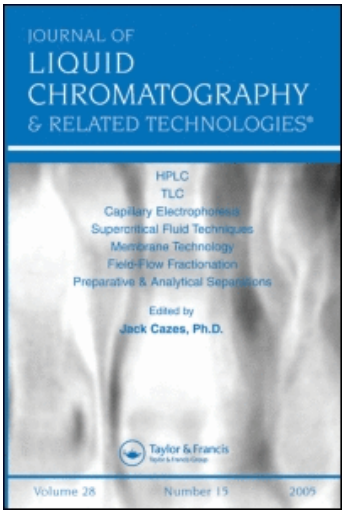

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Ineke Vlaanderen[a]; Rienk Van Grondelle[a]; Michael Bloemendal[b]
[a] Department of Biophysics, Free University, Amsterdam, The Netherlands [b] Department of General and Analytical Chemistry, Free University, Amsterdam, The Netherlands

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THE USE OF FAST PROTEIN LIQUID (SIZE EXCLUSION) CHROMATOGRAPHY FOR THE FRACTIONATION OF CRYSTALLINS AND THE STUDY OF β-CRYSTALLIN AGGREGATION

INEKE VLAANDEREN[1], RIENK VAN GRONDELLE[1], AND MICHAEL BLOEMENDAL[2]
[1]*Department of Biophysics*
[2]*Department of General and Analytical Chemistry*
Free University
De Boelelaan 1081
1081 HV Amsterdam, The Netherlands

ABSTRACT

The separation of bovine crystallins on two types of fast protein liquid chromatography® (Pharmacia) size exclusion columns, Superose-6 (fractionation range 5 - 5.10^3 kDa) and Superdex-75 (fract. range 3- 70 kDa), and on a combination of these was studied. This type of columns combines the biocompatibility of the conventional soft-gels with the performance characteristics of high performance liquid chromatography. Up to 20 mg protein in 100 µl could be separated within two hours on analytical columns without significant loss of resolution. On preparative columns we could fractionate 75 mg of protein within 6 hours. The effect of column load by variation of both concentration and injection volume is reported. It is shown that size estimation of β-crystallins by size exclusion chromatography, as is often done, gives inconsistent results. The possibility that native β-crystallin is significantly larger than sofar assumed cannot be excluded.

INTRODUCTION

The prevailing water-soluble proteins in lenses are the so-called crystallins. In mammals these occur mainly in three classes: α (Molecular weight ~ 800 kDa),

β (Mw 50 -300 kDa) and γ (Mw ~ 20 kDa) [1, 2, 3, 4]. α–Crystallins are complexes of 30 - 60 polypeptide chains of 20 kDa each [3, 5]. β-Crystallins are composed of subunits of 22-35 kDa, that form aggregates of different sizes [6, 7]. Based on their chromatographic behavior they are divided into two groups, $\beta_{H(igh)}$ and $\beta_{L(ow)}$. Sometimes β_L is split into two subfractions, β_{L1} and β_{L2} [3, 8, 9]. γ-Crystallins form a family of closely related monomeric polypeptides of about 20 kDa [3, 10]. The concentration of water-soluble crystallins in vivo is very high (200 - 600 mg/ml) [11], and these proteins can be dissolved very easily to 100 - 200 mg/ml, without raising the viscosity significantly. The supra-molecular organization of the crystallins and the proportions in which they occur vary greatly between species and also in different layers within the same lens [12, 13]. This suggests a specific role of different crystallins in transparency and/or refraction. Chemical and physical modifications or stress can lead to cataract or impaired vision [14]. In addition to their biophysical role, crystallins are often used as a model for developmentally regulated gene expression [13].

Isolation of crystallins from crude lensextract is usually based on size exclusion chromatography, which in a classical setup takes more than 30 hours [7, 8, 9, 15]. High performance liquid chromatography (HPLC) has also been used, but only analytical amounts of proteins have been separated by this technique [4, 8]. In general, these columns have no more than a moderate loading capacity [16]. A further disadvantage of the, usually silica-based, HPLC columns is their instability to alkaline pH. Finally, the possibility of artifacts induced by high pressure or adsorption to the column material can not be excluded [8, 16].

