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# Subunit Interactions of Vertebrate Lactate Dehydrogenase
## I. Immunochemistry of Subunits

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# 척추동물 젖산수소이탈효소 하부단위체의 상호작용
## Ⅰ. 하부단위체의 면역화학

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### 적 요

경골어류 드렁허리와 가물치에서 젖산수소이탈효소의 세가지 동질사량체를 순수 정제하였다. 이들에 대한 항혈청을 얻어 젖산수소이탈효소와 면역화학적 반응을 실시하였다.

하부단위체의 활성자리는 항원결정군에 속해 있지 않으며, 항혈청내의 항체 또는 미지의 물질이 하부단위체의 하전을 변화시키며, 두 하부단위체는 공통의 항원결정군을 갖고 있음이 확인되었다.

## INTRODUCTION

It seems to be clear that intracellular oligomeric proteins have to be continuously controlled in their metabolic pool with precise ways. Lactate dehydrogenase (EC. 1.1.1.27; LDH) which catalyzes the interconversion of pyruvate and L-lactate in the presence of NAD or NADH is suitable protein to get more careful scrutiny of those molecular manipulation because of the fairly abundant knowledge on the enzyme at the levels of biochemistry, phylogeny and ontogeny.

On the basis of a variety of observations (Appella and Markert, 1961; Cahn *et al.*, 1962; Heck, 1969), it is generally accepted that LDH is composed of four

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subunits, that two types of subunit are present in tissues of most species and that each subunit is capable of binding one molecule of coenzyme and substrate. These two monomers, the subunit M and subunit H, combine each other to produce five distinct tetramers: $M_4$, $M_3H$, $M_2H_2$, $MH_3$ and $H_4$.

The $M_4$ and $H_4$ isozymes differ in their amino acid composition, peptide map patterns and antigenic determinants (Markert and Appella, 1963; Pesce *et al.*, 1964; Pesce *et al.*, 1967). The three hybrid isozymes have intermediate properties which reflect their subunit composition. The two subunits are under separate genetic control (Dawson *et al.*, 1964; Kraus and Neely, 1964). In human being the gene for subunit M is located on the chromosome 11 (Boone *et al.*, 1972) and the gene for subunit H is on the short arm of chromosome 12 (Mayeda *et al.*, 1974).

In the present work with vertebrate LDH isozymes, we have purified three homotetrameric isozymes in teleosts and suggested possibility that the two subunits share common antigenic determinants.

## MATERIALS AND METHODS

Purification of isozymes

*Fluta alba* $H_4$ isozyme: Two hundred and fifty adult individuals of *F. alba* were decapitated immediately after purchase from commercial dealer. The one hundred and ten grams of pooled kidney tissue were ground in a ice-bathed homogenizer in 220 ml of cold glass distilled water followed by storage at −20°C for 12 hrs. The solution after thawing was gathered and passed through a glass wool column. The resulting solution was centrifuged in a PR-2 International refrigerated centrifuge at 5500×g for 1 hr, and the pellet was discarded.

Solid ammonium sulfate was added to the supernatant up to 30% saturation with the adjustment of pH at 7.0 by adding diluted ammonium hydroxide. After 24 hrs at 4°C, the mixture was centrifuged and ammonium sulfate was added to the supernatant until the solution was at 70% saturation. After 24 hrs at 4°C, the mixture was again centrifuged and the pellet was dissolved in 2 volumes of 0.01 M phosphate buffer, pH 7.0. The solution was dialyzed against 2 *l* of the same buffer, with four changes of buffer during 40 hrs at 4°C.

After centrifugation, the pH of the supernatant was adjusted to 5.0 by adding acetic acid. After 1 hr at 4°C, the solution was centrifuged and the supernatant was made 70% saturated with ammonium sulfate by the addition of the solid salts and, after 24 hrs at 4°C, the pellet was dissolved in minimum amount of phosphate buffer and desalted by thorough dialyzation.

