Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Luigi Bosco, Giorgio Venturini

Separation and characterisation of the lens proteins of the amphibian Rana esculenta

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **82** (1988), n.2, p. 353–359.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1988_8_82_2_353_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1988.

Atti Acc. Lincei Rend. fis.
(8), LXXXII (1988), pp. 353-359

Istologia. — *Separation and characterisation of the lens proteins of the amphibian* Rana esculenta (*). Nota di LUIGI BOSCO e GIORGIO VENTURINI, presentata (**) dal Socio A. STEFANELLI.

ABSTRACT. – In the present work the soluble lens proteins of *Rana esculenta* were studied by ion exchange chromatography, polyacrylamide gel electrophoresis and by immunoelectrophoresis. The column chromatography of total lens proteins, carried out with NaH_2PO_4-Na_2HPO_4-NaCl buffer system revealed the presence of three main fractions. The first eluted, and predominant fraction, corresponds to gamma crystallins. Alpha and beta fractions follow as abundance. Further chromatography with a Tris-HCl buffer system of the isolated and concentrated gamma crystallins fraction showed six distinguishable components: this result was confirmed by acrylamide gel electrophoresis. Immunoelectrophoretic analysis of total lens proteins demonstrated a complex pattern with the presence of a minor, fast component that can be identified with the pre-alpha crystallins.

KEY WORDS: Amphibia; Lens; Crystallins.

RIASSUNTO. – *Separazione e caratterizzazione delle proteine della lente in* Rana esculenta. Nella presente ricerca sono state studiate le proteine solubili della lente di adulti di *Rana esculenta* mediante cromatografia a scambio ionico su colonna, elettroforesi su gel di poliacrilamide ed analisi immunoelettroforetica. La cromatografia su colonna delle proteine totali della lente, effettuata con il sistema tampone NaH_2PO_4-Na_2HPO_4-NaCl, ha rivelato la presenza di sei frazioni. La prima frazione eluita corrisponde alle γ cristalline, e rappresenta la frazione predominante; seguono, in ordine di abbondanza, la frazione β e la frazione α. Una ulteriore cromatografia della frazione γ, isolata e concentrata, ha rivelato la presenza di sei distinte componenti, la cui presenza è confermata dall'analisi elettroforetica su gel di poliacrilamide. L'analisi immunoelettroforetica delle proteine totali ha evidenziato un quadro complesso con la presenza di una componente minore che può essere identificata come una frazione pre-α.

INTRODUCTION

The soluble lens proteins of various amphibian species have been widely investigated, and they are classified into alpha, beta and gamma crystallins (McDevitt, 1967; Campbell *et al.*, 1968; Brahma and Doorenmaalen, 1969; Polansky and Bennet, 1970; Brahma and Bours, 1971; McDevitt, 1972; Polansky and Bennet, 1973; Bours and

(*) Ricerca svolta col sussidio di finanziamenti del Ministero della Pubblica Istruzione.
(**) Nella seduta del 9 gennaio 1988.

Brahma, 1973; McDevitt and Brahma, 1973; Brahma and McDevitt, 1974a, 1974b; Brahma and Doorenmaalen, 1976; McDevitt and Brahma, 1977; McDevitt, 1982). In some species the sequential appearance of crystallins during development is also known (McDevitt *et al.*, 1969; McDevitt and Brahma 1973, 1977, 1979, 1981; Brahma and McDevitt 1974a, 1974b; Nothiger *et al.*, 1971), and the presence of these typical proteins in the lens cells is considered an indicator of lens differentiation. In particular, during the development of anuran species, the gamma crystallins appear in the inner cells of the lens rudiment and then remain restricted only to the lens fiber region (McDevitt *et al.*, 1969). The detection of soluble lens proteins is widely applied to determine the differentiative state of the lens cells during development, and of other tissues that, in some conditions, are able to undergo lens-trandifferentiative processes *in vivo* and *in vitro* (Yamada, 1982).