Fast protein liquid chromatography® (FPLC, Pharmacia) size exclusion matrices are known to show no interactions with biomolecules, to have high loading capacity, and to be stable over a large pH range. Furthermore, the FPLC columns can be operated at low pressure, while giving a high resolution in an elution time comparable to HPLC columns. Thus, they combine the biocompatibility of the conventional gels with the performance characteristics of HPLC [16-18]. We studied the separation of crystallins on two types of FPLC size exclusion columns, Superose-6 (fractionation range 5 - 5.10^3 kDa) and Superdex-75 (fract. range 3 - 70 kDa), and on a combination of these. Experiments were performed both on analytical and preparative columns. The combined system was then used to study the effect of column load on the aggregation state of the crystallins.

MATERIALS

Chemicals

All chemicals used were of analytical grade. Potassiumphosphate, KCl, sodiumethylenediaminetetraacetate (Na_2EDTA) were purchased from Baker, dithiothreitol from Sigma and NaN_3 from Merck. Chemicals used for electrophoresis were obtained by Serva (Acrylamid), Sigma (Sodiumdodecylsulfate), Baker (Tris-(hydroxy-methyl)aminomethane, Ammoniumpersulfate, Methanol and Acetic Acid) and Merck (Bisacrylamid, N,N,N^1,N^1-Tetramethylethylenediamine (TEMED)). The elution buffer was made with triple-distilled water, filtered through a 0.45 µm filter (Nalgene), and degassed.

Preparation of calf lensextract

Bovine calf eyes (8 - 11 months), obtained from the local slaughterhouse, were kept on ice and processed within 2 hours after slaughtering. Five lenses (average weight 2 grams) were removed, decapsulated and stirred during 30 minutes in 10 ml of the elution buffer used by Tardieu et al. [5] (40 mM potassiumphosphate (pH 6.8), 90 mM KCl, 0.01 %($^w/_w$) NaN_3, 1 mM Na_2EDTA and 0.2 mM dithiothreitol, ionic strength (I) = 0.15 eq). The homogenate was centrifuged at 30.000 g for 20 min. in a Beckman JB-21 centrifuge. The concentration of water-soluble proteins in the supernatant was 100-200 mg/ml (estimated from an average $A^{1\,\%}_{1\,cm}$ 280 nm of 18 [8]). The temperature was kept at 4 °C during the whole procedure. When necessary, the protein samples were concentrated by ultrafiltration using an YM 10 filter (Amicon).

METHODS

Chromatography

The chromatographic system consisted of a Waters-600 HPLC pump (Millipore), a P7 injection valve (Pharmacia), a prefilter (10 µm, Pharmacia), a Superose-6 and / or Superdex-75 size exclusion column (Pharmacia, prepacked, 30 x 1.0 I.D. cm), a Waters-990 diode-array detector (Millipore) and a Frac-100 fraction

collector (Pharmacia). The columns were equilibrated with at least 3 column-volumes elution buffer. Samples were centrifuged during 5 minutes at 10.000 g prior to injection. Elution was performed at a flowrate of 0.35 ml/min, unless indicated otherwise. The eluent was collected in aliquots and stored at 4 °C. For preparative applications a Superose-6-prep (Pharmacia, 60 x 1.6 I.D. cm) and a Superdex-75-prep column (Pharmacia, prepacked, 50 x 1.6 I.D. cm) were used. In these experiments the elution was performed at a flowrate of 0.5 ml/min. The chromatography was done at ambient temperature.

Gel electrophoresis

Fractionated proteins were identified and analyzed on purity by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of 5 - 15 µl (containing approximately 25 µg protein) of the collected fractions were denatured by heating to 100 °C in the presence of 2% SDS and 2.5% β-mercaptoethanol, and loaded onto a 12% SDS-polyacrylamidegel according to Laemmli [19]. A minigel system (Biorad) was used for electrophoresis. The gels were prerunned for 30 min. to get rid of free molecules. Electrophoresis was performed for 60 - 90 min. at 125 mV, 30 mA.

Molecular weight determination

The apparent molecular weights (Mw_{app}) of the crystallins were determined on the size exclusion columns by comparison of their retention times with the average (n=3) retention times of proteins from the Pharmacia HMW and LMW size exclusion calibration kit, assuming that the crystallins behave in the same hydrodynamically way as globular calibration proteins. Those with molecular weights of 669, 440, 232 and 158 kDa were used for the Superose-6, and those with Mw's of 67, 43, 25 and 13.7 kDa for the Superdex-75 columns.

