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Studies on biosynthesis of soluble proteins in early amphibian development by isoelectric focusing in thin layer polyacrylamide gel

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Isoelectric focusing is a relatively recent method for the separation of protein mixtures at their isoelectric points. This technique has a very high resolving power and proteins with a difference of 0.02 in their isoelectric points can be successfully separated [1, 2].

In the original method sucrose was used to

stabilize the pH gradient [1]. Recently, this method has been adapted to disc-gel and flat-gel electrophoresis and polyacrylamide has been used as a carrier to stabilize the pH gradient.

In recent years a number of articles have appeared on gel isoelectric focusing dealing with various protein samples ([3, 4, 5, 6] for references and [7] for a review.) Of the two, the flat-gel method has many advantages over the disc-gel [8].

In this note we present our results of isoelectric focusing of the radioactive soluble proteins from axolotl (*Ambystoma mexicanum*) gastrulae in thin layer polyacrylamide gel plate, and autoradiography of the same gel after staining and photography. By this method, we could detect a large number of protein bands and determine at the same time the number of proteins that are being synthesized at that stage of development.

Materials and Methods

Preparation of the radioactive sample: All solutions and glassware were sterilized and used under sterile conditions. Axolotl mid-gastrulae (stage 11, Harrison) were dejelled and decapsulated by hand, thoroughly washed with Steinberg solution [9] and subsequently incubated for 2 h at 21°C in a New Brunswick Gyration shaker in Steinberg solution containing 5 mM EDTA instead of Ca²⁺ and Mg²⁺ ions [10]. After this period, complete dissociation of the cells had taken place inside the vitelline membrane (unpublished data) and the embryos were washed 5 times with normal Steinberg solution. The dissociated embryos were cultured in Steinberg solution containing 1 mg/ml Human serum albumin (Kohn fraction IV, Koch-Light Labs. Ltd., Colnbrook, UK), 0.02 mg/ml streptomycin and 100 IU penicillin in presence of 5 µCi/ml protein hydrolysate ¹⁴C (52 mCi/mAtom Carbon, The Radiochemical Centre, Amersham) for 16 h under gentle shaking at 21°C. At the end of the labelling period, the reaggregated embryos were washed with Steinberg solution first and subsequently with distilled water and homogenized in a Teflon-glass Potter Elvehjem homogeniser with few strokes. The homogenate was centrifuged for 10 min at 10 000 g at 4°C and the supernatant was collected after careful removal of the lipid layer. This process was repeated once more and the supernatant was lyophilised and stored at -20°C until needed.

Acrylamide gel isoelectric focusing: Acrylamide, and N,N,N',N'-tetramethyl-1,2-diaminoethane were pur-

Fig. 1. Thin layer isoelectric focusing of the radioactive soluble proteins from axolotl gastrulae in 5% polyacrylamide gel containing 2% "Ampholine" carrier ampholyte with a pH range from 3–10. The sample was made radioactive by labelling with protein hydrolysate ^{14}C during 16 h. The protein bands were stained with Coomassie Brilliant Blue R-250 after fixation with 14% trichloroacetic acid.

Fig. 2. Autoradiogram of the gel shown in fig. 1. The print was made from Kodak No-Screen X-Ray transparency and has the same magnification as fig. 1. Though the radioactive bands run in coincidence with stained protein bands they are few in number. EL^+ and EL^- indicate the positions where the electrodes were placed. Distance between the electrodes was 7.5 cm, and the sample was 0.5 cm away from the anode. +, anode; -, cathode. The centimeter scale shown is magnified 1.56 times the original scale.

chased from BDH Chemicals Ltd. (Poole, UK), riboflavin from E. Merck A. G. (Darmstadt, BRD), "Ampholine" carrier ampholytes from LKB-Produkter (Stockholm), and Coomassie Brilliant Blue R-250 dye from Serva (Heidelberg, BRD).

Five percent acrylamide gel was prepared on a 8.2×8.2 cm cleaned glass plate by photopolymerization under two 20 W fluorescent daylight TL tubes in a similar manner already described [4]. This gel contained a 2% solution of the "Ampholine" carrier ampholytes covering a pH range from 3–10, and this was prepared by mixing Ampholines having pH ranges from 3–5, 5–7, and 7–10 in equal proportions.

We used 0.5 cm diameter stainless steel rods as the electrodes, and the anodic and cathodic electrode solutions were 5% phosphoric acid and 5% ethylenediamine, respectively. Isoelectric focusing was carried out at 4°C in an Immunoelectrophoresis tank No. 260 (Medical and Biological Instrumentation Ltd., Ashford, Kent, UK) on grooved perspex stands on which the electrodes were placed. Whatman 3MM paper strips 0.5×8 cm were soaked in the anode and cathode solutions and laid over the respective electrodes and the electrodes were set at a distance of 7.5 cm.