Comparative studies of the soluble lens proteins from different amphibian species have revealed that a different degree of lens antigens identity is present. Immunodiffusion tests showed a complete identity reaction of lens antigens of *Triturus cristatus*, *Ambystoma mexicanum*, *Rana esculenta* and *Bufo bufo* with respect to *Xenopus laevis* antiserum, while *Xenopus laevis*, *Triturus cristatus*, *Ambystoma mexicanum*, revealed only partial identity when tested against *Rana esculenta* and *Bufo bufo* antisera (Brahma and Doorenmaalen, 1969). These studies, however, have been done without the purification of lens proteins.

Brahma and Doorenmaalen (1976) prepared a specific antiserum against *Rana esculenta* lens pre-alpha crystallin, by injecting into a rabbit the antigen-antibody precipitate of this crystallin, obtained from immunoelectrophoresis of total lens proteins against homologous antiserum. De Jong and Goodman (1982) studying the mammalian phylogeny by sequence analysis of the eye lens protein α-cristallin, included *Rana esculenta* as an outgroup. However, the separation and complete characterization of *Rana esculenta* lens proteins has not been performed.

Materials and methods

Rana esculenta adults, obtained from local dealers, were sacrificed and the lenses were removed and freed from any foreign tissue. The lenses were then washed three times in ice-cold 5 mM phosphate buffer pH 7.0 and stored at - 20 °C.

Soluble lens proteins were obtained by homogenization in the same phosphate buffer and by centrifugation in a refrigerated centrifuge at 20000×g for 30 min. The supernatant was dialysed for 48 hours with repeated changes against the same homogenization buffer. The protein content was evaluated with the Lowry (Lowry *et al.*, 1951) method using bovine serum albumin as a standard, or with the E280/E260 method of Warburg and Christian (1942).

Total lens protein (500 mg in 2 ml) was applied to a 14 × 200 mm column of diethylaminoethyl (DEAE) Cellulose (Whatman DE 32) previously equilibrated with

5 mM phosphate buffer pH 7.0 and eluted with a NaH_2PO_4-Na_2HPO_4 - NaCl buffer system as proposed by McDevitt (1967), using the following sequence: 0.005 M phosphate pH 7, 30 ml; 0.0075 M phosphate pH 6.5, 70 ml; 0.01 M phosphate pH 6, 50 ml; 0.02 M phosphate pH 5.7, 70 ml; 0.02 M phosphate pH 5.7 + 0.1 M NaCl, 70 ml; 0.1 M phosphate pH 5.7 + 0.1 M NaCl, 50 ml; 0.1 M phosphate pH 5.7 + 0.3 M NaCl, 50 ml; 2.5 ml fractions were collected by drop counting. The γ crystallin fraction obtained from elution was concentrated using a dialysis bag covered with Aquacide (Calbiochem) and then reapplied to a DE 32 chromatographic column. Elution was achieved with a Tris-HCl buffer system using the following sequence: 0.01 M Tris pH 9.6, 75 ml; 0.02 M Tris pH 9, 75 ml; 0.04 M Tris pH 8.6, 75 ml; 0.06 M Tris pH 8.2, 75 ml; 0.08 M Tris pH 7.6, 75 ml; 0.1 M Tris pH 7.2, 75 ml; 3 ml fractions were collected. The 280 mμ absorbancy of fractions was measured using a Beckman DBG spectrophotometer.

Polyacrylamide gel electrophoresis of concentrated fractions was performed with 7.5% acrylamide, 0.2% bis-acrylamide in 0.37 M Tris HCl buffer pH 8.8. A 3% acrylamide stacking gel in 0.125 M Tris HCl buffer pH 6.8 was used.

To obtain anti-lens protein sera, female rabbits were injected subcutaneously with total soluble lens proteins emulsified with complete Freund's adjuvant (1:1). Injections were repeated three or more times every third week. Bleeding was performed one week after a booster injection, without adjuvant.

Immunoelectrophoretic analysis was performed using 1% agarose in 0.05 M veronal buffer, pH 8.4, on microscope slides, at 5 Volts/cm. Staining was performed with Coomassie Brillant Blue R-250.

