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Detection and identification of constitutive polypeptides of the third component of complement using microscale two-dimensional electrophoresis : Correlated with the separation by non-denaturing microscale two-dimensional electrophoresis

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SUMMARY

We have reported that the third component of complement (C3) is observed in the pattern of microscale non-denaturing two-dimensional electrophoresis (2-DE) after removal of IgG and IgA using protein A agarose and antibodies.¹⁾ For the investigation of constitutive polypeptides of the C3, the constituent polypeptides of human plasma proteins were analyzed using non-denaturing isoelectric focusing (IEF) in the first dimension and treated with mercaptoethanol/SDS, and then subjected to the second dimensional SDS electrophoresis (non-denaturing IEF/reduced-SDS PAGE). The α chain of C3 spot was detected in the pattern of non-denaturing IEF/reduced-SDS PAGE, but, the β chain of C3 spot was hidden by albumin spot. After albumin was removed using polyethylene glycol, both C3 α and C3 β spots were observed in the pattern of the non-denaturing IEF/reduced-SDS PAGE. The combining analysis of C3 in the non-denaturing 2-DE and the constituent polypeptides in the non-denaturing IEF/reduced-SDS PAGE can be useful for the analysis of C3 and its fragments during the activation of complements.

Key words: polyethylene glycol, 2-DE, immunoglobulin, albumin, C3 α , C3 β .

INTRODUCTION

The third component of complement (C3) is a key protein of the complement system which is common to both the classical and alternative pathways of activation.²⁾ The C3 molecule consists of an α -chain and β -chain linked by disulfide bonds. During the activation of the C3, the C3 molecule can be converted to the fragments of anaphylatoxin C3a and C3b (C3c). Accumulating evidences indicate that C3 spots are demonstrated on electrophoretic techniques,^{3,4)} but an applicable method for the investigation of the process of the C3 activation is not developed. In order to separate and investigate C3 and the fragments of C3, it might be necessary to establish the typical method to separate these proteins in the physiological condition and analyze constitutive polypeptides of these proteins.

Non-denaturing microscale two-dimensional electro-

phoresis (2-DE) can be applied to the investigation of C3 changes after the activation, because using this method human plasma proteins can be separated, retaining enzymatic activity and protein-protein interactions.⁵⁻⁷⁾ We have reported that these major proteins such as IgG and IgA can be selectively removed from the pattern of the non-denaturing 2-DE using protein A agarose and antibodies, and C3 spot is successfully detected in the pattern of non-denaturing 2-DE.¹⁾

Constitutive polypeptides in a small amount of human plasma can be separated, employing non-denaturing isoelectric focusing (IEF) in the first dimension and treated with mercaptoethanol/SDS and then subjected to second dimension SDS electrophoresis (non-denaturing IEF/reduced-SDS PAGE).⁸⁾ By non-denaturing IEF/reduced-SDS PAGE, both C3 α and C3 β were also separated in a complex at the same IEF. However, C3 β spot was hidden in the spot of albumin which is extremely

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abundant in human plasma. It has been reported that a nonionic polymer such as polyethylene glycol (PEG) is used in order to remove albumin.⁹⁾ Since PEG can be added to sample as liquid, PEG is thought to be applicable to proteins separation in a small amount of sample. And, using PEG, C3 β spot was successfully detected and identified in the pattern of non-denaturing IEF/reduced-SDS PAGE.

Present study indicates that both C3 α and C3 β spots are detectable in the pattern of the non-denaturing IEF/reduced-SDS PAGE, after albumin is removed by polyethylene glycol. And, the combining analysis of C3 in the non-denaturing 2-DE and the constituent polypeptides in the non-denaturing IEF/reduced-SDS PAGE can be useful for the analysis of C3 and its fragments during the activation of complements.

MATERIALS AND METHODS

Reagents and sample preparations were obtained as follows: ampholine pH 3.5-10 and pH 3.5-5.0 from Amersham Pharmacia Biotechnology (Uppsala); acrylamide from Daiichi Pure Chemicals (Tokyo); Coomassie Brilliant Blue R-250 (CBB) and polyethylene glycol #4000 (PEG) from Nacalai Tesque (Kyoto); Ammonium persulfate, N, N, N', N'-tetramethylethylenediamine (TEMED), Sodium dodecylsulfate (SDS), 2-mercaptoethanol (2-ME) and glycerol from Wako (Osaka); Polyvinylidene difluoride (PVDF) from Millipore (Bedford); Purified human immunoglobulin G, human albumin, human α -macroglobulin, bovine carbonic anhydrase from Sigma (St. Louis). These purified proteins were used as the relative molecular weight standard of SDS PAGE. Normal human plasma was obtained from healthy individuals and kept at 4°C. Sucrose was added to human plasma to give a concentration of 40% (w/v).