After centrifugation, the enzyme solution was subjected to gel filtration on a

Sephadex G-100 column (4.9×40 cm) previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. The eluate was collected in 10 ml fractions. Aliquot of each fraction was spotted on a cellulose acetate strip (Millipore) and stained for LDH isozymes by the method of Park and Cho (1972). LDH activity was appeared to be in 14-27 fractions. The fractions of LDH activity were pooled and concentrated by ammonium sulfate treatment. The pellet was subjected to subsequent dialyzation.

Small portion of the dialysate was layered on a DEAE-cellulose column (2.4× 21 cm) previously equilibrated with 0.01 M potassium phosphate buffer, and stepwise gradients of sodium chloride (0-200 mM) and pH (7.0—6.0) were established. The eluate was collected in 10 ml fractions. Aliquot of each fraction was electrophoresed on a cellulose acetate strip by the method of Park and Cho (1972). The fractions of $H_4$ isozyme were pooled, dialyzed against 0.01 M potassium phosphate buffer, and stored at−20°C until further studies.

*F. alba* $M_4$ isozyme and *Ophicephalus argus* $M_4$ isozyme: Each of these two isozymes was purified from pooled skeletal muscle tissue by a procedure similar to that used for the *F. alba* $H_4$ isozyme, except that a preferential heat denaturation at 60°C for 4 minutes was used instead of acid denaturation after the second ammonium sulfate treatment. The elution profile from the Sephadex G-100 column chromatography indicated that each of these two isozymes had molecular weight similar to that of *F. alba* $H_4$ isozyme.

Preparation and use of antisera

Protein concentration was measured by the method of Lowry et al. (1951). Ten mg protein with Freund's complete adjuvant (Difco) was injected into the dexter thigh muscle of adult male rabbits. One week later each rabbit received the second injection at the sinister thigh muscle. Third and fourth injections were made at the dexter and sinister dorsal muscle, respectively, every week.

The rabbits were bled one week after the last injection by the method of cardiac puncture (Campbell *et al.*, 1970) without any anticoagulant and anesthetic. The blood was stored at 4°C for 24 hrs and then the blood clot was removed by centrifugation. The antisera were stored at -20°C and used wihin a month.

A thorough mixturte of desired ratio of antigen solution and antiserum was incubated in moist chamber at 25°C for 1hr without any preservatives and then at 4°C for 1hr. The filtrate of the mixture through Whatman No. 1 filter paper was used for the electrophoresis.

## RESULTS

The $H_4$ and $M_4$ isozymes of a teleost *F. alba* were isolated from kidney and

skeletal muscle respectively (Fig. 1). The $M_4$ isozyme from *O. argus* skeletal muscle was purified with no contamination of other isozymes (Fig. 2).

Anti-*F. alba* $M_4$ or anti-*F. alba* $H_4$ was added to the mixture containing the $M_4$ and $H_4$ isozymes of *F. alba*. The $H_4$ isozyme was inactivated by the anti-*F. alba* $H_4$ extensively. The anti-*F. alba* $M_4$, however, inactivated not only the $M_4$ isozyme but also the $H_4$ isozyme to a large extent (Fig. 3). Unexpectedly, enhanced was the activity of the most cathodal LDH isozyme of rabbit serum (Fig. 3, B). Those phenomena were highly reproducible.

The electrophoretic pattern of LDH isozymes in *O. argus* skeletal muscle was severely influenced by the treatment of anti-*O. argus* $M_4$ to the crude extract of *O. argus* skeletal muscle. The disappearance of the abnormal LDH isozyme pattern was achieved as the antigen solution and the antiserum were mixed in a ratio of 1 to 6 in volume. The abnormality could not be encountered when the crude extract was diluted six times and then the antiserum was added to the solution (Fig. 4).

