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Isofocusing and Immunological Investigations on Cephalopod Lens Proteins

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Soluble lens proteins from *Octopus vulgaris*, *Sepia officinalis*, and *Loligo vulgaris* were analyzed by thin-layer isoelectric focusing and compared by various immunochemical methods using antibodies directed against total soluble lens protein antigens from the said three species. The results show close similarity between *Sepia* and *Loligo* lens proteins. Antibodies made against *Sepia* and *Loligo* lens proteins did not react against *Octopus* total lens soluble protein antigens. Our results suggest a common ancestry for *Sepia* and *Loligo* which is different from that of *Octopus*.

Key words: cephalopod; lens; soluble proteins; antigens; antibodies; isoelectric focusing; immuno-isofocusing.

1. Introduction

The eyes of the modern cephalopods are their most clamorously vertebrate like structure and every feature fundamental for their vision can also be found in the eyes of fish. The homology is known to be functional and this case of convergence has been discussed in detail by Packard (1972). Developmentally and in anatomical detail, cephalopod and vertebrate eyes differ widely. In the former, eyes appear as two thickened placodes on either side of the embryo, which invaginate to form the optic vesicles. The lens appears from a specialized group of cells lying in the outer surface of the eye primordium. The iris and cornea are formed by further surface invagination (Arnold and Williams-Arnold, 1977). In vertebrates, on the other hand, the optic vesicle appears as an evagination of the diencephalon and the lens develops from the overlying ectoderm in contact with it.

Although the cephalopod lens soluble proteins are called α-, β-, γ- and δ-crystallins as in fish (Bon, Dohrn and Batnik, 1967), they do not show any cross reaction against anti-vertebrate lens antisera (Wollman, Gonzales and Ducrest, 1938; Halbert and Fitzgerald, 1959). Halbert and Manski (1963) suggested that the "biochemical evolution of the analogous structure took place along quite distinct pathways". Brahma (1978) has shown that in cephalopods, ontogeny of the lens crystallins takes place simultaneously in the lens and in the lentigenic cells, a specialized group of cells from which the lens develops. In vertebrates, lens crystallins are detectable only in the lens itself.

There are, of course, relatively few studies on cephalopod eye lens proteins in comparison to the studies dealing with the physiological aspects of these animals (see Young, 1977).

The number of protein antigens reported so far in different cephalopod species also vary. Halbert and Fitzgerald (1958) obtained four antigenic fractions from *Loligo pealii*, while Papaconstantinou (1959) reported one major protein fraction in the lens

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of *L. pealii*. Van Dam (1967) obtained two protein fractions from *Sepia* lens. Bon, Dohrn and Batnik (1967) reported that the nuclear protein fraction of *Octopus vulgaris* lens contains α-, β-, γ- and δ-crystallins as in fish. Dohrn (1970), however, could not find any medium mobility (β-crystallin) in *O. vulgaris* and according to her, the cathodic fraction (δ-crystallin) disappears in the deep layer of the core of the lens. Swanborn (1971), on the other hand, reported that α-, β- and γ-crystallins are present in decapod (*Sepia* and *Loligo*) lenses; while the lens of one octopod (*Octopus*) contains α-, β- and δ-crystallins.

With these conflicting reports we decided to reinvestigate soluble lens proteins from three cephalopod species belonging to the sub-class Coeloidea by thin-layer isoelectric focusing, and to compare protein antigens from the three species by different immunochemical methods using antibodies directed against total soluble lens protein antigen from each species in order to determine evolutionary relationship between the species investigated here.

In this communication soluble lens proteins were used from *Octopus vulgaris* (Order-Octopodida), *Sepia officinalis* (Order-Sepoidea) and *Loligo vulgaris* (Order-Teuthida).

2. Materials and Methods

Protein preparation

Soluble proteins from *Octopus*, *Sepia* and *Loligo* lenses were prepared at the Zoological Station, Naples, from freshly killed adult animals. Whole lens homogenates were made over ice in cold distilled water in glass homogenizers fitted with motor driven Teflon pestles. The homogenates were centrifuged at 37 750×**g** for 20 min at 4°C. The supernatants were lyophilized and stored at −20°C until used.

