Volume 232, number 1, 39–45 FEB 05803 May 1988

Simultaneous separation of all lens crystallin subunits by chromatofocusing

P. Body* and H. Bloemendal

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands

Received 2 February 1988; revised version received 4 March 1988

The total population of water-soluble lens crystallin subunits has been separated in one run on a single chromatofocusing column. Dissociation of the polymeric α- and β-crystallin subunits occurred simultaneously on the column. Two components not described previously in the literature have been found in the γ-crystallin region.

Lens protein subunit; Chromatofocusing; Crystallin

1. INTRODUCTION

Vertebrate eye lens tissue has been recognized as a most useful tool for the study of fundamental biological processes such as development and growth [1–3]. This system has also been the subject of investigations involving evolutionary changes [4] and cataractogenesis [5]. In all of these processes the lenticular structural proteins, named crystallins, play major roles. Since lens cells, in contrast to other cell species, do not undergo cell death and virtually no proteolytic breakdown of the crystallins occurs during the life span of an animal, postsynthetic changes in protein structure are easily recognizable and may, in most cases, be due to aging [6]. Hence also in this respect the eye lens is an excellent model system. The most common procedure for the fractionation of crystallins is gel filtration, a method yielding four major fractions α-, β_H-, β_L- and γ-crystallins with molecular masses of approx. 800, 150, 50 and 20 kDa, respectively. In previous reports in the literature the individual fractions after gel filtration served as starting material for further separation into the composite subunits. Since with fractionation of the total water-soluble lens extract by several procedures, including gel filtration, losses of part of the original material are unavoidable we wondered whether it would be possible to circumvent the drawback of cut off and prolonged dialysis of separated fractions. We have found that chromatofocusing allows separation and dissociation into subunits on one and the same column provided an adequate choice of elution buffers with and without urea is made.

Correspondence address: H. Bloemendal, Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands

* *Permanent address:* Biochemical Division, Reanal Factory of Laboratory Chemicals, PO Box 54, 1441 Budapest 70, Hungary

2. MATERIALS AND METHODS

Decapsulated frozen calf eye lens cortices were dissolved in 25 mM ice-cold Tris-HCl buffer, pH 8.6, by gentle stirring for 70 min. A small part of the supernatant (8 ml containing about 500 mg protein), obtained after centrifugation at 15000 × *g* for 20 min, was dialyzed against the same buffer and applied to the column immediately after a second centrifugation at 25000 × *g* for 30 min. The remainder of the supernatant of the first centrifugation was dialyzed against distilled water, lyophilized and stored at −20°C. This part served also for the determination of the concentration of the sample.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

The ion-exchange resin PBE 94 and polybuffers 96 and 74 were obtained from Pharmacia (Uppsala, Sweden).

The resin was equilibrated in the above mentioned Tris-HCl buffer at 20°C. The column dimensions were 1.0 i.d. × 100 cm and the bed height after packing was 95 cm.

Buffers I and II were polybuffer 96 diluted 1:14, without and supplemented with 6 M urea, respectively, and adjusted with HCl to a final pH of 7.4. Buffer III was a mixture, with a composition of 10% polybuffer 96 and 90% polybuffer 74, diluted 1:10, containing 6 M urea and adjusted with HCl to a final pH of 5.0. All buffers were degassed before use.

The sample, in the case lyophilized lens extract was taken, was dissolved in the buffer used for the preparation. The final concentration was 100 mg/ml. Chromatofocusing was started by first running on 5 ml buffer I immediately followed by the sample. Thereafter elution was carried out at a flow rate of 13 ml/h. Fractions of 3.25 ml were collected and those containing protein were dialyzed against distilled water and lyophilized.

Analytical electrophoresis was carried out in 10% polyacrylamide gels containing 6 M urea, as described previously by Bloemendal [7], and in SDS slab gels containing 13% polyacrylamide as reported by Laemmli [8]. Both techniques were carried out on mini gels, allowing rapid analysis.

Amino acid analyses were carried out in an LKB 4541 Alpha Plus analyzer.

3. RESULTS

Details of the fractionation procedure have been described in section 2.

The use of either freshly made or lyophilized lens extract did not result in any difference in the shape of the elution curve. In the experiment shown, 400 mg of freshly prepared lens extract was applied to the column (fig.1). Sample amounts up to 750 mg did not result in decreased resolution.

