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Immunological Comparison of Reptilian Plasma Albumins and Hemeglobins

Sang Yoon Park, Sang Yeop Kim and Jung Joo Yum
(Department of Biology, Sung Kyun Kwan University)

파충류 혈장알부민 및 혈색소의 면역학적 비교

朴 相 允·金 相 曄·廉 貞 周
(성균관대 생물학과)

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적 요

유혈목이(*Rhabdophis tigrinus*)의 혈장알부민을 polyethylene glycol과 ethanol 침전 방법에 의해 정제하였고 혈색소는 agar-gel 전기영동방법에 의해 정제하였다. 이 두 단백질과 유혈목이 혈장을 각각 토끼에 주사하여 항혈청들을 얻었으며 포유류 1종, 조류 1종, 파충류 9종, 및 양서류 1종의 혈장 및 혈구파쇄물과 면역확산 및 면역 전기영동을 실시하였다.

혈장알부민과 혈장에 대한 항혈청들은 유혈목이 혈장알부민과만 반응을 나타냈고 다른종의 혈장알부민과는 반응을 나타내지 않았다. 혈색소에 대한 항혈청은 8종의 사류 혈색소와 강한 반응을 나타냈다. 파충류 혈장알부민의 구조는 상당히 빠르게 변형되어져 왔고 파충류 혈색소 분자는 강한 구조상의 상동성을 갖고 있는 것 같다. 그러므로 파충류의 경우 혈색소 분자의 구조는 혈장알부민의 구조보다 느리게 변형되어져 온 것으로 생각된다.

INTRODUCTION

It has been said that molecular biology was created by molecular geneticists whose interest in protein structure is essentially one-dimensional and structurisits who are concerned primarily with the precise geometry of biological molecules in three dimensions. There is, however, a dichotomy between these two schools of molrcular biologists. As Margoliash *et al*(1968) has stated, the reason for this parallelism is not merely our relative ignorance of the central dogma but that the large territory which provides the organic connection between the genetic and phenotypic fields is still largely unexplored in molecular terms.

Numerous attempts to clarify the temporal changes of biologically active proteins have been carried out with the gift of electrophoreses. These methods, however, preserve to some extent ambiguity because of the fact that the protein molecules are separated mainly due to their net charge (Guttman, 1973 ; Park *et al*, 1974). Next to the amino acid sequencing of homologous proteins, immunological approaches towards the study of protein structure have been continued to be of great value owing to the specificity of antibody against each antigenic determinant (Cocks and Wilson, 1969 ; Gorman *et al.*, 1971).

This report describes experiments with plasma albumins and hemoglobins in reptiles, in which we have demonstrated how many those homologous proteins are of immunological homogeneity.

MATERIALS AND METHODS

Live species were collected in South Korea or obtained from commercial dealers. These included: *Agkistrodon blomhoffii brevicaudus, A. saxatilis, A. caliginosus, Elaphe dione, E. schrenckii, E. rufodorsata, Dinodon rufozonatum rufozonatum, Rhabdophis tigrinus, Amyda maackii* (Reptilia); *Mus musculus musculus* (Mammalia); *Gallus gallus* (Aves); *Bombina orientalis* (Amphibia).

Blood with Na-EDTA collected by decapitating each individual of *R. tigrinus* was centrifuged and the cells were separated from the plasma. Each plasma was electrophoresed on cellulose acetate strips by the method of Park and Cho (1972). No individual differences in mobility or staining intensity were seen in the albumin region. The pooled plasma was fractionated using a minor modification of the polyethylene glycol and ethanol procedure (Jimenez *et al.*, 1974). Crude plasma, solution after treatment of polyethylene glycol, solution obtained in the final step of purification and commercial bovine serum albumin were electrophoresed on a cellulose acetate strip. Hemolysate of individual *R. tigrinus* was prepared by the method described elsewhere (Park *et al.*, 1974). An aliquot was electrophoresed on 1% agar(Difco) plate at pH 8. 6 and the gel slice of hemoglobin mobility was frozen for 24 hrs. After thawing, the solution was centrifuged and the supernatant was stored at −20°C until further studies.

