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HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY OF BOVINE EYE LENS PROTEINS IN COMBINATION WITH LOW-ANGLE LASER LIGHT SCATTERING

SUPERIOR RESOLUTION OF THE OLIGOMERIC β-CRYSTALLINS*

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SUMMARY

Calf lens extracts were subjected to high-performance gel permeation chromatography on TSK GEL G4000 SW and G3000 SW columns (fractionation range: $5 \cdot 10^6$–10^4 daltons) and resolved into thirteen crystallin fractions: HM-, α-, six β_H-, two β_L-, β_S- and two γ-crystallins. Molecular weights were determined using a low-angle laser light scattering detection system. The weight average and number average molecular weights for cortical α-crystallin, 860,000 and 740,000, respectively, reveal a polydispersity factor of 1.16 for this heterogeneous protein. The eight different β-crystallin fractions could be found with practically all possible oligomeric structures from dimers to aggregates larger than dodecamers. Different structures are found for the predominant β_H-crystallin fractions, *viz.*, hexamers and pentamers, in the extracts from cortex and nucleus. Additional identification of the fractions by sodium dodecyl sulphate gel electrophoresis and isoelectric focusing in the presence of urea also indicated that semi-preparative application of this high-performance technique is possible. The co-elution of putative cytoskeletal proteins with some β-crystallins was remarkable; moreover, co-elution of FM-crystallin with β_S-crystallin is discussed. A 23,000-dalton fraction, mainly found in the cortical region, most likely corresponds to the 24,000-dalton γ-crystallin preparation obtained from cattle lens cortices. It is questioned whether the similarities between this fraction and β_S-crystallin are merely coincidental.

INTRODUCTION

Aqueous chromatography based on gel filtration has become popular since the introduction of cross-linked dextran supports in 1959[1] where fractionation is dependent primarily on differences in molecular size. Björk[2,3] was the first to report gel

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filtration of eye lens proteins, applied to γ- and β-crystallin. Agarose-based matrices allowing fractionation of proteins with molecular weights up to several millions enabled Spector *et al.*[4] to characterize distinct macromolecular α-crystallin fractions, including HM-crystallins.

Attempts to design matrices which were more pressure-stable than the soft gel types, in order to reduce analysis times, were unsuccessful until the development of chemically modified macroporous silica supports where the negative surface charge was eliminated and hydrophilic groups were introduced[5]. The first paper reporting fractionation of proteins on TSK GEL SW type columns appeared in 1978[6], and although precise details concerning the structure of this support are not available, these packings exhibit most of the advantageous characteristics possessed by other commercially available matrices[7].

The soluble bovine lens proteins are usually size-fractionated into five crystallin groups: HM-, α-, β_H-, β_L- and γ-crystallin[8], sometimes with peak-doubling for the β_L- and γ-crystallins[9,10]. Subsequent characterization of these lens proteins on the basis of size and/or charge under native or denaturing conditions can be performed by several techniques. Size characterization under native conditions has been achieved using ultracentrifugation, light scattering, electron microscopy and gel permeation chromatography[11–15]. Determinations of subunit composition and their molecular weights were conducted by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis[16]. Isoelectric focusing has been used under native circumstances[17,18], as well as in the presence of 6 *M* urea to determine the subunit composition[16,19].

The usual method of molecular weight determination by gel permeation chromatography using the relative elution volumes obtained in calibration analyses has many drawbacks, such as non-linear calibration plots, variations in flow-rate and differences in molecular shape[20,21]. More reliable molecular weights can be obtained using a molecular weight sensitive detection system including a low-angle laser light scattering (LALLS) photometer equipped with a flow-through accessory[22,23].

In the present paper we describe the one-step fractionation of cortical and nuclear calf lens extracts on TSK GEL G4000 and G3000 SW type columns, revealing superior resolution in the β- and γ-crystallin range. Effluent molecular weight determination was achieved by applying LALLS. For further identification, the subunit compositions of the peak fractions were determined by SDS polyacrylamide gel electrophoresis and isoelectric focusing in the presence of 6 *M* urea.

MATERIALS AND METHODS

High-performance gel permeation chromatography (HPGPC), based on size exclusion, was carried out at room temperature in prepacked columns containing TSK GEL SW, which were connected in sequence of descending pore size; *viz.*, GSWP (precolumn, 10 × 0.75 cm); G4000 SW and 2 × G3000 SW (60 × 0.75 cm each) (Toyo Soda). The mobile phase composed of 0.10 *M* Na_2SO_4 and 0.02 *M* Na_2HPO_4–NaH_2PO_4 at pH 6.9 was passed through a Millipore filter and degassed under vacuum before use. Elution was performed at a constant flow-rate of 0.8 ml/min with pressures near 80 bar (pump: Beckman-Altex Model 100A). Protein detection was done with a differential refractometer (Melz: LCD 201).

The calf lens extracts were prepared as described earlier[24] and diluted with

elution buffer to obtain absorbances at 280 nm (1 cm) of 67 for cortical and 100 for nuclear extracts. The samples with protein concentrations near 35 mg/ml were centrifuged for 10 min at 10,000 × *g* and subsequently applied to the column system using a Valco loop injector equipped with a 200-μl loop.

