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 isoelectrofocusing 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 35000 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.

Immunoelectrophoresis

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

![Graphical representation of the gradient for zone centrifugation of bovine lens proteins. The sample was applied in an inverse gradient (hatched area).](image-url)
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 μmol/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 per­
formed 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 concen­
trations 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 53000 to 68000 rev./min at about 
20 °C. Sedimentation coefficients were corrected to 
water at 20 °C ($\bar{s}_{20,w}$).

**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 μg) were 
dissolved in 20 μl 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 μg/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.

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**RESULTS AND DISCUSSION**

In Fig. 2 the sedimentation pattern 
soluble lens proteins after zonal cent 
shown. A clear-cut separation between 
(1000—1400 ml) and the other water 
proteins is obtained. Fractions were listed in Table 1. Immunoelectropho­
ments revealed that fractions 1, 2 a 
mixtures of so-called pre-α-crystallin, 
and the β-crystallins. Fraction 4, how­
to contain only β-crystallins. From fra 
some overlap of β and α occurs. In fi 
10 and 11 only one precipitation arc car 
in the position characteristic for α-crys
Fig. 3. Immunoelectrophoretic characterization of protein fractions separated by zonal centrifugation. 1—11 are the fractions as listed in Table 1. Per hole 2 μl of 5% protein solution was applied. As reference 2 μl 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. Comparison of the amino acid composition of the various fractions reveals that from fractions 7 to 11 amino acid composition is similar whereas fraction 4 as expected are quite different (Table 2). The difference between the two latter fractions and those 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 an analytical ultracentrifuge. The sedimentation coefficients of the different fractions are listed in Table 3. From this table it can be seen that the “α-crystallin” fractions actually represent different aggregates. (A typical scanning pattern is shown in Fig. 4.) Electron microscopic observation of the different “α-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 the 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). The heterogeneity 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 experiments.
Table 2. Amino-acid composition of zonal-centrifuge fractions

<table>
<thead>
<tr>
<th>Amino-acid</th>
<th>mol/100 mol</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Aspartic</td>
<td>11.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.7</td>
</tr>
<tr>
<td>Serine</td>
<td>7.8</td>
</tr>
<tr>
<td>Glutamic</td>
<td>14.6</td>
</tr>
<tr>
<td>Proline</td>
<td>5.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8</td>
</tr>
<tr>
<td>Valine</td>
<td>5.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.1</td>
</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>6.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.2</td>
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</tbody>
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* Amino-acid composition as reported by Schoenmakers et al. [15].

Table 3. Sedimentation analysis of zonal-centrifuge fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein type</th>
<th>Sedimentation coefficient</th>
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<tbody>
<tr>
<td>4</td>
<td>β-crystallin</td>
<td>10.3</td>
</tr>
<tr>
<td>7</td>
<td>α-crystallin</td>
<td>16.8</td>
</tr>
<tr>
<td>8</td>
<td>α-crystallin</td>
<td>19.3</td>
</tr>
<tr>
<td>9</td>
<td>α-crystallin</td>
<td>21.1</td>
</tr>
<tr>
<td>10</td>
<td>α-crystallin</td>
<td>22.6</td>
</tr>
<tr>
<td>11</td>
<td>α-crystallin</td>
<td>24.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>αA₁ (100)</th>
<th>α₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

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 A₁, A₂, B₁ and B₂ by planimetry (compare Fig. 8) revealed that the major component A₂ occurs in a constant percentage of 43 ± 2%, whereas there seems to be a slight increase of the amount of the “minor” chains (A₁ and B₁, 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 values vary between 500000 and 1300000 highest values have consistently been found scattering, whereas the “average” value derived from analytical ultracentrifugation. These discrepancies are now understood. Only the light scattering method is sensitive enough to detect small amounts of highly aggregated view of our experiments we conclude that α-crystallin is a population of various aggregates which up by the same basic and acidic polypeptide. In this connection it might be misleading to speak of α-crystallin rather than speaking of poly peptide chain composition.

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

REFERENCES