Plasma albumin concentration was measured by the method of Lowry *et al.* (1951). Antisera to *R. tigrinus* plasma albumin were prepared by injecting one mg of the purified protein into each adult rabbits, those to *R. tigrinus* hemoglobin by injecting one fifth ml of hemoglobin sample and those to *R. tigrinus* crude plasma by injecting a half ml of crude plasma. The other conditions for preparation of antisera were described previously(Park *et al.*, 1976). Blood of the other eight species of reptiles were collected by decapitating the animals, those of *M. musculus musculus* and *G. gallus* by heart puncture and those of *B. orientalis* by

dissecting the tibiofibula. Crude plasma and hemolysate were prepared by the same methods as above. By the method of Ouchterlony and Nilsson (1973) were carried out the immunodiffusion tests between crude plasma of twelve species and anti-plasma albumin. Each crude plasma and hemolysate were electrophoresed on cellulose acetate strip by the method of Park and Cho (1972). With these strips placed on the 1% agar-gel plate, the immunoelectrophoreses were carried out with the anti-plasma and anti-hemoglobin.

RESULTS

The purity of *R. tigrinus* plasma albumin was characterized in terms of electrophoretic mobility (Fig.1). A faint band at the cathodal side of purified plasma albumin was revealed on the basis of densitogram to be a tailing of plasma albumin (Fig. 2). No further steps in plasma albumin purification were progressed because the plasma albumin was used only to elicit antibodies. Individuality in antibody formation was observed in rabbits injected with the plasma albumin and more than one precipitation lines were made in any antigen-antibody reactions (Fig.3). The anti-plasma albumin 1 was used throughout this experiment. The *R. tigrinus* hemoglobin was electrophoretically pure. No evidence of individuality in anti-hemoglobin formation in rabbits was established in immunodiffusion tests with *R. tigrinus* hemoysate.

In Fig. 4 are given the results of immunodiffusion tests between crude plasma of eight squamates and anti-plasma albumin. The anti-plasma albumin did not react not only with bovine serum albumin but also with other squamate albumins except that of *R. tigrinus*. Those are similar to the situations appeared in the immunodiffusion tests between the anti-plasma albumin and crude plasma of *A. maackii, B. orientalis, M. musculus musculus* and *G. gailus* (Fig.5). Immunoelectrophoreses of eight squamate crude plasma against anti-plasma demonstrated that the precipitation line corresponding to plasma albumin mobility was shown only in *R. tigrinus* crude plasma immunoelectrophoresis and that in each of seven squamate crude plasma immunoelectrophoresis were made two to three precipitation lines (Fig. 6) which was thought to be lines corresponding to the beta and gammaglobulin mobilities compared with the electrophoretic patterns of reptilian plasma proteins (Park and Cho, 1976).

In Fig. 7 are given the results of immunodiffusion tests between anti-plasma and crude plasma of *A. maackii, B. orientalis, M. musculus musculus* and *G.gallus*. At least seven precipitation lines were seen in immundiffusion of *R. tigrinus* crude plasma, one precipitation line in those of *A. maackii* and *G. gallus* crude plasma and no precipitation line in those of *B. orientalis* and *M. musculus musculus* crude plasma. Fig. 8 shows the immunoelectrophoreses of the eight squamate

hemolysates against anti-*R. tigrinus* hemoglobin, each of which reveals a distinct antigen-antibody reactivity. No reactivities, however, were made in those of four species of non-squamate vertebrate.

Since the anti-plasma albumin and anti-plasma have reactivity only with the *R. tigrinus* plasma albumin, it is likely that the reptilian plasma albumin has been experiencing rapid structural change. The reptilian hemoglobin molecule seems to be of high homogeneity on the basis of its immunological relationships. And thus the structure of the hemoglobin molecule is considered to have been changing slower that of the plasma albumin in reptiles.

