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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF LENS CRYSTALLINS AND THEIR SUBUNITS*

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SUMMARY

A high-performance liquid chromatography (HPLC) method for the separation of lens crystallins has been developed which utilizes molecular sieve HPLC. Also described is a rapid one-step separation of the lens crystallin subunits using a reversed-phase C_4 column.

INTRODUCTION

The lens crystallins have been the subject of investigations involving structure and aging[1]. organ development[2], evolutionary changes[3], and cataract formation[1,4]. In such studies it is often necessary to examine the crystallin distribution and subunit composition of many samples using individual lenses. Techniques using gel filtration[5], DEAE-cellulose[6], and even chromatofocusing[7] have been described for separation of the intact crystallins and subunits. These methodologies do not allow for quantitative measurement involving individual lenses. In this paper we describe a rapid and sensitive molecular sieve high-performance liquid chromatographic technique which easily resolves the α-, β_{high}-, β_{low}-, and γ-crystalline. A second reversed-phase methodology using a large pore C_4 column is described which allows one-step separation of the individual crystallin subunits from either isolated crystallins or the total water-soluble fraction.

MATERIALS AND METHODS

Lens crystallin preparation

Lens protein was obtained from 6-week-old and 2-year-old Sprague-Dawley rats. Animals were sacrificed and lenses removed. The lenses from each individual animal were homogenized in 50 m*M* sodium phosphate buffer pH 6.8. The resulting homogenate was centrifuged at 5000 *g* for 30 min. The supernatant is the crystallin containing water-soluble fraction.

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High-performance liquid chromatography (HPLC) techniques

Equipment. The instrument consists of a Beckman dual 110A pump gradient system with a 421 controller, Model 160 detector and a Hewlett-Packard 3390A recording integrator.

Molecular sieve HPLC. The molecular sieve columns used were Altex TSK 3000 SW, 600 × 7.5 mm, particle size 10 μm, coupled in series with an Altex TSK 4000 SW, 300 × 7.5 mm, particle size 10 μm. The sample size was 100–200 μg in 20 μl for analytical runs, while for semipreparative runs the sample size was 20 mg in 200 μl. All chromatograms were performed at ambient temperature. The mobile phase was 50 m*M* sodium phosphate, 50 m*M* sodium chloride, pH 6.8 with an isocratic flow-rate of 1.0 ml/min. Pressure remained at 45 bar. Absorbance was monitored at 280 nm.

Reversed-phase HPLC separation of crystallin subunits. The column used for the reversed-phase system was the Vydac-large pore (300 Å) C_4 column manufactured by The Separation Group, Hesperia, CA, U.S.A. The developers consisted of (A) 1% trifluoroacetic acid (TFA) in water and (B) 100% acetonitrile. All developers were HPLC grade, filtered and degassed under vacuum. The gradient system was as follows: 20 min equilibration with 20% B followed by a linear gradient 20% B to 50% B in 50 min. Isocratic 50% B for 10 min then 50% to 70% B in 1 min and isocratic for 10 min at 70% B. The column is then purged for 10 min at 100% B. The flow-rate was 1.5 ml/min and the pressure maintained at 165 bar. The sample size was 100–200 μg with chromatogram run at ambient temperature.

Amino acid analysis of crystallins and subunits

The crystallins and crystallin subunits were hydrolyzed in 6 *M* hydrochloric acid under vacuum at 100°C for 24 h. The amino acid composition was then determined with a Beckman 121M amino acid analyzer.

Calibration of molecular sieve HPLC columns

An amount of 200 μg of a molecular weight standard mixture consisting of thyroglobulin (mol.wt. 670 000) γ-globulin (mol.wt. 157 000), ovalbumin (mol.wt. 44 000), myoglobin (mol.wt. 17 000), and cyanocobalamine (mol.wt. 1250) was injected onto the molecular sieve HPLC system described previously. The results were plotted as log molecular weight *vs.* K_{av} [$K_{av} = (V_e - V_0)/(V_i - V_0)$ where V_e = elution volume of peak, V_i = column volume, and V_0 = void volume].