One hundred μ l of human plasma was added to microtube (5 mm i.d.) containing micro stirring bar (5 mm long and 2 mm i.d.). A liquid of PEG was added to the human plasma. The PEG concentration in human plasma was raised to 4% by adding 16% (w/v) PEG slowly. After 30 min with constant stirring at 4°C, the pellet and supernatant were obtained by centrifugation ($10,000 \times g$, 20 min). The pellet was dissolved in 100 μ l of 5 mM Tris-HCl, pH 7.2 (4% PEG fraction). The PEG concentration in the supernatant was raised to 16% (w/v) by the adding of 22% PEG (w/v), slowly stirring at 4°C. After 30 min with stirring, the pellet and supernatant (16% PEG fraction) were obtained by centrifugation ($10,000 \times g$, 20 min). The pellet was dissolved in 100 μ l of 5 mM Tris-HCl, pH 7.2 (4-16%PEG fraction) stirred for 1 h at 4°C, centrifuged ($10,000 \times g$, 20 min) to obtain supernatant and pellet. Each fraction was dialyzed against 500 ml of 5 mM Tris-HCl buffer (pH 7.2) overnight. Dialyzing apparatus was used (Desalyzer

AE-6590, ATTO corporation, Tokyo, Japan). Sucrose was added to each dialyzed fraction to give a concentration of 40% (w/v). The protein concentration in each fraction was estimated from their ultraviolet light absorption assuming the absorbance at 280 nm of an 1 mg/ml solution to be 1.0. Proteins in 10 μ l of human plasma were also fractionated by the method of PEG.

Micro non-denaturing 2-DE of plasma proteins was performed by the method previously reported.⁷⁾ The first-dimensional isoelectric focusing (IEF) was performed on gel columns (35 mm long and 1.3 mm i.d.). A mixed solution of 4% acrylamide and 0.2% bis-acrylamide (Bis) containing 2% ampholine pH 3.5-10, 1% ampholine pH 3.5-5, 0.05% ammonium persulfate and 0.029% TEMED was used to prepare the column gels. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H₃PO₄ (anode). The proteins of 4%, 4-16% and 16% PEG fraction (each 100 μ g in protein) were subjected to the first-dimensional IEF. The second-dimensional polyacrylamide gradient gel electrophoresis was performed in 4-17 % acrylamide linear gradient (0.2-0.85% Bis gradient) slab gels (38 $\times$ 38 $\times$ 1 mm). The gradient gel buffer was 0.14 M Tris-HCl (pH 8.9) and the electrode buffer was 0.05 M Tris-0.38 M glycine (pH 8.3). Microslab gels were stained in 0.1% CBB, 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.

After the separation of total proteins in human plasma or 4%, 4-16% and 16% PEG fraction of human plasma proteins by the first-dimension IEF, IEF gels were taken out from the gel columns, and soaked in the solution of 4% SDS, 5% 2-ME and 40% sucrose. The soaked gels were boiled for 5 min, set on the top of the second-dimension slab gel and equilibrated for 20 min with 0.02 M Tris-HCl buffer (pH 6.8) which contained 2% SDS, 5% (v/v) 2-ME and 10% glycerol. The second dimension slab gel was run on a 4-17% acrylamide linear gradient (0.2-0.85% Bis gradient) containing 0.1% SDS. The electrode buffer was 0.05 M Tris, 0.38 M glycine, 0.1% SDS (pH 8.3). Microslab gels which contained 0.1% SDS were washed in 7% (v/v) acetic acid, 50% (v/v) methanol for 15 min, stained in 0.1% CBB, 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.

Electrophoretic transfer of proteins from non-denaturing IEF/reduced-SDS PAGE gels to PVDF was performed by an apparatus which we devised for the simultaneous transfer of proteins from 4 microslab gels to 4 PVDF sheets.¹⁰⁾ Electrophoretic transfer was performed at 100 mA constant current in 0.05 M boric acid-NaOH (pH 9.0) and 20% methanol for 2 h. After blotting, each PVDF sheet was soaked in 0.1% CBB, 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. Amino acid sequence of the spots was analyzed in a gas-phase protein sequencer (LF-3400 D with high sensitivity chemistry, Beckman Coulter, Inc., Fullerton, CA, USA).

