

Thin layer Isoelectric Focusing of the Soluble Lens Extracts from Larval Stages and Adult *Xenopus laevis*

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Soluble lens extracts from different larval stages and also from adult *Xenopus laevis* were studied by thin layer isoelectric focusing. These lens extracts were also tested against anti-serum to adult *X. laevis* total lens proteins by two-dimensional antigen-antibody crossed electrophoresis. By isoelectric focusing the lens crystallins were resolved into 13 major components. In the adult lens some components of the γ -crystallins were lost, the β -crystallins formed the major fraction and the α -crystallin appeared to be the weakest of the crystallin fractions.

1. Introduction

Soluble lens crystallins of the vertebrates are classified into α -, β -, and γ -crystallins according to their mobilities in an electrical field and also according to their molecular weights. With the introduction of new methods of isolation of these crystallins, it is now known that all these crystallins are highly heterogeneous.

Immunofluorescence studies of the lens crystallins in *X. laevis* embryos with antibodies to adult *X. laevis* total lens proteins and chromatographically purified adult *Rana pipiens* γ -crystallins showed that γ -crystallins are among the first, if not the first crystallins to appear during development and distribution is restricted only to the cells where lens fibres will form (McDevitt and Brahma, 1971). McDevitt (1968) reported that in *R. pipiens* embryos γ -crystallins are one of the first lens specific proteins that could be detected by immunoelectrophoresis. This is supported by later observations of McDevitt, Meza and Yamada (1969) using immunofluorescence methods to detect lens specific antigens.

In other amphibian species (some urodeles) Yamada and his group (Yamada, 1967) have shown that γ -crystallins are restricted only in the fibre cells and α - and β -crystallins are always present in the cells which contains γ -crystallins.

In the bovine lens too, Papaconstantinou (1965) has shown that γ -crystallins are associated with fibre cells.

Electrophoretic and immunoelectrophoretic studies have revealed a difference between embryonic and adult lens crystallins in a number of vertebrates (Papaconstantinou, 1965; Rabaey, 1965; Génis-Galvèz and Maisel, 1967; Rabaey, 1968; Clayton, 1970; Polansky and Bennett, 1970; Rabaey and Lagasse, 1971).

We have made a comparative study of the soluble lens extracts from different larval stages and adult *X. laevis* by thin layer isoelectric focusing. We have included in our experiments two-dimensional antigen-antibody crossed electrophoresis using anti-serum to adult *X. laevis* total lens proteins against all these lens extracts.

2. Materials and Methods

Larval stages of *X. laevis* were collected from animals bred in the laboratory and reared and staged according to Nieuwkoop and Faber (1956). Lenses were removed from stages

52, 61, and 66 and also from adult animals. These tissues were then homogenized in cooled distilled water and centrifuged at 49,000 g for 30 min at 4°C. The supernatant was then lyophilized and stored at -20°C.

Thin layer isoelectric focusing

This was carried out on polyacrylamide gel which was cast on a 17.5 × 25 cm glass plate according to Bours (1971) with a 5% polyacrylamide gel containing 2% Ampholine carrier ampholytes (LKB-Produkter A.B. Bromma, Sweden). We used ampholines from pH 3-5, 5-7, and 7-10 mixed in the ratio of 1:1:1.

A 2% solution was prepared from the lyophilized lens extracts in distilled water and 50 µl of each of the samples was used in a single experiment. These samples were applied on 1 cm² Whatman No. 3 mm filter papers with an Eppendorf microliter pipette (Marburg, Germany). The filter papers were placed at a distance of 1.5 cm away from the anodic side and 1 cm apart from one another before the samples were applied on them.

Isoelectric focusing was carried out at 4°C for 22 hr with an initial current of 4 mA and 50 V. This was adjusted to 250 V after 16 hr. After the run the pH gradient along the gel was measured at 4°C with a flat membrane glass electrode (Radiometer G 242C) at places 20 mm apart on both right and left sides of the gel along the sample. The protein components were then fixed in 14% trichloroacetic acid overnight at the room temperature and subsequently stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) and photographed.

