Single-step purification of $\text{F(}ab')_2$ fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl-5PW

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Summary

Hydrophobic interaction high performance liquid chromatography (HPLC) using TSKgel Phenyl-5PW was applicable to single-step purification of $\text{F(}ab')_2$ fragments from pepsin digests of mouse monoclonal antibodies of IgG1 class. The digests were applied to the gel equilibrated with phosphate-buffered saline containing 1 M ammonium sulfate. $\text{F(}ab')_2$ fragments were adsorbed onto the gel using the same buffer, and eluted by reducing the ammonium sulfate concentration to 0 M. The fraction containing $\text{F(}ab')_2$ fragments was homogeneous (purity: higher than 98%) by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration HPLC. The recovery of the antigen binding site was 42–58%. The cycle time of the Phenyl-5PW HPLC was 45 min, and $\text{F(}ab')_2$ of up to 2200 mg was purified in a cycle. This method could be useful especially for large scale purification of $\text{F(}ab')_2$ fragments.

Key words: $\text{F(}ab')_2$ fragment; Hydrophobic interaction HPLC; Pepsin digestion; Immunoglobulin G1; Monoclonal antibody; Single-step purification

Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BSA, bovine serum albumin; CEA, carcinoembrionic antigen; DEAE, diethylaminoethyl; IgG, immunoglobulin G; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MW, molecular weight; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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Introduction

Mouse (Fab')₂ fragments of monoclonal antibodies (mAbs) are currently of great interests for both diagnostic and therapeutic agents [1,2]. They are more useful than the original mAbs, because they do not retain any biological functions due to Fc regions and interaction with non-specific proteins is reduced. Having a smaller molecular mass than the mAbs is another advantage. Some papers have reported the preparation of F(ab')₂ fragments by pepsin digestion of mouse IgG1 mAbs [3-5]. Generally, digestion proceeded for 12–48 h at the weight ratio, IgG1/pepsin = 40–100, and F(ab')₂ fragments were purified by size exclusion chromatography [4], ion-exchange chromatography [6,7] or protein A-Sepharose chromatography [7]. However, these methods are time-consuming, and cannot afford sufficient purity and recovery of F(ab')₂ fragments. Development of efficient procedures for F(ab')₂ preparation is an urgent necessity.

In this paper, we describe the single-step purification of F(ab')₂ fragments from pepsin digests of mAbs (IgG1 isotype) by hydrophobic interaction HPLC [8] using TSKgel Phenyl-SPW. We show that this method is suitable for large scale purification of F(ab')₂ fragments.

Materials and Methods

Monoclonal antibodies (mAbs)

Five mouse mAbs of the IgG1 class were used: CU203.2, EM89.6, FS42.7, FE138 and GC4. Their specific antigens are, respectively, human CEA, human myoglobin, human follicle stimulating hormone, human IgE and human growth hormone. The hybridomas secreting these mAbs were established in our laboratory, by fusing spleen cells from an antigen-immunized Balb/c mouse with NS-1 myeloma cells according to Köhler and Milstein [9]. The hybridoma cells were injected into pristane-primed Balb/c mice, and were grown in ascites [10]. mAbs were purified from the ascites at 4°C. The collected ascites were centrifuged at 3000 × g for 20 min to remove cells, and the supernatants were passed through Millipore filters (pore size: 0.8 μm; AA type). Ammonium sulfate (solid) was added to the filtrate to give 50% saturation, followed by centrifugation at 10000 × g after standing for 2 h. The precipitates were dissolved in PBS, pH 7.4. Ammonium sulfate (solid) was added to give 50% saturation, and the precipitates were collected by centrifugation as above, and dissolved in 100 mM citrate buffer (pH 3.5) (buffer A) to give a protein concentration of 10–20 mg/ml. Undissolved materials were removed by centrifugation at 10000 × g for 20 min. This centrifugation step was critical in reducing the period of pepsin digestion (see Discussion). The supernatant was adjusted to pH 3.7 with 1 M HCl or NaOH, giving partially purified mAb.

