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SUPERIOR RESOLUTION OF γ-CRYSTALLINS FROM MICRODISSECTED EYE LENS BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY A novel procedure is presented for the rapid quantitative analysis of eye lens γ-crystallins and β_S-crystallin by ion-exchange high-performance liquid chromatography on Synchropak CM300. At least six different γ-crystallin gene products can be resolved from the soluble fraction of calf lens extract. This method is applicable to the analysis of microsections from individual lenses, and can be used to rapidly characterize spatial variations in γ-crystallin composition which occur with aging and cataractogenesis.

Gamma-crystallins are eye lens-specific proteins which are reported to play an important role in modulating lens transparency (1-5). They consist of a variety of homologous gene products of similar molecular weight (M_r 20-22,000) but different surface charge distribution (6-9). Their relative proportions, which vary with age and location within the lens (5,10-12), may play a role in cataract formation (5,13). Separation and purification of the various γ-crystallins has been attempted by ion-exchange chromatography (6,10,11), isoelectric focusing (14-16), isotachophoresis (14) and chromatofocusing (17,18).

In recent years, the application of size-exclusion HPLC has provided a rapid, improved size fractionation of α-, β- and γ-crystallin subfractions (4,19,20) and allowed the analysis of microsections of single lenses (21,22). However, the potential for resolution by ion-exchange HPLC is far greater than by size exclusion (23,24).

In this paper, we report the successful application of HPLC on Synchropak CM300, a weak cation exchanger, to the rapid analysis of bovine γ-crystallins. In a one-step procedure, the multiple γ-crystallins in

Abbreviations: HPLC, high-performance liquid chromatography

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a lens microsection extract are separated, quantified and isolated, with a resolution superior to that of most previously employed methods.

MATERIALS AND METHODS

Lens cortices and nuclei from 1-week old calves were extracted separately in 0.05M Na-phosphate buffer pH=6.7, 0.02% Na-azide (5,25). Nuclear γ-crystallins and cortical β_S-crystallin were isolated by Sephadex G-75 chromatography of the nuclear and cortical extract, respectively, in the same buffer at room temperature (11,12). Subfractions γI, γII, γIII and γIV were separated on sulphopropyl-(SP)Sephadex C50 (15x400 mm) in Na-acetate buffer pH=5.0, with a linear 0.2-0.4 ionic strength gradient (6,11,12).

Lens microsections were homogenized in a 10-fold excess (w/v) of HPLC mobile phase buffer A, centrifuged for 15 minutes at 10,000xg and the supernatant analyzed by HPLC.

Ion-exchange HPLC was carried out at room temperature in a prepacked Synchropak CM300 column, 4.1x250 mm, connected to a Synchropak CSC precolumn, 4.1x50 mm (Synchrom, Inc., Linden, Indiana). This material consists of 6.5 micron silica beads with a polyamide coating, derivatized with carboxymethyl groups, and a 300 Å average pore size.

The HPLC system consisted of a Beckman 421 controller, two 110A pumps, a 160 detector (280 nm), an Altex 210 injector with 20 µl loop and a Hewlett-Packard 3390A integrator. Elution was at 1 ml/min, 1000-1200 psi, using mobile phase buffers 0.02M Tris-acetate (A) and 0.02M Tris-acetate, 0.5M Na-acetate (B), both containing 0.02% Na-azide, and both adjusted to the appropriate pH (range 5-7). Salt gradients are given in the text; each run was followed by 5 min. in 100% buffer B and reequilibration for at least 15 min. in starting mixture of A and B. HPLC-grade water (Milli-Q) was used throughout.

Gamma-crystallin solutions (but not microsection extracts) were dialyzed against HPLC buffer A and reconcentrated by ultrafiltration (Amicon, YM-10 membrane) prior to HPLC. Typically the 20 µl sample injected contained about 40 µg γ-crystallins or about 500 µg total lens proteins (containing about 100 µg γ-crystallins). Individual γ-crystallin subfractions in the HPLC eluate were collected and subjected to amino acid analysis after hydrolysis in 6N HCl, 110°C, 24 hours.

RESULTS

For the past twenty years the most conventional procedure for separating bovine γ-crystallins into fractions I, II, III and IV has been by ion-exchange chromatography on SP-Sephadex at pH=5.0, using the method and nomenclature of Björk (6). For further subfractionation of fractions III and IV additional chromatography steps using a different matrix and medium were required (2,6,11).