The reproducibility of our calibration curves and elution volumes was carefully checked. Seven to eleven separations on either the single or the combined columns yielded a standard deviation of about 0.5 ml in the elution volumes of all crystallins. For the calibration proteins this value was about 0.3 ml (n=3). Because of the logarithmic relation between molecular weight and elution volume this results in an error of 0.07 in $^{10}\log Mw_{app}$, which corresponds to uncertainties in ap-

parent molecular weights of about 100 kDa for α-, 30 kDa for β_H-, 10 kDa for β_{L1}-, 5 kDa for β_{L2}- and 2 kDa for γ-crystallins.

Coupled columns do not automatically result in a linear calibration curve, but our experiments yielded a straight line within the experimental error given above for standard proteins between 669 and 13.7 kDa.

RESULTS

Isolation

The separation of analytical amounts of cortical lensextract on a Superose-6 and a Superdex-75 FPLC size exclusion column is shown in figure 1 and 2, respectively. SDS-gel electrophoresis of selected fractions is shown in figure 3 and 4. On Superose-6 α-crystallin is virtually baseline-separated from the other proteins. The apparent molecular weight (Mw_{app}) at the peak maximum is about 785 kDa; rechromatography (figure 1 - insert) and SDS-PAGE (figure 3, lane 1) indicate that it is almost pure. The β-peaks show considerable overlap. The apparent molecular weights of the three peaks, 280, 150 and 90 kDa in the experiment shown, vary within different experiments. This will be discussed later. The 280 kDa-peak shows subunits of 35, 33, 27, 25 and 22 kDa (fig. 3, lane 2 - 4), which correspond to the pattern of β_H [6, 8], and a remnant of α-crystallin. Pure β_H was obtained after rechromatography on the same column. Lane 5 shows a fraction from the overlap of the 280 and 150 kDa peaks containing essentially a 25 kDa subunit. Both the 150 and 90 kDa-peaks contain subunits of 27, 25 and 22 kDa (lane 6, 7), which is typical for β_L-crystallin. However, the 90 kDa peak is significantly contaminated with γ-crystallin (lane 7, 8). The small peaks at the end of the chromatogram contain both proteins and low molecular weight non-protein (NP) components, as is indicated by the elution profile at 260 nm (fig. 1) and SDS-PAGE (not shown).

On the Superdex-75 column no separation of α- and β_H-crystallin is observed, as can be expected from its fractionation range (3-70 kDa). The shoulder on the right-hand side of the α/β_H-peak shows an intense 25 kDa band on the SDS-gel (fig. 4, lane 2). This indicates that it is enriched in β_L-crystallin [8, 9]. The next peak (Mw_{app} 45 kDa) contains reasonably pure β_L (lane 3). Based on their apparent molecular weights we define the two β_L-fractions as β_{L1} and β_{L2} respectively. The small peak at the right-hand side of β_{L2}, which has an apparent molec-