Since α-, β_H- and β_L-crystallins are multimeric proteins, whereas the γ-crystallins and β_S-crystallin are a population of monomeric 20 kDa proteins, we started equilibration of the column and the first elution in the absence of 6 M urea. Fortunately, γ-crystallins have pI values between 8 and 7.2, high enough above the pI values of the α- and β-crystallin aggregates to bind the latter proteins to the column.

The equilibration pH was chosen 0.2 units higher than that used in a previous separation of the subunits of γ-crystallins [9]. This modification resulted in an improved separation in the first part of the chromatographic pattern and in the appearance of a new γ peak (I/6), as a shoulder in front of peak I/7 (fig.1).

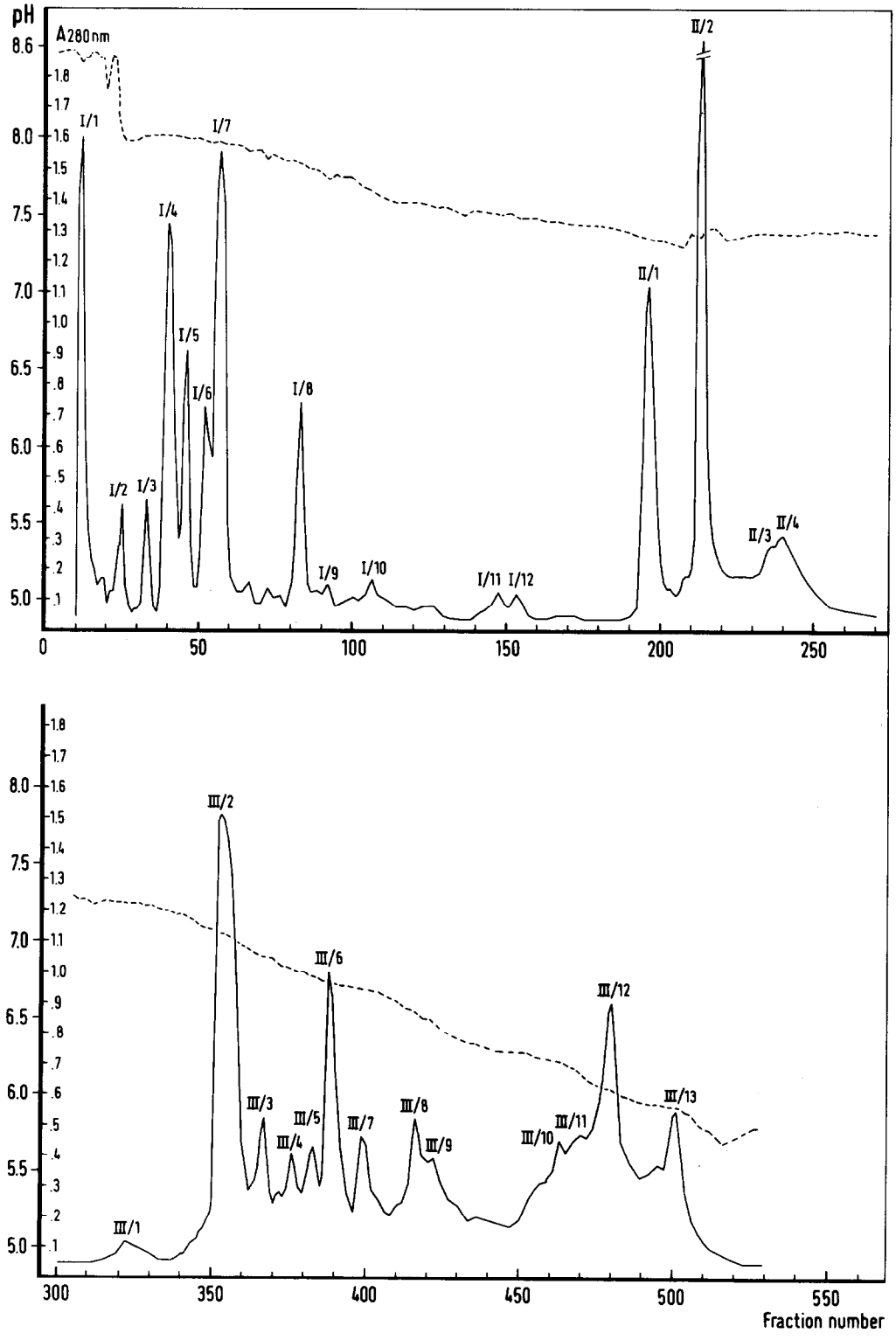
After the last γ-component emerged from the column we added urea to the elution buffer to a final concentration of 6 M and adjusted the pH again to 7.4. This manipulation served two aims. Firstly, the α- and β-crystallins become dissociated into subunits and components with an equal or higher pI than 7.4 (αB2, βs) emerge from the column (cf. fig.2A and B fractions II/1 and II/4).