Fig.1a presents the standard preparative separation we obtain for γ-crystallins from the calf lens nucleus after 24 hours chromatography

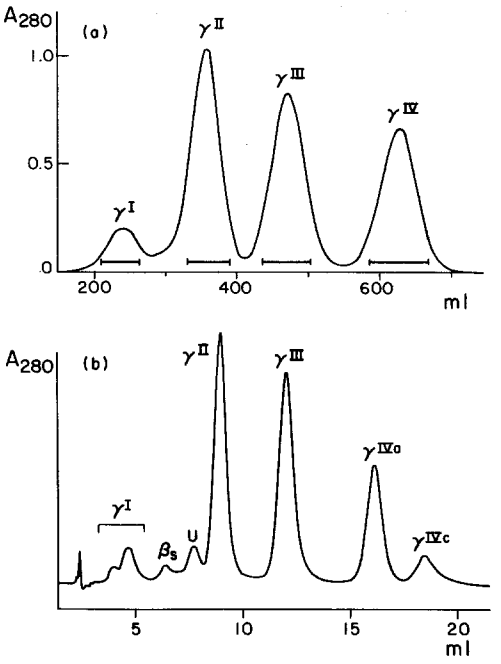

Fig.1 : Cation-exchange chromatography of calf lens nuclear γ-crystallins in acetate buffer pH=5.0. (a) SP-Sephadex, 800 ml linear gradient 0.2-0.4 ionic strength, 0.5 ml/min, 4°C, 70 mg sample applied. Solid bars indicate pooled fractions used for co-elution in HPLC. (b) Synchropak CM300, 20 ml linear gradient 0.15-0.27 ionic strength (25-50% buffer B), 1 ml/min, 20°C, 40 μg sample. U=unidentified peak. Full scale 280 nm absorbance is 0.05 units.

on SP-Sephadex at pH=5.0. We now demonstrate in Fig.1b that an analogous separation can be achieved in less than 20 min. by ion-exchange HPLC on Synchropak CM300 under similar experimental conditions. Moreover, the resolution of peaks is markedly improved in HPLC. Fraction IV splits into two peaks, IVa and IVc (nomenclature described later). Numerous small peaks are resolved prior to fraction II; several of these peaks correspond to fraction I which is known to be heterogeneous (6). In addition, one peak corresponds to pure β_S-crystallin. Table 1 shows that the weight percentages calculated from these two elution profiles in Fig.1 are essentially identical.

Fig.2 illustrates the effect of increasing pH on the separation of nuclear γ-crystallins by HPLC. In each case a short isocratic elution period, introduced to improve resolution of the numerous early peaks, was followed by a linear salt gradient of Na-acetate. No change in the elution

Table 1: Weight composition (%) of calf nuclear γ-crystallins

Column	γI	β_S	γII	γIII	γIV a	γIV c	Unknown (U)
SP-Sephadex	7		35	31	27		-
Synchropak CM300	7	2	30	30	19	7	5

profile occurs from pH=5.0-5.5, but at pH=6.0 and above two remarkable changes occur. First, subfractions IIIa and IIIb are clearly resolved, and secondly, the elution order of fractions II and III is reversed, the

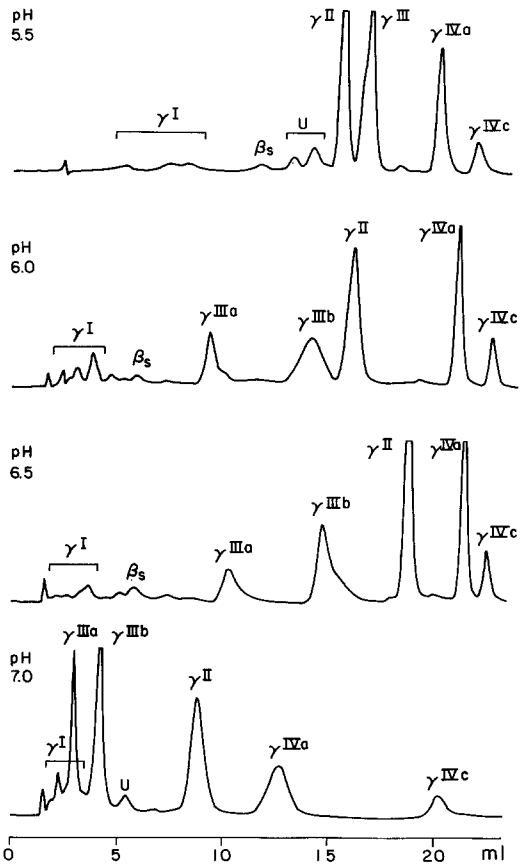

Fig.2 : pH-dependence of ion-exchange HPLC of calf lens nuclear γ-crystallins (40 μg) in Tris-acetate buffer. Elution conditions: (pH=5.5) 10% buffer B for 9 min, 10-40% B in 15 min; (pH=6.0) 10% B for 10 min, 10-40% B in 15 min; (pH=6.5) 0% B for 10 min, 0-30% B in 15 min; (pH=7.0) 0% B for 10 min, 0-25% B in 12.5 min. Peaks were identified by co-elution with purified individual γ-crystallins (as in Fig.1a) and β_S-crystallin, and by comparison of their amino acid composition with published values (6,7,26,27). U=unidentified peak. Full scale 280 nm absorbance is 0.05 units.