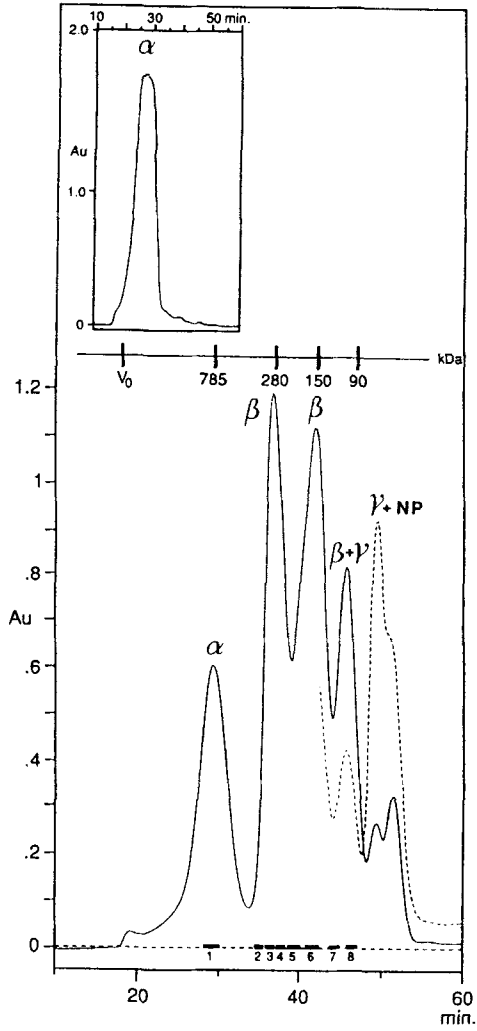

FIGURE 1. Chromatogram of bovine crystallins on a Superose-6 FPLC size exclusion column at 280 nm (——) and at 260 nm (- - - -). 25 µl of lensextract was injected and elution was performed at a flowrate of 0.35 ml/min. Horizontal bars indicate fractions for SDS-PAGE, vertical bars represent apparent molecular masses of peak maxima. Insert: chromatogram after rechromatography of fractionated α-crystallin.

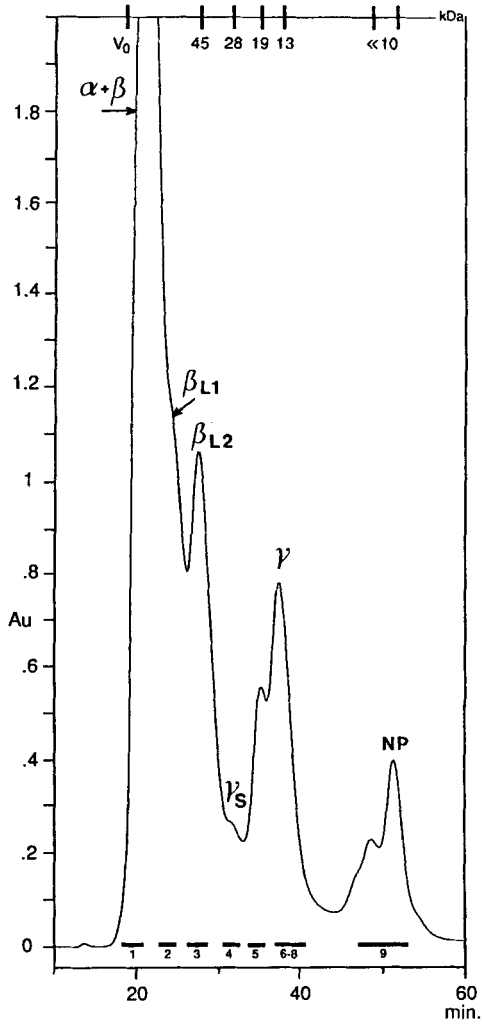

FIGURE 2. Chromatogram at 280 nm of bovine crystallins on a Superdex-75 FPLC size exclusion column. 25 µl of lensextract was injected and elution was performed at a flowrate of 0.35 ml/min. Horizontal bars indicate fractions for SDS-PAGE, vertical bars represent apparent molecular masses of peak maxima.

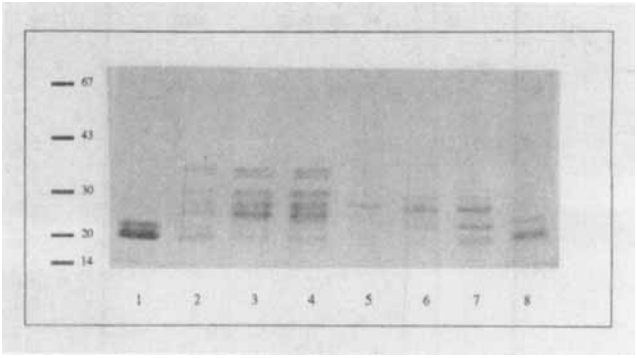

FIGURE 3. SDS-PAGE of fractions from the Superose-6 column. Lanes as indicated by bars in fig. 1.

