# ネコヘルペスウイルス1型赤血球凝集(HA)素の同定


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# Identification of Feline Herpesvirus Type 1-Hemagglutinin

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ABSTRACT. The crude hemagglutinin of feline herpesvirus type 1 (FHV-1), solubilized from infected fcwf-4 cells by detergents, was partially purified by three kinds of chromatographic methods. Lectin-affinity chromatography showed the hemagglutination (HA) activity in fractions, which was bound to Concanavalin A-sepharose and then eluted by $\alpha$-methyl D-mannoside, suggesting that the hemagglutinin might include a glycoprotein. Ion-exchange and gel-exclusion chromatographies were also capable of purifying the detergent-soluble crude hemagglutinin. When peak HA fractions, which were obtained from each of the three procedures, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel-exclusion chromatography was the most effective method. Electrophoreic analysis also showed only one band of 59,000 (59K) molecular weight protein, which was commonly observed in the three partially purified hemagglutinins with silver staining. In addition, the 59K protein band was clearly recognized in immunoblot analysis of the infected cell lysates using infected cat serum. These observations suggest that the FHV-1 detergent-soluble hemagglutinin from infected fcwf-4 cells may be closely related to a 59K immunogenic glycoprotein.—KEY WORDS: feline herpesvirus type 1, glycoprotein, hemagglutinin.

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Hemagglutination (HA) activity of feline herpesvirus type 1 (FHV-1), commonly known as the causative agent of feline viral rhinotracheitis, was initially reported by Gillespie *et al.* [2]. Later, Mochizuki *et al.* suggested that the FHV-1 hemagglutinin might be a glycoprotein, because it was inactivated by $\alpha$-amylase or $\beta$-glucosidase [11].

There are very few examples of HA-active viruses in *Herpesviridae*. As far as we know, equine herpesvirus type 1 [9] and bovine herpesvirus type 1 [4], in addition to FHV-1, would be all of such viruses which have HA activity. However, very little is known concerning immunogenicity of these herpesvirus hemagglutinins. Recently, bovine herpesvirus type 1-hemagglutinin was identified as a major glycoprotein, which was responsive to HA activity of the virus particles and carrying an important neutralization epitope [14].

As it is demonstrated in other herpesviruses, the glycoprotein content of FHV-1 has been demonstrated to include several glycoproteins [1, 8]. And major immunogenic proteins of FHV-1 were identified by immunoprecipitation as 60K, 68K, and 105K [8]. However, the specific roles of each glycoprotein in virus infection have not yet been made clear.

We previously indicated that high titer FHV-1 hemagglutinin could be solubilized by some detergents from fcwf-4 cells infected with the virus [3]. The present study was designed to identify a protein related to this hemagglutinin.

## MATERIALS AND METHODS

*Cell and virus:* Fcwf-4 cells were grown in Eagle's minimal essential medium (MEM, Nissui, Tokyo) supplemented with 8% heat-inactivated fetal calf serum, 10% tryptose phosphate broth, and antibiotics. After infection, cells were maintained in the same

medium without serum. FHV-1 C7301 strain [10] was propagated in monolayers of those cells.

*Preparation of the detergent-soluble hemagglutinins:* These were prepared from infected fcwf-4 cells according to the method as described previously [3].

*Purification of the detergent-soluble crude hemagglutinins:* Lectin-affinity chromatography was performed on an 11.5mm×25mm Concanavalin A (Con A) Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). Aliquoted 1.0 m*l* of 0.05% Triton X-100-solubilized crude hemagglutinin (128HAU/0.05 m*l*), pre-dialyzed against the eluent buffer consisting of 25 mM Tris-HCl, 0.3 M NaCl (pH 7.4) and 0.05% Triton X-100, was applied to the pre-equilibrated column, incubated for 30 min, and eluted at a flow rate of 0.14 m*l*/min at room temperature. Materials binding on Con A-column were eluted with 0.2 M $\alpha$-methyl D-mannoside in the same buffer at the same condition.

Anion-exchange chromatography was performed on a 143mm×44mm DEAE-cellulose column (DE52, Whatman, Kent, England). Aliquoted 2.0 m*l* of 0.05% Triton X-100-solubilized crude hemagglutinin (256HAU/0.05 m*l*), pre-dialyzed against 25 mM Tris-HCl (pH 8.0) containing 0.05% Triton X-100, was applied to the pre-equilibrated column. Elution was carried out by stepwise method with 0, 0.1, 0.2, and 0.5 M NaCl in the same buffer at a flow rate of 0.14 m*l*/min at room temperature.