Densitometric measurements

The photographs of the stained isofocused protein components of each of the samples were scanned with a Chromoscan recording reflectance densitometer (Joyce, Loebel and Company Limited, Gateshead, England).

The areas covering the α -, β -, and γ -crystallin peaks were recorded from the automatic integrator fitted with the machine and each of the lens crystallins was then expressed as a percentage of the whole by the expression:

$$\frac{\text{Reading obtained from the fractions}}{\text{Reading obtained for total recording}} \times 100.$$

Two-dimensional antigen-antibody crossed electrophoresis

We followed the modified methods of Laurell (1965) and Clarke and Freeman (1968) by Dr Ruth Clayton and her group introduced during the International Working Party on lens crystallins held in Edinburgh, 1970. These were carried out on 8.2 × 8.2 cm glass slides with Agarose A 37 (L'Industrie Biologique Française, S.A., France) in the high resolution buffer of Aronsson and Grönwall (1957) at pH 8.9.

The first dimensional electrophoresis was carried out at a constant voltage of 300 V for 45 min at 4°C. The second dimensional run was also at a constant voltage of 150 V for 22 hr against antiserum to adult *Xenopus laevis* total lens proteins at 4°C. Slides were then washed in saline, dried and stained with Amido Black.

3. Results

Thin layer isoelectric focusing of the lens extracts from different larval stages and adult *X. laevis* is shown in Plate 1(a, b, c, d). The focused protein components are sharp and altogether thirteen major components could be detected, though in none of the lens extracts could all of these thirteen components be observed.

A major difference is observed between stage 52 [Plate 1(a)] and adult lens proteins [Plate 1(d)]. In the latter, the concentrations of protein components at pH 4.75 and

