LETTER TO THE EDITORS

The Isolation of Lens Crystallins Using Lens Liquid as the Solvent

The prevailing water-soluble proteins in the lens are the so-called crystallins. In mammals these occur mainly in three classes: α (MW about 800 kDa), β (MW 50–300 kDa) and γ (MW about 20 kDa) (Bloemendal, 1982). Based on their chromatographic behaviour the β -crystallins are divided into two groups $\beta_{H(igh)}$ and $\beta_{L(ow)}$. In some cases the latter is further split into β_{L1} and β_{L2} (Bloemendal, 1981; Asselberg et al., 1979; Slingsby and Bateman, 1990). The exact molecular weight of native α -crystallin has been a source of, sometimes vigorous, debate (Tardieu et al., 1986; Walsh, Sen and Chakrabarti, 1991; Augusteyn, Parkhill and Stevens, 1992; Van Haeringen et al., 1993; Wistow, 1993; Groenen et al., 1994). Temperature and buffer conditions seem to play a role in the size of the aggregate obtained after isolation (Siezen, Bindels and Hoenders, 1980; Thomson and Augusteyn, 1983, 1984, 1988; Van den Oetelaar et al., 1985; Wistow, 1993; Groenen et al., 1994). Also the number of subunits in β -crystallins depends, among others, on solvent conditions (Li, 1978, 1979; Asselberg et al., 1979; Bindels, Koppers and Hoenders, 1981; Siezen, Anello and Thomson, 1986; Vlaanderen, Van Grondelle and Bloemendal, 1993).

The question has frequently been raised, whether or not the separation pattern of isolated crystallins reflects the in situ situation. Lasser and Balasz (1972) isolated crystallins in their own medium. However, for analysis they added water and buffer. Hence, their study could not give an answer to the question raised here. In order to study crystallins in their natural environment we have used the following procedure. (1) The lens liquid (lens homogenate without insoluble particles and water-soluble proteins) was isolated as described in the Appendix. (2) Total and cortical lens protein samples were obtained by homogenizing them in an equal mass of the lens liquid overnight and for an hour, respectively, and centrifugation as described (Vlaanderen, van Grondelle and Bloemendal, 1993). These samples will be denoted as T_{Liq} and C_{Liq} respectively. The cortical fraction contained about 70% of the mass of the lenses. (3) Similarly, total and cortical lens protein samples were prepared in a buffer of intermediate ionic strength [40 mM potassium phosphate at pH 6.8, 90 mM KCl, 0.01% (w/w) Na_2EDTA , 1 mM Na_2EDTA , 0.2 mM dithiothreitol (Tardieu et al., 1986)]. These will be denoted as T_{Buf} and C_{Buf} , respectively. (4) The isolation of the crystallins from

these samples by means of fast protein liquid chromatography using lens liquid and the buffer as eluent were compared. (5) The procedure was repeated with a high-salt buffer [0.1 M Na_2SO_4 , 0.02 M Na_2HPO_4 / NaH_2PO_4 , pH 6.9 (Bindels, de Man and Hoenders,

FIG. 1. Chromatograms of (A) bovine lens cortex and (C) total lens homogenized in lens liquid and eluted with lens liquid (---) and intermediate-salt-strength buffer (—), and of (B) lens liquid eluted with intermediate-salt-strength buffer (The Roman numerals are explained in Table 1). Chromatography according to Vlaanderen, van Grondelle and Bloemendal, 1993.

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FIG. 2. SDS-PAGE according to Laemmli (1974) of selected fractions of the chromatogram of total lens homogenized and eluted in lens liquid. Numbers refer to elution times in minutes, M denotes markers.

1982)], however the results with high- and intermediate-ionic-strength buffers gave comparable results.

Since strong absorption at 280 nm by the lens liquid, presumably due to small nucleotides and/or oligopeptides, precluded detection at this wavelength, we monitored the protein separation at 307 nm. As the various crystallins contain different numbers of aromatic residues, this will change the intensities of the chromatographic peaks compared to published results which were all obtained at 280 nm. However, for a comparison between buffers this has no consequences. The chromatograms at 280 nm of cortical and total lens proteins dissolved in and eluted with buffer (not shown) gave the normal pattern.

The use of either lens liquid or buffer as solvent did not affect the elution times of calibration compounds (not shown). In Fig. 1 we present the chromatograms of T_{Liq} and C_{Liq} eluted with buffer and lens liquid, as well as a chromatogram of lens liquid without proteins eluted with buffer. T_{Buf} and C_{Buf} eluted with buffer yielded chromatograms (not shown) that are similar to those of T_{Liq} and C_{Liq} in buffer. This shows that the solvent-induced changes to be discussed below, are reversible.

Gels of a number of selected fractions are shown in Fig. 2. Retention times of peaks in lens liquid and buffer with their assignments are given in Table I, and were compared with those of calibration proteins (Biorad gel filtration standard 151.190.1). Table I and

TABLE I

Retention times (in minutes) and assignments of bovine lens proteins in intermediate-salt-strength buffer (Buf) and lens liquid (Liq).

