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Short communication

Direct trapping and analysis of hemoglobin in flowing fluid using membrane-immobilized anti-haptoglobin antibody

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ABSTRACT

Haptoglobin is known to bind to hemoglobin during intravascular hemolysis. Membrane-immobilized anti-haptoglobin antibody, which was produced after antibody was isolated by non-denaturing two-dimensional electrophoresis, was transferred to a polyvinylidene difluoride membrane and was stained using Ponceau S. The proteins bound to the membrane-immobilized anti-haptoglobin antibody were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. Hemoglobin was specifically obtained when the membrane-immobilized anti-haptoglobin antibody was incubated with human serum obtained from hemolysis blood. Furthermore, hemoglobin in the flowing fluid was captured by the membrane-immobilized anti-haptoglobin antibody and analyzed directly. The results indicate that hemolysis can be examined by direct trapping and analysis of hemoglobin under physiological conditions.

1. Introduction

Hemoglobin is released into human plasma by erythrocytes during intravascular hemolysis, and this can cause human diseases such as hemoglobinuria and hemolytic anemia [1,2]. Hemoglobin released during intravascular hemolysis is known to bind to haptoglobin, to form stable hemoglobin–haptoglobin complexes in the serum [3,4]. We have previously reported that haptoglobin can be trapped by a membrane-immobilized anti-haptoglobin antibody after the antibody has been isolated by non-denaturing two-dimensional electrophoresis (2-DE), transferred to a polyvinylidene difluoride (PVDF) membrane, and then stained using a dye [5]. Because stable hemoglobin–haptoglobin complexes are formed in the serum obtained from hemolysis blood, not only haptoglobin but also hemoglobin is believed to be trapped by these immunoaffinity membranes. Furthermore, our previous study showed that a specific antigen is trapped by a membrane present on the inner wall of tubes because fluids, such as blood, flow without restrictions in the presence of the membrane [5]. When size of the immunoaffinity membrane is reduced, it can be inserted into a microspace such as silicon tube. Thus, flowing hemoglobin–haptoglobin complexes are believed to be trapped by the immunoaffinity membrane within a microspace. Further, this method can be applied for investigation of physiological changes of intact proteins such as protein–protein interactions.

2. Experimental

2.1. Chemicals and reagents

Polyvinylidene difluoride (PVDF) membrane and C_{18} Zip-Tip were purchased from Millipore (Bedford, MA, USA). Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10), and polyclonal rabbit anti-human haptoglobin antibody were purchased from Kishida Chemicals (Osaka, Japan), GE healthcare (Uppsala, Sweden) and Dako (Glostrup, Denmark), respectively. Bovine serum albumin, angiotensin II, adrenocorticotropic hormone (ACTH), Ponceau S, α-cyano-4-hydroxycinnamic acid and 3,5-dimethoxy-4hydroxy cinnamic acid (sinapinic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine trypsin (sequence grade) was purchased from Roche (Mannheim, Germany). All other reagents were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Osaka, Japan).

Abbreviations: 2-DE, two-dimensional electrophoresis; PVDF, polyvinylidene difluoride; IEF, isoelectric focusing; TEMED, N,N,N′N′-tetramethylenediamine; TFA, trifluoroacetic acid; CBB, Coomassie Brilliant Blue; MALDI TOF MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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2.2. Separation of antibodies by non-denaturing 2-DE and elecroblotting

Sucrose was added to the purchased antibody to a concentration of 40%. 5–10 μl of antibodies (10–20 μg) were subjected to non-denaturing 2-DE using a previously reported method [6,7]. After the proteins were separated using non-denaturing 2-DE, they were transferred to a PVDF membrane, using a semi-dry-type transblotting apparatus, to immobilize them [5,7]. To detect the proteins, the membrane was soaked in 0.5% Ponceau S in 10 mL of (0.1 M)-acetate buffer (pH 5.1) for 1 min, and destained with H_2O.

