Affinity chromatography of thiol ester-containing proteins

G. Houen[a,*], Ib Svendsen[b]

[a]*Department of Autoimmunology, Statens Serum Institut, Artillerivej 5, DK-2300 S Copenhagen, Denmark*
[b]*Department of Chemistry, Carlsberg Laboratory, Gamle Carlsbergvej 10, DK-2500 Valby, Denmark*

Abstract

A method is described for the affinity chromatographic purification of thiol ester proteins. These comprise the complement proteins C3 and C4 and the protease inhibitor α_2-macroglobulin (α_2M) and are known to contain an internal β-cysteinyl-γ-glutamyl thiol ester. The method employs aminoalkyl ligands coupled to a divinylsulfonyl-derivatized agarose matrix, and the length of the aminoalkyl spacer arm was found to be important for the effectiveness of the matrix. Optimal results were obtained with diaminododecyldivinylsulfonyl-agarose. Employing this matrix the thiol ester proteins C3, C4 and α_2M were isolated from human pregnancy serum. Application of the method to chicken and rainbow trout serum gave rise to isolation of several proteins including chicken and rainbow trout α_2M.   © 1998 Elsevier Science B.V.

Keywords: Proteins; Thiol ester proteins; Enzyme inhibitors

1. Introduction

The complement proteins C3 and C4 and the protease inhibitor α_2-macroglobulin (α_2M) have been found to contain an internal β-cysteinyl-γ-glutamyl thiol ester formed between a cysteine and a glutamine separated by only a few amino acids in the amino acid sequence [1]. The thiol ester proteins are part of an organisms primary defence against invading microorganisms and may represent an evolutionarily ancient defence against invading pathogens [2,3]. The thiol ester proteins have been found to react at the internal thiol ester with small primary amines [1,4–7]. Since primary amino groups and other nucleophilic groups are abundant in all organisms as part of proteins, amino acids and other molecules, it must be assumed that the internal thiol ester is not reactive with these primary amino groups under most circumstances. In agreement with this it has been found, that the thiol ester proteins require activation to induce a conformational change, which exposes the thiol ester and increases its reactivity [1,8–12]. For C3 and C4 this activation takes place as part of the classical complement cascade, initiated by binding of antibodies or mannan-binding lectin to foreign surfaces, or by activation of the alternative complement cascade, initiated by binding of C3 to foreign surfaces [13,14]. For α_2M the activation takes place as a result of protease binding to the inhibitor [1,12].

In this article we describe an affinity chromatographic method for purifying thiol ester-containing proteins. The method uses aminoalkyl-derivatized

*Corresponding author.

matrices and takes advantage of the affinity of these proteins for aliphatic amines.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS), tris(hydroxy-methylamino)methane (Tris), N,N,N′,N′-tetramethyl-ethylenediamine, glycerol, mercaptoethanol, tri-fluoroacetic acid (TFA), octylamine, 1,4-diaminobutane, 1,6-diaminohexane, 1,8-diaminooc-tane, ethylendiamine, octylamine, phenylmethylsul-fonylfluoride (PMSF), biotinhydrazide, *p*-nitro-phenylphosphate, bovine serum albumin (BSA), alkaline phosphatase-conjugated goat immuno-globulins against rabbit immunoglobulins, trypsin and Coomassie Brilliant Blue R-250 were from Sigma (St. Louis, MO, USA). Tween-20, NaCl, NaOH, NaH_2PO_4, Na_2HPO_4, 5,5-diethylbarbituric acid, dodecylamine, 1,10-diaminododecane, acetoni-trile, diethanolamine and ethylenediaminetetraacetic acid (EDTA) were from Merck (Darmstadt, Ger-many). Acrylamide, bisacrylamide, ammonium per-sulfate, bromophenol blue, and molecular mass standard proteins were from Bio-Rad (Richmond, CA, USA). AH-Sepharose, CNBr-Sepharose and Mono Q columns were from Pharmacia (Uppsala, Sweden). *o*-Phenylenediamine, diaminobenzidine and divinylsulfonyl-agarose (DVS-agarose) were from Kem-En-Tek (Copenhagen, Denmark). H_2O_2 was from Struers (Copenhagen, Denmark). Sheep blood, human immunoglobulin G (IgG) and H_2SO_4 was from Statens Serum Institut (Copenhagen, Den-mark). Rabbit immunoglobulins against complement proteins C3c, C3d, C4 and factor B, and peroxidase-conjugated swine immunoglobulins against rabbit immunoglobulins were from Dako (Copenhagen, Denmark). Rabbit immunoglobulins against human $\alpha_2 M$ were from Behringwerke (Marburg, Germany). Ethanol was from Danisco (Copenhagen, Denmark). Ethanolamine was from BDH Chemicals (Poole, UK). Aprotinin was from Bayer (Leverkusen, Ger-many). Dialysis bags were from Medicell (London, UK). Filters (0.22 μm pore size), polyvinyldifluoride (PVDF) membranes and Milli-Q plus apparatus for water purification were from Millipore (Boston, MA, USA). Chicken and rainbow trout serum were a kind gift from C. Koch, Statens Serum Institut.