Gel-exclusion chromatography was performed on a 960mm×26mm Sephacryl S-300 Superfine column (Pharmacia) by gel-filtration. The column was calibrated with the following molecular weight markers (Pharmacia): blue dextran (2000K), ferritin (440K), catalase (232K), aldolase (158K), bovine serum albumin (67K), ovalbumin (43K), and chymotrypsinogen A (25K). Aliquoted 5 m*l* of 0.5% DOC-solubilized crude hemagglutinin (128HAU/0.05 m*l*), pre-dialyzed against eluent buffer consisting of 50 mM Tris-HCl, 0.3 M NaCl, 1 mM EDTA with 0.25% DOC (pH 8.0), was applied to the column pre-equilibrated with the same buffer, and eluted at a flow rate of 0.33 m*l*/min at room temperature.

*Electrophoresis and immunoblot:* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the discontinuous Laemmli buffer system [5]. All samples were dissolved in the buffer (62.5 mM Tris-HCl, pH 6.8, 2.0% SDS, 5.0% 2-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue), then disrupted by heating for 2 min at 100°C. Polypeptides were separated on 7.5% gel and visualized by silver staining.

For immunoblot, the infected or uninfected cell lysates were separated by SDS-PAGE, and then electrophoretically transferred to nitrocellulose paper (BA83, Schleicher & Schuell, Dassel, W. Germany) by the method described by Towbin *et al.* [13]. The blotting paper was sliced and incubated for 2 hr at room temperature with appropriately diluted FHV-1-infected cat serum. Afterwards, they were washed three times, and incubated with anti-cat IgG peroxidase conjugate (Cappel Laboratories, PA, U.S.A) at room temperature for 1 hr. The reaction was visualized by the addition of a diaminobenzidine-hydrogen peroxide substrate.

## RESULTS

Fig. 1 presents the profile of lectin-affinity column using Con A-sepharose for the purification of the crude hemagglutinin solubilized by Triton X-100 and for the determination of the binding-ability of the hemagglutinin to Con A. Hemagglutinin was bound to Con A-sepharose and then eluted by 0.2M $\alpha$-methyl D-mannoside. The recovery of hemagglutinin was 22%.

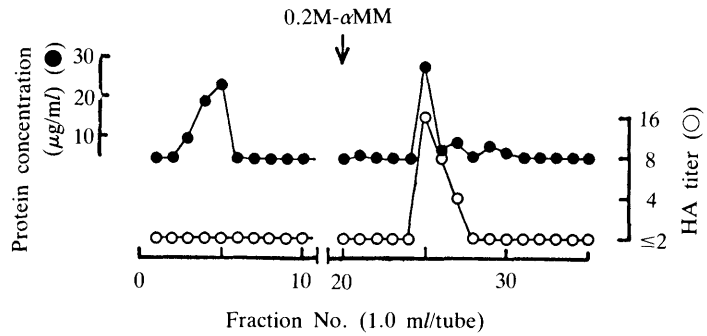


Fig. 1. Lectin affinity chromatography on Concanavalin A (Con A)-sepharose of Triton X-100-solubilized hemagglutinin from infected fcwf-4 cells. Con A-binding materials were eluted by a buffer which contained 0.2 M α-methyl D-mannoside (α-MM). Protein concentration (●) was determined by modifications [12] of the standard Lowry method [7]. HA titer (○) was expressed as the reciprocal of the highest dilution showing complete HA.

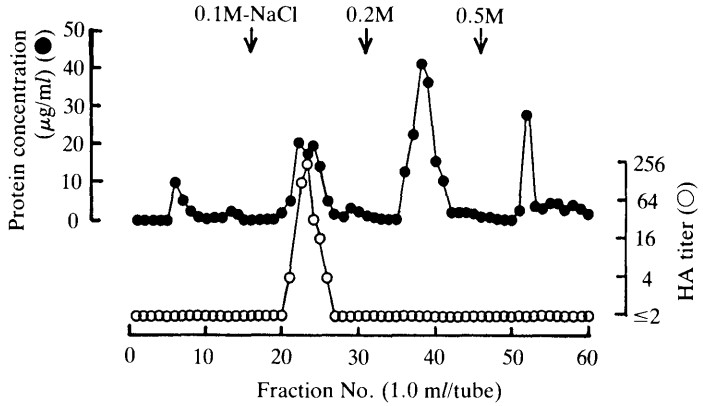


Fig. 2. Ion-exchange chromatography on DEAE-cellulose of Triton X-100-solubilized hemagglutinin from infected fcwf-4 cells. Elution was done using a buffer which contained stepwise concentration of NaCl (0, 0.1, 0.2, or 0.5 M). Protein concentration (●) was determined as described in legend for Fig. 1.