RESULTS

Molecular sieve HPLC separation of lens crystallins

The lens crystallins have been routinely separated in the past by Sephadex G-200 gel filtration into four classes; α-, β_{high}, β_{low}, and γ-crystallins[4]. The elution profiles obtained for soluble lens crystallins are shown in Fig. 1. Crystallin preparations from 6-week-old (Fig. 1A) and 2-year-old (Fig. 1B) rats were used for this study. By comparison to the calibration curve the resulting peaks are identified as a high-molecular-weight aggregate (HMW) (mol.wt. $1.5 \cdot 10^6$), α-crystallin (mol.wt. $8.0 \cdot 10^5$), β_{high} (mol.wt. $1.6 \cdot 10^5$, β_{low} (mol.wt. $4.2 \cdot 10^4$), and γ-crystallin (mol.wt. $2.0 \cdot 10^4$), eluted in that order. The results show excellent separation of each peak as

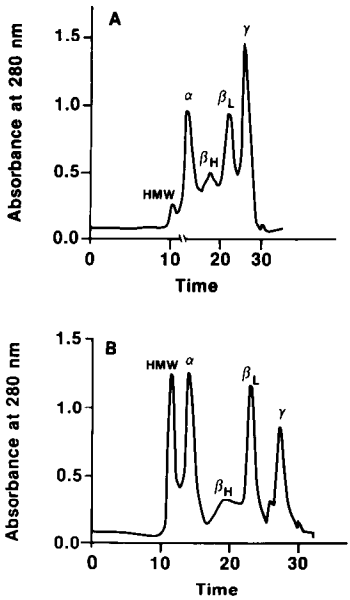

Fig. 1. Molecular sieve HPLC separation of lens crystallins from 6-week-old rat lens (A) and 2-year-old rat lens (B). Time is in minutes.

well as correlation with the established molecular weights of each protein[5].

Each peak was subsequently collected, dialyzed against water and lyophilized. The lyophilized protein was then subjected to amino acid analysis. The results shown in Table I confirm that the peaks correspond to α, β_{high}, β_{low}, and γ-crystallin.

Molecular sieve separations of lens crystallins from old and young rat lenses

Molecular sieve HPLC techniques provide a remarkable opportunity for micro-analytical experiments. An example of this capability is shown in Fig. 1 where lenses from individual animals were homogenized and 100 μg of the water-soluble fraction was injected onto HPLC. The resulting chromatogram depicts the different crystallin distribution that exists between 6-week-old rats *vs.* 2-year-old rats. The results show a dramatic decrease in the γ-crystallin fraction from 32% in the young animal to 16% in the older animal. Also shown is the increase in the high molecular weight fraction from 3% in the young rat to 18% in the older animals. Table II gives the summary of the results obtained for seven 6-week-old rats and seven 2-year-old rats. Similar changes have been reported previously where separations of crystallins was achieved by Sephadex G-200[1].

Reversed-phase separation of crystallin subunits

The α- and β-crystallins are composed of several individual subunits which have been isolated previously with CM-cellulose–urea chromatography[8], urea chromatofocusing[7], and urea isoelectric focusing[9]. Using the reversed-phase HPLC system it was possible to separate the major polypeptide subunits of the α- and β-crystallins in one step. Fig. 2 depicts the separation of the lens crystallin subunits using

TABLE I

AMINO ACID COMPOSITION OF LENS CRYSTALLINS OBTAINED FROM MOLECULAR SIEVE HPLC

Values in parentheses are expected values (ref. 1).

Amino acid	*Peak*			
	α	β_{high}	β_{low}	γ
Asp	75 (73)	71 (73)	88 (85)	165 (166)
Thr	34 (35)	24 (29)	30 (30)	29 (30)
Ser	106 (105)	79 (79)	79 (81)	77 (76)
Glu	112 (107)	150 (146)	148 (146)	126 (127)
Pro	78 (82)	58 (60)	56 (59)	48 (48)
Gly	65 (61)	96 (93)	93 (91)	55 (57)
Ala	42 (45)	54 (54)	49 (49)	31 (29)
Cys	– –	12 (17)	5 (9)	– –
Val	61 (59)	60 (61)	64 (63)	50 (50)
Met	13 (12)	17 (18)	9 (10)	31 (30)
Ile	48 (48)	31 (33)	37 (35)	36 (36)
Leu	86 (88)	53 (53)	64 (62)	70 (70)
Tyr	– (32)	30 (41)	– (41)	76 (83)
Phe	82 (77)	45 (48)	44 (43)	57 (57)
Trp	– –	42 (45)	41 (38)	– –
Lys	50 (49)	43 (43)	58 (54)	24 (25)
His	40 (39)	39 (41)	42 (44)	35 (35)
Arg	76 (74)	65 (67)	60 (61)	115 (111)

