

## Immunological Comparison of the Reptilian M<sub>4</sub>-LDH Isozyme

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파충류 M<sub>4</sub>형 젖산 수소이탈효소의 면역학적 연구

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### 적 요

살모사 근육조직의 M<sub>4</sub>형 젖산 수소이탈효소에 대한 항혈청을 토끼에서 얻어서 척추동물 15종의 젖산 수소이탈효소와 항원-항체 반응결과를 초산셀룰로즈 전기영동법에 의하여 얻었으며, 아울러 뱀목 4종에 대한 면역확산 실험도 실시하였다.

살모사, 쇠살모사, 까치살모사 및 유혈목은 면역학적으로 동일한 젖산 수소이탈효소를 가지고 있으며 쇠살모사는 살모사속의 다른 종과는 유연관계가 먼것 같았으며 젖산 수소이탈효소 단위체의 면역학적 상이성이 파충류에서도 재확인되었다.

### INTRODUCTION

Amino acid sequencing of homologous proteins from different taxa is most up to date but it preserves various difficulties. The electrophoresis is valuable for protein evolutionist. Its reliability at the lower category, however, is limited because of the fact that the protein molecules are separated mainly due to their net charge (Guttman, 1973; Park *et al.*, 1974). An intermediate choice between sequence determination and electrophoresis lies in immunological comparison of proteins (Gorman *et al.*, 1971). Estimation of relative reactivity of antigen and antibody provides more unambiguous analysis of homologous proteins.

On the Korean reptilian species, there have been limited reports (Gloyd, 1972; Kim *et al.*, 1973), and furthermore the chaos in establishment of reptilian

phylogeny is thought to be derived from the fact that only about ten species can be collected in this area (Park and Cho, 1976).

The present investigation is intended to make a comparison of the reptilian lactate dehydrogenase (LDH) isozymes and to find out an evidence to support the view of Gloyd (1972).

We thank Miss Jung Joo Yum for her help during the preparation of antisera.

## MATERIALS AND METHODS

The specimens used are listed together with their sources in Table 1. The partial purification of  $M_4$ -LDH isozyme from 50 grams skeletal muscle of one individual of *Agkistrodon blomhoffii brevicaudus* was carried out by the method of de Burgos *et al.* (1973). Aliquot of each 10 ml fraction from the DEAE cellulose column (170×23 mm) was electrophoresed on the cellulose acetate strip (Millipore) for 60 minutes and stained for LDH isozymes by the method of Park and Cho (1972). The  $M_4$ -LDH isozyme was appeared to be in fractions 6

**Table 1.** Specimens and their abbreviations. 1. Commercial dealer; 2. Yong Moon Area; 3. Byun San Peninsula.

| Species                                | Source | Abbreviation |
|--|--------|--------------|
| Class Aves                             |        |              |
| <i>Alauda arvensis</i>                 | 1      | Aa           |
| Class Reptilia                         |        |              |
| <i>Agkistrodon saxatilis</i>           | 2      | As           |
| <i>A. caliginosus</i>                  | 2      | Ac           |
| <i>A. blomhoffii brevicaudus</i>       | 2      | Ab           |
| <i>Rhabdophis tigrinus</i>             | 2      | Rt           |
| <i>Elaphe dione</i>                    | 2      | Ed           |
| <i>E. schrenckii</i>                   | 2      | Es           |
| <i>Zamenis spinalis</i>                | 2      | Zs           |
| <i>Dinodon rufozonatum rufozonatum</i> | 2      | Dr           |
| <i>Amyda mackii</i>                    | 1      | Am           |
| Class Amphibia                         |        |              |
| <i>Bufo bufo gargarizans</i>           | 3      | Bb           |
| <i>Bombina orientalis</i>              | 3      | Bo           |
| <i>Rana nigromaculata</i>              | 2      | Rn           |
| <i>R. rugosa</i>                       | 2      | Rr           |
| Class Pisces                           |        |              |
| <i>Ophicephalus argus</i>              | 1      | Oa           |

to 10, and the M<sub>2</sub>H<sub>2</sub>-LDH isozyme in fraction 18 to 19. The fractions 6 to 8 were pooled and stored at -20°C until further studies.