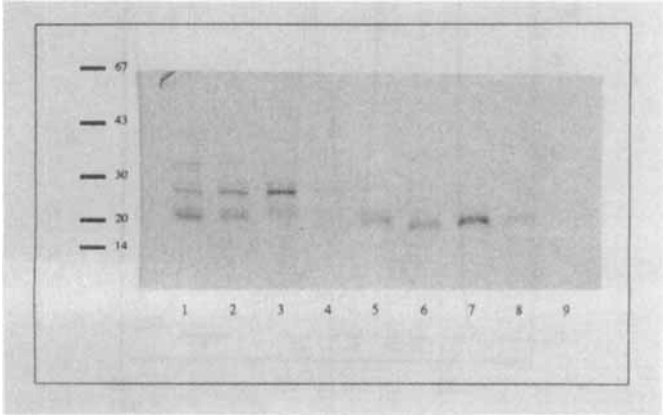

FIGURE 4. SDS-PAGE of fractions from the Superdex-75 column. Lanes as indicated by bars in fig. 2.

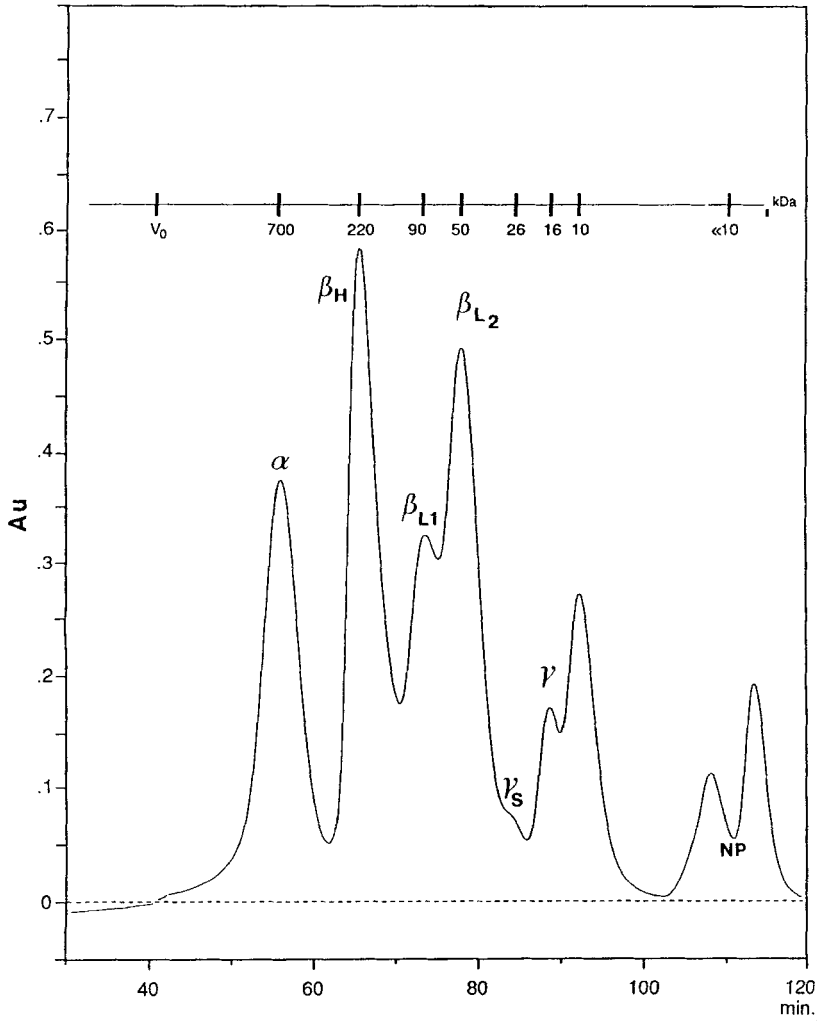

FIGURE 5. Chromatogram at 280 nm of bovine crystallins on a Superdex-75 + Superose-6 coupled system. 25 µl of lensextract was injected and elution was performed at a flowrate of 0.35 ml/min. Horizontal bars indicate fractions for SDS-PAGE, vertical bars represent apparent molecular masses of peak maxima.

ular weight of 28 kDa, is likely to be γ_S-crystallin [7, 20, 21], although it certainly is not pure and it can hardly been seen on gel (lane 4) due to the low concentration. Two other γ-peaks are found, with Mw_{app} of 19 kDa and 13 kDa. On SDS-PAGE (fig. 4, lane 5 - 8) these γ-crystallins have molecular weights of 22 and 20 kDa, which is in agreement with literature [7]. The non-protein compounds (Mw_{app} << 10 kDa) are split into two peaks. No protein is found on the SDS-gel (fig. 4, lane 9). When the lensextract is dialyzed against the buffer before elution, these peaks disappear.