DISCUSSION

Individuality of antibody formation in rabbits injected with the purified *R. tigrinus* plasma albumin was recognized in our experiment, which was validated by the nature that the ability to produce antibodies against specific antigen varies with different species, strains within a species. and even individuals within a given strain (Campbell *et al.*, 1974). The plurality of *R. tigrinus* plasma albumin seems to be brought about by the protein molecules of similar physicochemical properties on the ground of the facts that the bovine and human serum albumins are admitted to be of molecular heterogeneity with respect of sulfhydryl contents (Janatova *et al.*, 1968 ; Shrivastava *etal.*, 1972) and that while the plasma albumin concentration of reptiles (Masat and Desauer. 1968) is lower than that of human beings(Sober, 1974) our previous electrophoretic studies on the reptilian plasma proteins showed that the staining intensity was very strong to any other plasma proteins(Park and Cho, 1976).

The hypothesis that plasma albumin may well serve as an evolutionary clock was proposed to account for the results of immunological comparisons of primate albumins(Sarich and Wilson, 1967). This hypothesis has been substantiated by those immunological studies on plasma albumins of fissipeds and pinnipeds which are presently living in extremely diverse environments(Sarich, 1969), of iguanid lizards and crocodilians (Gorman *et al.*, 1971) and of some frogs experiencing convergent morphological evolution(Wallace *et al.*, 1971 ; Maxson and Wilson, 1975) Those facts are considered to verify our suggestion, though not from quantitative results, that the *R. tigrinus* plasma albumin has antigenic determinants which cannot be found in any other squamate plasma albumins.

Ingram (1961) suggested that cistrons specifying α-chains of hemoglobin would be conservative in evolution because the α_2 dimer had to combine with both β_2 and γ_2 dimer. This suggestion has been reaffirmed by the comparative studies on mammalian hemoglobins (Hill and Buettener-Janusch, 1964 ; Uzzell and Corbin, 1972). Hemoglobin has been reported to be evolving roughly half as fast as

albumin in fairly large number of vertebrate species(Wallace and Wilson, 1972 ; Maxson and Wilson, 1975). Electrophoretic patterns of squamate hemoglobins (Park and Cho, 1976) revealed that the proteins have similar net charge. Those facts on the hemoglobin molecules make our suggestion probable that every squamate hemoglobin examined has common antigenic determinant of almost identical structure in its tetramer conformation. Gorman *et al.* (1971) reported that the structure of the lactic dehydrogenase would be more conservative than that of the serum albumin in reptilian taxon and cogently discussed it in physiological terms. Our suggestion that the antigenic determinants of hemoglobin would be altered at slow rate compared to that of plasma albumin might be explained in physiological terms. While hemoglobin has a vital role in vertebrate respiratory physiology, plasma albumin is not essential for life. Although albumin probably has a role in transport of various small molecules and is reported to be used as a chief contributor of colloid osmotic pressure in reptiles (Masat and Dessauer, 1968), several species of vertebrate appear to lack albumin in their blood (Cohen and Stickler, 1958 ; Engle and Woods, 1960 ; Lewis, 1965).

SUMMARY

Plasma albumin and hemoglobin were purified from the blood of *Rhabdophis tigrinus*. Both purified proteins and *R. tigrinus* crude plasma were injected into rabbits. The resulting antisera were tested for reactivity with plasma albumin and hemoglobin from, eight species of squamate and four species of non-squamate-vertebrate. Reactivity was detected qualitatively by immunodiffusion tests and immunoelectrophoreses.

Antisera against plasma albumin and crude plasma reacted only with *R. tigrinus* plasma albumin. No antigen-antibody reactions were detected in plasma albumins of other species. Antiserum against *R. tigrinus* hemoglobin reacted strongly with eight squamate hemoglobins. It is likely that the reptilian plasma albumin has been experiencing rapid structural change and the reptilian hemoglobin molecules seem to be of high homogeneity. Thus, by immunological evidences, the structure of the hemoglobin molecule is considered to have been changing at slower rate than that of the plasma albumin in reptiles.