A third buffer change again in 6 M urea, but now at a final pH of 5.0, yielded residual β-crystallins and the αA-crystallin subunits.

To characterize further the subunits obtained we analyzed them by electrophoresis on minislab gels, a method which is much less time-consuming than the normal procedure. Electrophoresis was carried out in parallel on minipolyacrylamide slabs containing 6 M urea (fig.2A) versus 0.1% SDS (fig.2B). Most fractions are highly enriched in a particular component. Only in the case that a subunit from similar or different crystallins had both identical isoelectric points and molecular mass would it escape detection. However, small differences in electrophoretic mobility will be apparent after the dual electrophoretic analysis.

The amino acid compositions of each of the highly enriched protein fractions have been determined and are shown in table 1. Some of them have been found to be similar to values reported in the literature for components isolated by other methods. This is indicated by a reference number in the last row of table 1. Also, we detected two hitherto unknown crystallin fractions (cf. table 1 fractions I/2 and I/11) at pI values 7.9 and 7.5 and with molecular masses of 28 and 24 kDa, respectively. A computer search for possible homologous sequences was unsuccessful. Their identification awaits further investigation.

Fig.1. Chromatofocusing of the total water-soluble lens protein population. Absorbance at 280 nm (——); pH gradient (– – –). Numerals I, II and III correspond to the different buffers used for elution (see section 2). Buffer II started at fraction 170, buffer III at fraction 270.


pH
A280nm
I/1
I/2
I/3
I/4
I/5
I/6
I/7
I/8
I/9
I/10
I/11
I/12
II/1
II/2
II/3
II/4
III/1
III/2
III/3
III/4
III/5
III/6
III/7
III/8
III/9
III/10
III/11
III/12
III/13
Fraction number