Antibodies were raised against these lyophilized lens extracts in young rabbits. Each antiserum was then tested by immunoelectrophoresis against homologous antigens and stored at −80°C.

Isoelectric focusing

Thin-layer isoelectric focusing was carried out on 5% polyacrylamide gels (10×20 cm) containing 2% Ampholine (LKB) having a pH range from 3·5 to 11·0. Gels were cast according to methods described earlier (Brahma and Van der Starre, 1976). 0·5% H_3PO_4 and 0·5% ethylenediamine were used as the anodic and cathodic electrolytes respectively. The inner-electrode distance was 13·5 cm.

Two percent solutions were prepared from each lens extract in 2% Ampholine (pH 3–10) and 20 μl from each was applied over glass fibre papers (0·5×1 cm) placed at 0·5 cm away from the anode. Haemoglobin (1%) was applied at the anodic and cathodic ends of the same gel to act as a marker protein. Isofocusing was at 4°C for 19 hr with a constant voltage of 100 V which was then increased to 200 V. When the major marker protein bands from the two ends fused, the run was stopped.

The pH of the gel was measured at 4°C immediately after the run, at points 1 cm apart along the sample migration axis using a flat membrane glass electrode, starting from the anodic end. The gels were then treated with 14% TCA to precipitate the proteins and washed with TCA concentrations ranging from 10 to 3% and in this order to remove the Ampholine. The gels were then stained and photographed.

Immuno-isofocusing

This was carried out according to Brahma and Van der Starre (1976) on three gels (10×20 cm) containing 5% acrylamide and 2% Ampholine (pH range from 3·5 to 11). Ten-microlitre samples from freshly prepared 2% solutions of the lens extracts were used in

each gel. The gels were co-isofocused for 24 hr at 4°C using a constant voltage of 58 V. Each isofocused protein sample was then tested against homologous and two heterologous antisera by immunodiffusion for 72 hr in a humid chamber at room temperature. The gels were then washed in saline, dried over filter papers and stained.

Immunoelectrophoresis

This was performed on 2·6×7·6 cm glass slides in a Shandon apparatus according to Scheidegger (1955) using 1·5% Difco Bacto agar in high resolution buffer (Aronsson and Grönwall, 1957) at pH 8·9. Electrophoresis was done at 4°C for 90 min using a constant voltage (250 V). Immunodiffusion was carried out for 24 hr in a humid chamber at room temperature against homologous and two heterologous antisera. The slides were then washed in saline, dried and stained.

Osserman test

This method of Osserman (1960) is used mainly for the cross comparison of the protein constituents. The proteins are separated initially by electrophoresis as above followed by simultaneous interaction of the separated proteins with homologous immune serum and a heterologous lens protein sample by immunodiffusion. We used this method to test lens protein antisera against homologous and heterologous antigens. After immunodiffusion for 24 hr in a humid chamber at room temperature, gels were washed in saline, dried and stained.

Double diffusion test of Ouchterlony

This was performed with 1·5% Difco Bacto agar gel in high resolution buffer at pH 8·9 for 72 hr in a humid chamber at room temperature. Lens protein extracts were compared with homologous and heterologous antisera as in the three preceeding tests. The gels were washed in saline, dried and stained.

In all tests reported here gels were stained with Coomassie Brilliant Blue R-250 according to Weeke (1973).

3. Results

Thin-layer isoelectric focusing patterns of *Octopus*, *Loligo* and *Sepia* lens soluble proteins are shown in Fig. 1 (A, B, C). The pH gradient of the gel appeared to be linear from 4·2 to 9·7. Of the three samples, best resolution was obtained with the *Octopus* lens proteins and more than 12 components could be observed with isoelectric points lying between pH 6·09 and 9·04. *Loligo* and *Sepia* lens proteins did not show any such clear separation. In both, the components appeared as a broad band having isoelectric points between pH 7·14 and 8·76 in *Loligo* and 7·14 and 8·85 in *Sepia*. Some components below pH 7·14 were observed in both *Loligo* and *Sepia* lens proteins.

