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Note

Fast protein liquid chromatography–chromatofocusing of bovine and chicken α-crystallin subunits under denaturing conditions

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Chromatofocusing is an ion-exchange technique based on in-column formation of a pH gradient[1,2]. Isoelectric focusing of proteins during the development of this pH gradient causes the sample components to be eluted in order of their p*I* values and gives the method its high resolving ability. Bloemendal and Groenewoud[3] described chromatofocusing of the soft gel PBE 94 in 6 *M* urea of the subunits of bovine α-crystallin, a multimeric eye-lens protein of about 800 000 daltons[4]. Recently, chromatofocusing became available for high-performance liquid chromatography (HPLC) owing to the introduction of Mono P columns as part of the fast protein liquid chromatography (FPLC) system. The good results obtained by our group with this column in the fractionation of human immunoglobulins[5] prompted us to investigate whether its use offered advantages over PBE 94 for the isolation of α-crystallin subunits.

MATERIALS AND METHODS

A Mono P column HR 5/20 (Pharmacia) was integrated into a conventional stainless-steel HPLC system using Pharmacia SRTC-2 tubing-connectors. Gradient elution was carried out with two Beckman 100A pumps and a Beckman Model 420 controller. The two eluents were mixed in a zero-dead-volume Valco T-union without further mixing. The column was equilibrated at ambient temperature with 0.025 *M* triethanolamine hydrochloride, pH 8.0 containing 7 *M* urea and subsequently titrated with a Polybuffer mixture (70% Polybuffer 74, 30% Polybuffer 94; dilution 1:50) also containing 7 *M* urea and adjusted to pH 4.6 with hydrochloric acid. After application of the sample, dissolved in the equilibration buffer, the unbound material was washed off with the equilibration buffer in about 5 min at a flow-rate of 1.0 ml/min. Subsequently, the system was switched to the Polybuffer. Protein detection at 280 nm was performed with an LKB 2138 Uvicord S. Fractions were collected with an LKB 2070 Ultrorack. After each experiment, the Mono P column was regenerated as recommended[6].

Isoelectric focusing in 5 T, 3 C polyacrylamide gel rods was used for identifi-

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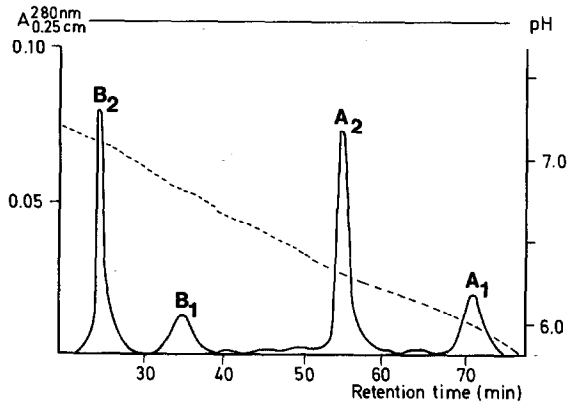

Fig. 1. Chromatofocusing of 7 mg bovine α-crystallin in 7 *M* urea. The pH gradient is indicated by the dashed line.

cation and to check the purity of the peaks. The recovery was calculated by comparing the absorbance of the eluted peaks with that of the sample applied.

Isolation of α-crystallin from bovine and chicken lenses was performed as described[4,7].

RESULTS AND DISCUSSION

The separation of the subunits A_1, A_2, B_1 and B_2 of bovine α-crystallin by chromatofocusing in 7 *M* urea on a Mono P column is depicted in Fig. 1. In contrast with chromatofocusing on PBE 94[3], baseline resolution was obtained. Furthermore, the fractionation time was reduced to about one sixth. The purity of the isolated peaks was analyzed by isoelectric focusing in polyacrylamide gel rods (Fig. 2). The high resolution could be obtained only by deviating from the manufacturer's recommendations[6] in two respects. First, the results were improved by using hydrochloric acid instead of acetic acid for the adjustment of the pH and, secondly, we found it necessary to dilute the Polybuffer fifty times, which is exceptionally high. As to the loading capacity, the shape of the largest peak, representing A_2, became irregular if the sample amount exceeded 20 mg. This indicates a maximum capacity of about 7