The protein was estimated by the method of Lowry *et al.* (1951). One mg protein with Freund's complete adjuvant (Difco) was injected at the dexter thigh muscle of adult male rabbits. One week later each rabbit received the second injection at the sinister thigh muscle. The third and fourth injections were made at the dexter and sinister dorsal muscles, respectively, every week. The rabbits were bled one week after the last injection by the method of cardiac puncture without any anticoagulant and anesthetic. The blood was stored at 4°C for 24 hours and then centrifuged for 20 minutes at 4,000 rpm (IEC PR-2). The antisera were stored at -20°C until further studies.

The heart and skeletal muscles of equiweight from 15 species were dissected and each pair was homogenated and centrifuged for 20 minutes at 4,500 rpm. One twentieth ml of each supernatant was thoroughly mixed with the same volume of antiserum. Then the mixture was incubated for an hour in moist chamber at 37°C and filtered through Whatman No. 1 filter paper. The filtrate was used for the electrophoresis. Each filtrate and untreated crude extracts were electrophoresed for 60 minutes at 19°C and then stained for LDH isozymes.

The skeletal muscle extracts from four species of Squamate were subjected to immunodiffusion tests with antisera on 1% agar (Difco) gel for 24 hours at 37°C and then 24 hours at 4°C.

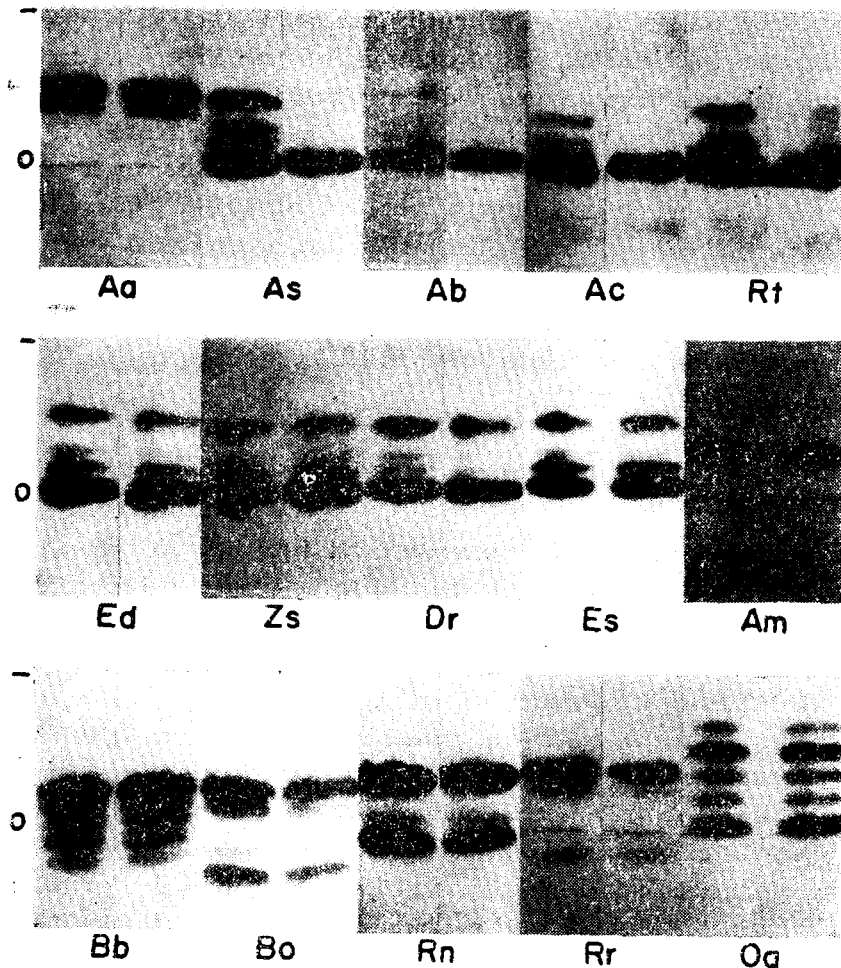
## RESULTS AND DISCUSSION

Zymogrammatic patterns of Squamate LDH isozymes on cellulose acetate electrophoresis at pH 8.6 do not fit the five isozyme hypothesis; M<sub>3</sub>H-LDH isozyme is absent or inactive in the skeletal muscle (Kim *et al.*, 1973). Our mixed homogenates of the skeletal and heart muscles revealed four isozymes except that of M<sub>3</sub>H heterotetramer (Fig. 1).