2.3. Hemoglobin capture on the membrane and its analysis by SDS-PAGE and MALDI-TOF MS

After the antibody was separated, blotted onto membranes and stained with the dye. These spots (circle with a diameter of 4 mm) were then transferred to a microcentrifuge tube. 20 μL of human plasma or serum obtained from hemolysis blood was added, and the tube was incubated for 1 h. To capture hemoglobin from flowing fluid directly, the spot on the membrane was placed in the tube of the flow system using a previously reported method [5]. For the purpose of control, the procedure was also performed utilizing a membrane of the same size that was soaked in 10 μL of 1.0 mg/mL human albumin which had not been immunoreacted to specific antigens. The spots were then transferred to a microcentrifuge tube, and washed three times with 100 μL of (50 mM)-Tris–HCl (pH 7.0) for 10 min. To extract the bound proteins, the spot was rinsed with 10 μL of 0.1% Trifluoroacetic acid (TFA) containing 0.2% sucrose without shaking. The extraction was applied to SDS-PAGE (12.5% acrylamide). After electrophoresis, the gel was stained in 0.1% CBB solution and then destained. For analysis of binding protein by MALDI-TOF MS, the following preparation was used as a previously reported method [5,7]: 1 μL of 0.1% TFA was added to the extraction, and then a 1 μL portion was spotted onto a stainless steel target plate and dried. In addition, 1 μL of a matrix solution containing 20 mg/mL of 3,5-dimethoxy-4hydroxy cinnamic acid (sinapinic acid) and 0.1% TFA in 60% acetonitrile was spotted at the same position of the sample on the plate. The target plate was then dried. MALDI-TOF MS analysis was performed using a Voyager DE PRO (Applied Biosystems, Framingham, MA, USA). The instrument was operated in positive ion linear mode at 25 kV accelerating voltage, with the nitrogen laser at 337 nm and 3 Hz. Internal or external calibration was performed using the average mass of human serum albumin (m/z 66,446).

2.4. Peptide mass fingerprinting for protein identification

The peptide mass fingerprinting (PMF) essentially followed a previously published protocol [8–10]. The protein spots were excised from the gel and digested in the gel by trypsin [7]. The digested polypeptides were recovered and desalted by C_{18} Zip-Tip, and were mixed with α-cyano-4-hydroxycinnamic acid. Mass analysis was done using MALDI-TOF MS, operating in a positive-ion reflector mode. The spectra were analyzed using the mass values for monoisotopic peaks that were used for searches (Mascot, http://www.matrixscience.com/) against the Swiss-Prot database. Monoisotopic peaks of angiotensin II (m/z 1046.5423) and ACTH 18-39 (m/z 2465.1989) were used for internal calibration. The database was searched using the following terms: taxonomy (*Homo sapiens*), trypsin digest (one missed cleavage allowed), cysteine modified by carbamidomethylation, and mass tolerance of 50 ppm, using internal calibration and oxidation of methionines. The criteria used to accept identification included the extent of sequence coverage, the number of peptides matched, and the probabilistic score (the required probability for a random match was <0.05).

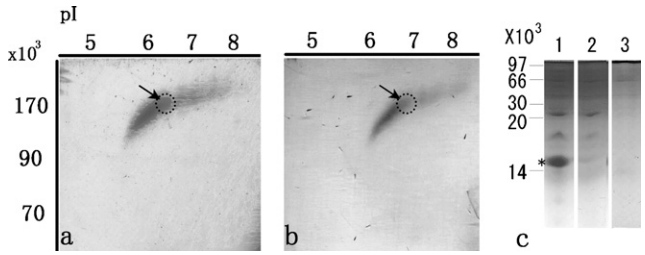

Fig. 1. CBB staining (a) after the polyclonal rabbit anti-haptoglobin antibody was isolated by non-denaturing 2-DE, and Ponceau S staining (b) after a polyclonal rabbit anti-haptoglobin antibody was isolated by non-denaturing 2-DE and electroblotted on a membrane. The spot (arrow in a and b) was identified as IgG by PMF using MALDI-TOF MS after separation by 2-DE. SDS-PAGE (c) of the proteins bound to the immunoaffinity membranes, after a membrane-immobilized anti-haptoglobin antibody (Lane 1) or the membrane-immobilized human albumin (Lane 2) was incubated with human serum obtained from hemolysis blood and after membrane-immobilized anti-haptoglobin antibody was incubated with human plasma obtained from nonhemolysis blood (Lane 3). The marked spot (* Lane 1 in c) was identified as hemoglobin using PMF.

