IMPROVED SEPARATION OF THE ALKALINE RNAse-INHIBITOR FROM EYE LENS AND ELECTROPHORETIC DEMONSTRATION OF ITS SUBUNITS

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SUMMARY

A purification procedure for the natural inhibitor of alkaline RNAse from calf lenses is described. Electro-elution has been introduced as purification step. Complex formation of the inhibitor with RNAse has been demonstrated by polyacrylamide gel electrophoresis. The complex resists dissociation by sodium dodecyl sulfate, whereas in this detergent the inhibitor splits into two subunits of about 30,000 molecular weight.

INTRODUCTION

A protein which inhibits the activity of alkaline RNAse *in vitro* has been found in many different tissues (1-3). Although this inhibitor appears to be a potent protective factor for the preparation of polyribosomes (4-8) and of messenger RNA (9-10), its actual function *in vivo* has not been established yet. Moreover, thorough characterization of the inhibitor has been hampered due to its instability with increasing purification, so that workers have had to rely exclusively on activity measurements.

The presence of the inhibitor could also be demonstrated in bovine eye lens (3, 11, 12) and in our hands lens tissue provides one of the most suitable sources for the isolation of RNAse inhibitor. Despite the fact that the inhibitor represents only a minute fraction (less than 0.1 part per 1000) of the total water-soluble lens protein it can be freed from the bulk of contaminants (mainly crystallins) in only two simple purification steps. We provide evidence that the inhibitor consists of two subunits.

Abbreviation: DTT = 1,4-dithiothreitol

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The inhibitor forms a complex with alkaline RNAse. We demonstrate that this complex resists dissociation in sodium dodecyl sulfate and that it is characterized by a slightly lower mobility than the free inhibitor upon polyacrylamide gel electrophoresis at alkaline pH.

MATERIALS AND METHODS

Preparation of the lens homogenate

Calf lenses were obtained fresh from the slaughter house. A ring of about 2 mm was removed from the lens periphery with the aid of a cork borer. In this way from 1000 lenses about 150 mg of wet material was obtained. Per gram of starting material 1.5 ml of a buffer was added which contained 0.05M Tris-Cl, pH 7.6, 0.05M NaCl, 0.001M EDTA, 0.001M DTT and 1% glycerol. The tissue was homogenized manually and stirred for 3 h. Thereafter the homogenate was centrifuged at 22,000 x g for 30 min and stored frozen. After thawing the 22,000 x g supernatant fraction was acidified to pH 6 by adding 1M HCl. Then the solution was dialyzed for 5 h against 10 l of a medium (A) containing 0.05M Tris-Cl, pH6, 0.15M NaCl, 0.001M EDTA, 0.001 DTT and 1% glycerol. The dialysate was centrifuged at 75,000 x g for 1 h.

DEAE-Sephadex chromatography

20,000 optical density units of protein were applied to a DEAE-Sephadex A-50 column (40 x 3 cm) which was equilibrated previously in medium (A). After application of the sample the column was washed with 2 l of medium (A). Elution was performed at 30 ml/h. Then a linear NaCl gradient was started ranging from 0.15M (600 ml) to 1M NaCl (600 ml). The inhibitor activity of the fractions was measured in tenfold solution as described previously (13). The active fraction was precipitated with ammonium sulfate at 65% saturation, resuspended in a small volume of water and dialyzed. This preparation was either lyophilized or used as such for gel filtration.

Gel filtration

Ultrogel AcA 34 was kept overnight in medium (A) from which sodium chloride had been omitted (medium B). Per column (100 x 3 cm) maximally 20 ml of the active fraction from the DEAE-Sephadex column was applied. Elution was performed with medium (B) at 30 ml/h. The active fraction was precipitated with ammonium sulfate at 65% saturation.