Electrophoretic results of the antigen-antibody reactivity are given in Fig. 1. Zymograms of the LDH isozymes of aves, amphibia and pisces revealed no reactivity. In the reptilian species studied only four, *A. saxatilis*, *A. blomhoffii brevicaudus*, *A. caliginosus* and *R. tigrinus*, showed that they had the M<sub>4</sub>-LDH isozymes of immunological identity and that anti-M<sub>4</sub>-LDH isozyme reacted not only with the M<sub>4</sub>-LDH isozyme but also with the M<sub>2</sub>H<sub>2</sub>- and MH<sub>3</sub>-LDH isozymes. Those are similar to the situations described in the chicken (Kaplan, 1964), in the lizard (Gorman *et al.*, 1971) and in the frog (Wright and Moyer, 1973).

The H and M lactate dehydrogenase subunits have been found to be immuno-

logically distinct. Immunological analyses of LDH isozymes in mammals (Markert and Appella, 1963; Rajewsky, 1964), in birds (Cahn *et al.*, 1962), in reptiles (Gorman *et al.*, 1971) and in amphibians (Wright and Moyer, 1973) have shown that the homotetramers,  $M_4$  and  $H_4$  are distinct antigens. In addition,  $M_4$  is a better immunogen than  $H_4$  (Burd and Usategui-Gomez, 1973). One mg protein contained in our every injection was analysed on the cellulose acetate electrophoresis at pH 8.6, and shown to consist of small quantity of  $M_4$ -LDH isozyme and other proteins. It is, thus, suggested that the reptilian  $M_4$ -LDH isozyme would have a strong immunogenicity.



**Fig. 1.** Zymograms showing the antigen-antibody reactivity. The electrophoresis was run for 60 minutes. The pattern of mixed homogenate (right) was compared to that of intact homogenate (left). For abbreviations, see Table 1.

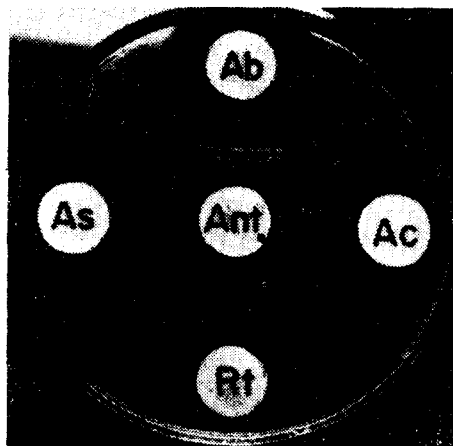
An additional very weak band was appeared just at the cathodal side of M<sub>4</sub>-LDH isozyme treated with immunized rabbit serum. This band was detected, but not shown in Fig. 1, in the zymograms from 9 reptilian species and 3 amphibian species, *R. rugosa*, *R. nigromaculata* and *B. orientalis*. On the Nothing Dehydrogenase tests, this band was not stained and believed to be another LDH isozyme band. Mixed with the unimmunized rabbit serum, the zymogram had no additional LDH isozyme band.

The cross-reaction tests showed one to two distinct precipitate lines (Fig. 2). For conveniences sake, PL-1 designates the precipitate line nearer the central well and PL-2 nearer the peripheral well. Distinct PL-1 and PL-2 were made in the cross-reactions of muscle extracts from *A. blomhoffii brevicaudus*, *A. saxatilis* and *R. tigrinus* but only PL-2 from *A. caliginosus*. This result is considered to support some extent view of Gloyd (1972) at the molecular level. Gorman *et al.* (1971) cogently discussed the probability that the biochemical differences between species of a genus could be more apparent than between those of different genera, and George and Dessauer (1970), furthermore, reported that there had been the distinguishable differences at the subspecies level.