Molecular weight determination was done with a low-angle laser light scattering (LALLS) photometer (Chromatix: KMX 6). The principles of the instrument, using a He–Ne source (λ = 632.8 nm), were presented by Kaye *et al.*[25] and coupling with HPGPC was described by Ouano and Kaye[22] and Takagi[23]. Between the column-system outlet and the 5-mm stainless-steel flow-through cell we used a filter holder equipped with two 0.22-μm filters (Millipore GS). The cell output was connected with a differential refractive index (DRI) detector thermostatted at 20.00°C. The molecular weights were determined according to the relative method[23] taking bovine thyroglobulin, serum albumin and chymotrypsinogen A (Sigma) as standards. The standard molecular weights and refractive index increments, dn/dc, used were 669,000 and 0.165 ml/g, 68,000 and 0.165 ml/g and 25,900 and 0.170 ml/g, respectively. The dependence of the light scattering intensity on the second virial coefficient was neglected because the effluent protein concentration was below 1 mg/ml; 0.17 ml/g was chosen as the refractive index increment for all lens proteins. Data sampling was performed with a Hewlett-Packard 3353 LAB DATA system using the area slice integration method and post analysis data processing; a list of the BASIC program will soon be available as a Hewlett-Packard "software note". Number average, $\bar{M}_n$, and weight average, $\bar{M}_w$, molecular weights were determined according to Baker[26]

$$\bar{M}_n = \Sigma N_i \cdot M_i / \Sigma N_i = \Sigma \text{DRI-area}_i / \Sigma(\text{DRI-area}_i / M_i)$$
$$\bar{M}_w = \Sigma N_i \cdot (M_i)^2 / \Sigma N_i \cdot M_i = \Sigma(\text{DRI-area}_i \cdot M_i) / \Sigma \text{DRI-area}$$

where N_i = the number of molecules having a molecular weight M_i:

$$M_i = (\text{LALLS-area}_i / \text{DRI-area}_i)\ [\text{calibration constant}/(\mathrm{d}n/\mathrm{d}c)]$$

Pooled fractions were precipitated at 0°C in 10% (w/v) trichloroacetic acid and the precipitates obtained after centrifugation were washed twice with cold ethanol prior to electrophoresis and isoelectric focusing. SDS polyacrylamide gel electrophoresis essentially according to Laemmli[27] was done using a vertical slab gel system (Bio-Rad 200). The thickness of the gels was 1.5 mm and the stacking and separation gels comprised 4% and 13% polyacrylamide, respectively; all reagents were of electrophoresis quality (Bio-Rad). Isoelectric focusing in 6 *M* urea was performed according to van Kleef and Hoenders[19] using 5% polyacrylamide rod gels. Urea (Merck; zur Analyse) was used without further purification and ampholytes (Pharmalyte 5-8) were obtained from Pharmacia.

RESULTS

Cortical and nuclear calf lens proteins were subjected to HPGPC using TSK GEL SW type columns. To obtain a one-step fractionation of the crystallins with molecular weights ranging from several millions to 20,000, it is necessary to use a combination of two different columns: the G4000 SW column which fractionates

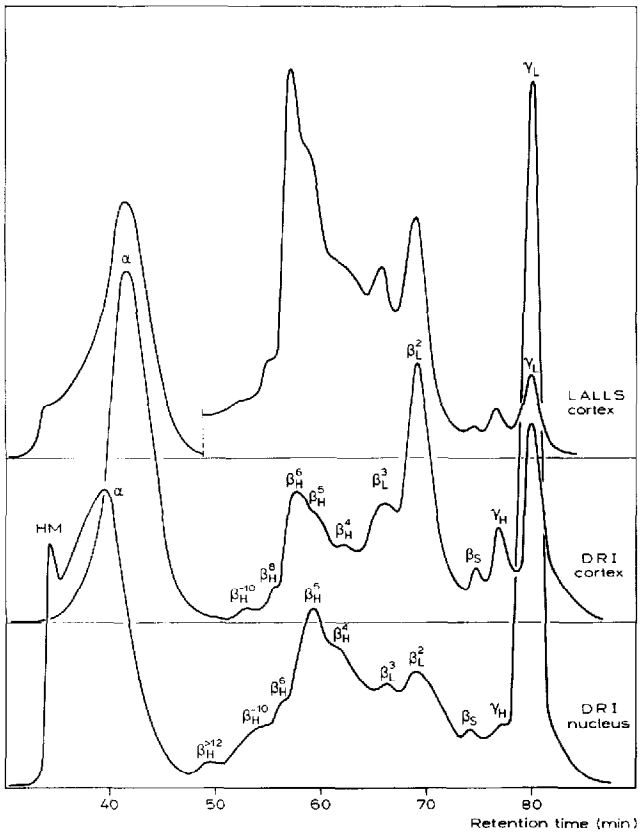

Fig. 1. High-performance gel permeation chromatography of calf cortical and nuclear water-soluble lens proteins. Elution profiles from bottom to top: differential refractive index (DRI) detection of nuclear crystallins; DRI detection of cortical crystallins and low-angle laser light scattering (LALLS) detection of cortical extract (note that the signal in the HM- and α-crystallin region is attenuated by a factor of 20).

globular proteins with molecular weights ranging from $5 \cdot 10^6$–$7 \cdot 10^6$ to 20,000 and the G3000 SW with a fractionation range from 500,000 to 10,000 daltons[6,7,21,28]. Some fine structure in the elution patterns obtained with a combination of these columns was enhanced using a combination of one G4000 SW and two G3000 SW columns (all columns were 60 cm long). The elution patterns are shown in Fig. 1. The upper tracing represents the LALLS signal recorded together with the middle trace (the DRI pattern) for the cortical lens extract elution. The LALLS profile correspons approximately to the product of the eluting crystallin molecular weight and its concentration (DRI signal). The lower tracing shows the DRI detection of the nuclear lens extract elution. Thirteen more or less discrete fractions with eight oligomeric β-crystallins can be discerned. For identification, the molecular weights of the peaks of the fractions were determined using LALLS detection (Table I); additionally, material obtained from pooled peak fractions was subjected to SDS electrophoresis and isoelectric focusing (Figs. 2–4).

