Application of two-dimensional electrophoresis without denaturing agents to comparative study of vertebrate plasma proteins

Youji Shimazaki and Takashi Manabe

SUMMARY

A comparative study of vertebrate plasma proteins was performed by two-dimensional electrophoresis (2-D PAGE) without denaturing agents in order to obtain a perspective of the variation of plasma protein composition in different vertebrates. Plasma from raccoon, chicken, turtle, newt, goldfish and human was subjected to the analysis. The 2-D PAGE patterns offered information on vertebrate plasma proteins in their physiological conditions: their isoelectric point (pI), approximate molecular weight (M.W.) and quantity. Each vertebrate plasma protein showed a characteristic protein distribution, reflecting the evolutionary changes in DNA sequence and the expression of plasma protein. On the other hand, for some plasma proteins, the structural relationships between human and other vertebrates were suggested immunochemically. A series of spots detected at pI 6.2-8.3, M.W. 850-kDa, observed in the 2-D PAGE patterns of raccoon, chicken and turtle plasma was immunochemically stained by anti-human immunoglobulin M antibody. And, a series of spots detected at pI 4.8-5.5, M.W. 500-kDa in the patterns of raccoon, chicken and newt plasma was immunochemically stained by anti-human α2-macroglobulin antibody. These results indicate that a perspective of the variation of plasma protein composition in different vertebrates may be obtained by 2-D PAGE without denaturing agents.

Key words: comparative study, electroblotting, α2-macroglobulin, immunoglobulin M, physiological condition.

INTRODUCTION

2-D PAGE without denaturing agents offers information on the separated proteins in their physiological condition: pI, M.W. and quantity. Since it is said that a protein which retains biological activity and the binding of other substances can be separated by this method, the obtained information from 2-D PAGE pattern in the absence of denaturing agents is different from that done in the presence of denaturing agents, as reported by O'Farrell. Therefore, accumulating evidence of the 2-D PAGE patterns in the absence of denaturing agents is thought to be necessary for researching proteins related to biological functions.

Only some mammalian plasma proteins have been analyzed by 2-D PAGE without denaturing agents (human, bovine, horse, mouse and rat):
llama and alpaca\(11\)), and there is no evidence of 2-D PAGE patterns of plasma proteins in lower vertebrates. All vertebrate plasma is thought to contain characteristic proteins which reflect the evolutionary change in DNA sequence and the expression of plasma protein. Thus, conservative proteins which possess the same function in all vertebrate plasma can exist. In fact, it has been reported that not only the plasma of mammals but also that of other vertebrates contains immunoglobulins, complement and protease inhibitors which might be related to biological functions.\(12\)

In the present study, we compared raccoon, chicken, turtle, newt and goldfish plasma proteins to human plasma proteins by 2-D PAGE without denaturing agents. We also indicate that proteins which have structural relationships with human can be immunochemically identified in the plasma of other vertebrates.

**MATERIALS AND METHODS**

Human *Homo sapiens* and raccoon *Procyon lotor* blood were obtained from veins. Raccoon plasma was provided from Tobe Zoo (Ehime prefecture, Japan). Newt *Cynos pyrrhogaster* and goldfish *Crassius auratus* blood was obtained from hearts, and turtle *Trachemys scripta* blood was obtained from legs. Each blood was centrifuged for 5 min at 2,200 × g. Chicken *Grallus gallus* plasma was purchased from Chemicon (USA). Sucrose was added to the supernatant plasma solution to give a concentration of 40\(\%\) (w/v). The native plasma without freeze was analyzed.

Reagents and chemicals were obtained as follows: Polyclonal anti-human immunoglobulin M antibody from Boeringer Mannheim (USA); anti-human \(\alpha\)-macroglobulin antibody and peroxidase conjugated goat anti-rabbit antibody from Dako (Denmark); polyvinylidene difluoride (PVDF) sheet from Applied Biosystems (USA); Ampholine pH 3.5-10 and pH 3.5-5.0 from Pharmacia Biotech (Sweden); Acrylamide from Daiichi pure chemicals (Japan); Bovine serum albumin from Seikagaku (Japan); Coomassie Brilliant Blue R-250 (CBB) from Nacalai Tesque (Japan); N,N'-methylene-bis (acrylamide)-HG, N, N, N', N'-tetramethylenediamine, ammonium persulfate, 3,3'-diaminobenzidine tetrahydrochloride and all other reagents from Wako (Japan).