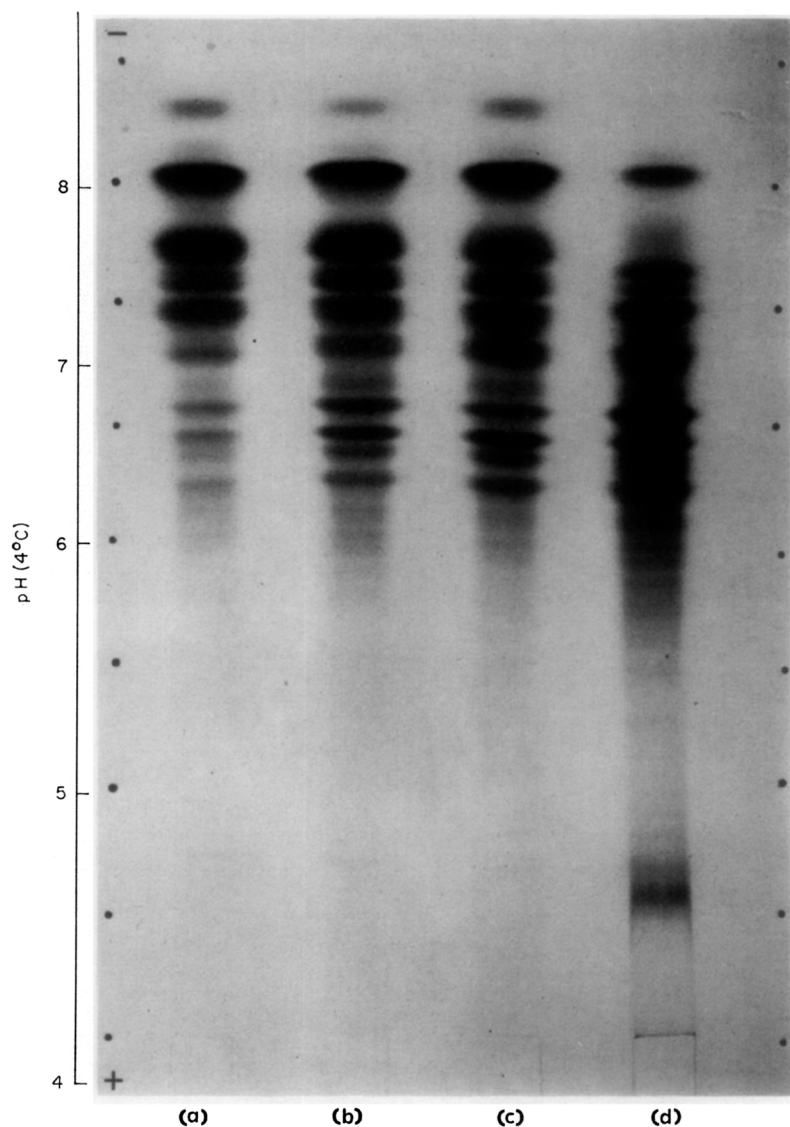


PLATE 1. Isoelectric focusing of lens extracts of (a) stage 52; (b) stage 61; (c) stage 66; and (d) adult *X. laevis*. We used ampholines from pH 3-5, 5-7, and 7-10 mixed in the proportion of 1:1:1. The isoelectric focusing was carried out at 4°C. The scale shows the pH values along the gel. Fifty microliters of a 2% solution in distilled water was used in each case and the gel was stained with Coomassie Brilliant Blue R-250.

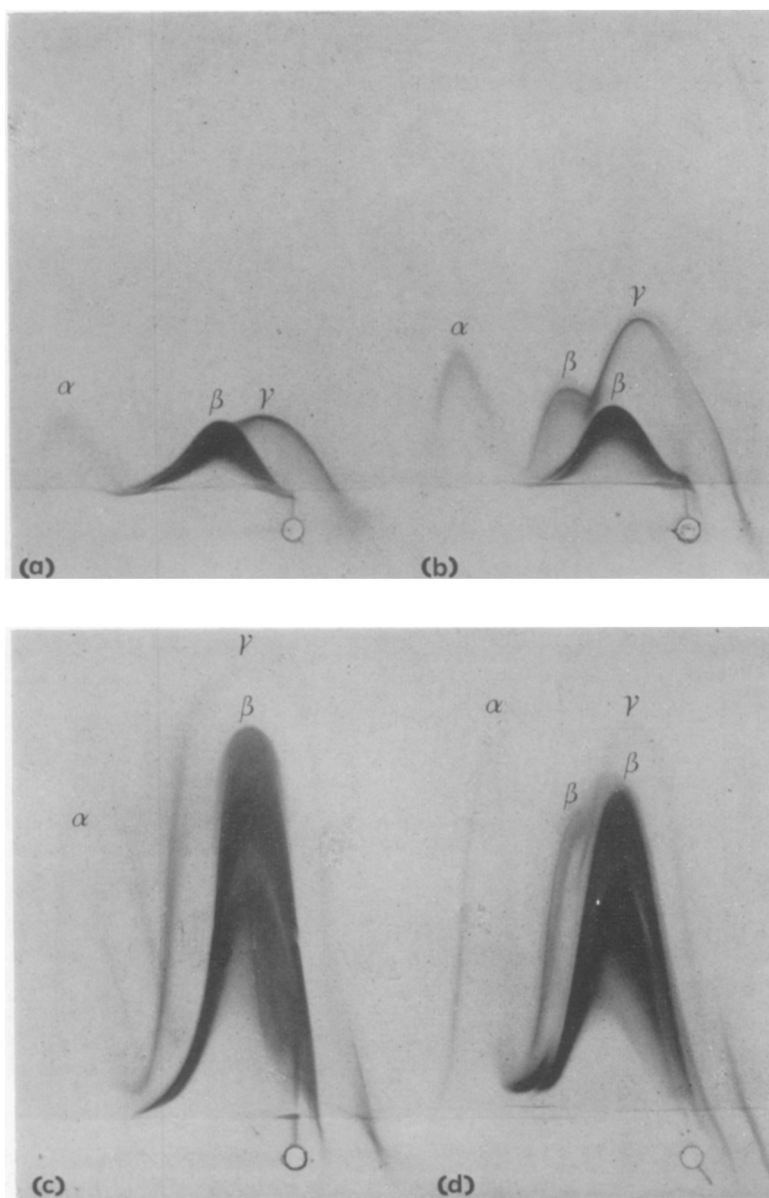


PLATE 2. 2D crossed electrophoresis of lens extracts of (a) stage 52; (b) stage 61; (c) stage 66; and (d) adult *X. laevis* tested with antiserum to adult *X. laevis* total lens proteins. The slides were stained with Amido Black. α - α -crystallin; β - β -crystallin; γ - γ -crystallin.