TABLE I

MOLECULAR WEIGHTS AND RETENTION TIMES OF THE WATER-SOLUBLE CALF LENS CRYSTALLINS

All molecular weights, determined at the peaks in the HPGPC–LALLS elution patterns, are accurate within 2–5% except where indicated otherwise. Retention times are accurate within 0.2 min.

Crystallin	*Cortex*		*Nucleus*	
	Mol. wt.	*Retention time (min)*	*Mol. wt.*	*Retention time (min)*
HM			$1.2 \cdot 10^7$*	34.9
α	780,000	41.5	1,100,000	39.9
$\beta_H^{>12}$			$>3 \cdot 10^5$*	49.8
$\beta_H^{\sim 10}$	$2 \cdot 10^5$–$3 \cdot 10^5$*	53.0	$2 \cdot 10^5$–$3 \cdot 10^5$*	53.0
β_H^8	200,000**	55.0		
β_H^6	160,000	57.8	165,000**	57.5
β_H^5	130,000**	60.2	135,000	60.0
β_H^4	100,000	62.5	110,000	62.4
β_L^3	80,000	65.9	80,000	66.1
β_L^2	50,000	69.9	50,000	69.9
β_S	28,000	74.4	28,000**	74.2
γ_H	23,000**	76.6		
γ_L	20,000	80.6	20,000	80.6

* Accuracy >10%.
** Accuracy 5–10%.

Size characterization of HPGPC–fractionated crystallins

The peak (Fig. 1) containing totally excluded protein aggregates with molecular weights larger than $5 \cdot 10^6$–$7 \cdot 10^6$ and virtually absent in the cortical extract elution pattern is called HM-crystallin. This designation, instead of polymeric α- or α_H-crystallin[24,29], is chosen because calf lens HM-crystallin definitely contains minor amounts of other lens proteins[30]; the non-α-crystallin constituents in the corresponding fraction isolated from other species are more abundant[18,31,32]. The value for the HM-crystallin molecular weight (Table I) is quite arbitrary because it represents the weight average molecular weight of a totally excluded fraction. It depends both on the exclusion limit of the column system and on the lens extract preparation procedure where the effectiveness of homogenization and the rate of subsequent centrifugation affect the HM-crystallin molecular weight distribution in solution.

The second peak represents α-crystallin and the Gaussian peak shape, especially in the pattern obtained from the cortical extract, must be attributed to the size heterogeneity of this protein, consisting of aggregates built up from 30–50 20,000-dalton subunits[33]. It must be emphasized that, with a polydisperse system, the molecular weight of the material eluting in the peak of the concentration profile lies between the weight average and the number average molecular weights[34]. In the case of the calf cortical extract elution it is possible to determine these parameters describing the α-crystallin molecular weight distribution; summation is performed for retention time 36 to 47 min. The weight average and number average molecular weights, $\bar{M}_w$ and $\bar{M}_n$, amount to 860,000 and 740,000, respectively, revealing a value of 1.16 for the polydispersity factor, $\bar{M}_w/\bar{M}_n$. Due to an enormous tailing of the HM-crystallin peak

obtained by LALLS detection for nuclear calf lens extract (pattern not shown), it is not feasible to determine $\bar{M}_n$ and $\bar{M}_w$ values for nuclear α-crystallins unless further purification (centrifugation) or application on other column systems is performed. Our $\bar{M}_w$ value for cortical α-crystallin is in excellent agreement with those obtained by Siezen and Berger[35] using various physico-chemical methods. The value of $1.1 \cdot 10^6$ found for nuclear α-crystallin at the peak of the DRI pattern is a good approximation and is in accordance with the values determined for isolated nuclear calf lens α-crystallin using static LALLS[36] and with sedimentation-based calculations[24].

All fractions eluting between 48 and 75 min after sample application are designated as β-crystallins. The previously found β-cyrstallin peaks (β_H-, β_L^3-, β_L^2- and the monomeric β_S-crystallin) using Sephadex G-200[9], G-75[10,37] or Sephacryl S200[38,39] are satisfactorily resolved. Moreover, for the first time, additional β-crystallin size-fractions can be observed (Fig. 1). The recently introduced nomenclature for β_L-crystallin[39], where a numerical superscript indicates the number of subunits, is also applied to the different β_H-crystallin fractions using the data presented in Table I and taking a value of 26,000 as the averaged subunit molecuar weight. It appears that, in addition to the preferential oligomeric structures for bovine β-crystallins, other possible architectures also occur. On comparing the elution profiles of nucleus and cortex, the differences in retention times for the predominant β_H-crystallin fractions