Results

DEAE (Diethyl aminoethyl) cellulose chromatographic analysis of total lens proteins of *Rana esculenta* reveals a typical and reproducible pattern. Six major fractions can be detected by 280 mμ absorbancy with the phosphate/NaCl buffer system (Fig. 1). According to previously observed elution patterns of *Rana pipiens* (McDevitt, 1967), the first eluted and predominant fraction (a), corresponds to gamma-crystallins, (b) and (c) fractions to beta-crystallins and (d) to (f) fractions, to alpha-crystallins. Further DEAE cellulose chromatography of the first fraction, performed after concentration, using a Tris/HCl buffer system, demonstrate the presence of five subfractions (Fig. 2). The more sensitive acrylamide electrophoretic analysis of the same gamma-crystallins concentrated fraction confirm the presence of at least six distinguishable components (Pl. I *a*).

Immunoelectrophoretic analysis of total lens proteins, against the anti-total lens proteins antiserum, demonstrates a complex pattern, with the presence of fast minor component that can be identified with the pre-alpha crystallins, observed in this species by Brahma and Doorenmaallen (1976).

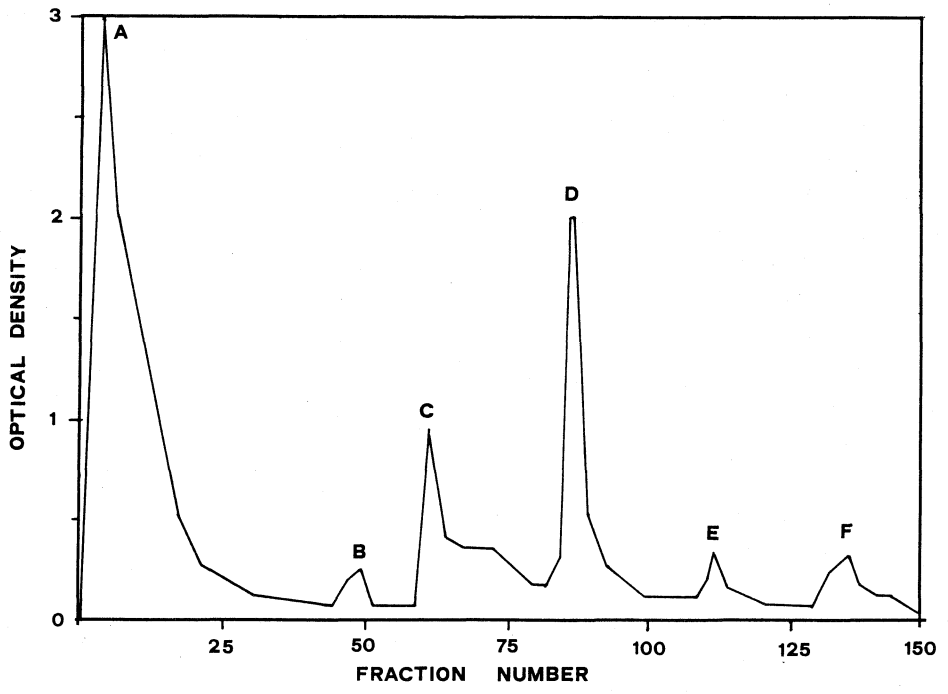


Fig. 1. - Elution pattern of adult *Rana esculenta* total lens protein. 2.5 ml fractions were collected. Optical density at 280 mμ in 1 cm light path cuvettes. A = γ cristallins; B + C = β cristallins; D + E + F = α cristallins.