200 μg of the water-soluble fraction. With the use of volatile solvents in this system, identification of each peak by amino acid analysis becomes a simple procedure. Table III gives the results of the amino analysis identification of the subunits obtained by reversed-phase HPLC. The slight variation between expected values and our results reflect species differences. However, there were no gross differences which would indicate a noncrystallin subunit.

The results show both separation and quantitation of the αA and αB subunits from α-crystallin. Using the area under each peak to quantitate the individual subunits a ratio of αA:αB = 3.4:1 is found which correlates to the approximate 3:1 ratio reported previously[10]. Similarly, the β-crystallins separate well into the major Bp

TABLE II

LEVELS OF THE VARIOUS LENS CRYSTALLINS AS DETERMINED BY MOLECULAR SIEVE HPLC IN 6-WEEK-OLD RATS (n = 7) AND 2-YEARS-OLD RATS (n = 7)

	Lens crystallin			
	HMW (%)	*α (%)*	*β (%)*	*γ (%)*
Young	3.8 ± 1.2*	26.7 ± 3.1	36.9 ± 4.2	32.6 ± 3.9
Old	18.9 ± 5.8	28.4 ± 4.4	30.8 ± 5.2	21.9 ± 4.7

* Mean ± S.D.

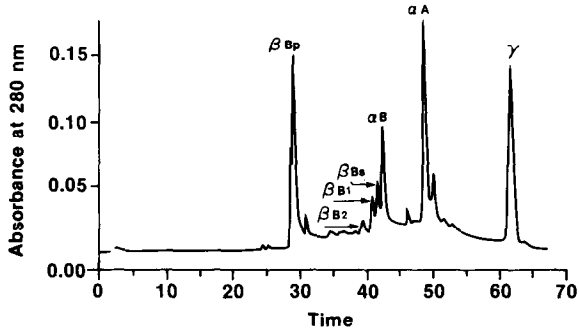

Fig. 2. Reversed-phase HPLC separation on a C_4 column of lens crystallin subunits from total soluble fraction. Time is in minutes.

subunit and the minor components B1, B2, and βs. Quantitation of the β-crystallin subunits indicate that the major subunit Bp comprises approximately 65% of the intact β-crystallin. The γ-crystallin consists of a single polypeptide and therefore elutes as a single peak.

Comparison between molecular sieve vs. reversed-phase HPLC system

Peaks were isolated from the molecular sieve HPLC and 100 μg of each α, β, and γ-crystallin was injected individually onto the reversed-phase HPLC system. The results depicted in Fig. 3 show that the polypeptide subunits of α-, β-, and γ-crystallins

TABLE III

AMINO ACID COMPOSITION OF CRYSTALLIN SUBUNITS OBTAINED BY REVERSED-PHASE HPLC

Values in parentheses represent expected values (ref. 1). Peak 1–6 are from bovine, peak 7 is from rat.

