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The State of Aggregation of α -Crystallin Detected after Large-Scale Preparation by Zonal Centrifugation

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The protein α -crystallin from calf eye lenses can be isolated in very high yield by zonal centrifugation. No other purification steps are required in order to obtain preparations the purity criteria of which allow sequence studies after dissociation into polypeptide chains. By this method evidence is provided that α -crystallin is a population of aggregates built up by four different polypeptide chains, occurring in an almost constant ratio independent on the size of the aggregate.

The possibility to fractionate lens proteins by electrophoretic techniques has been reviewed previously [1]. It appeared that with exception of isofocusing and free continuous electrophoresis all known electrophoretic procedures had been applied. Meanwhile the two latter methods have also been reported [2–4]. In most electrophoretic isolation procedures the yield of purified protein is moderate. However, for the study on the primary structure of lens proteins which has recently been started [5–8] rather large quantities of highly purified material are required. We have demonstrated earlier [9] that α -crystallin could be isolated by ultracentrifugation in a swinging bucket rotor. Also in this method the yield was not very high.

In the present paper we show that α -crystallin in a highly purified state and in large yield can be isolated by zonal centrifugation as sole purification step. A second advantage of this method is that partial purification for the other water-soluble lens proteins is achieved.

Part of this work has been communicated at the International Lens Symposium (Utrecht 1971).

MATERIALS AND METHODS

Lens Extract

The mixture of water-soluble proteins from calf lens tissue was isolated as described earlier [10].

Zonal Centrifugation

Zonal centrifugation was carried out in a B-XXIX rotor of an IEC ultracentrifuge. The total volume was 1440 ml. As we observed in a test run that the α -crystallin fraction sedimented in a range from 16 S to about 25 S we devised a gradient in which zone sharpening only for α -crystallin would occur.

This gradient is represented graphically in Fig. 1. Centrifugation was performed at 35 000 rev./min for 14 h and at 8 °C. All sucrose solutions contained 0.05 M Tris-HCl at pH 7.6. The sample (2.5 g protein) was pumped into the rotor as an inverse gradient in a volume of 70–100 ml. The rotor was emptied by pumping in 60% (w/w) sucrose solution at 30 ml/min. The absorbance was measured automatically at 280 nm in a Gilford spectrophotometer, adapted with an IEC 2-mm flow cell. The separated fractions were dialyzed against distilled water and lyophilized.

Immunelectrophoresis

Antibodies to total water-soluble lens protein were isolated from hyperimmunized white New Zealand rabbits. Immunelectrophoresis was carried out according to the micro-modification of Scheidegger [11] using LKB apparatus. The gels were

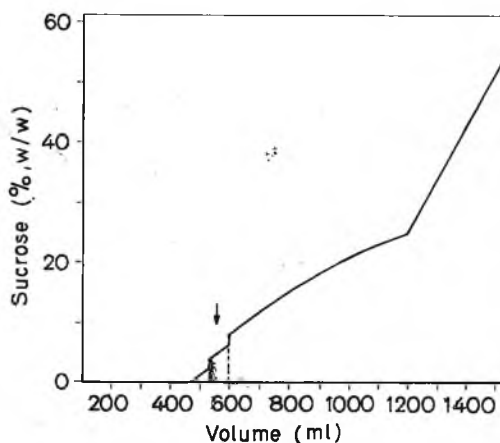


Fig. 1. Graphical representation of the gradient for zone centrifugation of bovine-lens proteins. The sample was applied in an inverse gradient (hatched area)

stained for 15 min in 0.1% Sudan black dissolved in 2% acetic acid. The staining was performed in 2% acetic acid to which 2% glycerol was added.

Amino-Acid Analysis

Protein samples were hydrolyzed in 6 N HCl (Merck, Suprapur) at 110 °C for 22 h in evacuated sealed Pyrex tubes. The subsequent analyses were carried out with a Phoenix amino acid analyser, equipped with micro-cuvettes (optical path 10 and 15 mm) and an Infotronics model CRS-10AB2 integrator. The apparatus was automated as described by Gerding [12]. Calibration runs were carried out with Beckman amino acid calibration mixtures (2.5 μ mol/ml of each amino acid). Some protein preparations were hydrolyzed during varying times in order to determine the following correction factors for certain amino acids: threonine 3%, serine 10%, valine 5%, isoleucine 2%.