The anti-*O. argus* $M_4$ also made the electrophoretic patterns of purified *O. argus* $M_4$ isozyme show the abnormality when the relatively small amount of the former was treated to the latter antigen solution (Fig. 5). When the plasma of unimmunized rabbits was added to the *O. argus* $M_4$ isozyme solution or skeletal muscle extract, no abnormality in electrophoretic patterns were established (Fig. 6).

The amino acid sequences of antigenic determinants in *F. alba* $M_4$ isozymes are considered to be almost similar to those of *O. argus* $M_4$ isozyme (Fig. 7). Each of the anti-*O. argus* $M_4$, anti-*F. alba* $M_4$ and anti-*F. alba* $H_4$ did not combine each of the one isozyme from *Parasilurus asotus*, *Hyla arborea japonica* and chicken.

## DISCUSSION

Common antigenic determinants

The immunochemical comparison of homologous proteins would provide strong evidences to the similarity of their primary structures (Cocks and Wilson, 1969: Gorman *et al.*, 1971: Park *et al.*, 1977). The anti-*F. alba* $M_4$ reacted strongly with the *F. alba* $M_4$ isozyme and to a large extent with the *F. alba* $H_4$ isozyme while the anti-*F. alba* $H_4$ reacted only with the *F. alba* $H_4$ isozyme. Holmes and Scopes (1974) in their immunochemical experiments with sardine (*Sardinops neopilchardus*) LDHs reported a result that $M_4$ and $H_4$ isozymes were shown to be immunochemically related in contrast to the results of other workers (Wilson *et al.*, 1964: Kaplan, 1964: Whitt, 1970: Gorman *et al.*, 1971). From the above results, it could be said

that several antigenic determinants on the subunit H are almost similar to those on the subunit M and that the $H_4$ and $M_4$ isozymes as immunogens are catabolized in different fashions in the internal environment of rabbit immunized.

Despite of possible different antigenic responses in animals, that is, the sheep in the work of Holmes and Scopes (1974) and the rabbits in this work, the common antigenic determinants in LDH has been identified by electrophoretic techinque. Homologies between LDH isozymes of fishes and those of higher vertebrates are strongly supported by physical, catalytic and immunological criteria (Bailey and Wilson, 1968). By analogy with the above and by consideration of the fact that the subunit M and H share common antigenic determinants, it is highly presumable that the two major lactate dehydrogenase loci arose from a single genetic locus by a duplication.

Electrophoretic abnormality

The abnormality arisen after electrophoresis of the mixture of anti-*O. argus* $M_4$ and purified $M_4$ isozyme of *O. argus* skeletal muscle might be resulted either from complex formation between antigen and antibody or from that between antigen and serum components of small molecular weight. Of the two possible alternatives, the former was demonstrated in the work of Burd and Usategui-Gomez (1973) who made the soluble antigen-antibody complexes move in electric field. Somewhat sophisticated abnormality, however, was observed in the zymogram resulting after electrophoresis of the mixture of anti-*O. argus* $M_4$ and crude extract of *O. argus* skeletal muscle in which all five isozymes were present. Furthermore, Park *et al.* (1976) revealed that when the antiserum against purified $M_4$ LDH isozyme of a squamate, *Agkistrodon blomhoffii brevicaudus*, was treated to each of the crude muscle extract of nine reptilian species and three amphibian species, another LDH isozyme band was appeared just at the cathodal side of $M_4$ isozyme. Although highly theoretical, Cann and Goad (1968) suggested that, under appropriate conditions, a single macromolecule interacting reversibly with an uncharged constituent of the solvent medium can give two zones despite instantaneous establishment of equilibrium.

The complex of *O. argus* $M_4$ and anti-*O. argus* $M_4$ shown as precipitates in origin (Fig. 4 and Fig. 5) exhibited the enzyme activity, suggesting that the configuration of active sites of the tetrameric LDH isozymes are not to be altered after combining the antibody. Similar and more definitive results could be found in the immunochemical studies on human erythrocyte $H_4$ LDH of Burd and Usategui-Gomez (1973) who demonstrated that the anti-acetylated $H_4$ isozyme formed soluble complexes with the non-acetylated $H_4$ isozyme without inhibiting or precipitating the enzyme in solution.