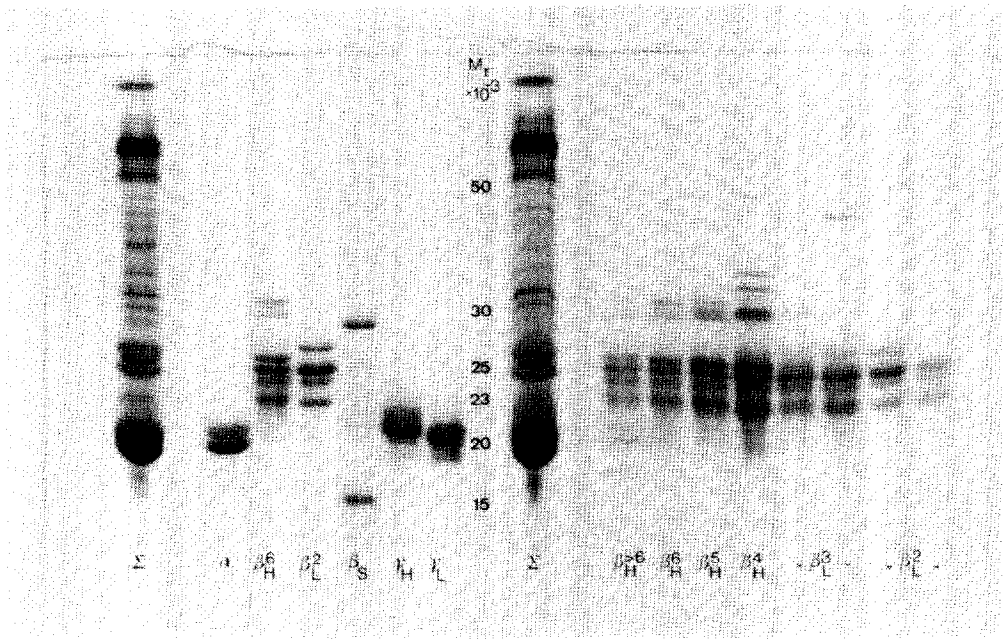

Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of total and HPGPC-fractionated cortical crystallins. Fractions applied and their retention times in HPGPC correspond to Fig. 1. From left to right: total crystallins (Σ), α-crystallin (38.0–45.0 min); β_H^6-crystallin (56.8–58.8 min); β_L^2-crystallin (67.2–70.5 min); β_S-crystallin (73.4–75.4 min); γ_H-crystallin (75.6-776. min); γ_L-crystallin (78.6–82.6 min); total crystallins; $\beta_H^{\geq 6}$-crystallin (52.0–56.5 min); β_H^6-crystallin, β_H^5-crystallin (59.0–61.5 min); β_H^4-crystallin (61.5–63.5 min); β_L^3-crystallin (64.0-66.0 min); β_L^3-crystallin (66.0–67.2 min); β_L^2-crystallin (70.5–73 min).

are remarkable. The most abundant nuclear β_H-crystallin aggregates have a pentameric structure, while in the cortical extract the molecules eluting in the peak fraction are built up from six subunits. The differences which can be observed in the β_H-/β_L-crystallin ratio for cortex and nucleus are in accordance with the results of van Kamp and Hoenders[8].

The three monomeric crystallins found on HPGPC are designated β_S-, γ_H- and γ_L-crystallin. The molecular weight value of 28,000 obtained under native conditions and by SDS electrophoresis (Figs. 2 and 4) is a convincing argument that our β_S-crystallin fraction is identical to the one isolated by Van Dam[40]. Two peaks are found for the cortical γ-crystallins with clearly different molecular weights, *viz.* 23,000 and 20,000. The 23,000-daltons fraction corresponds most probably with the 24,000-dalton cattle γ-crystallin fraction isolated by Kabasawa *et al.*[10]; because we obtained this fraction from calf lenses, the designation of γ_H-crystallin, used by the same authors for the corresponding human fraction[41], seems more appropriate. The observation that the highest content of the 20,000-dalton γ_L-crystallin is found in the nuclear part of calf lenses agrees with earlier results[8].

Electrophoretic characterization of HPGPC-fractionated crystallins

To assess the application of HPGPC for semi-preparative purposes, pooled fractions obtained from several analyses were subjected to SDS polyacrylamide gel electrophoresis (Figs. 2 and 4a) and isoelectric focusing in the presence of 6*M* urea (Figs. 3 and 4b). It is remarkable that in the total cortical crystallin preparation high-molecular-weight bands, absent in any of the crystallin fractions, can be seen (Fig. 2). Most likely we are dealing with "water-insoluble" lens proteins since possible oligomerization by sulphydryl oxidation can practically be ruled out because an excess of reducing agent was present in our sample buffer. The presence of only two bands in the α-crystallin lane implies that no degradations occurred during the HPGPC fractionation and subsequent manipulations. The patterns of the β_H^6-, β_L^2- and γ_L-crystallin fractions are similar to those presented earlier[39]. The relative mobilities of the major band in β_S-, γ_H- and γ_L-crystallin, corresponding to molecular weights of 29,000, 22,000 and 20,000, agree fairly well with the data of Kabasawa *et al.*[10]. The minor band, seen in the β_S-crystallin lane, and migrating as a 15,000-dalton polypeptide, is marked. It might correspond to half β_S-crystallin molecules, assuming for this protein a two-domain structure as found for γ_L-crystallin and the βB_P-subunit[42,43]. Co-elution of FM-crystallin with β_S-crystallin would also be conceivable. This minor fraction with "acidic" mobility on electrophoresis and composed of 14,500-dalton polypeptides[44] was found to elute on Sephadex G-75 between β_L- and γ-crystallin[37]. The acidic band observed in Fig. 3a corresponds to the established electrophoretic mobility of FM-crystallin.