### SUMMARY

M<sub>4</sub>-LDH isozyme was partially purified from the skeletal muscle of *Agkistrodon blomhoffii brevicaudus*. The protein was injected into rabbits and the resulting antiserum was tested for reactivity with crude preparations of LDH isozymes of fifteen vertebrate species.

Antisera against M<sub>4</sub>-LDH isozyme of *A. blomhoffii brevicaudus* reacted very strongly with the LDH isozymes, except the H<sub>4</sub>-LDH isozyme, of *A. saxatilis* and *A. caliginosus* but weakly with those of *Rhabdophis tigrinus* at fixed conditions. *A. caliginosus* showed a difference in the immunodiffusion test and was considered to be a species less related to others of genus *Agkistrodon*. The suggestion that the H and M lactate dehydrogenase subunits are immunologically distinct has been reaffirmed in the present study.



**Fig. 2.** Immunodiffusion test with the antiserum (Ant) prepared against *Agkistrodon blomhoffii brevicaudus* muscle protein fraction. For abbreviations, see Table 1.

## REFERENCES

- Burd, J.F. and M. Usategui-Gomez, 1973. Immunological studies of lactate dehydrogenase. *Biochim. Biophys. Acta* **310**:238-247.
- Cahn, R.D. N.O., Kaplan, L. Levine and E. Zwilling, 1962. Nature and development of lactic dehydrogenases. *Science* **136**: 963-969.
- de Burgos, N.M., C. Burgos, M. Gutierrez and A. Blanco, 1973. Effect of temperature upon catalytic properties of lactate dehydrogenase isoenzymes from a poikilotherm. *Biochim. Biophys. Acta* **315**:250-258.
- George, D.W. and H.C. Dessauer, 1970. Immunological correspondence of transferrins and the relationships of colubrid snakes. *Comp. Biochem. Physiol.* **33**:617-627.
- Gloyed, H.K., 1972. The Korean snakes of the genus *Agkistrodon* (Crotalidae). *Proc. Biol. Soc. Wash.* **85**:577-578.
- Gorman, G.C., A.C. Wilson and M. Nakanishi, 1971. A biochemical approach towards the studies of reptilian phylogeny: Evolution of serum albumin and lactate dehydrogenase. *Syst. Zool.* **18**:286-295.
- Guttman, S.I., 1973. Biochemical techniques and problems in anuran evolution. *In: Evolutionary Biology of Anurans*(J.L.Vial, editor). Univ. Mo. Press, Columbia, USA, pp. 183-203.
- Kaplan, N.O., 1964. Lactate dehydrogenase:Structure and function. *Brookhaven Natl. Lab. Sym. Biol.* **17**:131-153.
- Kim, S.O., D.H. Cho and S.Y. Park, 1973. Comparative studies of isozymes in vertebrate. Lactate and malate dehydrogenase isozymes of Korean Squamate. *Korean J. Zool.* **16**: 55-66.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Markert, C.L. and E. Appella, 1963. Immunochemical properties of lactate dehydrogenase isozyme. *Ann. N.Y. Acad. Sci.* **103**:915-928.
- Park, S.Y. and D.H. Cho, 1972. Separation of dehydrogenase isozymes by cellulose acetate electrophoresis. *Korean J. Zool.* **15**:101-104.
- Park, S.Y. and D.H. Cho, 1976. On the isozymes of reptilian blood proteins. In press.
- Park, S.Y., D.H. Cho, S.Y. Kim, S.K. Kim and C.H. Kim, 1974. Electrophoresis of the hemoglobins and the serum proteins of Korean anuran. *Korean J. Zool.* **17**:159-162.
- Rajewsky, K., S. Averameas, P. Grabar, G. Pfeleiderer and E.D. Wachsmuth, 1964. Immunologische Spezifitaet von lactatdehydrogenase Isozymen dreier Saeugetier-Organismen. *Biochim. Biophys. Acta* **92**:248-259.
- Wright, D.A. and F.H. Moyer, 1973. Immunochemistry of frog lactate dehydrogenase (LDH) and the subunit homologies of amphibian LDH isozymes. *Comp. Biochem. Physiol.* **44**: 1011-1016.