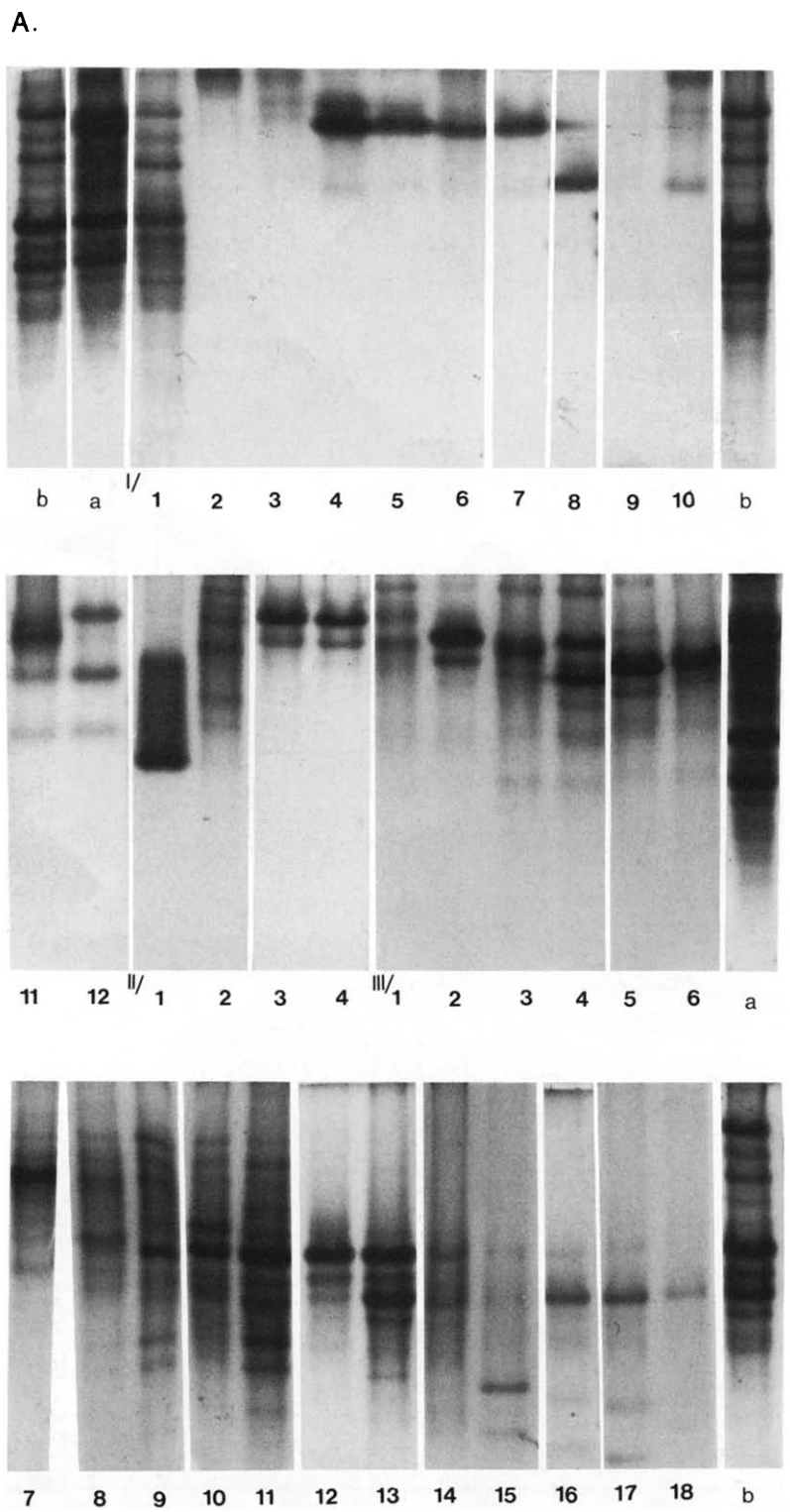
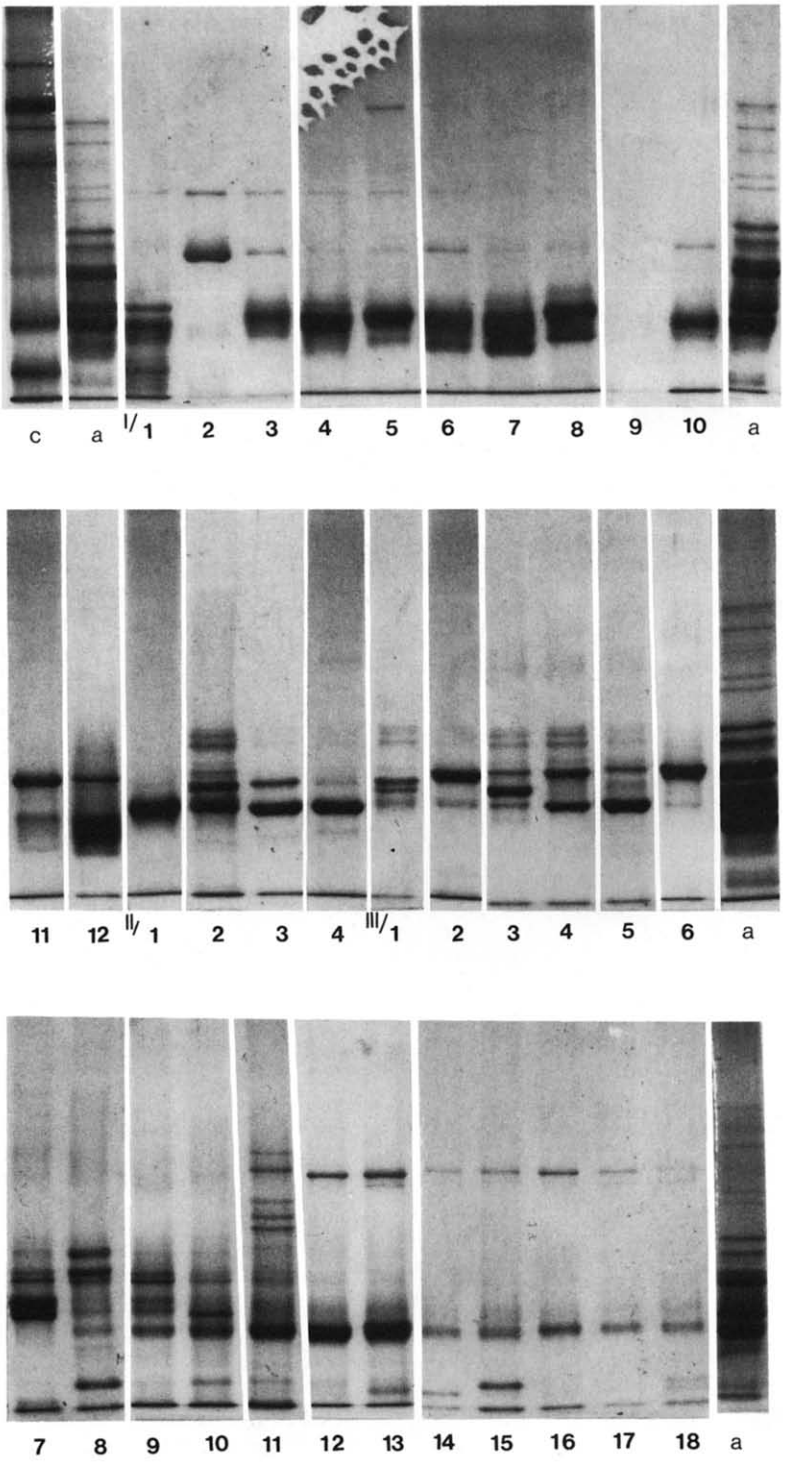