To separate all crystallins in one chromatographic experiment we connected both columns in series. The resulting chromatogram is given in figure 5. The separation of α and β_H is like that obtained with the single Superose-6 column, but their apparent molecular weights are just 700 kDa and 220 kDa respectively. The β-subgroups are remarkably well resolved in the coupled system. The β_L-peaks have apparent molecular weights of 90 and 50 kDa, which are also lower than those found with the single Superose-6 column. The γ-peaks (Mw_{app} 26, 16 and 10 kDa) and the non-protein compounds show the same pattern as on the single Superdex-75 system. In general the resolution was slightly better when the Superdex-75 column was used in front of the Superose-6.

On the coupled preparative columns the separation was similar to that on the analytical columns. In the preparative mode 75 mg of protein could be separated within 6 hours.

After rechromatography of single β_H- and β_{L1}-peaks we detected a new, irreversible peak with a Mw_{app} of about 1000 kDa, which might be the supra-aggregates reported before [8]. We did not see those supra-aggregates after rechromatography of the β_{L2}-fractions.

When the lensextract was stored at 4 °C for 16 days, a small gradual shift of β_{L2} from 50 to 90 kDa occurred at a protein concentration of 140 mg/ml. At a lower concentration (10 mg/ml) we did not see any noticeable effect.

The effect of column load

The effect of column load on the separation was studied on the coupled system. The concentration of the lensextract was varied from 175 to 1.75 mg/ml at a constant injection volume of 25 µl resulting in a loaded mass of protein between 4.4 mg and 44 µg. Alternatively the mass of loaded protein was changed by vary-

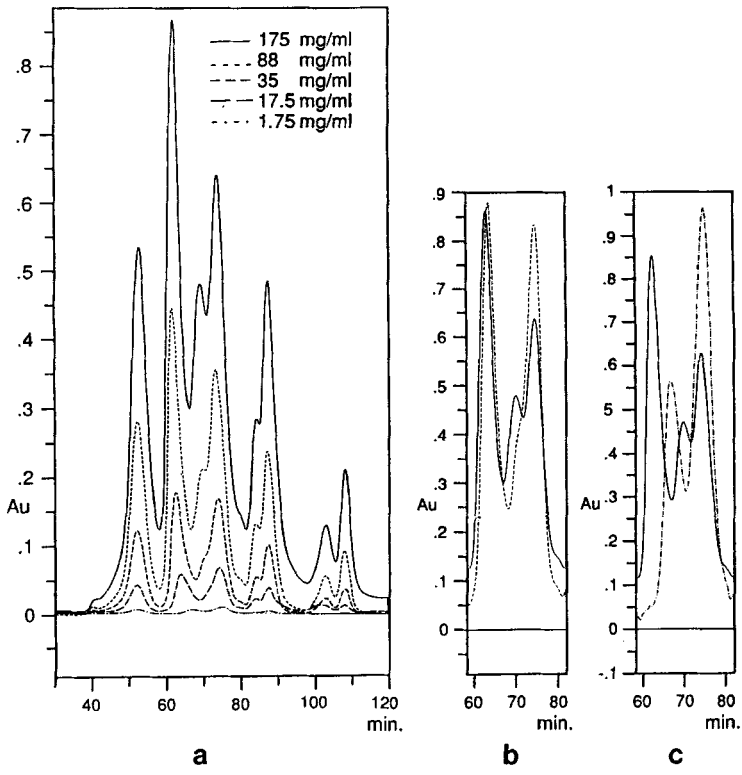

FIGURE 6. Chromatograms at 280 nm of 25 µl lensextract on a coupled system of Superdex-75 and Superose-6 at different protein concentrations as indicated in the figure. (a) Untreated chromatograms, (b) β-peaks for 175 mg/ml (——) original size and 35 mg/ml (- - -) five times enlarged, (c) β-peaks for 175 mg/ml (——) original size and 1.75 mg/ml (·····) hundred times enlarged.

ing the injection volume from 900 to 25 µl at a constant protein-concentration of 15 mg/ml. The viscosity of the protein solution did not seem to increase significantly, not even at the very high concentration of 175 mg/ml.