RESULTS AND DISCUSSION

Figure 1 shows a non-denaturing 2-DE (a) and the non-denaturing IEF/reduced-SDS PAGE (b) pattern of human plasma proteins. We can easily compare both patterns since the relative location of proteins in the IEF axis is the same. In the pattern of the non-denaturing IEF/reduced-SDS PAGE as shown in Fig. 1b, IgG and IgA dissociated into constituent polypeptides, heavy chain (50 kDa) and light chain (25 kDa). C3 molecule dissociates into α chain and β chain in the presence of denaturing and reducing agents. But, the C3 β spot was not detected in the pattern of the non-denaturing IEF/reduced-SDS PAGE because of the disturbance with albumin which is located at 67 kDa as shown in Fig. 1b. In order to detect C3 β spot, albumin must be removed.

Plasma proteins in 4% PEG (a), 4-16% PEG (b) and 16% PEG (c) fraction were separated by the non-denaturing 2-DE as shown in Fig. 2. Using this non-denaturing 2-DE, human plasma proteins in each PEG fraction can be identified, because Manabe et al. reported that 84 spots of human plasma proteins are separated and identified in the pattern of non-denaturing 2-DE.¹¹⁾ Several protein spots at pI 6.0-7.0, less than 170 kDa were observed in the pattern of 4% PEG frac-

tion (Fig. 2a). These proteins are thought to be degradation products during the process of PEG fractionation preparation. The spots of IgG, IgM and low density lipoprotein (LDL) were observed in the pattern of 4% (a) and 4-16% (b) PEG fraction as shown in Fig. 2a and 2b, and these protein spots were not observed in the pattern of the 16% PEG fraction as shown in Fig. 2c. The spots of IgA was specifically observed in the pattern of 4-16% PEG fraction (b). Fibrinogen (Fg) spot was specifically observed in the pattern of the 4% PEG fraction (a). A large amount of albumin was observed at the position of pI 4.5-5.8/69 kDa in the pattern of 16% PEG fraction (Fig. 2c), whereas the amount of albumin was clearly decreased in the pattern of 4% (a) and 4-16% PEG fraction (b). These results indicate that 4-16% PEG fraction contains immunoglobulins, and 16% PEG fraction mainly contains albumin and high density lipoprotein (HDL). The other major proteins such as α_2 -macroglobulin (α_2 -M), and transferrin (Tf) spots were detected in the pattern of 4%, 4-16% and 16% PEG fractions (Fig. 2a-c).

Figure 3 shows that the pattern of the non-denaturing IEF/reduced-SDS PAGE of human plasma proteins in 4% (a), 4-16% (b) and 16% (c) PEG fraction. Immunoglobulin gamma heavy chain and immunoglobulin light chain were clearly observed in the pattern of 4-16% PEG (b) fraction. A protein spot at 75 kDa was detected in the pattern of the 4-16% PEG fraction. The polypeptide spot was not observed in the pattern of human plasma proteins without PEG fractionation prep-

Fig. 1. Non-denaturing 2-DE (a) and non-denaturing IEF/reduced-SDS PAGE (b) pattern of human plasma proteins.

A sample of human plasma was subjected to the isoelectric focusing (IEF) in the absence of denaturing agents, the IEF gel was set on the micro slab gel of polyacrylamide pore gradient, and the second dimensional gel electrophoresis was run in the absence of denaturing agents (a). After the IEF in the absence of denaturing agents, the IEF gel was soaked in the solution containing 4% SDS, 5% 2-ME and 40% sucrose. After the soaked gels were boiled for 5 min, second-dimensional electrophoresis was run in the presence of denaturing agents (b). Abbreviations: α_2 M, α_2 -macroglobulin; IgG, immunoglobulin G; IgA, immunoglobulin A; l, light chain of IgG; h, heavy chain of IgG; Alb, albumin.

Fig. 2. Non-denaturing 2-DE pattern of human plasma in the 4% PEG (a), 4-16% PEG (b) and 16% PEG (c) fractions.

Human plasma proteins in the 4% PEG (a), 4-16% PEG (b) and 16% PEG (c) fractions were subjected to the IEF in the absence of denaturing agents, the IEF gel was set on the micro slab gel of polyacrylamide pore gradient, and the second dimension gel electrophoresis was run in the absence of denaturing agents. Abbreviations: α_2 M, α_2 -macroglobulin; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; Alb, albumin; LDL, low density lipoprotein; HDL, high density lipoprotein; Fg, fibrinogen; Tf, transferrin.