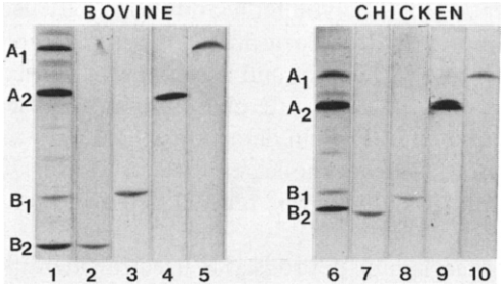

Fig. 2. Isoelectric focusing of bovine and chicken α-crystallin subunits, isolated by FPLC–chromatofocusing. Bovine α-crystallin, αB_2, αB_1, αA_2 and αA_1 are shown in rods 1–5, respectively, chicken α-crystallin, αB_2, αB_1, αA_2 and αA_1 in rods 6–10, respectively.

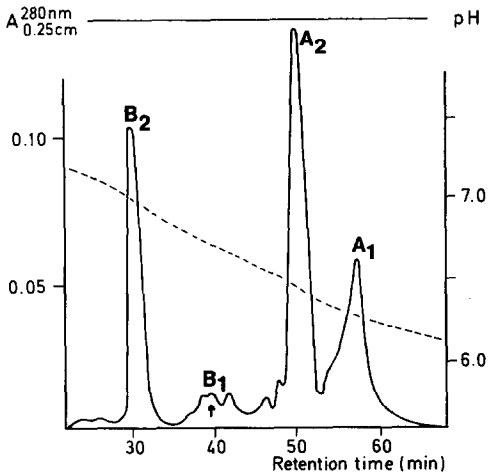

Fig. 3. Chromatofocusing of 10 mg chicken α-crystallin in 7 *M* urea. The pH gradient is indicated by the dashed line.

mg per component. The recovery was somewhat disappointing since a yield of only 25% was found. Repeated use of the same column indicated that the use of highly concentrated urea solutions did not noticeably affect its resolving power.

The high resolving power of the Mono P column is even better demonstrated by the fractionation of chicken α-crystallin. In bovine α-crystallin, both A_1 and A_2 as well as B_1 and B_2 differ by two units of charge due to phosphorylation[8], whereas for the subunits of chicken α-crystallin this difference is only one unit of charge caused by deamidation[7]. Therefore, the difference in isoelectric points for the chicken subunits is smaller, but nevertheless a very satisfactory resolution could be obtained with the same gradient programme (Fig. 3).

Bloemendal and Groenewoud[3] had noted that the pH at which the bovine αA_1 and αA_2 subunits are eluted from a PBE 94 column differs substantially from the p*I* values as calculated from isoelectric focusing. This behaviour of the bovine αA chains is also found with the Mono P column (Table I), and is not surprising since both

TABLE I

p*I* VALUES OF BOVINE α-CRYSTALLIN SUBUNITS IN CONCENTRATED UREA SOLUTIONS

Subunit	*Mean from isoelectric focusing**	*FPLC–chromatofocusing on Mono P*	*Difference*
A_1	5.56 ± 0.21	5.97	0.41
A_2	5.90 ± 0.19	6.31	0.41
B_1	6.81 ± 0.22	6.81	0.00
B_2	7.17 ± 0.18	7.08	−0.09

* These means ± standard deviations are calculated from p*I* values obtained by various isoelectric focusing experiments summarized in ref. 9.

gels carry the same charged groups[6]. The different behaviour of the αA and αB chains must be attributed to a difference in the slope of the titration curves, dQ/dpH, where Q is the protein charge of these polypeptides near their isoelectric points. The deviation of the apparent from the real pI in chromatofocusing has been examined theoretically by Sluyterman and co-workers[1,2].

In conclusion, we believe that chromatofocusing on Mono P, despite a low yield, is very well suited to the purification of α-crystallin subunits.

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