Pepsin digestion

The partially purified mAb was digested by porcine pepsin (EC 3.4.23.1) (Lot No. 117F-8080; 3900 U/mg according to the supplier) purchased from Sigma
Chemical Co. (St. Louis, MO). The starting concentration of mAb was 10–20 mg/ml in buffer A. Pepsin was added to the mAb solution at a weight ratio of 1:100 (pepsin: IgG1). Digestion proceeded with gentle stirring at 37°C for 2 h, and was stopped by adding 10 vols. of 3 M Tris to give a pH around 7.

**Analytical studies**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12 or 16% slab gel under reducing conditions and a 6% slab gel under non-reducing conditions according to the method of Laemmli [11]. Proteins were reduced by treatment with 2.5% 2-mercaptoethanol at 100°C for 10 min. Proteins were stained with Coomassie brilliant blue R-250. The molecular mass marker kit containing rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), chicken ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk α-lactoalbumin (14.2 kDa) is a product of Pharmacia (Uppsala, Sweden).

Concentrations of F(ab')2 and mAb were estimated using an extinction coefficient at 280 nm, \( A \ (1 \text{ mg/ml}) = 1.4 \) [12].

Antigen binding activities of F(ab')2 and mAb were measured by a solid-phase enzyme immunoassay [13]. A microtiter plate (96 wells; Nunc-Intermed, Maxi Sorp; Roskilde, Denmark) was coated with the antigen by adding 100 μl antigen solution at various concentrations (2.0, 1.0, 0.5, 0.125, and 0 μg/ml) in 50 mM carbonate buffer (pH 9.5) to each well, and incubated for 1 h at 37°C. Plates were blocked by incubation with 0.2% BSA in PBS overnight at 4°C. The plate was washed with PBS, and incubated with F(ab')2 or mAb for 1 h at 37°C. After washing once more with PBS, excess goat anti-mouse IgG (F(ab')2 specific) antibody conjugated with horseradish peroxidase (Tago, Inc., Burlingame, CA) was added to the plate and incubated for 1 h at 37°C. The plate was washed with PBS, then the enzyme reaction was started by adding 100 μl substrate (0.03% ABTS and 0.03% H₂O₂ in 100 mM citrate buffer, pH 4.1) to each well, and then terminated by 100 μl oxalic acid (0.1 M) after reaction for 5 min at 25°C. The absorbance at 415 nm was measured by a microtiter plate reader MPR-A4 (Tosoh, Tokyo, Japan).

**High performance liquid chromatography (HPLC)**

The HPLC apparatus composed of a solvent-delivery system CCPM, UV monitoring system UV-8010, a fraction collector FC-8000 and a computer control system SC-8010 was purchased from Tosoh. Throughout this study, the elution was monitored by absorbance at 280 nm, and fractions (1 ml) were collected.

DEAE-HPLC was performed on a TSKgel DEAE-5PW column (7.5 mm (inner diameter) × 75 mm) (Tosoh), equilibrated with starting buffer, 20 mM Tris-HCl buffer (pH 8.0) containing 40 mM NaCl. The pepsin digests were dialyzed against the starting buffer, and applied to the column. A linear gradient of NaCl from 40 to 500 mM in the same buffer was generated in 30 min at a flow-rate of 1 ml/min.

Hydrophobic interaction HPLC was performed on a TSKgel Phenyl-5PW column (7.5 mm (inner diameter) × 75 mm) (Tosoh). Pepsin digests of mAbs were
salted out with 60% saturated ammonium sulfate, and the precipitates were immediately dissolved in PBS containing 1 M ammonium sulfate (pH 7.4). The solution was applied to the column equilibrated with the same buffer, and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in PBS (pH 7.4), for 30 min at a flow-rate of 1 ml/min at room temperature.

Gel filtration HPLC was performed using a TSKgel G3000SWxL (7.8 mm (inner diameter) × 30 cm) (Tosoh) with 50 mM phosphate buffer containing 150 mM sodium sulfate (pH 6.5) at a flow-rate of 1 ml/min.