Fig.2. (A) Polyacrylamide gel electrophoresis in 6 M urea of samples taken from the peak fractions shown in fig.1. (a) Starting material (total lens extract). (b) α-Crystallin for comparison. (B) SDS-gel electrophoretic pattern of samples taken from the peak fractions

B.



shown in fig.1. (a) Starting material (total lens extract). (c) Marker proteins (93 kDa, phosphorylase *b*; 68 kDa, bovine serum albumin; 54 kDa, leucine aminopeptidase; 45 kDa, ovalbumin; 20 kDa, αA2-crystallin; 17 kDa, myoglobin; 12.4 kDa, cytochrome *c*).

Table 1

Amino acid composition of total lens extract protein fractions separated by chromatofocusing, shown in fig.1

	Peak						
	I/2	I/4	I/5	I/6	I/7	I/8	I/11
Asp	10.8	11.3	10.1	10.9	11.1	10.5	10.7
Thr	4.6	1.4	2.9	2.4	2.9	2.2	2.8
Ser	7.2	6.9	7.0	7.2	7.0	8.3	5.9
Glu	9.5	13.2	12.8	13.0	12.5	14.0	11.0
Pro	7.5	4.7	4.1	4.6	7.4	4.4	5.6
Gly	7.9	9.4	8.8	8.2	8.3	8.3	8.0
Ala	7.7	2.2	2.4	2.6	1.5	2.4	5.0
Cys	1.0	1.1	1.2	2.2	0.4	1.4	0.9
Val	4.6	3.6	3.7	4.3	3.6	4.0	4.0
Met	1.1	2.1	2.7	3.2	4.1	2.4	3.4
Ile	4.2	4.7	4.3	4.1	3.5	4.4	5.5
Leu	9.0	9.4	7.3	9.0	7.7	6.7	11.1
Tyr	3.8	8.8	8.3	7.2	8.4	7.9	4.6
Phe	4.2	3.3	5.8	4.8	5.5	5.4	5.8
His	4.5	4.1	4.8	2.6	2.9	4.4	2.1
Lys	8.1	1.6	1.1	1.9	1.4	1.0	7.0
Arg	4.4	12.3	12.8	11.9	11.7	12.1	6.7
Sub.		γ1	γ2		γ3	γ4	
Ref.		[9]	[9]		[9]	[9]	

	Peak						
	II/1	II/4	III/2	III/5	III/6	III/12	III/13
Asp	9.4	7.7	9.3	7.9	8.8	9.1	9.2
Thr	2.9	4.0	3.6	3.6	2.3	2.9	2.9
Ser	6.2	9.0	8.3	8.6	7.5	11.2	11.2
Glu	14.1	11.2	17.2	12.3	15.6	11.4	11.7
Pro	4.4	9.5	7.3	8.9	5.9	6.6	6.8
Gly	8.6	5.5	9.6	6.0	9.8	6.2	6.6
Ala	5.5	5.8	4.2	5.3	6.1	3.8	4.0
Cys	1.6	n.d.	0.7	n.d.	0.5	0.6	0.6
Val	4.3	5.7	6.4	5.5	5.8	5.2	5.3
Met	3.5	1.1	1.2	1.2	1.3	1.3	1.2
Ile	3.7	5.0	2.9	4.8	2.8	4.5	4.4
Leu	6.0	8.3	5.1	7.9	7.2	7.5	7.8
Tyr	6.7	1.6	4.4	1.8	4.1	3.5	3.4
Phe	6.0	7.2	4.2	6.8	4.7	7.6	7.9
His	4.1	4.8	4.0	4.8	4.7	4.3	4.1
Lys	5.5	5.7	6.5	5.9	4.9	4.5	4.5
Arg	7.7	7.9	5.1	8.7	8.1	9.9	8.3
Sub.	β_S	αB2	βB2	αB1	βB3	αA2	αA2
Ref.	[13]	[14]	[15]	[13]	[16]	[17]	[17]

