186. *Partial Purification of a Factor Promoting Chicken Myoblast Multiplication in vitro*

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Carrel (1924) found that embryo extract added to culture medium was very effective in promoting the growth of fibroblasts; the plasma or extracts of adult tissues was also shown to have some effect, but their activities were much less than that of embryo extract.

Embryo extract is now widely used as an important constituent of media for embryonal primary culture of various kinds of cells including muscle cells. Some properties of a factor in embryo extract concerning the growth of muscle cells were described by De La Haba and Amundsen (1972), but no pronounced success in purifying the factor has been reported.

We found that adult chicken serum could exert an effect comparable to that of chick embryo extract on the growth of muscle cells *in vitro*. An attempt was then made to purify the factor in adult chick serum and, as a result, about 80 fold purification was attained.

This communication is mainly concerned with the method of purification of the factor. Its properties related to its function will be described in the next paper (Kohama and Ozawa, 1973).

Experimental. Hens and roosters were purchased from a local farm and were sacrificed by cutting the carotid arteries. Arterial blood was collected and left standing until blood clot retracted. It was then centrifuged to remove the clot. The supernatant, the serum, was stored in a deep freezer (-80°C).

The activity of the serum and the purified factor was assayed by the method of which the details will be published elsewhere. Briefly, 12 day chick myogenic cells, which had been purified by differential adhesion technique (Kaighn, Ebert and Stott, 1966), were cultured in 2.4 ml of the medium composed of 85% Eagle's minimum essential medium (MEM, Nissui Co.) and 15% horse serum. To each plate, 0.1 ml of appropriately diluted factor was added, unless specifically mentioned. The number of cells plated was 3×10^4 per 35 mm Falcon plastic plate. After 3 or 4 days' incubation, the cells were fixed and

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the number of the nuclei of myoblasts and myotubes were counted as described before (Ozawa, 1972). Using these values, the activities were then calculated using 5- or 3-point assay of slope ratio assay method (Burn, 1950).

The concentration of protein was determined by biuret reaction and/or by optical density at 280 nm standardized by bovine serum albumin.

Results. The factor which promotes the growth of myogenic cells was purified as follows:

Step 1. Frozen serum was thawed and diluted with twice volumes of 0.9% NaCl solution. To 100 ml of the diluted serum, 27.7 g of solid ammonium sulfate was added and dissolved. After several tens of minutes, the resulting precipitates were removed by centrifugation. To the supernatant, further 13.4 g of solid ammonium sulfate per 100 ml of the original diluted serum was added. The resulting flocculated material was collected by centrifugation. Usually, the material was found to be packed on the top layer of the content of the centrifugation tube. The clear solution underneath the packed material was removed with a siphon. The packed material remaining in the tube was dissolved in a small amount of distilled water, and the resulting solution was then dialysed against 5 mM phosphate buffer (pH 7.0).

Step 2. Solid urea was carefully added to the dialysed solution described above, the final concentration being 1 M. To 100 ml of the resulting solution 44 g of solid ammonium sulfate was added and dissolved. The solution was then allowed to stand for about 30 minutes. The resulting flocculated material was removed by centrifugation. If the resulting solution had reddish tone, 0.5 or 1 g of ammonium sulfate per 100 ml was further added. Then the solution was centrifuged again to remove the flocculated material. The resulting clear solution was dialysed against 5 mM phosphate buffer (pH 7.0) containing 60 mM NaCl.

Step 3. DEAE-Sephadex resin, swollen in 5 mM phosphate buffer (pH 7.0) containing 60 mM NaCl, was poured into a column and washed with the same solution. The dialysed solution of the Step 2 was centrifuged to remove the denatured materials, if necessary, and the supernatant was applied to the column. The column was then washed with a large volume of the same buffer containing 60 mM NaCl. The factor was eluted with the same buffer containing 155 mM NaCl.

Step 4. The eluate in the Step 3 was concentrated with collodion bag SM 132 00 of Sartorius Membrane Filter GmbH. A column of Sephadex G 200, swollen in 5 mM phosphate buffer (pH 7.0) contain-

ing 155 mM NaCl, was prepared. The concentrated solution was applied to the column, which was then eluted with the same buffer containing 155 mM NaCl. Active principle appeared in the eluate of which the apparent partition coefficient (K_v) ranged from 0.33 to 0.67.

Table I. Comparison of activities of original serum and purified factor

	Protein concentration of the factor in culture medium	Mean of myogenic cell number of four plates (25 fields were counted per each plate)	Relative specific activity
Control	0 $\mu\text{g/ml}$	41.8	—
Original serum	195	143.5	(1.0)
Purified factor	1.0	83.5	80.5

4×10^4 cells were cultured in a Falcon plastic 35 mm plate containing 2.2 ml of 85% MEM and 15% horse serum and 0.3 ml of purified factor, 24 times diluted serum or 0.9% NaCl. In each case, four plates were incubated for 4 days at 37.5°C. After fixation and staining, nuclei of myogenic cells were counted.

Fig. 1. Photograph of the chick myogenic cells cultured in the medium containing 85% MEM, 15% horse serum, and 1 $\mu\text{g/ml}$ of the purified factor. The cell number plated was 4×10^5 per 35 mm Falcon plastic plate. The volume of the culture medium was 2.5 ml per plate. The cells were incubated for 53 hours. The inserted bar indicates 100 μm .

The specific activity of an eluted fraction which showed the highest activity was about 80 times higher than that of the original serum on the protein basis (Table I). Cultured cells in the media with the purified factor (1 μ g of protein per ml) were shown in Fig. 1. The condition of culture is described in the legend to the figure.

The factor was destroyed on boiling for 1 minute, whereas its activity was well retained at 38°C for at least 30 minutes. The factor lost its activity irreversibly at pH 2.5 or lower. The activity was reduced intensely when the factor was shaken with chloroform. Treatment with 1% sodium dodecyl sulfate or 6 M urea eliminated the activity irreversibly. The factor was resistant to a fairly high concentration of trypsin or neuraminidase so far examined.

The molecular weight of the factor was estimated by the method of Martin and Ames (1961) modified by Kato *et al.* (1971). According to a preliminary experiment, it was about 1.1×10^5 , if we assume that the molecule would be spherical.

The human, horse, or mouse serum scarcely replaced the chicken serum in promoting the growth of chicken muscle cells under the condition of the present assay system.

Discussion. In spite of the presence of abundant horse serum in the assay system, a distinct difference in the degree of growth of chicken myogenic cells was noticed between the plates with or without chicken serum. Not only the horse serum, but also the human or mouse serum was scarcely effective in promoting chicken myogenic cell growth. These findings indicate that the factor promoting myogenic cell growth has species specificity.

Although the fraction containing the factor is purified 80 fold on the protein basis, it is far from being homogeneous. If this factor is a kind of protein hormone in its nature, like insulin or growth hormone, the concentration of the factor in the serum should be very low such as 10^{-9} g per ml (Williams, 1968; Daughaday, 1968). If this is true, we must accomplish further several 1,000 fold purification. Thus the purification of the factor is still in a preliminary stage and will require further painstaking effort.

Summary. The method of purification of the factor promoting myoblast multiplication was described. The highest specific activity of a fraction thus purified was about 80 times higher than that of the original serum on the protein basis.

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