also between 6.40 and 6.95 are greatly increased; the concentration of protein component at pH 8.10 is reduced; the component at pH 7.85 is reduced almost to nothing; and the component at pH 8.30 is absent.

In the stages 61 and 66 [Plate 1(b) and (c)] distribution patterns of the protein components and their concentrations are nearly the same all along the pH range. These two stages also show difference from stage 52 [Plate 1(a)] in the pH regions between 6.40 and 6.95 by the fact that the concentration of protein components in these regions is greatly increased in these two stages.

The pH gradient obtained after the run was nearly linear between pH 4.2 and 9.5 (Fig. 1).

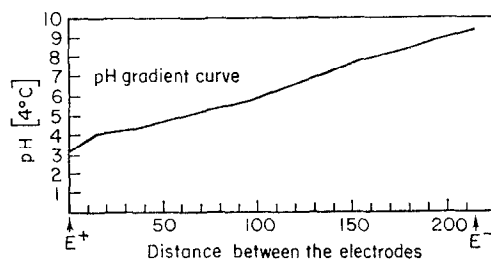


FIG. 1. pH gradient curve of the gel plate measured with a Radiometer surface glass electrode at 4°C after isoelectric focusing also at 4°C. The gradient was nearly linear between pH 4.2 and 9.5. E denotes position of the electrodes; + = anode; - = cathode.

TABLE I

*Isoelectric points of the different components of the lens crystallins from various larval stages and adult *X. laevis* as revealed by thin layer isoelectric focusing*

Lens crystallin components	Isoelectric points (pH = pI)	Crystallins
1	4.75	α
2	6.40	
3	6.60	
4	6.70	} β
5	6.85	
6	6.95	
7	7.15	
8	7.40	} γ
9	7.50	
10	7.60	
11	7.85	
12	8.10	
13	8.30	

Protein components at their different isoelectric points are shown in Table I and are numbered in arabic numerals from the anodic to the cathodic side.

Densitometric tracings of the lens protein samples are shown in Fig. 2(a, b, c, d). The number of peaks, which are also marked with arabic numerals, correspond with the components shown in Table I.

The components between pH 7.15 and 8.30 appear to be the γ -crystallins as these correspond with the isofocused components of isolated γ -crystallins from *R. pipiens*

lens (McDevitt and Brahma, unpublished). Since lens α -crystallins from different vertebrate lens have an isoelectric point between 4.75 and 4.85 (Bours, 1971) it appears that the component at pH 4.75 in our experiment represents the α -crystallin. The rest of the components between pH 6.40 and 6.95 are very likely the β -crystallins.

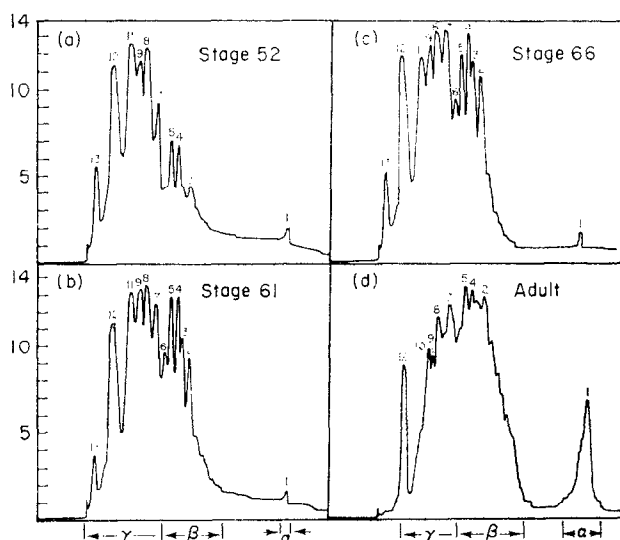


FIG. 2. Densitometric tracings of the lens extracts of (a) stage 52; (b) stage 61; (c) stage 66 and (d) adult *X. laevis*. These tracings are from the photograph shown in Plate 1. Each peak is marked with arabic numerals which corresponds with the components shown in Table I.