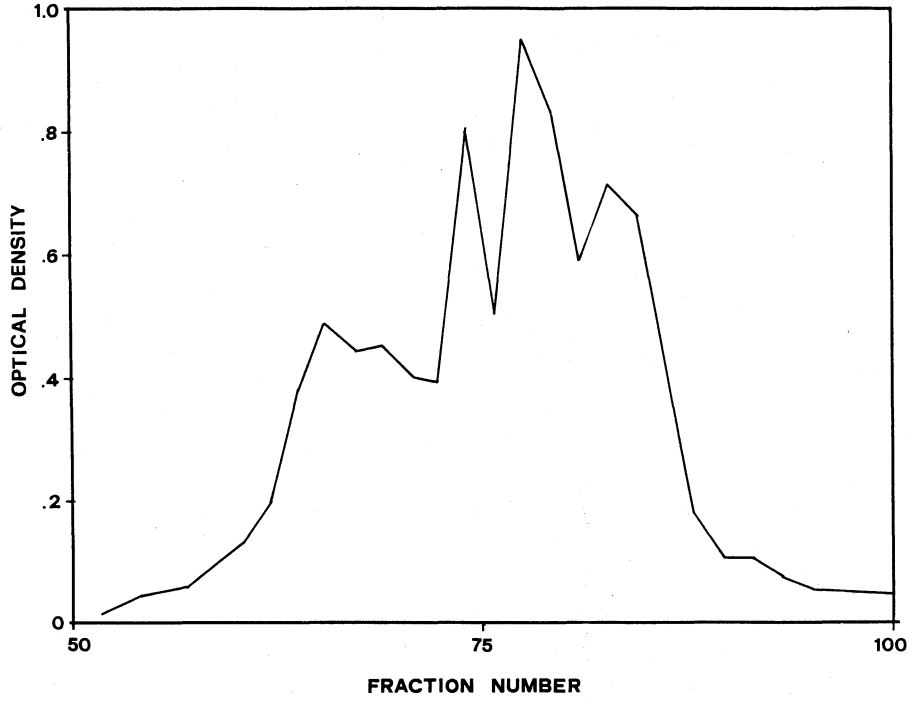


Fig. 2. - Elution pattern of adult *Rana esculenta* isolated γ cristallins (Fractions A of Fig. 1). 3 ml fractions were collected. Optical density at 280 mμ in 1 cm light path cuvettes.

Discussion

The results obtained in this investigation show that the soluble lens proteins of adult *Rana esculenta*, can be separated by DEAE cellulose chromatography in the typical α, β and γ crystallins of the amphibians eye lens. In this species the more abundant class of lens proteins is represented by γ crystallins (56.2%), followed by α crystallins (26.4%) and β crystallins (17.4%).

The isolated γ crystallins fraction, re-chromatographed on DEAE cellulose with a Tris-HCl buffer elution system, demonstrated the presence of at least four subfractions. Polyacrylamide disc electrophoresis of the same fraction reveals the presence of six distinct proteins.

The immunoelectrophoretic analysis of total lens proteins, carried out using rabbit anti-total lens proteins antiserum, allowed the detection of a pre-α precipitation arc, not evident on chromatography. The presence of a pre-α crystallin was already demonstrated in *Rana esculenta* by Brahma and Doorenmaalen (1969), in *Xenopus laevis* by Campbell *et al.*, (1968) and in mammals by Van Dam and Ten Cate (1966) and by Swanborn (1966).

The lens protein pattern of amphibians has been considered, from the phylogenetic point of view (McDevitt 1967; McDevitt and Brahma 1977), in an intermediate position between the pattern of fish and birds. In fish in fact the γ crystallins are the predominant lens proteins, and α and β crystallins are present in small amounts, (Lerman *et al.*, 1965; Rabaey 1965; Maisel and Goodman 1965), whereas in birds a very small quantity of γ crystallins is present (Papaconstantinou and Resnik 1959, Rabaey 1962, Rabaey *et al.*, 1972, Maisel and Goodman 1965, McDevitt and Crof 1977). The present data concerning adult *Rana esculenta* confirm this phylogenetic evaluation, even if for a more significant discussion further investigations are necessary, especially in Reptiles.

REFERENCES

BOURS J. and BRAHMA S.K. (1973) - Isoelectric focusing of embryonic cow lens crystallins. "Exp. Eye Res.", *16*, 131-142.

BRAHMA S.K. and BOURS J. (1971) - Thin layer isoelectric focusing of the soluble lens extracts from larval stages and adult *Xenopus laevis*. "Exp. Eye Res.", *13*, 309-314.

BRAHMA S.K. and VAN DOORENMAALEN W.J. (1969) - Study of the soluble lens proteins from different amphibian species. "Exp. Eye Res.", *8*, 168-171.