Figure 6 shows the chromatograms of the coupled columns at different concentrations. Only the β-crystallin peaks are influenced. When the concentration was lowered, the original β_{L2}-peak (~50 kDa) increased in size at the expense of β_{L1} (fig. 6b). When the concentration was further decreased, the β_H peak shifted from about 220 kDa to 130 kDa (fig. 6c). A very small fraction of the original peak

TABLE 1

Apparent Molecular Weights and Relative Peak Areas of β- Crystallins at different Protein Concentrations and Injection Volumes.

Protein Concentration	Injection Volume	Loaded Mass	β_H Mw_{app}	{%}[a]	β_{L1} Mw_{app}	{%}	β_{L2} Mw_{app}	{%}
175 mg/ml	25 µl	4.4 mg	220	{24}	90	{12}	52	{18}
88	25	2.2	220	{24}	87	{ 9}	52	{21}
35	25	0.88	205	{25}	86	{ 8}	52	{23}
17.5	25	0.44	180	{26}	-	{ -}	51	{32}
1.75	25	0.044	220	{ 1}	-	{ -}	49	{37}
			130	{21}				
15 mg/ml	900 µl	14 mg	220	{27}	88	{13}	48	{16}
15	200	3.0	205	{26}	82	{10}	49	{20}
15	100	1.5	205	{25}	86	{ 9}	49	{22}
15	25	0.38	180	{25}	-	{ -}	48	{31}

[a] {%} is the relative contribution of to the chromatogram at 280 nm ($\%A_{280}$) estimatd according to Siezen et al. [8] by dropping verticals at valley points.

always remained. The same effect was observed when the injection volume was varied at constant protein concentration. No flowrate effect was seen between 0.15 to 0.5 ml/min for a single amount of protein loaded (4.4 mg). Since only the β-peaks are effected by column load, we interpret the changes as a variation in size of the aggregates rather than as a chromatographic effect.

In order to quantify these results we estimated the relative contribution of β_H, β_{L1} and β_{L2} to the chromatogram at 280 nm ($\%A_{280}$) as done by Siezen et al. [8] by dropping verticals at valley points. The results are given in table 1. The table suggests that the β_{L1}-peak disappears abruptly at 0.4 mg column load. However, this is misleading since the contribution to A_{280} of shoulders in the chromatogram is overestimated by dropping of verticals. Figure 6a indicates that the dissociation process is gradual. The aggregation behavior is reversible, as was checked by chromatography of a concentrated, diluted and reconcentrated sample.

DISCUSSION

Isolation

Cortical lensextract with protein concentrations as high as 100 - 200 mg/ml was used. α-Crystallin can be isolated within one hour on Superose-6 using a flow-rate of 0.35 ml/min. β_H can be obtained pure after rechromatography only. β_L- and γ- crystallins cannot be separated on this column, notwithstanding the fractionation range (5 - 5.10^3 kDa) given by the manufacturer. From the chromatogram at 280 nm and SDS-PAGE one might get the impression that the last two peaks are pure γ-crystallins. However, when the absorption at 280 nm is compared to the absorption at 260 nm a non-protein compound, possibly a polynucleotide [3], is discovered. Measurement of both $A_{280\ nm}$ and $A_{260\ nm}$ during the run appears to be important in this type of separation! To get rid of these compounds the lensextract is often dialyzed against the elution buffer before size exclusion [8]. In our system these small compounds can be completely separated from the γ-crystallins on a Superdex-75 column.

By coupling the Superose-6 and Superdex-75 columns the fractionation range of the size exclusion system becomes 3 - 5.10^3 kDa and this seems sufficient to separate all compounds. Although one would expect a loss of resolution by diffusion of the proteins, this is hardly the case. On the contrary the β- and γ-crystallins are better resolved. Apparent molecular weights found by means of the coupled system seem to be smaller than those based on retention times on the single Superose-6 column, although their errors do overlap.

Up to 20 mg protein in 100 µl can be injected on our analytical columns without significant loss of resolution. On preparative columns we can separate 75 mg of protein within 6 hours. Especially for biophysical experiments, which often need relatively high amounts of fresh protein, and for the further separation of the main classes of crystallin into their elements this is an important result.