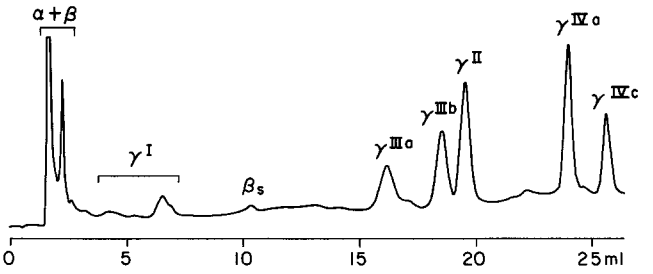

Fig.3 : Ion-exchange HPLC on Synchropak CM300 of a microsection extract from calf lens inner nucleus. Tris-acetate buffer pH=6.0 : 5-10% buffer B in 2.5 min, 10% B isocractic for 10 min, 10-40% B in 15 min. Approx. 500 µg protein injected. Full scale 280 nm absorbance is 0.25 units.

separation increasing as pH increases. Differences in the pK values of exposed histidine residues in these different gene products are presumably responsible for these effects. It is clear that the pH range 6.0-6.5 is ideal for the complete separation of all γ-crystallin subfractions and β_S-crystallin; the resolution of any two peaks can be improved even further, is so desired, by subtle variations of pH and salt gradients (not shown) or buffer ions (24).

Lens microsections were extracted in pH=6.0 HPLC buffer and the water-soluble proteins analyzed directly by ion-exchange HPLC. Fig.3 shows the elution profile of a section from the calf lens inner nucleus. The α- and β-crystallins are not retarded and they elute in the break-through volume well ahead of all γ-crystallins. Table 2 presents the γ- and β_S-crystallin compositions determined for this inner nuclear section, and for three other radial sections. These preliminary results indicate that

Table 2: Weight composition (%) of γ-crystallins in extracts of microdissected calf lens

Microsection	γI	β_S	γII	γIII a	γIII b	γIV a	γIV c	Unknown (U)
Inner Cortex	8	10	32	11	17	14	5	3
Outer Nucleus	6	6	29	12	20	18	8	1
Middle Nucleus	8	4	27	12	17	21	10	1
Inner Nucleus	8	2	23	13	16	24	12	2

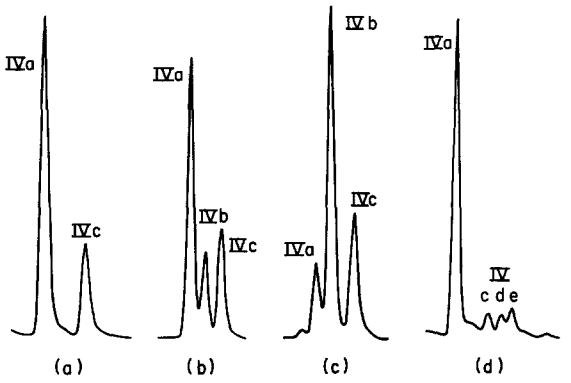

Fig.4 : Effects of in vitro aging on γIV-crystallin. Partial HPLC elution profiles at pH=6.0 of nuclear γ-crystallins : (a) freshly prepared, (b) stored frozen at pH=5.0, (c) stored frozen at pH=6.7, (d) stored at 4°C at pH=6.0 for two weeks.

the relative proportion of (a) γII and β_S decrease towards the nucleus, (b) γIII remains constant, as does the IIIa/IIIb ratio, and (c) γIV increases towards the nucleus, with a concomitant shift in the IVa/IVc ratio. Elution profiles of outer cortical extracts contain a number of additional unknown peaks. The identification of these peaks and the complete radial distributions of bovine γ-crystallins as a function of lens age will be presented in detail elsewhere.

Our nomenclature for γIV-crystallin subfractions (i.e. a,b,c,d,e in order of elution) relates to in vitro modification we have observed following extraction, as shown in Fig.4. Fresh samples contain only components IVa and IVc, in a ratio of about 3:1 (Fig.4a). Therefore, only subfractions γIVa and γIVc are different gene products. Upon storage below 0°C peak IVa slowly converts to IVb (Fig.4b,c); at 4°C peak IVc converts to IVd and IVe (Fig.4d). The exact storage conditions responsible for these modifications are not clear at present. Our amino acid composition of γIVa (not shown) is nearly identical to that found previously for the main γIV component (6,26). Our minor component γIVc has significantly more Asx, Leu and Tyr than γIVa, and less Thr, Glx and Phe, which is consistent with partial sequence data (7,26). Interestingly, similar compositional differences are found between γIIIa and γIIIb (6; our unpublished results).

DISCUSSION

The single-step separation of multiple γ-crystallins and β_S-crystallin by ion-exchange HPLC, as reported in this communication, has enormous potential for the rapid analysis of compositional variations which arise through differential synthesis, aging and cataractogenesis. In addition, it should now be possible to determine which of the numerous human, mouse and rat γ-crystallins, most of which have only been deduced from nucleotide sequences (8,9,28), are actually expressed at various stages of development.

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