Electro-elution

Electro-elution was performed with an apparatus constructed according to the design of Popescu et al (14) with some modifications (15). The gel dimensions were 4 x 1.2 cm. Buffers and gel concentration have been described earlier (16). The sample, dissolved in 0.3 ml of buffer, contained 20,000 inhibitor units. First a pre-electrophoretic run was carried out at 200 V (20mA) for 4 h. Thereafter the sample was applied and electrophoresis continued till the buffer front reached the bottom of the tube (ca. 1 h). The current should drop to 6mA. Then the gel was connected to a pump system (pump speed: 1 ml per 5 min). One ml fractions were collected in tubes containing 2M Tris-Cl, pH 6 and 0.005M DTT, (0.2 ml). The final pH was 7.6.
All manipulations were carried out at $4^{o}C$.

Determination of inhibitor activity in alkaline polyacrylamide gels

Polyacrylamide gels, pH 8.9, were prepared as described earlier (16). Per gel 50 units of inhibitor were applied. When the buffer zone reached the

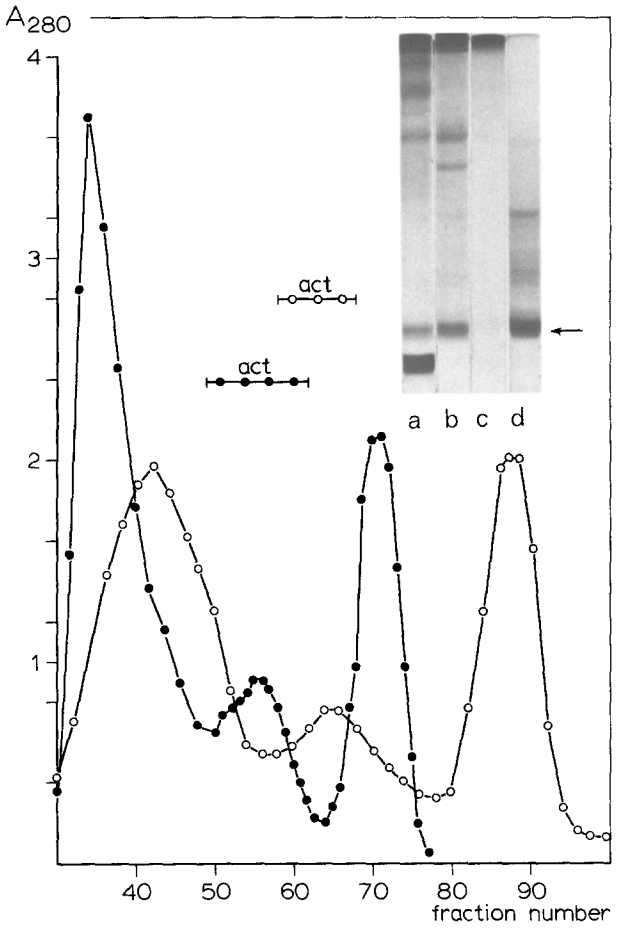

Fig. 1 *Fractionation of calf lens homogenate (75,000 x g supernatant fraction) on DEAE-Sephadex A50 at pH 7 (-●-●-●-) and at pH 6 (-o-o-o-).* The localization of the activity is indicated by the abbreviation "act". Insert: Polyacrylamide gel electrophoresis at pH 8.9 of the isolated active fractions a) at pH 7, b) at pH 6, c) α-crystallin (for comparison), d) after additional purification on Ultrogel (see also fig. 2). The position of the inhibitor is indicated by an arrow.

bottom of the gel tubes one of the gels was stained with Amido black after the electrophoretic run. The other gels were cut into 2 mm pieces. To each slice 0.1 ml water, 0.1 ml RNAse solution (0.05 µg/ml) and 0.2 ml buffer containing 0.5M Tris-Cl, pH 7.8 was pipetted. Incubation was for 30 min. Thereafter 2 mg of yeast RNA (0.2 ml) was added to each tube. The activity was determined virtually as described previously, but at room temperature (13).