The right-hand part of Fig. 2 shows the electrophoretic patterns of the several oligomeric β-crystallins. It can be seen that only the cortical β_L-crystallins are lacking the 30,000–34,000 dalton bands; in the intermediate β_H-crystallin fractions several bands in addition to the βB_{1a}- and βB_{1b}-crystallin subunits[39] are observed in this area. Remarkable is the presence of 50,000- and 44,000-dalton bands in the β_H^4-crystallin and the trailing edge of the β_L^3-crystallin fraction, respectively. Probably these bands represent the water-soluble cytoskeletal proteins vimentin and actin[45] which co-migrate with the β-crystallins on size-exclusion. It is uncertain whether these proteins

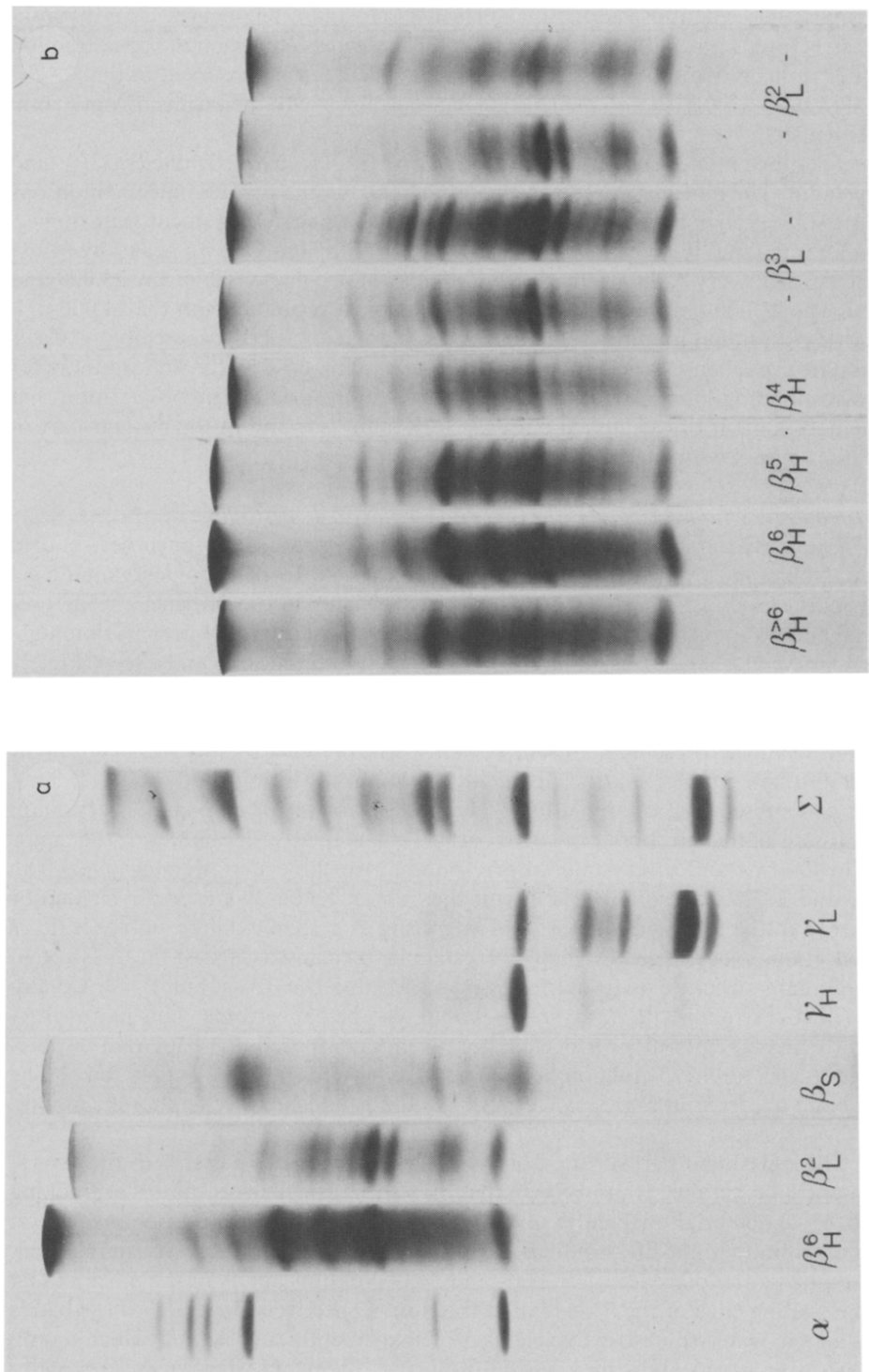

Fig. 3. Isoelectric focusing in polyacrylamide gels (pH range 5–8, top to bottom) in the presence of 6 *M* urea of total and HPGPC-fractionated cortical crystallins. Fractions correspond to those in Fig. 2.

have oligomeric structures since they elute together with 100,000- and 75,000-dalton crystallin aggregates or they exhibit an asymmetrical monomeric structure in solution.

Fig. 3 shows the calf cortical crystallin subunit compositions, obtained by isoelectric focusing in the presence of 6 *M* urea. The patterns for the β-crystallins are similar to those obtained before[39], albeit that the basic βB_1-crystallin subunits (30,000–34,000 dalton bands in Fig. 2) did not focus this time; most likely this is due to aggregation which prevents them entering the gels. The relatively pure β_S-crystallin fraction as judged from Fig. 2 appears to complex on isoelectric focusing. The acidic band might arise from FM-crystallin and the basic band from a contamination with γ_H-crystallin, thus leaving the middle band to correspond with β_S-crystallin. γ_H-Crystallin appears to be rather pure; in γ_L-crystallin additional bands can be observed.