Micro 2-D PAGE of each vertebrate plasma protein without denaturing agents was performed by the method previously reported.\(7\) In each experiment, human plasma proteins were used as a control of pI and M.W. Proteins on the gel were stained in 0.1\% Coomassie Brilliant Blue-7\% (v/v) acetic acid-50\%(v/v) methanol for 15 min, and destained in 20\%(v/v) methanol-7\% (v/v) acetic acid for 2 h. In order to identify hemoglobin, the gel was incubated with 10 ml of dianamobenzidine solution (10 mg dianamobenzidine dissolved in 10 ml 0.5 \(\mu\)l acetate buffer, pH 6.0) and 0.1 ml 3\% \(\mathrm{H}_2\mathrm{O}_2\) at room temperature for 20-30 min.

Electrophoretic transfer of proteins from micro 2-D PAGE gels to PVDF was performed by an apparatus which we devised for the simultaneous transfer of proteins from 4 micro-slab gels to 4 PVDF sheets.\(13,14\) Electrophoretic transfer was performed at 150 mA constant current in 0.02 M Tris-0.19 M glycine, pH 8.3 for 1 h. After blotting, each PVDF sheet was soaked in 5 ml of 3\% bovine serum albumin (BSA) in Tris-saline (0.9\% NaCl-10 mM Tris-HCl, pH 7.4) at room temperature for 2 h. The anti-human immunoglobulin M or \(\alpha\)-macroglobulin antibody was added to the BSA solution and PVDF sheet was incubated overnight at 4\(^\circ\)C. After washing in Tris-saline, the PVDF sheet was incubated with peroxidase conjugated goat anti-rabbit antibody (\(\mu\)l in 5 ml Tris-saline, 1/1000 dilution) at room temperature for 1 h. After washing in Tris-saline, immunoreactive spots were stained with 0.2 mM dianamobenzidine-\(\mathrm{H}_2\mathrm{O}_2\) in Tris-saline for 15 min at room temperature.

**RESULTS**

**Comparative study of vertebrates plasma proteins**

Figure 1 shows 2-D PAGE patterns of human (a), raccoon (b), chicken (c), turtle (d), newt (e) and goldfish (f) plasma proteins without denaturing agents. Each spot of human plasma proteins in the 2-D PAGE patterns have already been immunochemically identified.\(14\) In the present study, human plasma proteins, with information on their pI and M.W., were used as controls when 2-D PAGE patterns of other vertebrate plasma proteins were examined. As for the positions of immunoglobulins (Igs), low density lipoprotein (LDL), \(\alpha\)-macro-
globulin (α2M), transferrin (Tf) and albumin (Alb) in human plasma protein patterns (Fig. 1a), the same positional spots were observed in raccoon plasma proteins (Fig. 1 b). As shown in Fig. 1 c, the spots located at the same positions of IgM, IgA, α2M, Tf, and Alb in human plasma protein (Fig. 1 a) were observed in chicken plasma. As shown in Fig. 1 d, e and f, except for the positions of IgM, IgA, LDL, glycoprotein and fibrinogen in human plasma (Fig. 1 a), no spots with the same pI and M. W. were observed in the patterns of the turtle plasma protein (Fig. 1 d). In the patterns of the turtle plasma protein, characteristic spots were abundant in the area of pI 4.0-5.0 (Fig. 1 d). Spots located at the same positions of IgM, LDL, α2M and Tf in human plasma protein patterns (Fig. 1 a) were observed in newt plasma proteins as shown in Fig 1 e. As for the position of IgM, LDL and Alb in human plasma protein patterns (Fig. 1 a), the same positional spots were detected in goldfish plasma proteins (Fig. 1 f). Characteristic spots were observed in an area of less than M. W. 70-kDa in the patterns of the goldfish plasma proteins. The spots resided at pI 7.0, M. W. 87-kDa as shown in Fig. 1 b, and those that resided at region of pI 7.0, less than M. W. 90-kDa, as shown in Fig. 1 d and f were identified as hemoglobin because of the red color and peroxidase activity.

Figure 2 shows 2-D PAGE patterns of chicken plasma proteins (a) and a mixture of human and chicken plasma proteins (b). Whereas no spots of IgG in human plasma proteins were observed in chicken plasma as shown in Fig. 2a, one was detected at pI 6.0-7.0, M. W. 170-450-kDa. The positional difference of the spot was not attributable to mismanipulation when first dimensional gel was attached to the second dimensional slab gel, but to the pI difference, because the area of pI 6.0-8.0, M. W. 170-450-kDa possesses two distinct spots in a mixture of human and chicken plasma proteins (Fig. 2 b).

The experiments shown in Figs. 1 and 2 indicated that a series of spots which resided at pI 6.2-8.3, M. W. 850-kDa was observed in the patterns of raccoon, chicken, turtle, newt and goldfish plasma proteins. The spots were identified to be IgM in the pattern of human plasma proteins. In addition, a series of spots which resided at pI 4.8-5.5, M. W. 500-kDa was observed in the patterns of raccoon, chicken and newt. They were also identified to be α2M in the pattern of human plasma proteins.