## SUMMARY

Two homotetrameric lactate dehydrogenase isozymes from *Fluta alba* and one from *Ophicephalus argus* were purified by combination of gel filtration and DEAE-cellulose chromatography. The final preparations were isozymically pure and used to elicit antibodies in rabbits.

The immunochemical reactivities demonstrated that the amino acids of active site is not to be included in the antigenic determinants, that antibodies or unknown component of immunized rabbit serum might be responsible for the electrophoretic abnormality and that two subunits share common antigenic determinants, reflecting that these polypeptides have a common evolutionary origin.

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## ILLUSTRATIONS OF FIGURES

**Fig. 1.** Final purification of the $H_4$ and $M_4$ LDH isozymes from *F. alba* kidney (left half) and skeletal muscle (right half) respectively.

**Fig. 2.** Final purification of the $M_4$ isozyme of *O. argus* skeletal muscle. CE means crude extract; PLI, purified lactate dehydrogenase isozyme.

**Fig. 3.** Effect of anti-*F. alba* $M_4$ and anti-*F. alba* $H_4$ upon the *F. alba* LDH isozymes. RS means the rabbit anti-*F. alba* $M_4$ serum; A, the mixture of purified *F. alba* $M_4$ and $H_4$ isozymes; B, the mixture of A and anti-*F. alba* $M_4$ (1 : 2); C, the mixture of A and anti-*F. alba* $H_4$ (1 : 2).

**Fig. 4.** Effect of anti-*O. argus* $M_4$ upon the LDH isozymes of *O. argus* skeletal muscle. CE means the crude extract; RS, rabbit serum; A, the mixture of CE and anti-*O. argus* $M_4$ (4 : 1 to 1 : 5); B, the mixture of CE and anti-*O. argus* $M_4$ (1 : 6); DCE, six times diluted crude extract; C, the mixture of DCE and anti-*O. argus* $M_4$ (1 : 1).

**Fig. 5.** Effect of anti-*O. argus* $M_4$ upon the purified *O. argus* $M_4$ isozyme. PI means the purified $M_4$ isozyme; A, the mixture of PI and anti-*O. argus* $M_4$ (4 : 1); B, the mixture of PI and anti-*O. argus* $M_4$ (2 : 1); C, the mixture of PI and anti-*O. argus* $M_4$ (1 : 2); D, the mixture of PI and anti-*O. argus* $M_4$ (1 : 4).

**Fig. 6.** Effect of intact rabbit plasma upon the purified *O. argus* $M_4$ isozyme and the crude extract of *O. argus* skeletal muscle. A means the purified *O. argus* $M_4$ isozyme; C, the rabbit intact plasma; B, the mixture of A and C; E, the crude extract of *O. argus* skeletal muscle; D, the mixture of E and C.

**Fig. 7.** Effect of anti-*O. argus* $M_4$ upon the *F. alba* LDH isozymes. RS means rabbit serum; A, the mixture of purified *F. alba* $M_4$ and $H_4$ isozymes; B, the mixture of A and anti-*O. argus* $M_4$ (4 : 1); C, the mixture of A and anti-*O. argus* $M_4$ (1 : 1); D, the mixture of A and anti-*O. argus* $M_4$ (1 : 2).