Immuno-isofocusing results showed two precipitin lines when *Octopus* lens proteins antigens reacted with homologous antiserum (Fig. 2A). The same antiserum did not react against *Loligo* lens protein antigens (Fig. 2B), but produced one weak precipitin line against *Sepia* lens protein antigens (Fig. 2C). Anti-*Sepia* lens protein antiserum, on the other hand, did not react against *Octopus* lens protein antigens (Fig. 3A), while it produced one precipitin line against *Loligo* as well as against homologous lens protein antigens (Fig. 3B,C). Likewise, anti-*Loligo* lens protein antiserum did not react against *Octopus* lens protein antigens (Fig. 4A), while it produced one precipitin line against homologous as well as against *Sepia* lens protein antigens (Fig. 4B,C).

Results from immunoelectrophoresis showed that *Octopus*, *Sepia* and *Loligo* total

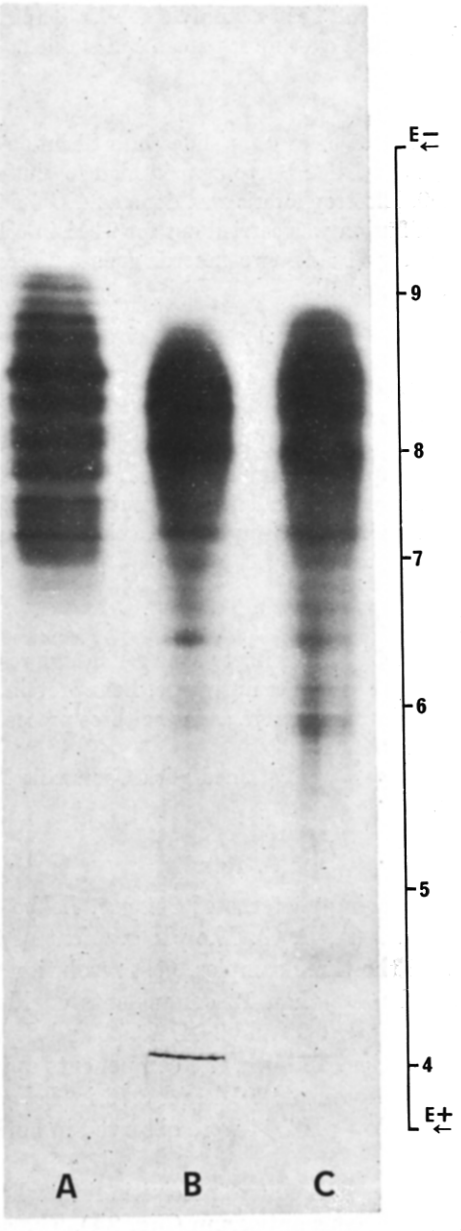


FIG. 1. Thin-layer isoelectric focusing of soluble lens proteins from *O. vulgaris*, *L. vulgaris* and *S. officinalis*. The gel contained 5% acrylamide and 2% Ampholine with a pH range from 3·5 to 11·0. The protein samples were dissolved in 2% Ampholine (pH 3–10), and 20 μl was used from each sample. The electrodes were placed 13·5 cm apart. The electrolyte buffers were 0·5% H_3PO_4 and 0·5% ethylenediamine respectively. The proteins were precipitated with 14% TCA, washed in different concentrations of TCA from 10 to 3% and in this order to remove the ampholine. It was then stained with Coomassie Brilliant Blue R-250 and photographed. A, *Octopus*; B, *Loligo*; and C, *Sepia* lens protein samples. E+ and E− indicate anodic and cathodic ends of the gel. The arrows show the position of the electrodes. The pH gradient is shown on the right side of the gel.