Peak	Total proteins		Cortical proteins		MW	Assignment
	<i>t</i> _{ret} (Buf)	<i>t</i> _{ret} (Liq)	<i>t</i> _{ret} (Buf)	<i>t</i> _{ret} (Liq)		
I	—	26	—	~ 24	> 10 MDa	?
II	42	41	41	41	~ 5MDa	α _{HM}
III	55	54	55	55.5	~ 700 kDa	α
IV	65.5	64.5	64.5	65.5	~ 150 kDa	β _H
V	~ 73	69	72.5	70.5	~ 75 kDa	β
VI	77	76	76	77	~ 50 kDa	β _L
VII	86	86	86	86	~ 17 kDa	γ
VIII	90.5	91	—	90.5	~ 14 kDa	γ

the chromatograms show that most retention times, and hence aggregational states of the crystallins, are very similar in lens liquid and buffer. The molecular mass of α-crystallin (peak III) is found to be ~ 700 kDa in both cases. A significant difference, however, is observed for the β-crystallins. The low-molecular-weight fraction of the β-crystallins (β_L, peak VI) appears to be increased in lens liquid at the expense of the higher-molecular-weight β-crystallins (peaks IV and V). This suggests that β_L-crystallin is present in intact lenses in higher concentrations than after isolation in buffers. The incidently observed intermediate fraction (sometimes called β_{H2} or β_{L1}) is virtually absent in the lens liquid, inferring that this form is an isolation artefact. In other words, in the intact lens β-crystallin seems to exist essentially in the high- (MW ~ 150 kDa) and low- (MW ~ 50 kDa) molecular-weight forms.

After 106 min (MW ~ 3 kDa) there is a pronounced peak in the lens liquid, which is absent in both buffers used (with and without dithiothreitol and EDTA). This cannot be caused by a component of the lens liquid as such, since this should be equally reflected in the whole chromatogram (lens liquid is used for both dissolving and eluting the crystallins). Neither can it be a contaminant of the sample, as in that case it would show up after elution with buffer as well. Hence, this peak must be due to the formation of a complex between lens-liquid and sample components, that is unstable in buffer. As the maximum absorption of this compound was at 310 nm, it might be a charge-transfer complex formed by metal-ion binding to one of the smaller lens compounds.

Finally, we like to draw attention to peaks I and II in the chromatograms, although we cannot give an unequivocal interpretation. Peak II (*t*_{ret} = 41 min, MW ~ 5 MDa) presumably is high-molecular-weight α-crystallin, α_{HM}, the higher aggregation state caused by chemical modifications during ageing (Spector et al., 1971; Hoenders and Bloemendal, 1981). Peak I (MW > 10 MDa) is only present in the lens-liquid chromatograms. SDS-PAGE suggests that this is not a

protein. However, the amount of protein might be below detection level. The relatively high intensity of this peak in the chromatogram when measured at 307 nm might be due to oxidation (Borkman, Hibbard and Dillon, 1986; Bloemendal et al., 1989). In the latter case peak I might represent a weakly aggregated form of some unidentified compound that is unstable, when not in its native environment.

In conclusion, this study shows that the aggregation of crystallins, with exception of β-crystallins, is barely influenced by a change of solvent. β-Crystallin in the lens liquid exists essentially as high- (~ 150 kDa) and low- (~ 50 kDa) molecular-weight aggregates, but not in intermediate forms. However, low- and high-molecular-weight complexes are found, that are only stable in lens liquid. The low-molecular-mass complex might be a metal ion bound to a small polypeptide or polynucleotide.

Appendix: Isolation of Lens Liquid

Bovine calf eyes were obtained from the local slaughterhouse and kept on ice. The lenses were removed (G₀ grams), and were homogenized in G'₀ grams (G'₀ ≈ G₀) of distilled water using a blender. The homogenate was centrifuged in a Beckman preparative ultracentrifuge at 35 000 rpm for 20 min using a Ti45 or Ti50.2 rotor. The pellet was rehomogenized in G_{rh} grams of water (G_{rh} is 20 to 30% of G₀) and centrifuged at 35 000 rpm for 30 min. The supernatant of this run was collected, combined with that of the first one and once more centrifuged at 35 000 rpm for 30 min. The masses of the pellet (G_p) and of the total supernatant (G_s) were determined. The combined supernatant was then ultra-filtered on a Filtron Minisette over two L ω5K filters (two blocks of ten filters), applying a pressure of 2 bar. This yielded G_f grams of filtrate. As this filtrate still contained significant amounts of γ-crystallin, it was further filtered over a 3k Amicon filter. SDS-PAGE revealed that with this procedure all the proteins were removed from the lens liquid. [Compare also the chromatogram

shown in Fig. 1(B), the peaks of which are presumably due to oligonucleotides and/or small polypeptides (apparent MW < 7.5 kDa)]. Small samples of the pellet, the supernatant and the filtrate were taken, and the fractions of water therein (f_p , f_s and f_f) were determined by lyophilization. Finally, the filtrate was reconcentrated by lyophilization to reach native concentrations as outlined in the next paragraph. The whole procedure was performed at 4°C. The pH was checked after each step, and appeared to vary from 7.2 to 7.8.

The only assumption in the calculation of the reconcentration factor is that small solutes (not retained by the filter) and water molecules show no preference for the pellet, the supernatant, the filtrate or the residual protein solution. The amount of water in G_0 grams of lens material started with is defined as X_L . After centrifugation this is distributed between pellet and supernatant. The total amount of water therein is $f_s G_s$ and $f_p G_p$, respectively. The amount of water for homogenization added to the system is ($G'_0 + G_{rh}$). Hence

$$X_L = f_s G_s + f_p G_p - (G_0 + G_{rh}) \quad (1)$$

After ultrafiltration $f_f G_f$ of the total mass of water is found in the filtrate. The fraction in it originating from the lenses is given by $X_L / (G_0 + G_{rh} + X_L)$. Defining this quantity as f_L , the amount of water to be removed from the filtrate is $(1 - f_L) f_f G_f$.

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