BRAHMA S.K. and VAN DOORENMAALEN W.J. (1976) - Preparation of specific antiserum against *Rana esculenta* pre α crystallin. "Experientia", *32*, 930-931.

BRAHMA S.K. and MCDEVITT D.S. (1974a) - Ontogeny and localization of the lens crystallins in *Xenopus laevis* lens regeneration. "J. Embryol. Exp. Morphol.", *45*, 271-281.

BRAHMA S.K. and MCDEVITT D.S. (1974b) - Ontogeny and localization of gamma crystallin in *Rana temporaria*, *Ambystoma mexicanum* and *Pleurodeles waltlii* normal lens development. "Exp. Eye Res.", *19*, 379-387.

CAMPBELL J.C., CLAYTON R.M. and TRUMAN D.E.S. (1968) - Antigens of the lens of *Xenopus leavis*. "Exp. Eye Res.", *7*, 4-10.

DE JONG W.W. and GOODMAN M. (1982) - Mammalian phylogeny studied by sequence analysis of the eye lens proteins α-crystallins. "Z. Sauget", *47*, 237-276.

LERMAN S., ZIGMAN S., TUTTLE J. and FORBES W. (1965) - A study of gamma crystallin in the dogfish lens. "Biol. Bull.", *129*, 412.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. and RANDALL R.J. (1951) - Protein measurement with the Folin phenol reagent. "J. Biol. Chem.", *193*, 265-275.

MAISEL H. and GOODMAN M. (1965) - Comparative electrophoretic study of vertebrate lens proteins. "Amer. J. Ophthal.", *59*, 697-794.

MCDEVITT D.S. (1967) - Separation and characterization of the lens proteins of the amphibian, *Rana pipiens*. "J. Exp. Zool", *164*, 21-30.

MCDEVITT D.S. (1972) - Sequential appearnce of α, β and γ crystallins in embryonic and metamorphic *Rana pipiens*. "Experientia", *28*, 1215-1217.

MCDEVITT D.S. (1982) - Ontogeny and localization of the crystallins in Eye lens developement and regeneration. In "Cell Biology of the eye", (McDevitt D.S. edit.), Academic Press, New York, pp. 143-191.

MCDEVITT D.S. and BRAHMA S.K. (1973) - Ontogeny and localization of the crystallins during *Xenopus laevis* embryonic development. "J. Exp. Zool.", *186*, 127-140.

MCDEVITT D.S. and BRAHMA S.K. (1977) - Eye development and crystallins in *Discoglossus pictus*. "Experientia", *33*, 1087-1089.

MCDEVITT D.S. and BRAHMA S.K. (1979) - Embryonic appearance of α, β and γ crystallins in the periodic albinism (a^p) mutant of *Xenopus laevis*. "Differentiation", *14*, 107-112.

MCDEVITT D.S. and BRAHMA S.K. (1981) - Ontogeny and localization of the α, β and γ crystallins in newt eye lens development. "Dev. Biol.", *84*, 449-454.

MCDEVITT D.S. and CROFT L.R. (1977) - On the existence of γ crystallin in the bird lens. "Exp. Eye. Res.", *23*, 473-481.

MCDEVITT D.S., MEZA I. and YAMADA T. (1969) - Immunofluorescence localization of the crystallins in amphibian lens development, with special reference to the gamma crystallins. "Dev. Biol.", *19*, 581-607.

NOTHINGER R., MCDEVITT D.S. and YAMADA T. (1971) - Comparison of crystallins from frog and newt. "Exp. Eye Res", *12*, 94-98.

PAPACOSTANTINOU J. and RESNIK R.A. (1959) - Characterization of the lens proteins. As reported by: James D. Ebert in Ann. Rep. Dir. Dept. Embryol., Carnegie Inst., Washington, 1958-1959, pp. 379-385.

POLANSKY J.R. and BENNETT T.P. (1970) - Difference in the soluble lens proteins from tadpole and adult bull frogs. "Biochem. biophys. Res. Commun.", *39*, 197-204.