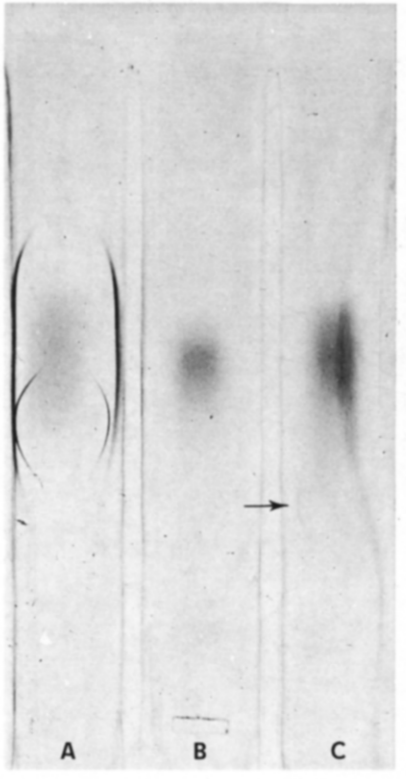


FIG. 2.

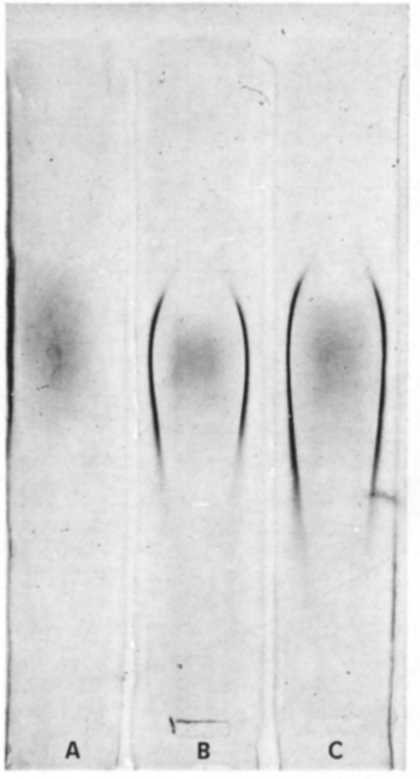


FIG. 3.