The profile of anion-exchange chromatography of the Triton X-100-solublized crude hemagglutinin showed that the hemagglutinin could be purified effectively in this manner, because it was eluted in the fractions using only eluent containing 0.1 M NaCl (Fig. 2). The recovery of HA activity was 86%.

Gel-filtration on a column of Sephacryl S-300 was done by using the DOC-solubilized crude hemagglutinin. As shown in Fig. 3, hemagglutinin fractions formed one peak. However, the recoverd HA titers were unexpectedly low (9%). The molecular weight of the peak HA fraction was estimated to be approximately 110K to 130K.

The hemagglutinin partially purified by Con A-column was visualized with silver staining under denatured condition as three clear bands of proteins (59K, 65K, and 105K) and as an unclear band (69K) (Fig. 4, lane 2). The partially purified hemagglutinins by ion-exchange column or gel-filtration were also visible, respectively, as two major (59K and 105K) and two minor (44K and 95K) bands (Fig. 5, lane 2), and as a major (58K) and a minor (37K) band (Fig.

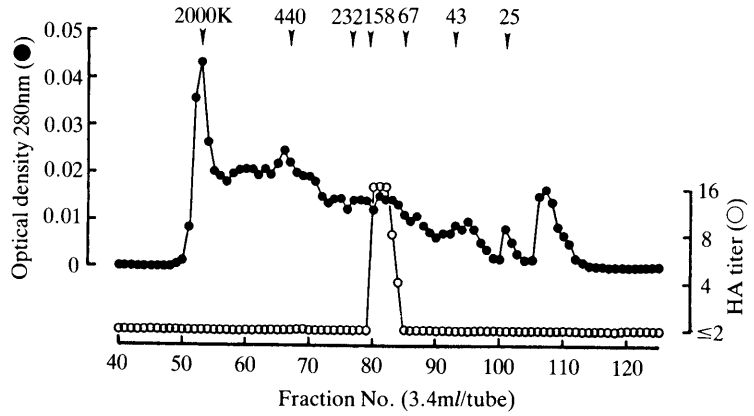


Fig. 3. Gel-filtration on Sephacryl S-300 of DOC-solubilized hemagglutinin from infected fcwf-4 cells. Protein concentration (●) was monitored in optical density 280 nm.

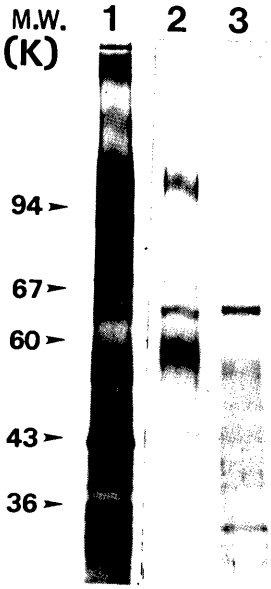


Fig. 4. SDS-PAGE analysis of crude hemagglutinin solubilized from infected fcwf-4 cells (lane 1); hemagglutinin partially purified by Con A-chromatography, which bound to Con A and then eluted from it by 0.2 M α-methyl D-mannoside (lane 2); or Con A-unbinding materials (lane 3).

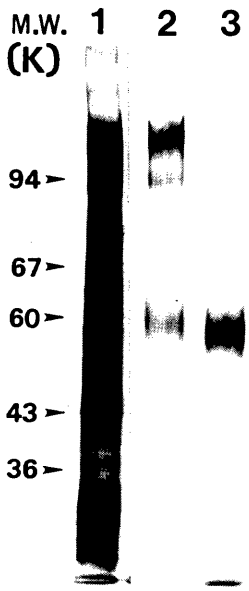


Fig. 5. SDS-PAGE analysis of crude hemagglutinin solubilized from infected fcwf-4 cells (lane 1); hemagglutinin partially purified by ion-exchange chromatography (lane 2); or by gel-filtration (lane 3).

5, lane 3). There was one major band (58–59K) observed commonly in each hemagglutinin partially purified by the procedures described above.

As shown in Fig. 6, immunoblot analysis showed that several immunogenic protein bands were specifically detected on the blotting paper. They consisted of two major bands (58K and 105K) and other two minor bands (44K and 65K).