Results

Pepsin digestion

Time dependence of pepsin digestion of IgG1 (CU203.2; mouse anti-CEA mAb) was monitored by SDS-PAGE (Fig. 1). After the incubation time indicated in Fig. 1, the reaction was stopped. Half of the reaction mixture was applied to SDS-PAGE (6% gel) under non-reducing conditions (Fig. 1A), and the other half to SDS-PAGE (12% gel) under reducing conditions after incubation with 2-mercaptoethanol (Fig. 1B). The ascites, from which the mAb was partially purified by ammonium sulfate, were also applied to SDS-PAGE under both conditions. As shown in Fig. 1A, a 160-kDa band corresponding to IgG disappeared completely after digestion for 120 min. On the other hand, a 110-kDa band which is considered to be F(ab')2 appeared during the reaction. Under reducing conditions (Fig. 1B), IgG shows two bands before the reaction, of 50 kDa and 28 kDa, which correspond to heavy (H) and light (L) chains, respectively. The H chain disappeared in the reaction, and a new band of 30 kDa appeared. The L chain appeared not to be degraded in the reaction. After a 2-h digestion, the H chain band disappeared completely, and only 30-kDa and 28-kDa bands remained. The new 30-kDa band must be derived from cleavage of the H chain.

Similar time courses of pepsin digestion were obtained for four other mAbs (IgG1) examined (data not shown). Throughout this study, mAb solutions were prepared from mouse ascitic fluids by centrifugation at 10,000 × g for 20 min after precipitation by 50% saturated ammonium sulfate and dissolution of the precipitates with buffer A. The mAb solutions prepared without centrifugation required up to 12 h for completion of the pepsin digestion. This centrifugation is critically significant in reducing the digestion time.

Purification of F(ab')2 fragments by DEAE-HPLC

Products of the pepsin digestion for 2 h were separated by DEAE-HPLC using a TSKgel DEAE-5PW column, and fractions (1 ml each) were collected every 1 min. An elution pattern for pepsin digests of mAb CU203.2 is shown in Fig. 2. F(ab')2 fragments were eluted from DEAE-HPLC in the void volume at 4 min. Although they were separated clearly from intact mAbs (elution time: 26 min), pepsin and other peptides were also eluted in the void volume [5,12]. In fact, the fractions eluted in the void volume were heterogeneous in protein composition as
verified by SDS-PAGE (data not shown). This suggests that DEAE-HPLC is not sufficient for purification of F(ab')₂ fragments and that further purification steps are required.

**Hydrophobic interaction HPLC**

The pepsin digests were separated by hydrophobic interaction HPLC using a TSKgel Phenyl-5PW column. Chromatograms for the pepsin digests of mAb
CU203.2 obtained by the digestion for 0, 15, and 120 min are shown in Fig. 3. A linear gradient of ammonium sulfate from 1.0 to 0 M was generated over 30 min, and the elution was continued using ammonium sulfate free PBS. The mAb was eluted at 34 min, and as pepsin digestion progressed, the mAb peak decreased. The peak disappeared completely after digestion for 120 min, and inversely, a new peak corresponding to F(ab')_2 fragments appeared at 30 min. Fractions from 28 to 31 min were collected for further analyses.

**Purity of F(ab')_2 fragments**

The fractions collected from 28 to 31 min in the hydrophobic interaction HPLC (Fig. 3) were applied to gel filtration HPLC on a TSKgel G3000SW_{XL} column (Fig. 4A). The proteins were eluted as a single peak, showing that the purity of the F(ab')_2 fragments was more than 98%. SDS-PAGE (Fig. 4B) of the fraction showed only two bands corresponding to H and L chains (lane 2), and contaminating proteins observed before the HPLC (lane 1) were removed.