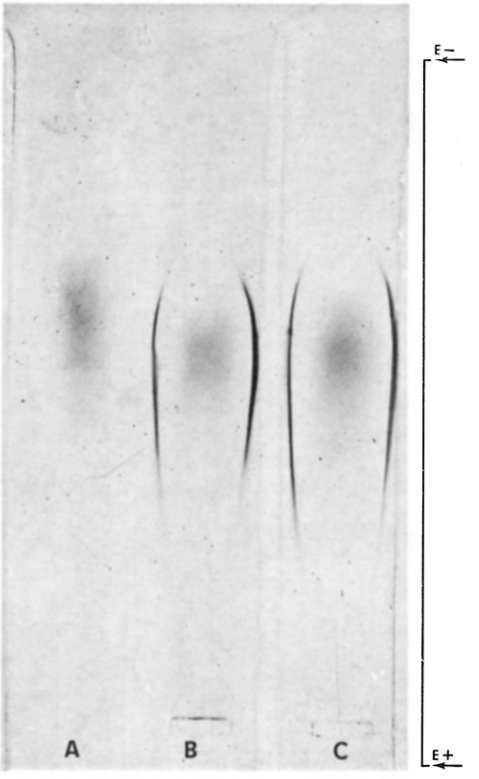
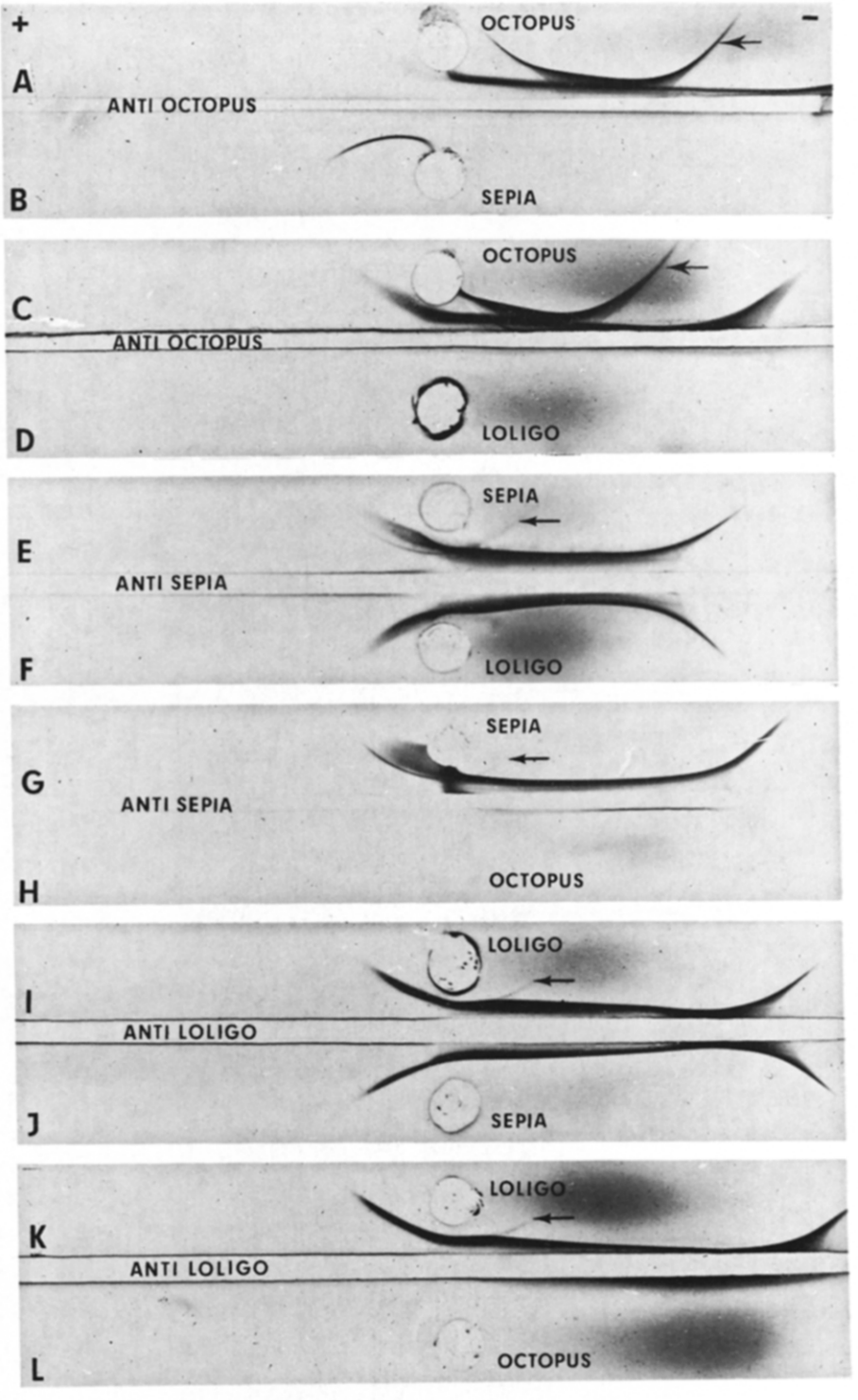


FIG. 4.