Identification of IgM and α2M

As shown in Fig. 3, several spots which react to anti-human IgM antibody were observed in the patterns of raccoon (a), chicken (b) and turtle (c) plasma proteins. The spots at pI 6.2-8.3, M. W. 850-kDa in the patterns of raccoon, chicken and turtle plasma proteins was immunochemically
stained by anti-human IgM antibody since the same position in the 2-D PAGE patterns of human plasma proteins was already identified. In the patterns of newt and goldfish plasma proteins, the spots detected at pI 6.2-8.3, M.W. 850-kDa did not react to anti-human IgM antibody. Figure 4 shows that several spots which react to anti-human αM antibody were observed in the 2-D PAGE patterns of raccoon (a), chicken (b), turtle (c) and newt (d) plasma proteins. The spots resided at pI 4.8-5.5, M.W. 500-kDa in the patterns of raccoon, chicken and newt plasma proteins were immunochemically stained by anti-human αM antibody since the same positional spot in the 2-D PAGE patterns of human plasma proteins was already identified. In the pattern of turtle, the spot reacted to anti-human αM antibody located at pI 4.5-6.0, M.W. 500-kDa (Fig. 3c). As shown in Fig. 3a, c and Fig. 4a, c, d, hemoglobin was stained by diaminobenzidine.

**DISCUSSION**

We compared several vertebrate plasma proteins by 2-D PAGE without denaturing agents, and each pattern was compared to that of human plasma proteins. For the IgM position in the human plasma proteins pattern (Fig. 1a), the same positional spot was observed in all vertebrates plasma proteins in the present study. Since Putnam\(^{15}\) reported that
IgM exists in all vertebrate plasma, it is possible that the spot in other vertebrates is IgM. As for the LDL position in human plasma proteins patterns (Fig. 1a), the same positional spot was detected in the patterns of raccoon, turtle, newt and goldfish plasma proteins. Since it was reported that lipoproteins exist in the plasma of all vertebrates, including lamprey and other fish, the same lipoprotein spot might be detected in the patterns of raccoon, turtle, newt and goldfish plasma proteins. The same positional spots of Tf and α2M in the human plasma proteins pattern (Fig. 1a) were observed in raccoon, chicken and newt plasma proteins. Since both proteins exist in all vertebrate plasma, it may be possible that these proteins possess the same pI and M. W. in the patterns of raccoon, chicken and newt. The IgA spot in the pattern of human plasma proteins was observed in the raccoon, chicken and turtle plasma proteins. Mammalian and chicken plasma contains IgA, but there is no evidence that IgA exists in turtle plasma. Therefore, the spot detected in the pattern of turtle plasma proteins is thought to be other protein. At present, the spot has not been identified, and the reason for possessing the same pI and M. W. is unknown. On the other hand, several characteristic spots such as the area of pI 4.0-5.0 in turtle plasma proteins patterns (Fig. 1d) and the area of less than M. W. 70-kDa in the goldfish plasma proteins pattern (Fig. 1f), were detected. These proteins might be related to its biological role. In the present study, hemoglobin was detected in raccoon, turtle and goldfish plasma (Figs. 1b, d and f). Since it is unquestionably accepted that hemoglobin is contained in the red blood cells of vertebrates, the hemoglobin in the plasma samples detected might come from hemolysis in the course of taking plasma from individuals.

The spots which resided at pI 6.2-8.3, M. W. 850-kDa in the 2-D PAGE patterns of raccoon, chicken and turtle cross-reacted with anti-human IgM antibody. However, the spots which resided at the same position, pI 6.2-8.3, M. W. 850-kDa in newt and goldfish patterns did not show cross-reactivity. The differences in cross reactions might reflect the differences in amino acid sequences. In fact, it has been reported that the amino acid sequences of human and mouse IgM μ chains are more than 85% identical, and the chicken, turtle and toad sequences are 30–40% identical to mammals, whereas shark and mammalian sequences are only 30–35% identical. The anti-human IgM antibody also reacted with a series of spots which corresponded to IgG position in the patterns of human plasma (Figs. 3a–c). This fact might be explained by the fact that the anti-IgM antibody reacted with several epitopes of IgGs, which exists in IgG position. We indicated that a spot detected at pI 4.8–5.5, M. W. 500-kDa in the patterns of raccoon, chicken and newt was immunochemically stained by anti-human α2M antibody (Fig. 4). Since Starkey and Barrett reported that the same M. W. of α2M, which retains papain-binding activity, exists in amphibians, reptiles and birds in much the same form that it assumes in mammals, α2M, which possesses the similar structure and function, might exist in all tetrapod plasma. Furthermore, since the existence of α2M is reported in invertebrates such as horseshoe crab and crayfish, 2-D PAGE without denaturing agents might be applied to a comparative study of α2M.
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REFERENCES