3. Results and discussion

3.1. Production of membrane-immobilized anti-haptoglobin antibody

CBB staining (a) after the polyclonal rabbit anti-haptoglobin antibody was isolated by non-denaturing 2-DE, and Ponceau S staining (b) are shown in Fig. 1 after polyclonal rabbit anti-haptoglobin antibody was separated by non-denaturing 2-DE and electroblotted on a membrane. The spot (arrow in Fig. 1a and b) was identified as IgG by PMF using MALDI-TOF MS after separation by 2-DE. The separated IgG exists within the non-denaturing gel (Fig. 1a), whereas it exists on the membrane surface after elecroblotting to the membrane (Fig. 1b). Because the antigen–antibody interaction is performed on the membrane, macromolecule complexes such as haptoglobin–hemoglobin complexes can be trapped by the membrane-immobilized antibody in the present study.

3.2. Trapping and analysis of hemoglobin

As shown in Fig. 1c, a15-kDa protein band on SDS-PAGE was obtained when the membrane-immobilized anti-haptoglobin antibody was incubated in human serum obtained from hemolysis blood, whereas the band was not obtained when it was incubated in human plasma obtained from nonhemolysis blood or when membrane-immobilized human albumin was incubated in human serum obtained from hemolysis blood. The band was identified as the hemoglobin subunit α by PMF using MALDI-TOF MS. Fig. 2 shows the MALDI-TOF MS spectra of the extraction using 0.1% TFA, after membrane-immobilized anti-haptoglobin antibody or membrane-immobilized human albumin was incubated with human serum obtained from hemolysis blood or human plasma obtained from nonhemolysis blood. Peaks at m/z 15,095 and 15,840 – corresponding to the α and β subunit of human hemoglobin, respectively – were obtained when the membrane-immobilized anti-haptoglobin antibody was incubated with human serum obtained from hemolysis blood (Fig. 2a). These peaks were faintly detected when the membrane-immobilized anti-haptoglobin antibody was incubated with human serum obtained from hemolysis blood (Fig. 2b), and were not visible when this antibody was incubated with human plasma (Fig. 2c). These results