FIGS 2, 3, 4. Immuno-isofocusing of lens proteins from *O. vulgaris*, *L. vulgaris* and *S. officinalis* against homologous and heterologous antisera. The gel contained 5% acrylamide and 2% Ampholine (pH range from 3·5 to 11·0). The electrodes were placed 13·5 cm apart. Immunodiffusion was continued during 72 hr in a humid chamber at room temperature. After washing in saline the gels were stained with Coomassie Brilliant Blue R-250. Fig. 2A, B and C; *Octopus*, *Loligo* and *Sepia* lens proteins antigens. The gutters were filled with anti-*Octopus* lens proteins antiserum. Fig. 3A, B and C; *Octopus*, *Loligo* and *Sepia* lens proteins antigens. The gutters were filled with anti-*Sepia* lens proteins antiserum. Fig. 4A, B and C; *Octopus*, *Loligo* and *Sepia* lens proteins antigens. The gutters were filled with anti-*Loligo* lens proteins antiserum.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soluble lens protein antigens produced two immunoprecipitin lines on the cathodic side, one very extensive in each, and the second one strong in *Octopus*, prominent in *Sepia* and rather weak in *Loligo* (Fig. 5A,C,E,G,I,K, shown by arrow). It is not clear why the same antisera did not produce two immunoprecipitin lines against homologous antigens from *Loligo* and *Sepia* in immuno-isofocusing.

Anti-*Octopus* lens protein antiserum produced a precipitin arc against *Sepia* lens proteins antigens (Fig. 5B) but did not react against *Loligo* lens protein antigens (Fig. 5D).

Anti-*Sepia* lens protein antiserum produced a major precipitin line against *Loligo* lens protein antigens (Fig. 5F), but did not react against *Octopus* lens protein antigens (Fig. 5H). Similarly, anti-*Loligo* lens protein antiserum produced a major precipitin line against *Sepia* lens protein antigens (Fig. 5J), and did not react against *Octopus* lens protein antigens (Fig. 5L).

Identical results were obtained with Osserman's test and Ouchterlony's double diffusion test.

4. Discussion

In determining evolutionary relationships, electrophoretic and serological methods are frequently used to make biochemical comparisons. Since the immunological cross-reaction between antiserum and various antigens is proportional to the chemical similarity of the antigens tested, this is a literal interpretation of "blood relationship" (Boyden, 1940). Our immunological findings show a close relationship between *Loligo* and *Sepia* lens proteins. It also appears from the present experiment that the soluble lens proteins from *L. vulgaris* and *S. officinalis* differ immunologically from those of *O. vulgaris*. Our results support the view of Young (1977) and Donovan (1977) that *Sepia* and *Loligo* have a common ancestor, distinct from that of *Octopus*. Young's view is based on striking similarities between the nervous system and other soft tissues in *Loligo* and *Sepia*, and Donovan's view is based on palaeontological evidence, although according to him this is "hopelessly inadequate".

Our results also suggest that *Octopus* may have originated from a stock different from the one that gave rise to *Loligo* and *Sepia*, supporting the speculation of Donovan (1977) that there may have been two different phragmatuthid groups, one ancestral to the *Decapods* and the other to the *Octopods*.

Unlike Bon et al. (1967), Dohrn (1970) and Swanborn (1971), we failed to distinguish different classes of proteins in our materials. Our gel filtration analysis showed two protein fractions in *S. officinalis* and *L. vulgaris*, and like Van Dam (1967), we observed no high molecular weight lens protein fraction in these two materials we examined.

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FIG. 5. Immunoelectrophoresis of *O. vulgaris*, *S. officinalis* and *L. vulgaris* lens proteins antigens against homologous and heterologous antisera. + = Anode; — = Cathode. Immunoelectrophoresis was carried out at 4°C during 90 min with a constant voltage. Immunodiffusion was carried out for 24 hr in a humid chamber at room temperature. A, *Octopus* vs. anti-*Octopus*; B, *Sepia* vs. anti-*Octopus*; C, *Octopus* vs. anti-*Octopus*; D, *Loligo* vs. anti-*Octopus*; E, *Sepia* vs. anti-*Sepia*; F, *Loligo* vs. anti-*Sepia*; G, *Sepia* vs. anti-*Sepia*; H, *Octopus* vs. anti-*Sepia*; I, *Loligo* vs. anti-*Loligo*; J, *Sepia* vs. anti-*Loligo*; K, *Loligo* vs. anti-*Loligo*; L, *Octopus* vs. anti-*Loligo*. The arrows indicate minor (second) immunoprecipitin line.

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