Fig. 3. Non-denaturing IEF/reduced-SDS PAGE pattern of human plasma in the 4% PEG (a), 4-16% PEG (b) and 16% PEG (c) fractions.

Human plasma proteins in the 4% PEG (a), 4-16% PEG (b) and 16% PEG (c) fractions were subjected to the IEF in the absence of denaturing agents, the IEF gel was soaked in the solution containing 4% SDS, 5% 2-ME and 40% sucrose. After the soaked gels were boiled for 5 min, second-dimension electrophoresis was run in the presence of denaturing agents. Two polypeptides spots at 115 kDa (spot b) and 75 kDa (spot a) were observed in the pattern of 4-16% PEG fraction (b). Abbreviations: l, light chain of IgG; h, heavy chain of IgG.

aration (Fig. 1b). This spot is thought to be hidden by albumin spot in Fig. 1b. These results indicate that the 75 kDa spot as shown in Fig. 3b was detected after removing the spots of albumin. The N-terminal amino acid sequence of the 75 kDa polypeptide spot (spot a in Fig. 3b) and 115 kDa polypeptide spot (spot b in Fig. 3b) were SPMYSITPNIL and SVQLTEKRM, respectively. These polypeptides of 75 kDa and 115 kDa were identified as human C3 β and C3 α , respectively.

We have already reported that C3 spot is detected in

the pattern of non-denaturing 2-DE, after removal of IgG and IgA using protein A and antibodies.¹⁾ Present study indicates that both C3 α and C3 β spots were obtained in the pattern of the non-denaturing IEF/reduced-SDS PAGE, after albumin was removed by PEG fractionation. The combining analysis of C3 in the non-denaturing 2-DE and the constituent polypeptides in the non-denaturing IEF/reduced-SDS PAGE can be useful for the analysis of C3 and its fragments during the activation of complements.

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REFERENCES

- 1) Shimazaki Y, Ohara S, Manabe T. Removal of specific protein spots on the patterns of non-denaturing two-dimensional electrophoresis using protein A agarose and antibodies. *J Biochem Biophys Methods* 1998; 37: 1-4.
- 2) Dodds AW, Law SKA. The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and α_2 -macroglobulin. *Immunol Rev* 1998; 166: 15-26.
- 3) Allemann E, Gravel P, Leroux JC, Balant L, Gurny R. Kinetics of blood component adsorption on poly (D, L-lactic acid) nanoparticles: Evidence of complement C3 component involvement. *J Biomed Mater Res* 1997; 37: 229-34.
- 4) Acevedo F. Simple quantification of complement factors C3 and C3b using separation by isotachopheresis. *Electrophoresis* 1999; 20: 469-72.
- 5) Emes AV, Latner AL, Martin JA. Electrofocusing followed by gradient electrophoresis: A two-dimensional polyacrylamide gel technique for the separation of proteins and its application to the immunoglobulin. *Clin Chim Acta* 1975; 64: 69-78.
- 6) Manabe T, Tachi K, Kojima K, Okuyama T. Two-dimensional electrophoresis of plasma proteins without denaturing agents. *J Biochem* 1979; 85: 649-59.
- 7) Manabe T, Hayama E, Okuyama T. Microscale multisample two-dimensional electrophoresis of proteins in human serum, cerebrospinal fluid and urine. *Clin Chem* 1982; 28: 824-7.
- 8) Manabe T, Mizuma H, Watanabe K. A nondenaturing protein map of human plasma proteins correlated with a denaturing polypeptide map combining techniques of micro two-dimensional gel electrophoresis. *Electrophoresis* 1999; 20: 830-5.
- 9) Fried M, Chun PW. Water-soluble nonionic polymers in protein purification. *Methods Enzymol* 1971; 22: 238-49.
- 10) Manabe T, Takahashi Y, Okuyama T. An electroblotting apparatus for multiple replica technique and identification of human serum proteins on micro two-dimensional gels. *Anal Biochem* 1984; 143: 39-45.
- 11) Manabe T, Kojima K, Jitzukawa S, Hoshino T, Okuyama T. Normalization of two-dimensional electrophoretic patterns of human plasma proteins and cerebrospinal fluid and urine patterns in terms of the normalized map. *J Biochem* 1981; 89: 841-53.