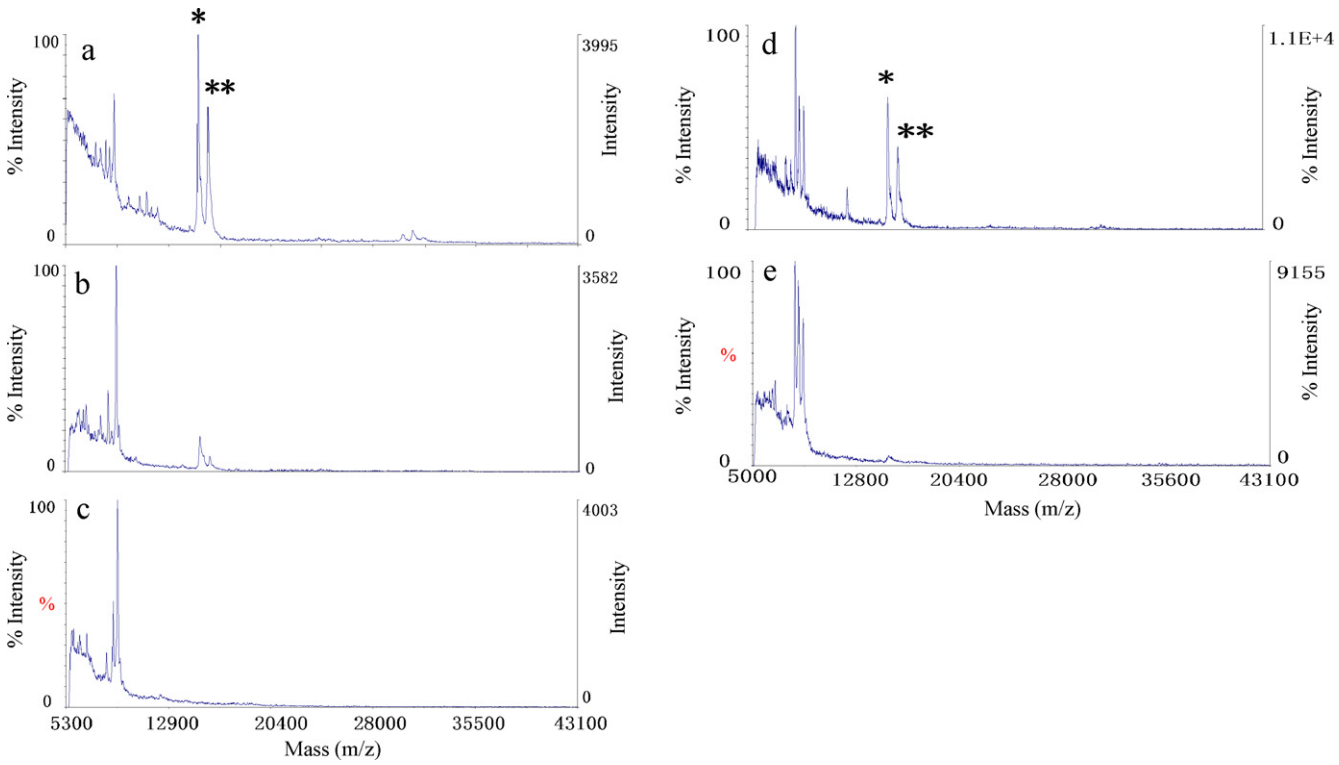

Fig. 2. MALDI-TOF MS spectra of extraction by a 0.1% TFA solution after the membrane-immobilized anti-haptoglobin antibody (a) and the membrane-immobilized human albumin (b) was incubated with human serum obtained from hemolysis blood, as well as after membrane-immobilized anti-haptoglobin antibody was incubated with human plasma obtained from nonhemolysis blood (c). Further, MALDI-TOF MS spectra of the protein bound to the membrane immobilized anti-haptoglobin antibody (d) and the membrane-immobilized human albumin (e) when human serum obtained from hemolysis blood was applied to a flow system equipped with immunoaffinity membranes. The peaks at *m/z* 15,095 (*) and 15,840 (**) were bound to the membrane-immobilized anti-haptoglobin antibody.

indicate that because haptoglobin–hemoglobin complexes are believed to bind to the membrane-immobilized anti-haptoglobin antibody, hemoglobin is obtained on SDS-PAGE and MALDI-TOF MS, when the membrane-immobilized anti-haptoglobin antibody is incubated with human serum obtained from hemolysis blood. Further, the MALDI-TOF MS spectra of the protein bound to the membrane-immobilized anti-haptoglobin antibody (d) and the membrane-immobilized human albumin (e) are shown in Fig. 2 when serum from hemolysis blood was applied to a flow system equipped with the immunoaffinity membrane. The spectrum indicates that hemoglobin was specifically captured by the membrane-immobilized anti- haptoglobin antibody (asterisks in Fig. 2d), and not by membrane-immobilized human albumin. As shown in Fig. 2, peaks of the α and β subunit of human hemoglobin were directly obtained by MALDI-TOF MS after extraction of the binding proteins to the membrane-immobilized anti-haptoglobin antibody. It has been reported that posttranslational modification of hemoglobin are examined by MALDI-TOF MS [11]. Thus, changes of hemoglobin subunits related to hemolysis can be investigated by MALDI-TOF MS after extraction of the binding hemoglobin to the membrane-immobilized antibody.

These results suggested that haptoglobin–hemoglobin complexes in fluids are believed to be captured by the immunoaffinity membrane in the flowing system. Macromolecules such as xenoreactive antibodies are selectively removed and analyzed using immunoaffinity adsorption on hollow-fiber microporous or dialysis membranes [12,13]. Therefore, the method in the present study can be applied to trap and analyze other flowing macromolecules or macromolecule complexes within a microspace such as capillary vessel.

4. Conclusion

According to the results of the present work, protein complexes in the flowing fluid are captured by membrane-immobilized antibody and analyzed directly. Further, this method can be applied for investigation of physiological changes of intact proteins.

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