In Fig. 4 the polypeptide compositions of the nuclear crystallins as obtained by SDS electrophoresis and isoelectric focusing under dissociating conditions are presented. As found earlier[4,19,24], the subunit compositions of HM- and α-crystallin are very similar. The low-molecular-weight bands in Fig. 4a, absent in the cortical α-crystallin patterns (Fig. 2), originate from C-terminally degraded polypeptide chains. The several nuclear β-crystallin subunit compositions analyzed by both methods all look alike and therefore only the predominant fractions are shown. The finding that there is much less difference in subunit composition between β_H- and β_L-crystallin in the calf lens nucleus than in the cortex is remarkable. On thorough examination of Fig. 4a some bands migrating in the molecular weight range of cytoskeletal proteins are seen in the β_L^2-crystallin fraction; moreover, the βB_1-crystallin-like bands (30,000–34,000 daltons) are present in the β_H^5- as well as in the β_L^2-, α- and HM-crystallin fractions. In the β_S-crystallin fraction only a very faint 29,000-dalton band can be found due to strong tailing of the β_L^2-crystallin fraction; the 15,000-dalton band, present in the cortical β_S-crystallin lane (Fig. 2), can also be detected. Although the γ_H- and γ_L-crystallin fractions show similar patterns on SDS electrophoresis, differences can be seen on the isoelectric focusing gels. It seems that the least basic bands (Fig. 4b) correspond with cortical γ_H-crystallin (Fig. 3) and that the major basic band is identical with the most intense band observed in cortical γ_L-crystallin (Fig. 3).

DISCUSSION

It is shown that HPGPC with TSK GEL SW type columns is an excellent method for analytical and semi-preparative fractionation of lens proteins. Molecular weight determinations were performed using a flow-through molecular weight-dependent detection system including a LALLS photometer and a differential refractometer[22,23]. Direct HPGPC molecular weight determination by LALLS has advantages over the usual method using relative retention times from known proteins obtained in separate analyses. The calibration curves generally presented are constructed by plotting the logarithm of the molecular weight *versus* the elution volume and are linear over a reasonable range[46]. However, assuming that the gel network is made up of rigid cylindrical rods[47] or describing a statistical function based on a random distribution of penetrable volume elements[48] leads to a more linear relationship between functions of the molecular (Stokes) radius and the distribution coefficient. Several other, more or less different approaches to obtain linear calibration

curves have been reported but, due to the non-ideality of the system, none of them is clearly advantageous over the others. Although calibration curves for high-performance size exclusion systems are mostly constructed by the logarithmic treatment, Himmel and Squire[21] obtained better linear correlation by plotting a function of the elution volume against $\sqrt[3]{M_r \bar{v}}$. The accuracy of such molecular-weight determinations, expressed as the standard deviation, was 14% which is slightly better than obtained in the logarithmic method (16%). Structural factors, such as variations in shape, hydration and partial specific volume of the calibration and unknown proteins, as well as chromatographic ones (adsorption, ion-exclusion, flow-rate variations), may all contribute to the errors in calculating molecular weights from calibrated columns[7,20,21]. Takagi[23] was the first to report protein molecular weights obtained by the HPGPC–LALLS method using a TSK GEL G3000 SW column. The accuracy can be within 1% when the exact differential refractive index increment, dn/dc, and the second virial coefficient are known. Because of small variations in dn/dc for proteins and the negligible concentration dependence of the scattering intensity at low protein concentrations, accuracies between 2 and 5% are routinely obtained without further parameters.

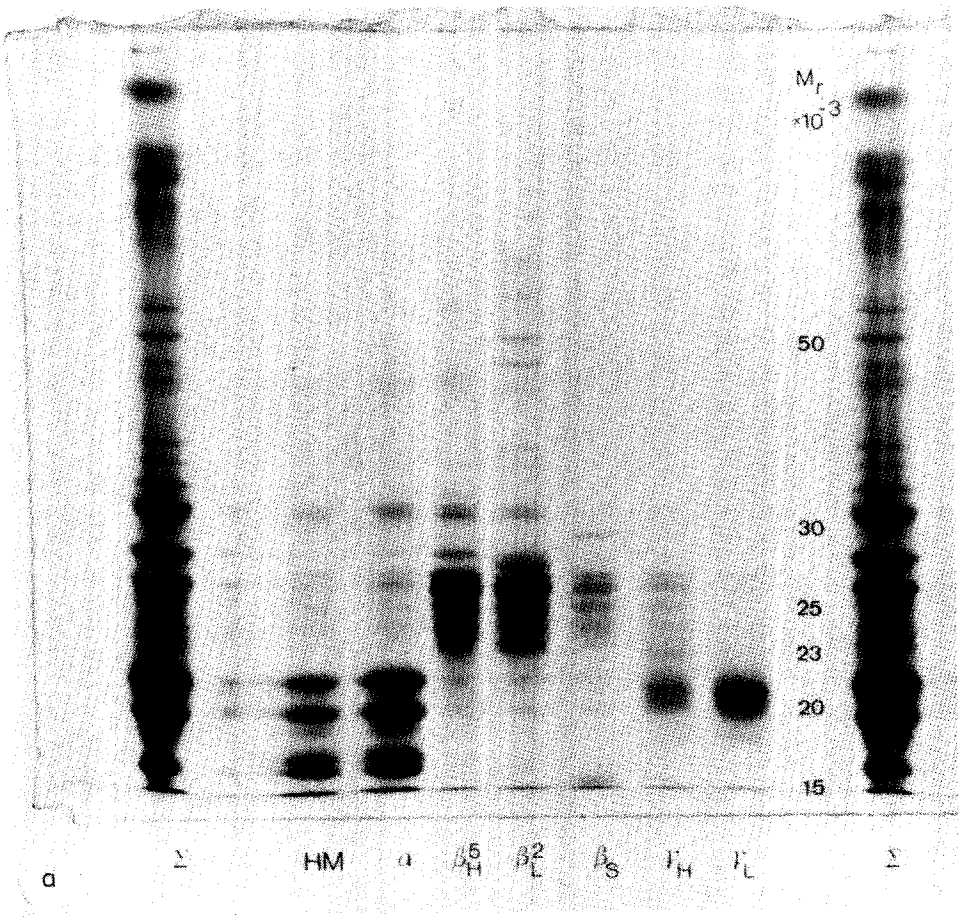

Fig. 4.