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ILLUSTRATIONS OF FIGURES

Fig. 1. Cellulose acetate electrophorphoresis of crude plasma (SCP), solution after treatment of polyethylene glycol (PGA), solution obtained in the final step of purification (PSA) and bovine serum albumin (BSA).

Fig. 2. Densitogram showing the purity of plasma albumin (PSA).

Fig. 3. Immunodiffusion test with antisera (1 and 2) prepared against purified *Rhabdophis tigrinus* plasma albumin.

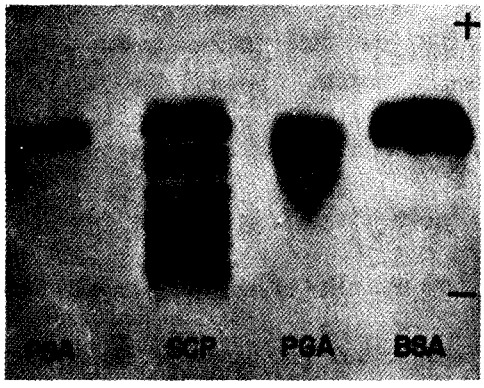
Fig. 4. Immunodiffusion test with antiserum 1 prepared against purified *R. tigrinus* plasma albumin. Rt means *Rhabdophis tigrinus*, Ab; *Agkistrodon blomhoffii brevicaudus*, As; *A. saxatilis*, Ac; *A. caliginosus*, Ed; *Elaphe dione*, Es; *E. schrenckii*, Er; *E. rufodorsata*, Dr; *Dinodon rufozonatum rufozonatum*, B; bovine serum albumin.

Fig. 5. Immunodiffusion test with antiserum 1 prepared against purified *R. tigrinus* plasma albumin. Am means *Amyda maackii*, Bo; *Bombina orientalis*, Gg; *Gallus gallus*, Mm; *Mus musculus musculus*.

Fig. 6. Immunoelecteophoresis of eight squamate plasma against anti-*R. tigrinus* plasma serum.

Fig. 7. Immunodiffusion test with anti-*R. tigrinus* plasma serum.

Fig. 8. Immunoelectrophoresis of eight squamate hemolysates against anti-*R. tigrinus* hemoglobin serum.



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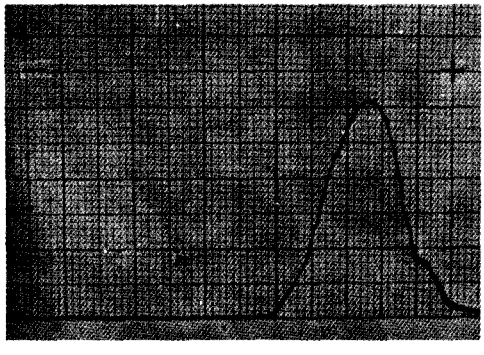
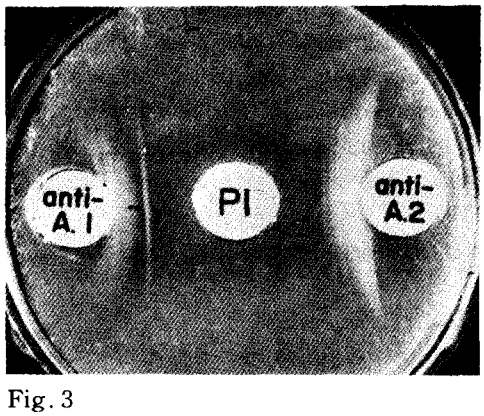


Fig. 2



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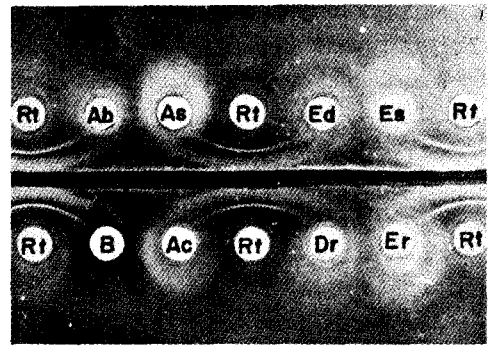