POLANSKY J.R. and BENNETT T.P. (1973) - Alterations in physical parameters and proteins of

lens from *Rana catesbiana* during development. "Devel. Biol.", *33*, 380-402.

Rabaey M. (1962) - Electrophoretic and immunoelectrophoretic studies on the soluble proteins in the developing lens of birds. "Exp. Eye Res.". *1*, 310-316.

Rabaey M. (1965) - Lens proteins during embryonic development of different vertebrates. "Invest. Ophthalmol.", *4*, 560-578.

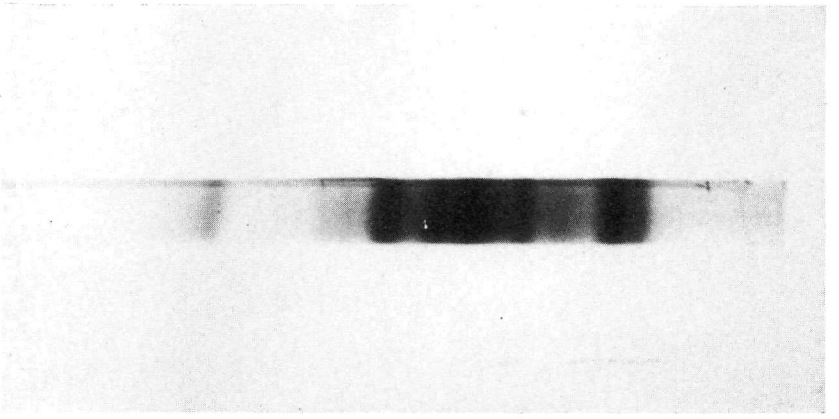
Rabaey M., Rikkers I. and De Mets M. (1972) - Low molecular weight protein (γ-crystallin) in the lens of birds. "Exp. Eye Res.", *14*, 208-213.

Swanborn L. (1966) - The method of simultaneous electrophoretic of antiserum and antigen (immuno-osmophoresis) applied to lens antigens. "Exp. Eye Res.", *5*, 302-308.

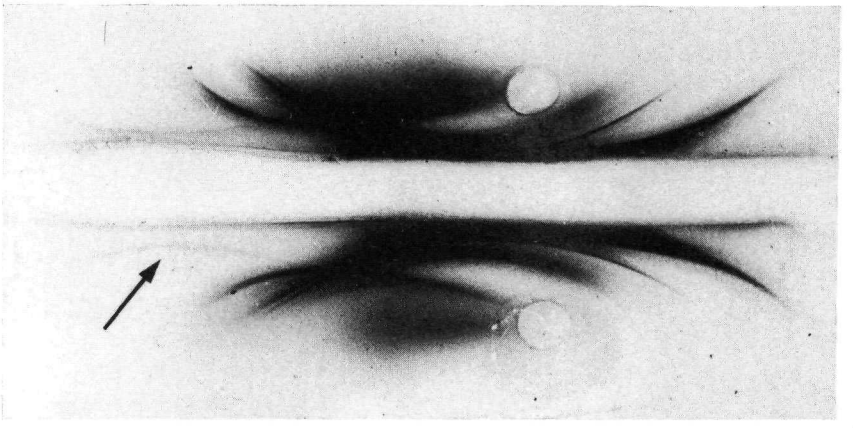
van Dam A.F. and Ten Cate G. (1966) - Isolation and some properties of α-crystallin. "Biochim. Biophys. Acta", *121*, 183-186.

Warburg O. and Christian W. (1942) - Isolierung und kristallisation des garungsferments enolase. "Biochem. Zeit", *310*, 384-421.

Yamada T. (1982) - Transdifferentiation of lens cells and its regulation. In "Cell Biology of the eye" (McDevitt D.S. edit.), Academic Press, New York, pp. 193-242.



I,*a* – Immunoelectrophoretic pattern of adult *Rana esculenta* total lens protein against rabbit anti-total lens protein antiserum. Acrow indicates pre-alpha fractions.



I,*b* – Acrylamide gel electrophoresis of adult *Rana esculenta* isolated γ cristallins (Fractions A of Fig. 1).