Amino acid	*Peak 1 βBp*	*Peak 2 βBs*	*Peak 3 βB2*	*Peak 4 βB1*	*Peak 5 αB*	*Peak 6 αA*	*Peak 7 γ*
Asp	85 (83)	78 (80)	82 (80)	90 (89)	80 (75)	93 (97)	165 (166)
Thr	27 (34)	30 (28)	37 (35)	25 (28)	42 (44)	30 (37)	29 (30)
Ser	86 (84)	77 (75)	88 (85)	61 (61)	92 (96)	125 (126)	77 (76)
Glu	155 (158)	142 (148)	152 (158)	120 (126)	110 (100)	99 (95)	130 (127)
Pro	68 (68)	72 (76)	51 (49)	37 (37)	86 (94)	65 (67)	50 (48)
Gly	91 (93)	78 (80)	99 (106)	83 (87)	58 (56)	63 (60)	55 (57)
Ala	40 (42)	54 (58)	49 (53)	52 (57)	49 (53)	36 (35)	30 (29)
Cys	10 (09)	– –	14 (13)	30 (37)	00 (00)	05 (06)	– –
Val	72 (75)	58 (59)	68 (67)	47 (49)	52 (55)	54 (55)	50 (50)
Met	11 (09)	21 (18)	07 (09)	27 (33)	19 (13)	13 (12)	28 (30)
Ile	25 (31)	33 (32)	30 (26)	37 (41)	38 (47)	45 (50)	32 (36)
Leu	48 (50)	45 (47)	62 (59)	57 (57)	84 (84)	82 (84)	70 (70)
Tyr	47 (44)	45 (43)	50 (47)	59 (65)	17 (14)	29 (33)	70 (83)
Phe	42 (40)	52 (50)	60 (55)	57 (57)	73 (70)	77 (78)	60 (57)
His	44 (41)	39 (37)	46 (47)	43 (41)	50 (47)	38 (41)	37 (35)
Lys	59 (60)	59 (60)	50 (52)	47 (49)	54 (58)	38 (41)	24 (25)
Arg	52 (50)	87 (90)	68 (62)	74 (73)	82 (78)	80 (73)	110 (111)

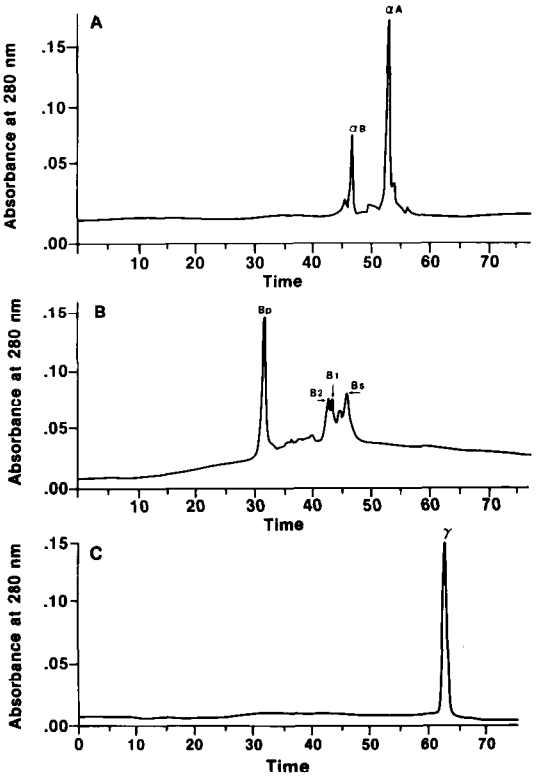

Fig. 3. Reversed-phase HPLC separations on a C_4 column of the subunits of α-crystallin (A), β-crystallin (B), and γ-crystallin (C). Time is in minutes.

elute in the exact position expected from the amino acid analysis data. This helps to demonstrate both the identity of the peaks obtained by reversed-phase HPLC as well as the relative purity of the lens crystallins obtained from the molecular sieve HPLC system.

DISCUSSION

The lens crystallins have been routinely separated by gel filtration or DEAE-cellulose into four classes: α-, β_{high}-, β_{low}-, and γ-crystallins. Each method, however, has its own inherent problems. To separate such large molecular weight proteins as the lens crystallins, molecular sieve gels such as Sephadex G-200 or Bio-Gel A5M must be used. These large pore gels are very fragile requiring a great deal of care in packing and slow flow-rates. DEAE-cellulose requires extensive equilibration of the column as well as sample equilibration. With the advent of HPLC molecular sieve columns the separation of proteins has become simplified. We have demonstrated excellent separations of the lens crystallins and their subunits using molecular sieve HPLC and reversed-phase HPLC. Molecular sieve HPLC only recently became available through the use of macroporous silica based supports. Bindels *et al.*[11,12] originally used these TSK columns to separate the bovine lens crystallins. Their system

employed a guard column, one TSK 4000 SW column, and two TSK 3000 SW columns connected in series with a low-angle laser light scattering detection system. Their results showed separation of all the lens crystallins and resolved the β-crystallins into up to eight discernable fractions. However, they were not able to resolve adequately the high-molecular-weight aggregate (HM-crystallin) from the α-crystallin and the α-crystallin showed excessive tailing. In our earlier experience with the TSK columns we were not able to ressolve the HM-crystallin from the α-crystallin when a guard column was included in the system. Presumably the HM-crystallin is too large to pass through the guard column and the loose packing possibly results in a dead volume which would cause the peak tailing.