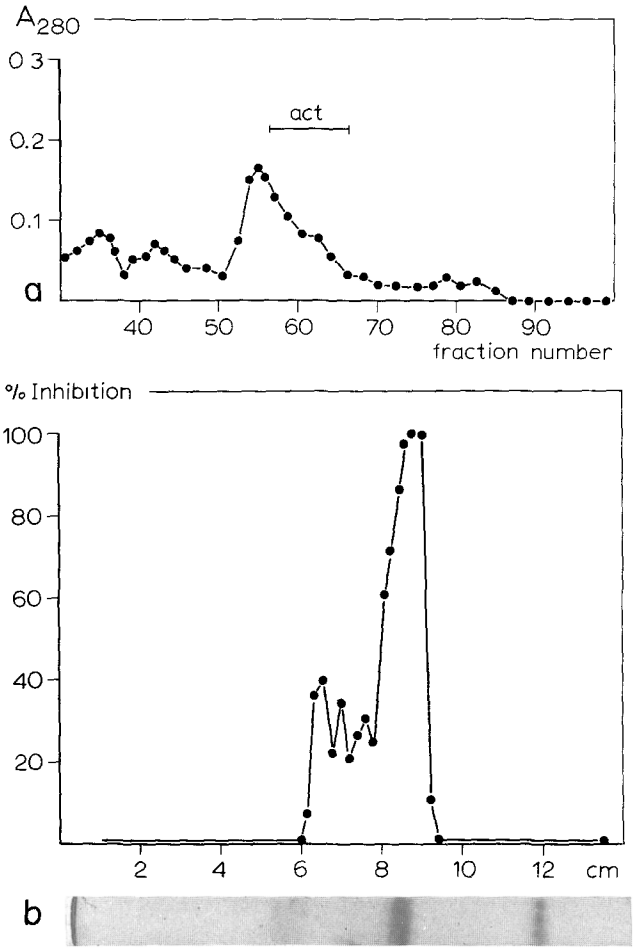

Fig. 2 a) *Gel filtration pattern on Ultrogel AcA 34 of the RNAse inhibitor purified by DEAE-Sephadex chromatography.* The active fraction is indicated by "act". The positions of the water-soluble lens crystallins on such a column are: fraction 30-40: α; 40-55: β_H; 60-75; β_L; 80-95 γ. The molecular weight estimate of the inhibitor is 55,000.

b) *Polyacrylamide gel electrophoresis at pH 8.9 of the active fractions from fig. 2a.* After electrophoresis the gel was cut and the activity measured in each slice.

RESULTS AND DISCUSSION

Ninety per cent of the wet weight of the calf lens consists of structural proteins named crystallins. Numerous hitherto unidentified proteins constitute the remaining ten per cent. Among these proteins the natural inhibitor of alkaline RNAse was demonstrated some years ago (3, 11, 12). In contrast to the previously reported purifications of the inhibitor

TABLE I

Recovery of the RNAse-inhibitor activity
after various purification steps

fraction	absorbance (280 nm)	total activity	specific activity	per cent recovery
30,000 x g supernatant	20,800	171,000	8.4	100
DEAE				
wet	51.6	64,500	1,290	40
lyophilized	22.8	37,000	1,740	20
Ultrogel				
wet	51.1	44,700	10,000	32
lyophilized	3.9	15,300	3,290	10.4

which have been performed at pH 7 or higher, we fractionated the crude lens extract at pH 6. This lowering of the pH from 7 to 6 results in a remarkable improvement of the first purification step by DEAE-Sephadex chromatography. In Fig. 1 separations at both pH values are compared. Gel electrophoretic analysis of the isolated active fractions reveals that besides some β-crystallins also a rapidly migrating major contaminant has completely been removed at pH 6 (compare in insert of Fig. 1, 1a with 1b). The position of the inhibitor was determined by slicing the analytical gel and assaying the individual slices (see Fig. 2b).
A second minor change in the routine procedure was the use of Ultrogel AcA 34 instead of Sephadex G100 in the second purification step. Gel filtration on this material allows a more rapid separation (Fig. 2a). Polyacrylamide gel electrophoresis of the active fraction shows that the major contaminant α-crystallin has completely been removed (Fig. 1 insert d). These results demonstrate that under well-chosen conditions a fairly highly purified inhibitor preparation can be obtained after as few as two purification steps. A quantitative evaluation of the results is summarized in Table I. During removal of ammonium sulfate from the DEAE-preparation by dialysis some inactive protein precipitates,