Fig. 4

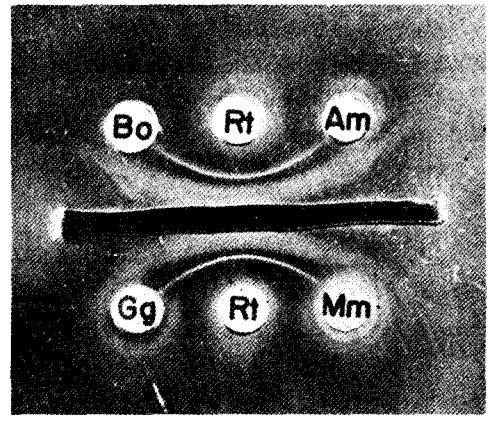

Fig. 5

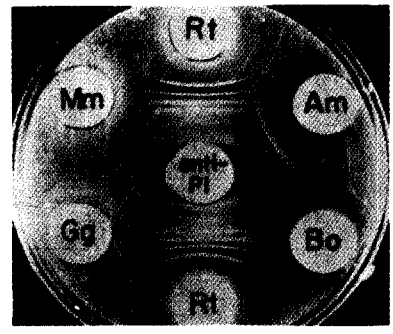

Fig. 7

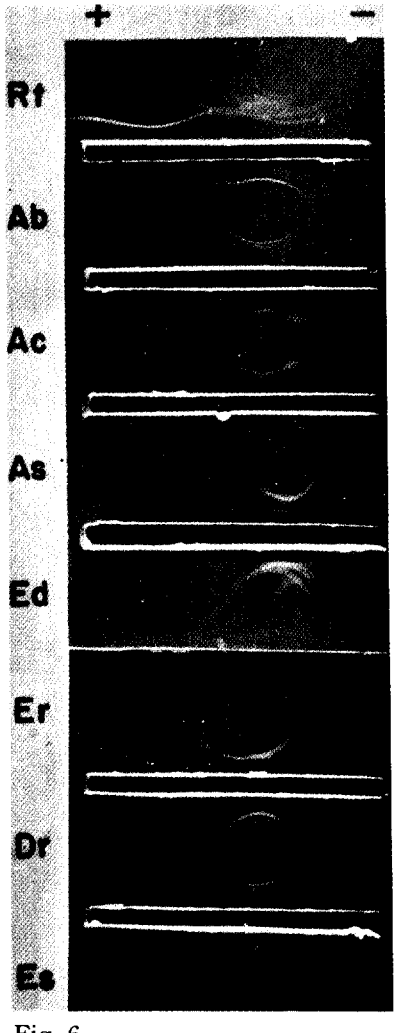

Fig. 6

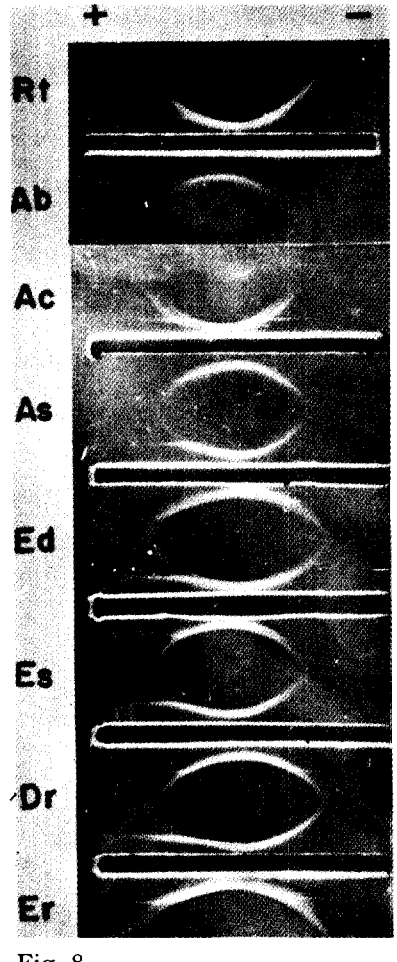

Fig. 8