The purification processes are summarized in Table 1. In the case of mAb CU203.2, the total protein in 10 ml of ascites was 208 mg and after ammonium sulfate precipitation, it was 24 mg, estimated from the mAb peak at the elution time of 8.6 min in the gel filtration HPLC (Fig. 4A). When the total mAb was theoretically entirely converted to F(ab')_2 by pepsin digestion, the ratio of F(ab')_2 should be two thirds of the mAb quantity, namely 16 mg. The quantity of the latter obtained from the hydrophobic interaction HPLC was 9 mg, thus, the yield of F(ab')_2 was 56%. The material balances for other mAbs are listed in Table 1. The mAb concentrations in the ascites were all different. However, the purity of F(ab')_2 was greater than 98%, and the yield was in a narrow range, 42–58%.
Fig. 3. Purification of F(ab')\textsubscript{2} fragments from pepsin digests of mAb CU203.2 by hydrophobic interaction HPLC on a TSKgel Phenyl-5PW column. Conditions of the pepsin digestion were as described in Fig. 1. Incubation time with pepsin: a, 0 min; b, 15 min; c, 120 min.

TABLE 1

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Volume of ascites (ml)</th>
<th>Protein in ascites (mg)</th>
<th>IgG for pepsin digestion ( ^{\text{a}} ) (mg)</th>
<th>F(ab')\textsubscript{2} purified ( ^{\text{b}} ) (mg)</th>
<th>Purity of F(ab')\textsubscript{2} ( ^{\text{b}} ) (%)</th>
<th>Recovery yield of F(ab')\textsubscript{2} ( ^{\text{c}} )</th>
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<td>208</td>
<td>24</td>
<td>9</td>
<td>98</td>
<td>0.56</td>
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<td>56</td>
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<td>3000</td>
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<td>99</td>
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<tr>
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<td>1330</td>
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</tr>
<tr>
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<td>13020</td>
<td>5700</td>
<td>2200</td>
<td>98</td>
<td>0.58</td>
</tr>
</tbody>
</table>

\( ^{\text{a}} \) Quantity of IgG1 obtained after precipitation of ascitic fluids with 50% saturated ammonium sulfate. The quantity was estimated by absorbance at 280 nm after gel filtration HPLC using TSKgel G30000SW\textsubscript{XL}.

\( ^{\text{b}} \) Quantity of F(ab')\textsubscript{2} fragments purified by hydrophobic interaction HPLC using TSKgel Phenyl-5PW. The quantity was estimated by absorbance at 280 nm.

\( ^{\text{c}} \) (Quantity of F(ab')\textsubscript{2} (mg))/(Quantity of IgG1 (mg)\times2/3).
Fig. 4. Characterization of F(ab')$_2$ fragments purified from pepsin-digests of mAb CU203.2 by hydrophobic interaction HPLC on a TSKgel Phenyl-SPW column. (A) Analytical gel filtration HPLC on a TSKgel G3000SWXL column. (B) SDS-PAGE under reducing conditions. Lane 1, pepsin digests of mAb CU203.2; lane 2, purified fractions of F(ab')$_2$ fragments by the Phenyl-5PW HPLC.

**Solid-phase enzyme immunoassay**

Fig. 5 shows immunoreactivities of mAb CU203.2 and its F(ab')$_2$ against the specific antigen, CEA. Each well of a microtiter plate was coated with 100 µl of CEA solutions at various concentrations; 2.0, 1.0, 0.5, 0.25, 0.125, and 0 µg/ml, in
Fig. 5. Immunoreactivities of mAb CU203.2 and its F(ab')₂ fragment against their specific antigen, CEA. Each well of a 96-well microtiter plate was coated with CEA by adding 100 μl CEA at the concentrations shown in the horizontal axis. 100 μl of 12 nM mAb (○) or F(ab')₂ (●) was added, followed by goat anti-mouse IgG (F(ab')₂ fragment specific) antibodies conjugated with peroxidase. Absorbance at 415 nm generated by the reaction with ABTS for 5 min was observed.