Sedimentation Analysis

Sedimentation velocity experiments were performed in a Beckman Spinco model E analytical ultracentrifuge using ultraviolet absorption optics (280 nm) in combination with an automatic scanner as described by van Es and Bont [13]. The solvent was 0.05 M Tris-HCl buffer pH 7.6. Protein concentrations were in the order of magnitude of 1 mg/ml. The absence of sucrose after dialysis was checked by means of refractive index determinations. Runs were made at 53 000 to 68 000 rev./min at about 20 °C. Sedimentation coefficients were corrected to water at 20 °C ($s_{20,w}$).

Gel Electrophoresis in 6-M Urea

Electrophoresis was performed in polyacrylamide gels containing 6 M urea, pH 8.9 according to Bloemendal [14]. Protein samples (50 μ g) were dissolved in 20 μ l of 6 M urea-Tris-EDTA-boric acid buffer at pH 8.9. In order to obtain optimal resolution of zones the run was performed in 10-cm tubes for 150 min at 3.5 mA per tube.

Electron Microscopy

The electron microscopical investigation was performed using fraction 7 and fraction 11 of α -crystallin. Observations were made at pH 7.8. Samples containing 1 μ g/ml 0.001 M ammonium formate were spread on carbon grids and shadowed with carbon and platinum. Electron micrographs have been taken with the EM-300 Philips operating at 60 kV.

A specimen cooling device has routinely been used.

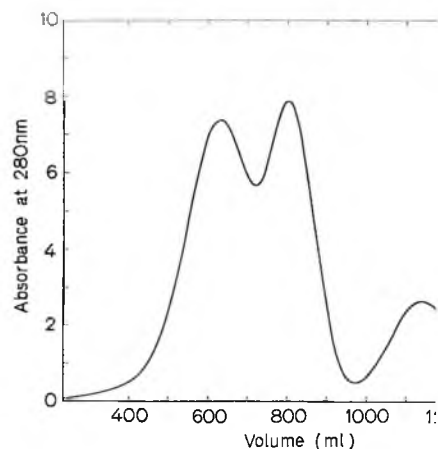


Fig. 2. Sedimentation pattern of a typical run in the zone centrifuge of bovine-lens proteins. Centrifugation at 35 000 rev./min for 14 h; 2.5 g water soluble protein was applied.

Table 1. Fractions after zone centrifugation of lens proteins. The amount of protein was determined by the amount of protein lysed and lyophilized material.

Fraction no.	Volume ml
1	500—600
2	600—700
3	700—800
4	800—900
5	900—970
6	970—1040
7 ^a	1040—1100
8 ^a	1100—1200
9 ^a	1200—1300
10 ^a	1300—1350
11 ^a	1350—1420

^a Immuno-electrophoretic analysis (compare Fig. 1). Fractions 7—11 contained α -crystallin freed from the other proteins.

RESULTS AND DISCUSSION

In Fig. 2 the sedimentation pattern of soluble lens proteins after zonal centrifugation is shown. A clear-cut separation between the α -crystallin and the other water soluble proteins is obtained. Fractions are listed in Table 1. Immunoelectrophoresis revealed that fractions 1, 2 and 3 contain mixtures of so-called pre- α -crystallin and the β -crystallins. Fraction 4, however, contains only β -crystallins. From fraction 5 some overlap of β and α occurs. In fractions 10 and 11 only one precipitation arc can be seen in the position characteristic for α -crystallin.