The percentage of the different lens crystallins present in each of the samples isofocused, and calculated from the densitometric tracings is shown in Table II. It shows a gradual decrease of the γ -crystallins from stage 52 to the adult lens accompanied by an increase of the β -crystallins, while the α -crystallin shows an increase only in the adult lens.

Results from antigen-antibody crossed electrophoresis are shown in Plate 2(a, b, c, d). Here the α -crystallin appeared as a single and weak peak; the γ -crystallin appeared also as a single but strong peak, while the β -crystallins appeared as two prominent peaks.

We obtained similar results with immunoelectrophoresis as well.

TABLE II

Concentration* of α -, β -, and γ -crystallins in the lens of different stages and adult *X. laevis*

Stages	α -crystallin	β -crystallin	γ -crystallin
52	0.5	23.0	76.5
61	0.5	35.0	64.5
66	0.5	37.8	61.7
adult	7.3	52.1	40.6

* The concentration is expressed as a per cent of the total reading obtained from densitometric tracings of the isofocused lens crystallin components.

4. Discussion

Thin layer isoelectric focusing resulted in a separation of the lens crystallins into thirteen major components according to their isoelectric points. It has already been shown by Hayes and Wellner (1969), Susor, Kochman and Rutter (1969), Drysdale (1970), and Righetti and Drysdale (1971) how a homogeneous protein sample shows its apparent heterogeneity when subjected to isoelectric focusing, and the heterogeneity is due to a difference in the primary structure of various protein bands.

From our experimental data it appears that development and growth of the lens is accompanied by an acquisition and loss of some of its crystallin components. In the γ -crystallin region we found more than one component to be missing. Clayton (1970) also reported a loss of a single component in the adult *X. laevis* lens. This difference is most likely due to a difference in the two methods applied. Loss of γ -crystallins during development of *Rana catesbeiana* has also been reported by Polansky and Bennett (1970). Like the said authors we also found an increase of the α -crystallin in the adult lens but we did not observe two α -crystallins as in *R. catesbeiana* (Polansky and Bennett, 1970) and this is probably due to a species difference. The observation of a single α -crystallin supports the earlier report of Brahma and van Doorenmaalen (1969).

We observed a gradual increase of the β -crystallin components from stage 52, and like Campbell, Clayton and Truman (1968) we also found that in the adult *X. laevis* lens β -crystallins form the bulk of the lens proteins. The two peaks we obtained in the β -crystallin region by antigen-antibody crossed electrophoresis and immunoelectrophoresis show that this crystallin is heterogeneous and bears different antigenic properties. Campbell et al. (1968) have also reported that in *Xenopus* lens β -crystallin fractions are complex molecules containing several antigens that exhibit partial immunological similarity with a line in the anodal end, and could be partially identical to some lines at the cathodal end in immunoelectrophoresis.

It appears that, unlike the β -crystallins, all the components of the γ -crystallin are immunologically similar. In the rat lens Zigman, Schultz, and Yulo (1970) have shown that, though the γ -crystallins exhibited seven components by sucrose density-gradient isoelectric focusing, all these components showed reaction of identity by the agar diffusion test.

A gradual loss of γ -crystallin with an increase of β - and α -crystallins has also been reported by Lerman, Zigman and Forbes (1966) in rat lens. According to these authors, in 11 month old rat lens the β -crystallins form the bulk of the lens proteins.

It can be concluded from the present investigations that in the *X. laevis* lens there is a gradual loss of γ -crystallins from stage 52 to the adult lens accompanied by a slight increase of the α -crystallin, while the β -crystallins form the major fraction of the lens proteins.

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