Results are expressed as the number of residues per 100 residues. n.d., not detectable

4. DISCUSSION

Hitherto the structural lens proteins have routinely been fractionated into four classes by gel filtration [1,10]. Further separation of the isolated aggregates into the composite subunits requires additional purification and dissociation steps. We have demonstrated previously that excellent fractionation of γ- and α-crystallin subunits can be achieved by chromatofocusing [9,11]. Here we have shown that our attempt to separate the complete population of α-, β- and γ-subunits starting with a single sample of total water-soluble lens extract and one column load appeared to be rather successful. We were able to separate the monomeric βs completely from the γ components (fig.1, fraction II/1) just before urea emerges. In another series of experiments (not shown) we observed that, in the absence of urea, the β_H aggregate starts to elute at a pH of 7.4. This is the reason why undissociated β_H appears immediately following β_S (fig.1, fraction II/2) where the urea concentration is still too low to dissociate the aggregate (fig.2A and B, fraction II/2). On the other hand, the two major β-crystallin subunits, βB2 and βB3, are obtained in a highly purified state (cf. III/2 and III/6). Some difficulty is encountered with the basic β-crystallin subunits, βB1a and βB1b. They appear in several fractions ranging from II/2 to III/8. This was also proven by immunoblotting (not shown). However, in fraction III/8 βB1b is highly enriched. In fractions III/12 and III/13, there is a 42 kDa protein (presumably actin) emerging together with the αA2 polypeptides. The same behavior of the αA2 chain has been observed in previous experiments [12]. It suggests that soluble actin is associated in some way with α-crystallin in situ.

The advantage of the method described is two-fold. (i) It can be utilized for preparative purposes too. (ii) It may be used to discriminate between primary gene products and those proteins that arose from postsynthetic modification, if applied to newly synthesized crystallins after incubation of lenses with radioactive precursors.

Acknowledgements: The authors wish to thank Miss M. Versteeg for performing the amino acid analyses. One of the authors (H.B.) is the recipient of the Alcon Award 1986 that in part enabled these studies to be carried out.

REFERENCES

[1] Bloemendal, H. (1977) Science 197, 127–138.
[2] Harding, J. and Crabbe, M.J.C. (1984) in: The Eye (Davson, H. ed.) chapter 3, pp.207–212, Academic Press, London.
[3] Piatigorsky, J. (1981) Differentiation 19, 134–153.
[4] De Jong, W.W. (1981) in: Molecular and Cellular Biology of the Eye Lens (Bloemendal, H. ed.) chapter 6, pp.221–278, John Wiley & Sons, New York.
[5] Spector, A. (1986) in: The Ocular Lens (Maisel, H. ed.) chapter 11, pp.405–433, Marcel Dekker, New York.
[6] Hoenders, H. and Bloemendal, H. (1983) J. Gerontol. 38, 278–286.
[7] Bloemendal, H. (1963) Zone Electrophoresis in Blocks and Columns: Elsevier Monographs: Chemistry Section 28, Elsevier, Amsterdam, New York.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Bloemendal, H., Groenewoud, G., Versteeg, M., Kibbelaar, M. and Berbers, G. (1983) Exp. Eye Res. 36, 537–542.
[10] Bloemendal, H. (1982) CRC Crit. Rev. Biochem. 12, 1–38.
[11] Bloemendal, H. and Groenewoud, G. (1981) Anal. Biochem. 117, 327–329.
[12] Kibbelaar, M., Selten-Versteegen, A.M.E., Dunia, I., Benedetti, E.L. and Bloemendal, H. (1979) Eur. J. Biochem. 95, 543–549.
[13] Van Dam, A.F. (1966) Exp. Eye Res. 5, 255–266.
[14] Van der Ouderaa, F.J., De Jong, W.W., Hilderink, A. and Bloemendal, H. (1974) Eur. J. Biochem. 49, 157–168.
[15] Herbrink, P. and Bloemendal, H. (1974) Biochim. Biophys. Acta 336, 370–382.
[16] Herbrink, P., Van Westreenen, H. and Bloemendal, H. (1975) Exp. Eye Res. 20, 541–548.
[17] Van der Ouderaa, F.J., De Jong, W.W. and Bloemendal, H. (1973) Eur. J. Biochem. 39, 207–222.