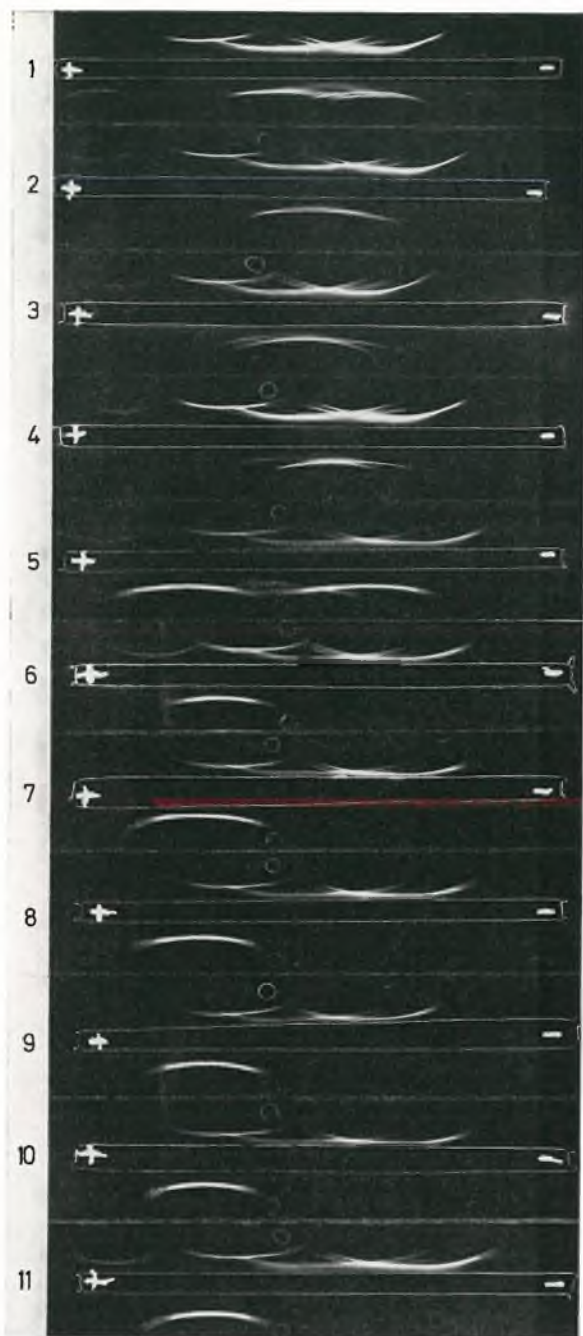


Fig. 3. Immunoelectrophoretic characterization of protein fractions separated by zonal centrifugation. 1–11 are the fractions as listed in Table 1. Per hole 2 μ l of 5% protein solution was applied. As reference 2 μ l of 5% total water-soluble lens protein was electrophoresed parallel to each sample. Electrophoresis was performed at 6 V/cm and 4 mA per slide for 3 h. Slides 1–11 were developed with antiserum against total water-soluble lens protein for 16 h

Hence it can be concluded that these fractions contain highly purified α -crystallin. This conclusion obtained further support by other criteria. Com-

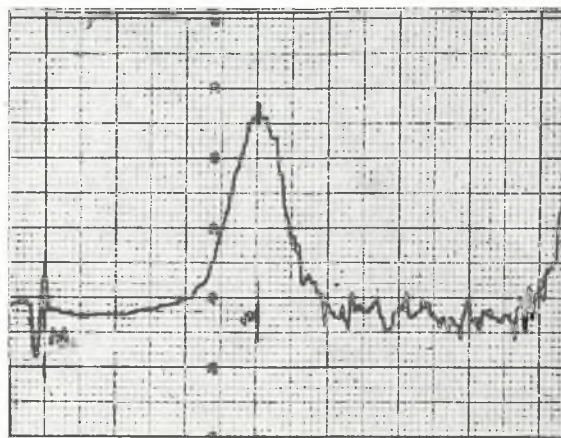


Fig. 4. Sedimentation characteristics of α -crystallin (fraction 7). This recording of the differentiated absorbance was taken 8 min after reaching maximum speed (59780 rev./min). M = meniscus; B = bottom

parison of the amino acid composition of the various fractions reveals that from fractions 7 to 11 amino acid composition is similar whereas fractions 1 and 4 as expected are quite different (Table 2). The difference between the two latter fractions and that from the α -crystallin region is more clearly demonstrated by the electrophoretic experiments which will be discussed later on (Fig. 7).