Ample evidence has now accumulated that the new HPLC molecular sieve columns are weakly anionic and slightly hydrophobic. It is often overlooked that this deviation from the ideal behavior of a size exclusion column may be manipulated to the point where great selectivity between select molecular species is obtained. Kopaciewicz and Regnier[13] demonstrated that by varying the pH and the ionic strength of the mobile phase, the retention behavior of myoglobin and ovalbumin may be adjusted for optimum separations. Similar results may be obtained with the lens crystallins. By varying the pH from 6.0 to 7.0 and the ionic strength from 0.25 to 0.1 we were able to achieve a variety of separations of the lens crystallins. Since our current research involves the HM-crystallins we chose the conditions described here for our experimental separations. However, ideal separation based solely on molecular weight may be obtained by merely maintaining an ionic strength above 0.2. Our experience has been that after over 300 runs the separations are still excellent with no tailing of peaks or changes in elution patterns. Sample size has been varied from 100 μg to 20 mg with no effect on results. The only limitation is that the ionic strength be high enough to obviate any ionic or absorption effects (50 m*M* salts are sufficient) and a pH between 2 and 8. Manufacturers claim that sample sizes of up to 60 mg may be applied without overloading the column. This capacity allows for semipreparative operations with extremely short turn-around time.

In the past the subunits of α- and β-crystallins have been separated by CM-cellulose urea chromatography, urea chromatofocusing or urea isoelectric focusing[7–9]. The largest drawback to these procedures is the extensive sample preparation before and after. Desalting, removal of urea and ampholines, requires long term dialysis or gel filtration both of which involve time and sample losses. The Vydac C_4 column has a large pore size and short hydrocarbon reactive side chains which practically eliminate the irreversible binding found in conventional C_{18} columns. Described here is a one-step separation of the polypeptide subunits of the lens crystallins. The TFA–water–acetonitrile gradient has the advantage not only of simplicity but also of volatility so that subsequent operations such as amino acid analysis, sequencing, etc. may be done after evaporation of the solvent. Additionally, with only slight modifications of the buffers and gradient it is possible to achieve separation of the post-translationally modified crystallins such as the deaminated αA_2 and αB_2 polypeptides from the αA_1 and αB_1 counterparts. These simple and rapid techniques provide methods for effective and sensitive examination of progressive changes which occur within the lens during aging, differentiation, and disease states.

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REFERENCES

1 J. J. Harding and K. J. Dilley, *Exp. Eye Res.*, 22 (1976) 1.
2 H. Bloemendal, *Science*, 197 (1977) 127.
3 W. W. de Jong, in H. Bloemendal (Editor), *Molecular and Cellular Biology of the Eye Lens*, Wiley, New York, 1981, p. 221.
4 A. Spector and D. Roy, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 3244.
5 F. A. M. Asselberys, M. Koopmans, W. J. van Venrooij and H. Bloemendal, *Exp. Eye Res.*, 28 (1979) 223.
6 A. Spector, L. K. Li, R. C. Augusteyn, A. Schneider and T. Freund, *Biochem. J.*, 124 (1971) 337.
7 H. Bloemdal and G. Groenewood, *Anal. Biochem.*, 117 (1981) 327.
8 R. C. Augusteyn and A. Spector, *Biochem. J.*, 124 (1971) 345.
9 P. Herbrink and H. Bloemendal, *Biochim. Biopohys. Acta*, 336 (1974) 370.
10 J. Delcour and J. Papaconstantinou, *Biochem. Biophys. Res. Commun.*, 57 (1974) 134.
11 J. G. Bindels, B. M. De Man and H. J. Hoenders, *J. Chromatogr.*, 252 (1982) 255.
12 J. G. Bindels and H. J. Hoenders, *J. Chromatogr.*, 261 (1983) 381.
13 B. Kopaciewicz and F. E. Regnier, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, p. 150.