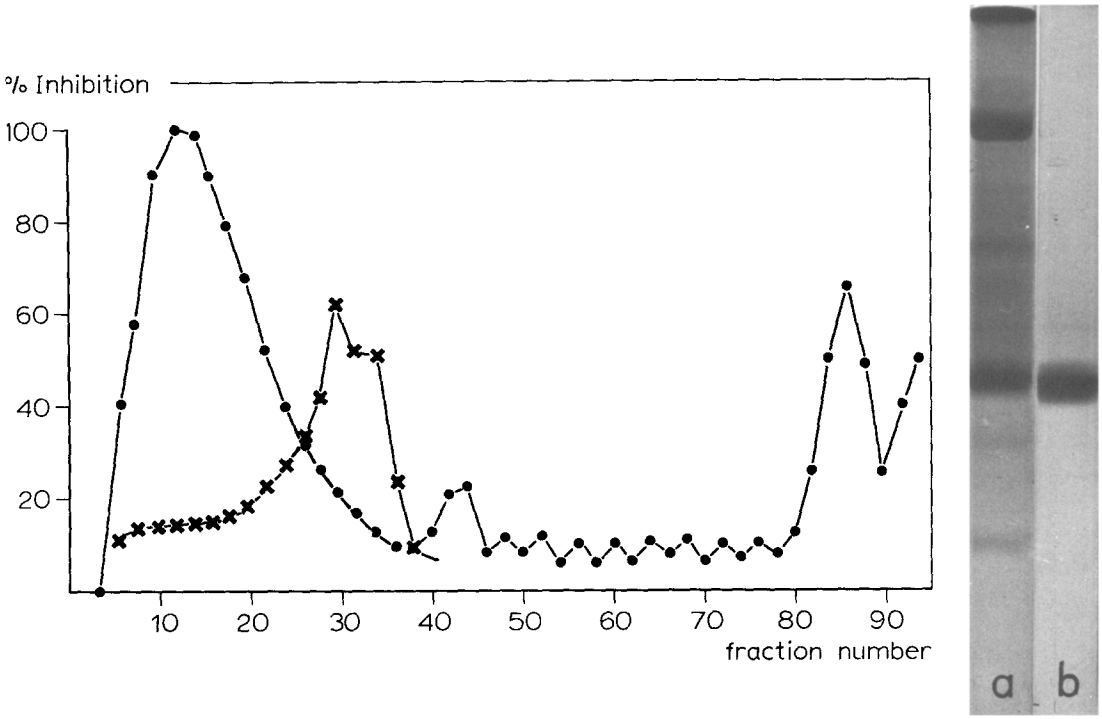

Fig. 3 *Electro-elution of the purified inhibitor and its complex.*
(-●-●-●-) inhibitor activity determined in the absence of *para*-chloromercuribenzoate
(-*-*-*-) inhibitor activity determined in the presence of *para*-chloromercuribenzoate.
a) protein pattern after polyacrylamide gel electrophoresis of the starting material purified on DEAE-Sephadex; b) protein pattern after electro-elution.

giving rise to a higher specific activity of the remaining soluble fraction, albeit a considerable proportion of the total activity is lost (Table I, DEAE, lyophilized). This confirms earlier observations that, in general, the inhibitor activity diminishes after dialysis and lyophilization. In previous papers it was always claimed that upon gel filtration the activity of the inhibitor is completely lost when the preparations are dialyzed and lyophilized. We now succeeded in preserving a considerable part of the activity by the following procedure. (i) After gel filtration the active fractions were immediately precipitated by addition of ammonium sulfate at 65% saturation. (ii) The solution was deaerated and then centrifuged at 75,000 x g. (iii) The sediment was resuspended in a minimal volume of water, dialyzed during 2 h at 4^{o}C four times against 1 l water and twice against 1 l water supplemented with 2 ml medium (A)

in order to keep the inhibitor solubilized. (iiii) The dialyzed solution was lyophilized as short as possible. Apparently the shorter time required for dialysis and lyophilization due to the decrease in sample volume by ammonium sulfate precipitation resulted in the partial maintenance of activity. As after gel filtration no inactive protein precipitates during dialysis the decrease in total activity is paralleled by a decrease in specific activity of the lyophilized preparation (Table I).