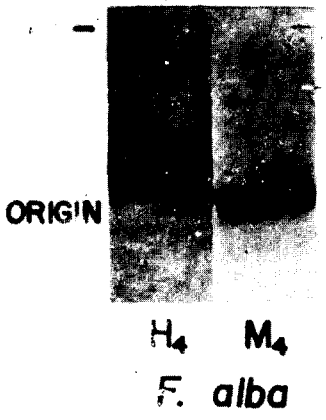


Fig. 1

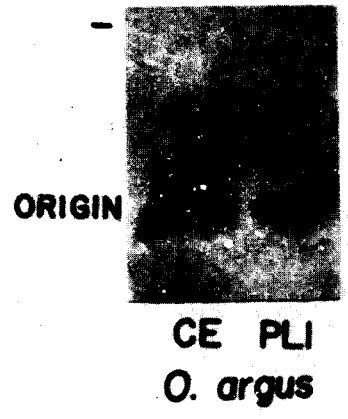


Fig. 2

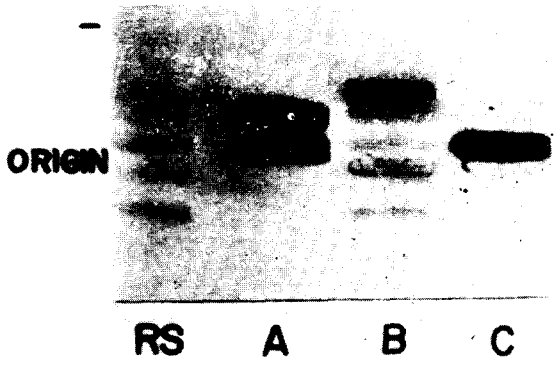


Fig. 3


Fig. 4

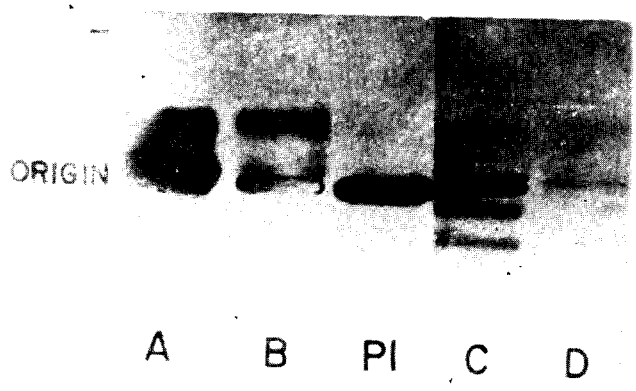


Fig. 5

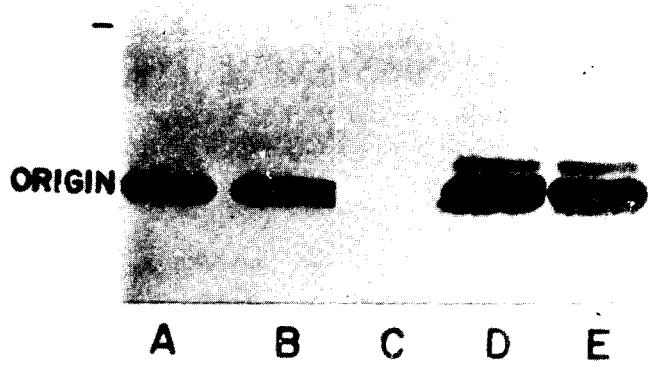


Fig. 6

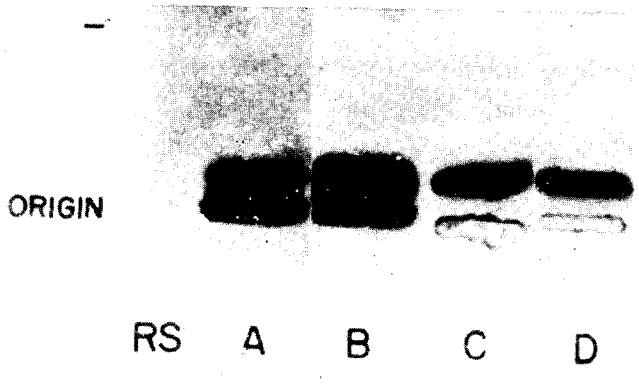


Fig. 7