Fifteen μl of a 5% solution of the lyophilised extract dissolved in deionised water was applied on a 0.5×1 cm Whatman 3MM paper which was placed 0.5 cm away from the anode. We used water to dissolve the sample in order to minimise the salt concentration as high salt causes a disturbance in the pH gradient [11]. The gel plate was then inverted

and carefully placed on the filter paper strips lying over the electrodes. The lid of the apparatus was then put over and the voltage was applied.

Isoelectric focusing was carried out for little over 16 h with 50 V and an initial current of 2 mA. After 16 h, the voltage recorded was 75 V and the current dropped to about 0.2 mA. The voltage was then increased to 250 V for 10 min to sharpen the protein zones [4]. The protein zones in the gel were then precipitated with 14% trichloroacetic acid (TCA) overnight at the room temperature followed by washing in 10, 5, and 3% TCA to remove the ampholine. The gel was then stained with Coomassie Brilliant Blue R-250 [4]. Recently, quick methods for the protein staining with Coomassie Blue after isoelectric focusing have been described where stain is applied directly to the gel without prewashing of the ampholine [8, 12, 13].

Results

As a result of the staining with Coomassie Blue dissolved in methanol acetic acid water the gel shrunk, it was then reswollen in acetic acid water (9:91) and photographed using a red filter to enhance the contrast [14].

After photography, the gel was immersed in 30% formic acid for few minutes and then

Fig. 3. Densitometric tracing of the stained protein bands shown in fig. 1. In the anodic end the protein bands are not well separated.

Fig. 4. Densitometric tracing of the radioactive protein bands shown in fig. 2. Though the peaks in fig. 3, 4 run in coincidence only few proteins show labeling. +, anode; -, cathode. The centimeter scale shown is magnified 1.07 times the original scale.

carefully laid over a closely grained hard wood (9×12 cm) which was coated with resin [15] and then dried at the room temperature for 48 h. This method of drying did not cause any shrinkage of the gel.

Autoradiography of the dried gel was made with Kodak No-Screen X-Ray film under Kodak Wratten series 6B safelight filter by placing the film over the dried gel. Another piece of hard wood of (9×12 cm) was placed over the film and the whole assembly was then tied with thick rubber bands to ensure firm contact between the gel and the film. It was then wrapped in black cloth and exposed for 7 days at 4°C . The film was then processed with DX-80 developer and FX-40 fixer (both from Kodak) according to the manufacturer's instructions.

Prints were made from the negative showing the stained protein patterns and from the positive transparency in equal magnification. The former one is fig. 1 and autoradiogram of the fig. 1 is designated as fig. 2. These two prints were placed side by side for comparison. The stained gel showed at least 20 bands which is more than what other authors have so far obtained using various methods of separation of the soluble proteins in different amphibian materials [16–18].

Densitometric tracing of the photographs of the gel was carried out in a Chromoscan recording reflectance densitometer (Joyce, Loebel and Co. Ltd., Gateshead, UK) and are shown in figs 3 and 4.

Discussion

From figs 1, 2, 3 and 4 it appears that though the radioactive bands run in coincidence with stained protein bands, not all stained bands show radioactivity. It can therefore be assumed that within the limits of detection methods we applied here, not all the protein components detectable by the staining method are being synthesised at this stage of axolotl gastrulae.

Further work is in progress for a detailed comparative study of the protein biosynthesis during amphibian development using thin layer isoelectric focusing and radioautography in combination.

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Influence of the iris upon the sorting out of mitotic and non-mitotic cells in cultured vertebrate lenses

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In an account from our laboratory recently published in this journal [1], we reported that when leopard frog (*R. pipiens*) lenses are explanted to a serum-containing culture medium, the epithelial cells are somehow stimulated to divide. Division figures all appear in the polar region of the lens which is characterized by a marked migration of cells from outlying areas. The area invaded by and thus rich in cells was termed the accumulation zone (AZ); the area which, because of emigration, has a low cell density, was termed the depletion zone (DZ). Not only is mitosis itself confined to the AZ in the leopard frog lenses, but also the increments in RNA, protein and DNA syntheses which are required for division to occur. As judged by ³H-actinomycin D uptake gene activation also occurs mainly in the AZ. Thus genetic and mitotic activation are coextensive, both being confined to the AZ.

In our last report on this subject we were unable to provide any reasons for the appearance of an AZ and DZ. Furthermore, it was pointed out that these two zones were not found in the cultured bullfrog (*Rana catesbeiana*) lens. Over the last few months we have collected evidence which unambiguously implicates the iris in the process of AZ-DZ evolution. Furthermore, we have proven the response to be one which is not species specific.

As in the past [2], lenses were cultured in a 23 % serum-containing medium with an osmotic pressure of 225–235 mOsm. They were usually removed from enucleated eyes and cultured for a period of 4–5 days at 24°C.