Electro-elution

Examination of the profile of the partially purified inhibitor on polyacrylamide gels at alkaline pH showed that the inhibitor migrates with high mobility (Fig. 1 insert). This strongly suggests electro-elution as a favorable tool for further purification. Attempts to isolate the RNAse inhibitor by semi-preparative electrophoresis have previously been reported by Bont et al (17) and Van den Broek et al (3). These workers sliced the gel after electrophoresis. Subsequent extraction of the inhibitor is accompanied by a serious loss of both activity and protein. In a search for a more satisfactory procedure we found the system of Popescu et al (14) to give relatively high recoveries of the protein. Model experiments with serum albumin revealed that 70% of the applied protein could be recovered (15). In Fig. 3 the result obtained after electro-elution of 20,000 inhibitor units from DEAE-Sephadex (pH 6) material is shown. It has to be mentioned that after the electrophoretic purification step the activity is rapidly lost. Variations from 20 - 60% recovery may occur depending upon the moment of activity determination after the run. However, for physico-chemical characterization this loss of activity is less significant than the purity of the protein which is high as shown by the gel electrophoretic pattern (Fig. 3b).

Subunit composition of the inhibitor

The molecular weight of the inhibitor from a variety of sources has been estimated to be about 55,000. This value was obtained by molecular weight determinations on gel filtration columns. Ortwerth and Byrness were the only workers who reported a molecular weight of 32,000 (12). We wondered whether this discrepancy could be due to a subunit structure of the inhibitor. Therefore we subjected the inhibitor isolated by electro-elution to polyacrylamide gel electrophoresis under dissociating conditions. On polyacrylamide gels containing 6M urea three bands were observed (Fig. 4a), two of which migrated with higher mobility than the most acidic subunit of α-crystallin (Fig. 4b) (αA_1: iso-electric point