100 mM carbonate buffer (pH 9.5). After blocking with BSA, 100 μl of 12 nM mAb or F(ab')₂, namely 1.9 μg/ml mAb and 1.3 μg/ml F(ab')₂, respectively, was added. Their immunoreactivities against CEA were in good agreement, suggesting that the immunoreactivity of mAb CU203.2 is maintained entirely in the F(ab')₂ fragments.

Discussion

There have been some papers reporting the purification of F(ab')₂ fragments by gel filtration [4] or ion-exchange chromatography [6,7] after pepsin digestion of IgG. However, F(ab')₂ cannot be purified to homogeneity by single-step chromatography as reconfirmed here using DEAE-HPLC (Fig. 2). In the present paper, we described the application of hydrophobic interaction HPLC using TSKgel Phenyl-5PW to the single-step purification of F(ab')₂ fragments of mouse mAbs of the IgG1 isotype. Generally in this HPLC, proteins of interest are adsorbed on the resin by hydrophobic interaction at high salt concentration, and removed by decreasing the concentration. One of the features of this HPLC is that ammonium sulfate can be used as the salt for controlling the adsorption and removal of the proteins. Ammonium sulfate is widely used as a salting-out reagent in protein purification, because its effects on proteins are mild and the solubility is considerably high. In our experience, mAbs (IgG1) and F(ab')₂ fragments are stable in the presence of ammonium sulfate, although it has been reported that the immunoreactivity of a mAb was reduced significantly by precipitation with ammonium sulfate [14]. We showed here that F(ab')₂ fragments treated by 60% saturated ammonium sulfate, could be applied to hydrophobic interaction HPLC
after adjusting the ammonium sulfate concentration to that of the starting buffer, namely 1 M.

The advantage of the purification of F(ab′)2 fragments described here is not only that the procedure takes only a single-step, but also that no buffer exchange, such as dialysis, is required. These advantages can lead to a simple and rapid process with high recovery. In general, mouse ascites containing mAbs are precipitated with 50% saturated ammonium sulfate (1.9 M). The precipitates are collected and dissolved with the HPLC starting buffer, which contains ammonium sulfate (generally 1 M), and applied to hydrophobic interaction HPLC. After completely washing the column with the same buffer, the ammonium sulfate gradient is applied. By reducing the concentration of ammonium sulfate, F(ab′)2 fragments and mAbs elute separately from the gel at 0.3 M and 0.2 M, respectively. The fraction containing F(ab′)2 fragments is homogeneous (Fig. 4), and can be used directly for immunological reactions. If necessary, the remaining ammonium sulfate can be removed by gel filtration or dialysis.

The pepsin digestion time can be greatly reduced by centrifugation (10000 × g, 20 min) prior to digestion after ascitic fluids are precipitated with 50% saturated ammonium sulfate. It takes 12–48 h for the digestion in the methods previously reported [3,4], however, we showed here that 2 h are sufficient. The effect of the centrifugation is supposedly that lipids and contaminating proteins which prevent the pepsin action are removed.

It is noteworthy that all IgG1 mAbs elute at almost the same retention times (32–35 min) as well all F(ab′)2 fragments (27–30 min) under the conditions shown in Fig. 3. This suggests that the IgG1 and F(ab′)2 are almost identical with regard to hydrophobicity. On the other hand, the elution times of IgG1 and F(ab′)2 in ion exchange chromatography are different reflecting their hydrophilicity. Therefore, hydrophobic interaction with the gel matrix would be a useful way to identify their isotypes. Recently, we reported a HPLC method to separate IgG isotypes using TSKgel G3000SW in the presence of 0.3–1.0 M NaCl [15]. This separation is based upon slight differences in hydrophobicity among IgG isotypes.

By using the procedures provided here, the cycle-time of the hydrophobic interaction HPLC was 45 min, and 9–2200 mg of F(ab′)2 fragments were obtained from each cycle. It takes 30 h to purify F(ab′)2 from a collection of ascites. The procedures are thought to be suitable for large scale preparation of F(ab′)2 fragments of IgG1 mAbs. We are currently developing procedures for the single-step purification of F(ab)2 fragments of other immunoglobulin isotypes.

References