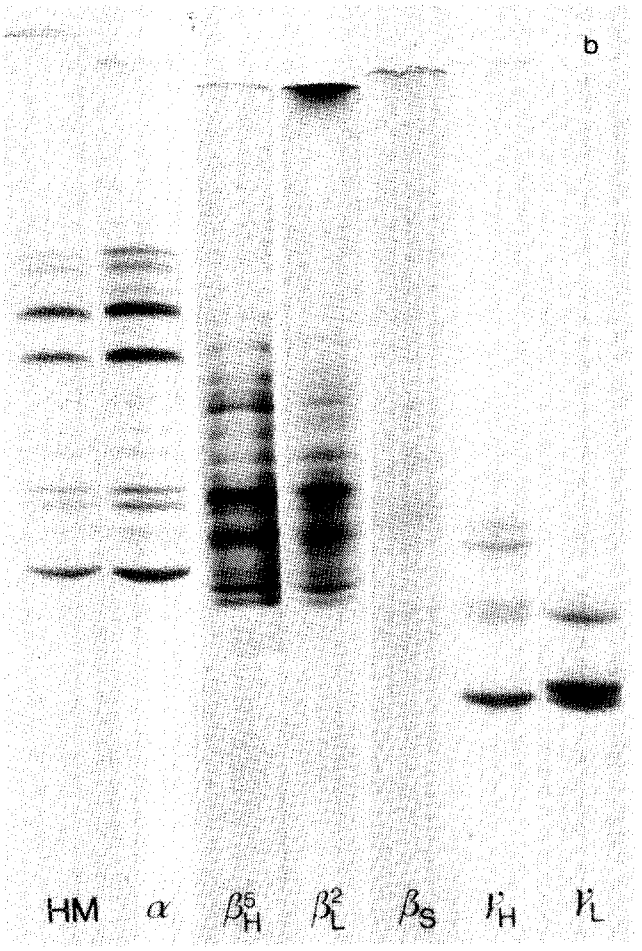

Fig. 4. SDS electrophoresis (a) and isoelectric focusing (pH range 5–8, top to bottom) in urea (b) of total and HPGPC-fractionated nuclear crystallins. Fractions applied and their retention times, corresponding to Fig. 1, were: total crystallins (Σ), HM-crystallin (34.0–35.5 min); α-crystallin (36.5–43.0 min); β_H^5-crystallin (59.0–61.5 min); β_L^2-crystallin (68.0–72.0 min); β_S-crystallin (73.5–75.0 min); γ_H-crystallin (75.5–77.0 min) and γ_L-crystallin (78.6–82.6 min).

The resolution of proteins in high-performance liquid chromatography can be improved upon by lowering the elution rate[49]; for TSK GEL SW HPGPC this was demonstrated by Sjödahl[50]. An elution rate of 0.08 ml/min revealed virtually the same resolution in the β- and γ-crystallin range with only one TSK GEL G3000 SW column (chromatogram not shown). However, we use high flow-rates enabling the performance of numerous analyses each day. The fractionation of lens proteins on TSK GEL G3000 SW at very low flow-rate and pressure (4 bar), revealing similar resolution for the β-crystallins compared with the one obtained at high flow-rate (Fig. 1), argues against pressure-induced aggregation–dissociation effects.

It is interesting to speculate whether the differently sized oligomeric β-crystallins are present in the lens "*in vivo*". In a previous paper[39] we compiled several factors

which determine association–dissociation equilibria of β_H- and β_L-crystallins, such as protein concentration, temperature, medium density, ionic strength and pH. Although it is not possible to judge which factors will predominate, better insight into these reactions may enable one to do so in future investigations.

On comparing cortical and nuclear calf lens crystallins, it can be inferred that the older (nuclear) proteins are more heterogeneous with respect to size (Fig. 1) and charge (Figs. 2 and 4b). Therefore, the analysis of fractions separated by HPGPC or any other technique is hampered by interference and overlap with other fractions. For instance, the overlap of β_S- with β_L-crystallin cannot be diminished by improving the resolution. The presence of mainly βB_1-crystallin-like subunits in both α- and HM-crystallin is another complication; perhaps some very large β-crystallin aggregates co-elute with α-crystallin. In the case of HM-crystallin it is unclear whether the β-crystallins are present as separate high-molecular-weight aggregates or whether they are part of mixed protein clusters[51].

In our previous report on bovine β-crystallins[39] we had several reasons to assign the fraction eluting on Sephacryl S200 between β_L^2- and γ-crystallin to β_S-crystallin; only the mobility on SDS electrophoresis, corresponding to a molecular weight of 23,000, puzzled us. However, in the light of the present results, where β_S-crystallin can be found next to γ_H-crystallin, this assignment would appear to be incorrect. The designation of γ_H-crystallin by Kabasawa *et al.*[10] was formerly not generally accepted due to its close resemblance with β_S-crystallin[52]. In this respect, we as well as Bloemendal and Zweers[37] attributed the 22,000 band to β_S-crystallin. Nevertheless, because of several similarities, it is hard to consider both fractions to be part of different crystallin classes. A thorough structural study of both fractions may end this confusion and will, perhaps, result in a better nomenclature.