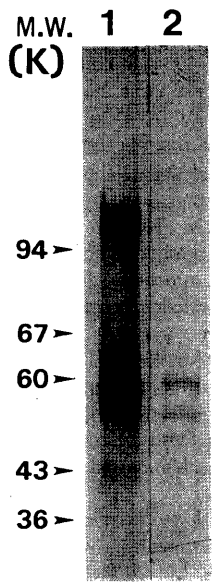


Fig. 6. Immunoblot analysis of infected (lane 1) or mock-infected (lane 2) fcwf-4 cell DOC-lysates with infected cat serum.

## DISCUSSION

We previously reported that the FHV-1 hemagglutinin was successfully solubilized by detergents from infected fcwf-4 cells [3]. The present study was designed to identify the detergent-soluble hemagglutinin by ordinary biochemical methods, chromatography and SDS-PAGE.

The hemagglutinin was partially purified by either Con A affinity, ion-exchange, or gel-exclusion chromatography. In general, gel-exclusion chromatography gives rather poor results when applied to membrane proteins solubilized by non-ionic detergent, *e.g.*, Triton X-100, because of formation of large micelles. Therefore, DOC, a bile salt, was used in this study for solubilizing hemagglutinin from cell membranes and was added in the eluent for gel filtration. On the other hand, for Con A and ion-exchange chromatography, Triton X-100 was utilized to repress non-specific ionic-binding of protein-containing micelles. As a result, profiles of the three chromatographic methods used here and each SDS-PAGE analysis of peak HA fraction indicated that they were effective for purification of the detergent-soluble hemagglutinin.

The analysis by Con A affinity chromatography suggested that the hemagglutinin might include glycoproteins. SDS-PAGE analysis of peak HA fraction from each column revealed only one band which was commonly visible, and its molecular weight was estimated as 59K. In addition, an identical protein was recognized to be virus-specific by immunoblot. These findings indicated that the hemagglutinin must be related to the immunogenic 59K glycoprotein.

Gel-filtration has the important potential advantage for separating differently sized homopolymers. However, mixed micelles of phospholipid and detergent cannot always be separated from the detergent-solubilized protein. Thus, when gel-filtration is used as a tool for estimating the molecular weight of detergent-solubilized membrane proteins, they sometimes tend to be eluted prior to the elution of water-soluble proteins of the same size [6]. SDS-PAGE analysis of HA fraction with only one peak indicated that the hemagglutinin was purified very effectively by gel-filtration. This finding has led us to believe that the molecular weight (110K–130K) of the HA peak fraction estimated here are worthy of consideration. It was also observed that urea (4 M) did not affect HA activity, but a chaotropic reagent, NaSCN (2 M), inhibited it. Moreover, the HA peak fraction was identically visualized as a major 59K band under conditions of SDS-PAGE with or without 2-mercaptoethanol (data not shown). From these observations, we propose that the monomer of FHV-1 hemagglutinin subunit is a 59K glycoprotein and it forms homopolymers, probably a dimer and/or a trimer, mainly by hydrophobic bond in the detergent-solubilized state to appear as HA

activity. However, further experiments are needed to prove this point.

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要　約

ネコヘルペスウイルス1型赤血球凝集(HA)素の同定：堀本泰介・笠岡達彦・土屋耕太郎・高橋英司（東京大学農学部家畜微生物学教室）――ネコヘルペスウイルス1型(FHV-1)感染fcwf-4細胞より界面活性剤を用いて可溶化した粗HA素を，3種のクロマトグラフィーを用い部分精製した．レクチンアフィニティークロマトグラフィーでは，コンカナバリンAに結合した後α-メチルD-マンノシドにより溶出された分画にHA性が見い出され，HA素には糖蛋白が含まれることが考えられた．イオン交換クロマトグラフィーおよびゲル濾過もまた界面活性剤可溶化粗HA素の精製には有効であった．これら3種のクロマトグラフィーより得た各HA分画をSDS-PAGEを用いて分析した結果，ゲル濾過が最も効果的な精製法であると考えられた．またSDS-PAGE分析では，各部分精製HA素に共通に59,000の分子量を示す蛋白バンドが銀染色で認められた．さらに，この蛋白はイムノブロット分析において猫の感染血清と強い反応性を示した．これらの結果は，FHV-1感染fcwf-4細胞からの界面活性剤可溶化HA素は分子量59,000の免疫原性糖蛋白に関連することを示唆する．