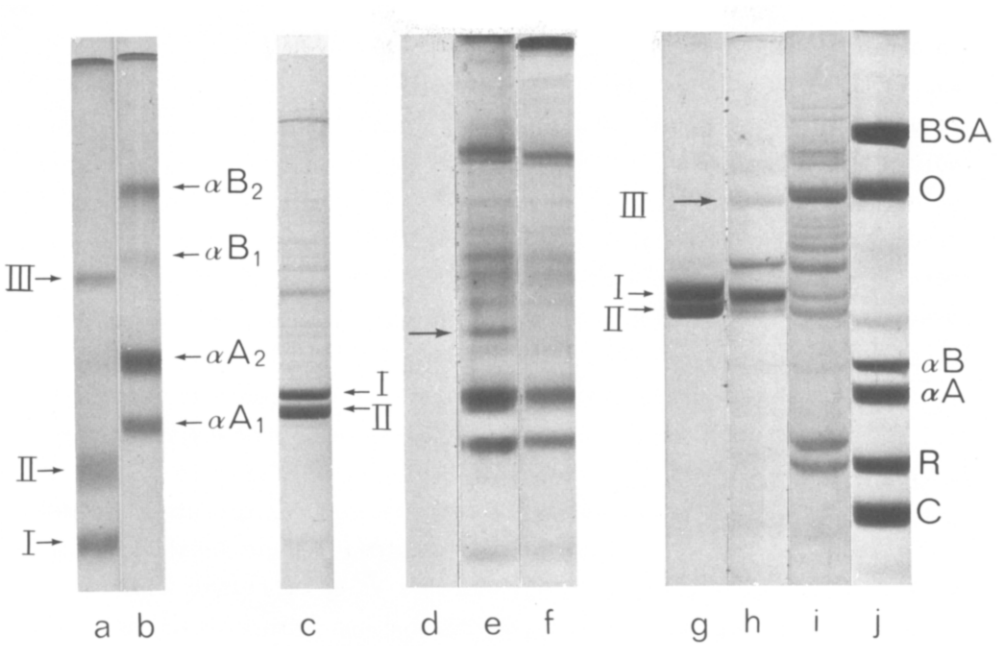

Fig. 4 *Polyacrylamide gel electrophoretic patterns of the subunits of the RNAse inhibitor and of the inhibitor-RNAse complex under different conditions.* In 6M urea: a) subunit I, subunit II and complex III; b) α-crystallin subunits (for comparison). In 0.1% sodium dodecyl sulfate: c) the subunits of the inhibitor. Without addition of detergents: d) RNAse (control); e) complex (arrow); f) DEAE-Sephadex preparation used as inhibitor material for complex formation (control). In 0.1% dodecyl sulfate: g) inhibitor isolated by electro-elution; h) complex formation with excess of inhibitor (the band above subunit I is occasionally found in the starting material); i) complex formation with excess of RNAse; j) marker proteins: C = cytochrome c (12,400) R = ribonuclease (13,700), αA = calf α-crystallin A chain apparent mol. weight (19,000), αB = calf α-crystallin B chain apparent mol. weight (21,000), O = ovalbumin (45,000), BSA = bovine serum albumin (67,000).
(The complex migrates just below ovalbumin.)

in 6M urea at 24°C = 5.6 (18)). On polyacrylamide gels containing sodium dodecyl sulfate the purified inhibitor yields the pattern shown in Fig. 4c. The two subunits have an approximate molecular weight of 28,000 and 30,000, respectively. The bands from the urea gel were cut out and subsequently applied to sodium dodecyl sulfate gel. In that case subunit I yields the 30,000 band, whereas subunit II corresponds with the 28,000 component. (Band III is due to native inhibitor-RNAse complex, see below).

The inhibitor-RNAse complex

Several authors provided indirect evidence that the inhibitor exerts its activity by complex formation with RNAse. For instance when the putative complex is treated with *para*-chloromercuribenzoate the inhibitor activity is destroyed and RNAse can be measured (19). We were able to visualize the complex electrophoretically. For this purpose an inhibitor preparation purified on DEAE-Sephadex was incubated with RNAse and the mixture subjected to polyacrylamide gel electrophoresis at pH 8.9. From Fig. 4e it can be seen that a new band appears which migrates less fast than free inhibitor. With increasing concentration the complex band becomes more intensive. If only RNAse is loaded onto the gel, no band at all can be detected (Fig. 4d).

Like free inhibitor the complex can be isolated by electro-elution (compare Fig. 3). It resists dodecyl sulfate treatment and is localized in a region that corresponds to about 44,000. This value represents the sum of the molecular weights of RNAse (13,700) and one inhibitor subunit (approximately 30,000).

The latter finding together with the notion that free inhibitor consists of two subunits still leaves two possibilities open. (i) RNAse interacts with only one of the subunits. (ii) RNAse interacts with both subunits but the total inhibitor-RNAse complex is dissociated into two half complexes upon treatment with sodium dodecyl sulfate. Van den Broek (20) attempted to estimate the molecular weight of the complex by gel filtration. Surprisingly he found the artificially prepared complex almost coinciding with free inhibitor in the 55,000-60,000 region. This observation may indicate that under certain non-dissociating conditions two RNAse molecules interact with one of the inhibitor subunits. We have the following experimental evidence which may favor the latter explanation. In order to achieve complex formation incubation of excess RNAse (1 µg) was carried out with 32 inhibitor units. In a parallel experiment an excess of inhibitor (160 units) was incubated with 1 µg of RNAse. Both incubations and a control without RNAse were subjected to electro-elution at pH 8.9. Since under this condition unreacted RNAse cannot migrate, its presence as "free" contamination can be excluded.

The complex and free inhibitor emerging in the fast moving fractions (see Fig. 3) were assayed in the presence and absence of *para*-chloromercuribenzoate, respectively. Thereafter analytical polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed.

Fig. 4g shows the free inhibitor consisting of 2 subunits. If incubation was carried out with excess of inhibitor, only one subunit seems to be

involved in complex formation (Fig. 4h). When, on the other hand, RNAse was present in excess during incubation, both subunits seem to have interacted with RNAse, whereas one RNAse molecule is released (Fig. 4i). This means that one RNAse molecule is firmly bound and resists the action of sodium dodecyl sulfate, while the other molecule(s) is (are) dissociated from the complex.

The elucidation, however, of the exact mechanism of interaction between the enzyme and the inhibitor requires more quantitative studies.

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