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REFERENCES

1 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657–1659.
2 I. Björk, *Exp. Eye Res.*, 1 (1961) 145–154.
3 I. Björk, *Exp. Eye Res.*, 3 (1964) 248–253.
4 A. Spector, L.-K. Li, R. C. Augusteyn, A. Schneider and T. Freund, *Biochem. J.*, 124 (1971) 337–343.
5 F. E. Reigner and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1–25.
6 K. Fukano, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 166 (1978) 47–54.
7 E. Pfannkoch, K. C. Lu, F. E. Reigner and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430–441.
8 G. J. van Kamp and H. J. Hoenders, *Exp. Eye Res.*, 17 (1973) 417–426.
9 F. A. M. Asselbergs, M. Koopmans, W. J. van Venrooij and H. Bloemendal, *Exp. Eye Res.*, 28 (1979) 223–228.
10 I. Kabasawa, Y. Tsunematsu, G. W. Barber and J. H. Kinoshita, *Exp. Eye Res.*, 24 (1977) 437–448.
11 W. F. Bon, *Proc. K. Acad. Wet. Amsterdam*, 58c (1955) 344–355.

12 J. Francois and M. Rabaey, *AMA Arch. Ophthalmol.*, 53 (1955) 481–486.
13 H. Bloemendal, *Exp. Eye Res.*, 1 (1962) 300–305.
14 I. Björk, *Exp. Eye Res.*, 3 (1964) 254–261.
15 A. F. van Dam, *Thesis*, University of Nijmegen, 1967, pp. 49–86.
16 P. Herbrink and H. Bloemendal, *Biochim. Biophys. Acta*, 336 (1974) 370–382.
17 J. Bours, *J. Chromatogr.*, 60 (1971) 225–233.
18 K. N. Liem-The and H. J. Hoenders, *Exp. Eye Res.*, 18 (1974) 143–152.
19 F. S. M. van Kleef and H. J. Hoenders, *Eur. J. Biochem.*, 40 (1973) 549–554.
20 D. D. Bly, H. J. Stoklosa, J. J. Kirkland and W. W. Yau, *Anal. Chem.*, 47 (1975) 1810–1813.
21 M. E. Himmel and P. G. Squire, *Int. J. Pept. Protein Res.*, 17 (1981) 365–373.
22 A. C. Ouano and W. Kaye, *J. Polym. Sci. Part A-1*, 12 (1974) 1151–1162.
23 T. Takagi, *J. Biochem. (Tokyo)*, 89 (1981) 363–368.
24 R. J. Siezen, J. G. Bindels and H. J. Hoenders, *Exp. Eye Res.*, 28 (1979) 551–567.
25 W. Kaye, A. J. Havlik and J. B. McDaniel, *J. Polym. Sci., Polymer Lett. Ed.*, 9 (1971) 695–699.
26 D. R. Baker, *Application Note AN 232-10*, Hewlett-Packard, Palo Alto, CA, 1979.
27 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
28 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297–304.
29 J. Bours, A. Wieck and O. Hockwin, *Interdiscip. Top. Gerontol.*, 12 (1978) 205–220.
30 J. G. Bindels, B. M. de Man and H. J. Hoenders, in preparation.
31 P. J. Ringens, K. N. Liem-The, H. J. Hoenders and J. Wollensak, *Interdiscipl. Top. Gerontol.*, 13 (1978) 193–211.
32 L. H. Cohen, L. W. Westerhuis, W. W. de Jong and H. Bloemendal, *Eur. J. Biochem.*, 89 (1978) 259–266.
33 R. J. Siezen, J. G. Bindels and H. J. Hoenders, *Eur. J. Biochem.*, 91 (1978) 387–396.
34 H. L. Berger and A. R. Schultz, *J. Polym. Sci., Part A*, 2 (1965) 3643–3648.
35 R. J. Siezen and H. Berger, *Eur. J. Biochem.*, 91 (1978) 397–405.
36 J. G. Bindels, unpublished results.
37 H. Bloemendal and A. Zweers, *Doc. Ophthalmol. Proc. Ser.*, 8 (1976) 91–104.
38 J. A. Jedziniak, H. Baram and L. T. Chylack, Jr., *Exp. Eye Res.*, 26 (1978) 377–388.
39 J. G. Bindels, A. Koppers and H. J. Hoenders, *Exp. Eye Res.*, 33 (1981) 333–343.
40 A. F. M. van Dam, *Exp. Eye Res.*, 5 (1966) 255–266.
41 I. Kabasawa, G. W. Barber and J. H. Kinoshita, *Jap. J. Ophthalmol.*, 21 (1977) 87–97.
42 T. Blundell, P. Lindley, L. Miller, D. Moss, C. Slingsby, I. Tickle, B. Turnell and G. Wistow, *Nature (London)*, 289 (1981) 771–777.
43 G. Wistow, C. Slinbsby, T. Blundell, H. Driessen, W. de Jong and H. Bloemendal, *FEBS Lett.*, 133 (1981) 9–16.
44 W. G. M. van den Broek, J. N. Leget and H. Bloemendal, *Biochim. Biophys. Acta*, 310 (1973) 278–282.
45 F. C. S. Ramaekers and H. Bloemendal, in H. Bloemendal (Editor), *Molecular and Cellular Biology of the Eye Lens*, Wiley, New York, 1981, pp. 85–136.
46 P. Andrews, *Biochem. J.*, 96 (1965) 595–606.
47 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317–330.
48 G. K. Ackers, *J. Biol. Chem.*, 242 (1967) 3237–3238.
49 B. N. Jones, R. V. Lewis, S. Pääbo, K. Kojima, S. Kimura and S. Stein, *J. Liquid Chromatogr.*, 3 (1980) 1373–1383.
50 J. Sjödahl, *Sci. Tools*, 27 (1980) 54–56.
51 K. N. Liem-The, A. L. H. Stols and H. J. Hoenders, *Exp. Eye Res.*, 20 (1975) 307–316.
52 J. J. Harding and K. J. Dilley, *Exp. Eye Res.*, 22 (1976) 1–73.