One may ask why in a zonal centrifuge in which band sharpening occurs α -crystallin does not appear as one sharp peak. In order to answer this question we investigated the separated fractions in analytical ultracentrifuge. The sedimentation coefficients of the different fractions are listed in Table 3. From this table it can be seen that "alpha-crystallin" fractions actually represent different aggregates. (A typical scanning pattern is shown in Fig. 4.) Electron microscopic observation of different "alpha-fractions" are in agreement with this finding. Fig. 5B shows that fraction 7 (16.8 S) consists of aggregates displaying a great variety of sizes ranging from 5 to 18 nm. On the contrary, aggregates in fraction 11 (24.4 S) have an almost uniform size with mean value of 20 nm (Fig. 5). The histogram illustrates a comparative size distribution for both fractions 7 and 11 (Fig. 6). Microheterogeneity of α -crystallin has already been postulated by van Dam [17].

The nature and meaning of these aggregates still remain obscure. We have earlier shown that α -crystallin is composed of four different polypeptide chains, designated A₁, A₂, B₁ and B₂ (A stands for acidic and B for basic) [6,16]. One might speculate that the aggregates differ in quantity and type of polypeptide chains. Evidence against this assumption was obtained by a series of electrophoretic exper-

Table 2. Amino-acid composition of zonal-centrifuge fractions

Amino-acid	1	4	6	7	8	9	10	11
	mol/100 mol							
Aspartic	11.5	8.4	10.0	8.7	9.3	8.6	8.8	9.8
Threonine	3.7	3.3	3.8	3.2	3.3	3.6	3.4	3.8
Serine	7.8	8.2	11.0	11.2	10.9	10.7	10.8	10.9
Glutamic	14.6	15.5	11.6	10.2	11.1	10.6	10.0	11.1
Proline	5.7	7.3	6.7	8.6	8.0	7.4	7.8	7.3
Glycine	9.8	10.8	7.0	6.1	6.1	6.6	6.1	6.6
Alanine	4.8	7.0	5.5	4.6	5.0	5.0	4.7	5.0
Valine	5.5	7.1	5.7	6.1	7.2	6.2	6.6	8.1
Methionine	2.1	1.4	1.0	1.1	1.4	1.0	1.3	1.0
Isoleucine	3.2	3.0	4.5	4.8	4.6	4.8	5.0	4.3
Leucine	6.7	4.9	7.4	8.0	7.9	7.9	8.4	7.6
Tyrosine	5.4	3.4	3.0	2.9	2.6	2.5	2.9	2.4
Phenylalanine	4.2	5.1	6.7	7.8	7.9	7.7	8.1	6.8
Lysine	4.0	4.5	4.4	4.5	4.3	5.1	4.6	4.5
Histidine	3.2	3.5	4.0	4.4	4.0	4.5	4.3	4.0
Arginine	7.2	6.7	7.8	7.8	7.5	8.0	7.5	7.1

* Amino-acid composition as reported by Schoenmakers *et al.* [15].

Table 3. Sedimentation analysis of zonal-centrifuge fractions

Fraction number	Protein type	Sedimentation coefficient
		S
4	β -crystallin	10.3
7	α -crystallin	16.8
8	α -crystallin	19.3
9	α -crystallin	21.1
10	α -crystallin	22.6
11	α -crystallin	24.4

with isolated aggregates. The isolated fractions were run in polyacrylamide gels containing 6 M urea (Fig. 7). Under this condition dissociation into the individual polypeptides occurs. Visual examination reveals immediately that each aggregate yields the same kinds of polypeptides. Tracings of the electrophoretic patterns and estimation of the percentages of A_1 , A_2 , B_1 and B_2 by planimetry (compare Fig. 8) revealed that the major component A_2 occurs in a constant percentage of $43\% \pm 2\%$, whereas there seems to be a slight increase of the amount of the "minor" chains (A_1 and B_1 , Table 4). Therefore the state of aggregation of α -crystallin should be interpreted in terms of differences in the total number of composing polypeptides, rather than in marked differences in ratios of the individual polypeptide chains. At any rate the finding that there is no detectable chemical or immunological difference in the various size classes allows pooling of all α -fractions to serve as pure starting material for the preparation of the individual polypeptides and subsequent structural studies.