β-crystallin aggregation

It has been shown that the aggregation state of β-crystallins depends on medium density, temperature, ionic strength and protein concentration [7, 8, 15, 22, 23]. Siezen et al. [8] using HPLC, have indicated that it is not influenced by the amount of protein loaded onto the column, provided that the concentration of the

sample is constant. On FPLC we obtain a different result. The size of the β-aggregates changes with the mass of protein loaded onto the column in the same way, whether the amount of protein was changed by injection volume or by protein concentration.

The results of the size exclusion chromatography can be used to estimate the number of subunits in the different aggregates. Using an average subunit mass of 26 kDa [4,8] the 220 kDa aggregate of β_H, found at a protein load of at least 0.88 mg, corresponds to an octa- or nonamer, and the 180 kDa aggregate at 0.44 mg to particles containing 7 subunits. When the injected mass of protein is less than 45 μg only 1% of the original 220 kDa-peak can be seen. (In the 0.44 mg experiment the remaining 220 kDa peak is overlapped by the 180 kDa main peak.) The 180 kDa aggregate falls apart into particles of 5 subunits with an Mw_{app} of 130 kDa and of 2 subunits with an average Mw_{app} of 50 kDa. β_{L1} is found as a trimer and/or tetramer (87 kD) at higher protein masses and splits into dimers when less protein is loaded onto the column. The position of β_{L2} remains always the same, whereas also the supra-aggregates found after rechromatography of single β_H- and β_{L1}-fractions are not observed for β_{L2}. This suggest that β_{L2} is very stable and can be used as internal standard.

Bindels et al. [4], using HPLC (TSK G4000 SW and 2 x TSK G3000 SW columns, coupled) with low angle laser light scattering (LALLS) detection, identified eight different β-crystallin fractions with oligomeric structures from dimers to aggregates larger then dodecamers. In cortical extracts they found that the hexameric and dimeric forms predominate and occur in the ratio 1 : 2, when 7 mg (200 μl, 35 mg/ml) protein was loaded. Siezen et al. [8] using a single TSK G3000 SW or a conventional Sephacryl S200 SF column, also reported a hexamer as the largest aggregate of β_H, but with a ratio $\beta_H : \beta_L$ of about 3 : 2 at a column load of 5 mg (concentration 20-249 mg/ml). We find octa- and/or nonamers as the dominating form of β_H and a β_H:β_L ratio of 0.8 : 1 when 4.4 mg crystallin (concentration 175 mg/ml) is loaded. Our results on the single Superose-6 column even suggest the predominance of undecamers for β_H (MW_{app} 280 kDa) and hexamers for β_L (MW_{app} 150 kDa). When we eluted with the buffer used by Bindels et al. [4], we observed no differences from our previous results. A few explanations are possible for the discrepancies. It is possible that interconversion of aggregation states takes place during the dilution of the sample on the column. This dilution will depend on the size of the columns, the elution time and on the type of column. In our case it is

clear from the magnitude of the absorption in figure 1, 2 and 5 that the samples are more diluted on the coupled columns. This could explain the lower molecular masses on the coupled system. Alternatively, the occurrence of interaction (attractive or repulsive) with size exclusion gels can influence the aggregational state. As pointed out by Regnier [24] aggregated proteins occasionally present problems upon chromatography by disruption of the quaternary structure due to concurring interactions between the different subunits on one hand and the protein with the column on the other hand. This renders questionable the assumption, that FPLC gels have less interaction with proteins than the silica based HPLC gels [16-18] and are more suitable for studies of the aggregation of proteins.

The inevitable conclusion is that molecular weights of β-crystallin aggregates based on size exclusion chromatography are inconsistent and thus unreliable. Regrettably most studies on the size of β-crystallin have been based on such chromatography, and to our best knowledge analytical ultracentrifugation has only been performed at low protein concentrations [1, 2, 4, 7, 8, 15, 22, 23]. Since samples are always diluted during chromatography and β-crystallin tends to dissociate upon dilution, whereas also interaction with the column may disrupt the quaternary structure, the possibility that native β-crystallin is significantly larger than sofar assumed cannot be excluded.

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