizes can be detected. The results obtained by gel filtration clearly indicate that α -crystallin is composed of at least three distinct populations of macromolecules. An observed elution peak of α -crystallin from the Agarose columns may be produced by the concentration distribution of a single molecular-weight species. It may also be caused by a population of species of different molecular weights which approximates a Gaussian distribution. The sedimentation-equilibrium studies suggest that the latter possibility is probably a more likely interpretation of our results. The present work clearly suggests that a symmetrical elution peak obtained by gel filtration, combined with sedimentation-equilibrium studies of the entire peak under only one set of conditions, may not be sufficient to describe the molecular-weight distribution of the population. These results might be surprising for a physiologically active protein such as an enzyme, where structure and activity are intimately associated. However, it appears that a physiological system can accept a wider variation of macromolecular structure in the case of a structural protein such as α -crystallin.

Fig. 7. Gel electrophoresis of α -crystallin fraction in the presence of sodium dodecyl sulphate. (a) Peak 2 from a Bio-Gel A-5m column; (b) peak 1 from a Bio-Gel A-5m column; (c) high-molecular-weight fraction from a Bio-Gel A-15m column. Migration was from top to bottom and towards the positive pole. See legend to Fig. 6 and the text for further information.

The α -crystallin population in which the majority of macromolecules have molecular weights in the range 6×10^5 – 8×10^5 has been examined over a wide range of concentrations and did not show any tendency for the macromolecules to dis-aggregate. If macromolecules considerably smaller than 600000 daltons are present, their concentration is too low to observe with our methodology. Aggregates of α -crystallin outside the three observed populations have not been detected. Further, it would appear that there is no observable equilibrium between members of the same population or between populations. In regions of the lens where there is no protein synthesis (Wannemacher & Spector, 1968), there is with age an increase in the higher-molecular-weight populations and a concomitant decrease in the lowest-molecular-weight population. This suggests that some as yet undefined reaction causes a marked increase in the molecular weight of the α -crystallin

macromolecule (A. Spector, T. Freund, L.-K. Li & R. C. Augusteyn, unpublished work). No difference in amino acid composition, subunit profile or end groups has been observed between these fractions. However, there is a significant difference in the hexose content of these α -crystallin fractions. It is possible that the observed physical heterogeneity may be caused by the hexose.

There have been previous reports of fractionation of α -crystallin, but not on the basis of size. On DEAE-cellulose it is possible to separate the α -crystallin into a number of components, all of which are physically heterogeneous and have similar amino acid compositions (Spector, 1965). Similar experiments reported by Björk (1963) indicated that although the amino acid compositions of the α -crystallin components were similar, they exhibited a different mobility in free electrophoresis at pH 8.0. In none of these experiments were different sized populations of α -crystallins observed.

Recently, it has been reported that prolonged exposure to 6M-urea, 4M-urea plus 3M-guanidine or to 2% sodium dodecyl sulphate resulted in the production of subunits of α -crystallin with mol. wt. approx. 11500 (Hoenders, De Groot, Gerding & Bloemendal, 1969; De Groot, Hoenders, Gerding & Bloemendal, 1970). In this laboratory, incubation in 2% sodium dodecyl sulphate at 37°C for 3 days by the method of Weber & Osborn (1969) did not change the sodium dodecyl sulphate-gel electrophoresis patterns. Only two major components similar to those noted in the present paper were observed. Further, in sedimentation-equilibrium experiments in 5.5M-guanidinium chloride or 7M-urea no changes were observed for 48h after equilibrium was reached. In our sodium dodecyl sulphate-gel-electrophoresis experiments, small amounts of components of lower molecular weight were noted in some experiments, but in no case could their content be increased. Neither incubation at pH 12, in 70% (v/v) formic acid or with hydroxylamine has any effect on the gel pattern. Also, when a purified subunit with a molecular weight of approx. 22500 was treated with cyanogen bromide, two fragments with molecular weights of approx. 12500 and 9000 were detected (Augusteyn & Spector, 1971). Such results would not be expected if the original subunit was in the 10×10^3 – 11×10^3 dalton range. The evidence that α -crystallin contains two molecular sizes of subunits does not prove that only two chemically different subunits are present in the macromolecule. Indeed, it appears clear that there are a number of different subunits (Spector, 1965; Schoenmakers,

Gerding & Bloemendal, 1969; Augusteyn & Spector, 1971).

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