Furthermore, zone centrifugation elucidates the question of the discrepancies in molecular weights which have been reported in the literature. These

Table 4. Comparison of the amount of polypeptides in the different size-classes of α -crystallin

These data were obtained from duplicate experiments on two separate zonal runs. The maximal deviation of the individual values obtained by planimetry was

Fraction	$\frac{\alpha A_1 (100)}{\alpha A_1 + \alpha A_2}$	$\frac{\alpha B_1}{\alpha B_1 + \alpha B_2}$
7	30	
8	30	
9	34	
10	34	
11	36	

values vary between 500000 and 1300000. The highest values have consistently been found in the light scattering, whereas the "average" value derived from analytical ultracentrifugement. These discrepancies are now understood. Only the light scattering method is sensitive to small amounts of highly aggregated polypeptides. In view of our experiments we conclude that α -crystallin is a population of various aggregates which are composed of the same basic and acidic polypeptides. In this connection it might be misleading to speak of "subunits" of α -crystallin rather than of polypeptide chain composition.

It is tempting to speculate that the process of aggregation of increasing size may be some molecular process during lens fiber formation, a process which continues from the state throughout the entire life cycle. Consistent with this view is the observation of Delcourt and Constantinou [19] that differentiation of cells into lens fibers is characterized by a change in the relative polypeptide chain composition of α -crystallin.

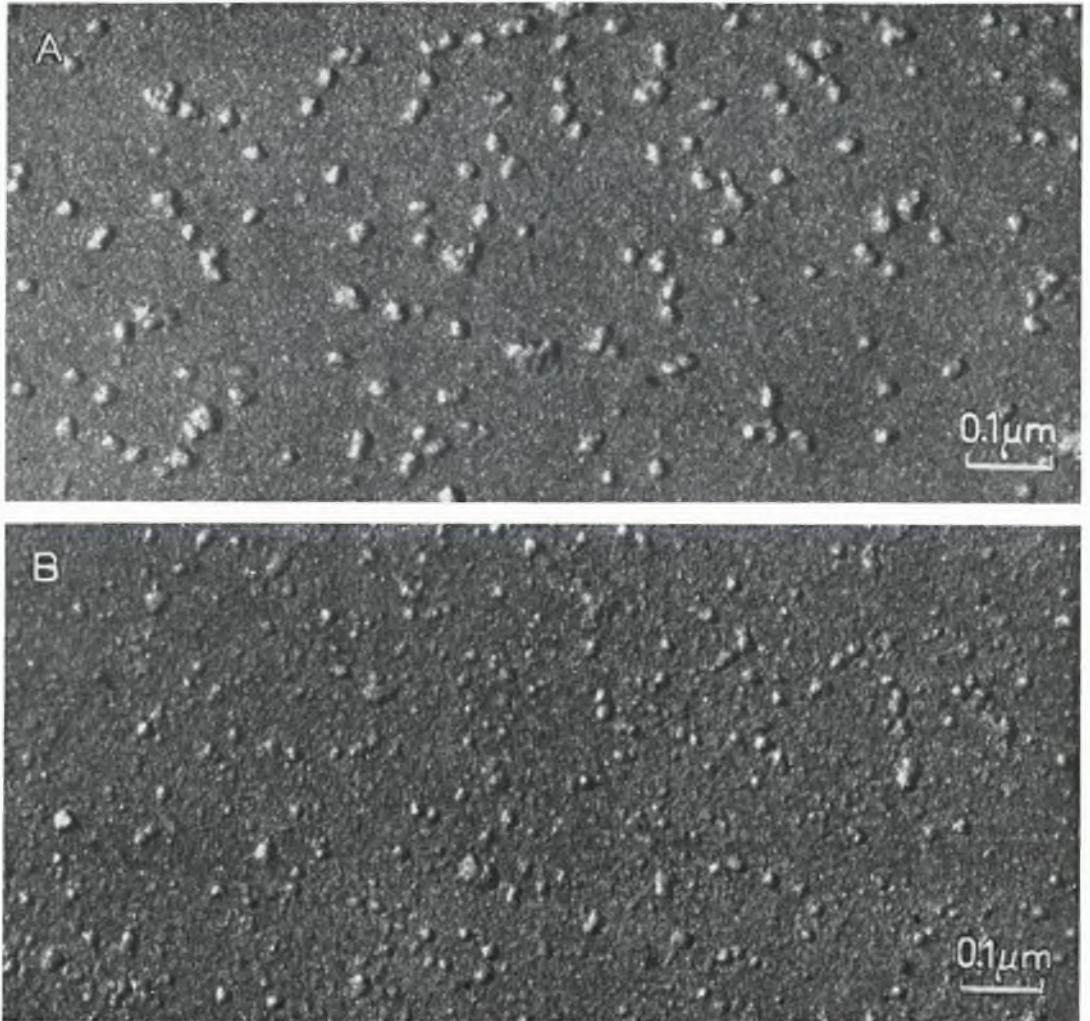


Fig. 5. Electron micrograph of fractions of α -crystallin. (A) Fraction 11 with sedimentation coefficient 24.4 S; (B) fraction with sedimentation coefficient 16.8 S

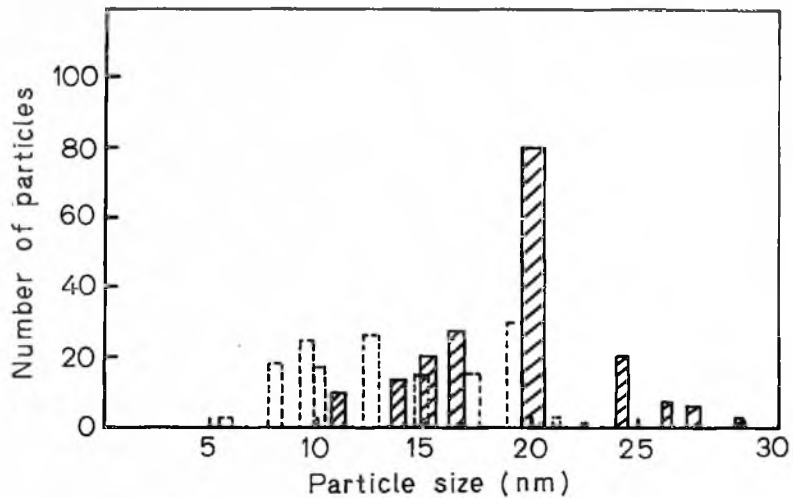


Fig. 6. Size distribution of particles in fraction 7 (open blocks) and fraction 11 (hatched blocks) from α -crystallin purified zonal centrifugation

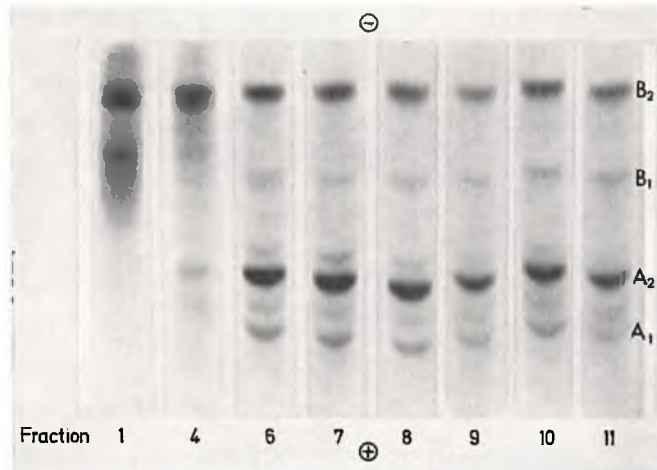


Fig. 7. Polyacrylamide-gel electrophoresis of lens proteins in 6 M urea. Fractions 7–11 reveal the gel pattern known as α -crystallin after dissociation into polypeptides

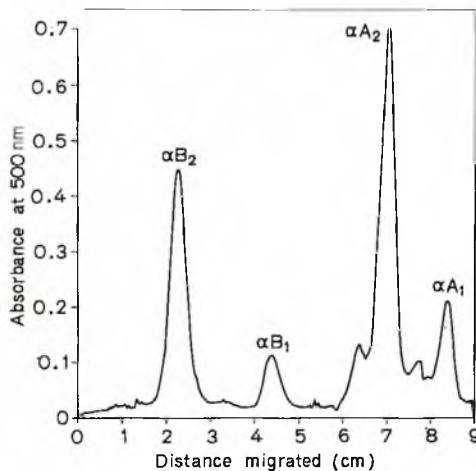


Fig. 8. Typical profile of α -crystallin in 6-M urea obtained after scanning gels 7–11 (compare Fig. 7) in a Gilford recording-spectrophotometer model-240. The area under each peak was determined with a planimeter

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