2.2. Synthesis of affinity matrices

The matrices were prepared by washing DVS-agarose or CNBr-Sepharose twice in equal volumes of 0.1 *M* sodium carbonate, pH 9, followed by incubation overnight at 4°C with an equal volume of 0.05 *M* amine in the same buffer. After coupling with the amine the matrices were washed overnight in 0.1 *M* Tris, 1 *M* glycine, pH 8.8 to block unreacted divinylsulfonyl groups. The matrices were then washed extensively in 50 m*M* sodium phos-phate, 0.9% NaCl, 0.05% Tween 20 before use, except for the matrix with dodecylamine which was first washed extensively with MeOH to remove undissolved dodecylamine and then washed with 50 m*M* sodium phosphate, 0.9% NaCl, 0.05% Tween 20. A control matrix was prepared in the same way without amine added during the overnight incuba-tion.

2.3. Preparation of placental extract

One placenta was cut into small pieces, mixed with 0.5 liter 0.1 *M* sodium phosphate, pH 7.4, and homogenized for 15 min at 4°C using an ordinary food blender (Moulinex). The homogenate was cen-trifuged at 10 000 *g* and the supernatant stored in aliquots at −20°C.

2.4. Ammonium sulfate precipitation

Ammonium sulfate was added to 400 g/l and the solution was stirred overnight at 4°C. The resulting precipitate was isolated by centrifugation at 13 000 *g*, redissolved in water (one tenth of the starting volume) and dialysed against 5 m*M* sodium phos-phate, pH 7.4.

2.5. Affinity chromatography of human serum on AH-Sepharose

Samples were dialysed against 5 m*M* sodium phosphate, pH 7.4 before being applied to the column (60×1 cm). Following application of sample the column was washed with 5 m*M* sodium phos-

phate until the absorbance was below 0.05, and eluted with a gradient of increasing sodium phosphate concentration to 200 m*M* before being eluted with 10 m*M* octylamine in 200 m*M* sodium phosphate, pH 7.4. The flow-rate was 1 ml/min and the chromatography was monitored at 280 nm.

2.6. Ion-exchange chromatography

Samples were chromatographed on a Mono Q column using an FPLC system, controlled by the ''FPLC director'' software. One or 5-ml samples were dialysed against 50 m*M* sodium phosphate, pH 7.6 before application to the column, which was equilibrated in the same buffer and eluted by a linear gradient to 1.5 *M* NaCl. Flow: 1 ml/min.

2.7. Binding to affinity matrices

For batch experiments samples were incubated with an equal volume of the matrices overnight at 4°C with or without the inclusion of EDTA (10 m*M*) and protease inhibitors (1 m*M* PMSF, 400 KIE/ml aprotinin). The matrices were then washed extensively with 50 m*M* sodium phosphate, pH 7.4 and then incubated with 50 m*M* ethanolamine in 50 m*M* sodium phosphate, pH 7.4.

2.8. SDS–polyacrylamide gel electrophoresis (PAGE)

SDS–PAGE was performed as described by Laemmli [15] using 7% gels. Twenty μg of antigen were applied per lane and electrophoresis was carried out at 40 V. Gels were stained with 0.1% Coomassie Brilliant Blue in 10% CH_3COOH, 20% EtOH, and destained with 10% CH_3COOH.

2.9. Immunoblotting of SDS–PAGE gels (Western blotting)

SDS–PAGE gels were electroblotted overnight to nitrocellulose in a semidry electroblotting apparatus (Trans-Blot SD, Bio-Rad, Hercules, CA, USA) using 10-times diluted SDS–PAGE running buffer as transfer buffer and a current of 0.1 mA/cm^2. Following transfer the membrane was washed in 50 m*M* sodium phosphate, 0.9% NaCl, 0.5% Tween 20, and

then incubated with primary antibody diluted 1:1000 in 50 m*M* sodium phosphate, 0.9% NaCl, 0.5% Tween 20. After 1 h incubation the membrane was washed three times in 50 m*M* sodium phosphate, 0.9% NaCl, 0.5% Tween 20, and incubated for 1 h with conjugate diluted 1:1000 in the same buffer as the primary antibody. After three washes of the membrane in 50 m*M* sodium phosphate, 0.9% NaCl, 0.5% Tween 20, bound antibodies were visualized by incubation with 1 mg/ml diaminobenzidine, 0.03% hydrogen peroxide in 20 m*M* sodium phosphate, pH 7.

2.10. Immunoelectrophoresis

Rocket immunoelectrophoris was carried out as described in [16]. The electrophoreses were performed at 2 V/cm overnight in 1% agarose gels in 20 m*M* 5,5-diethylbarbituric acid, 40 m*M* Tris, 0.5 m*M* calcium acetate, 3 m*M* sodium azide, pH 8.6, with antisera added.

2.11. Tryptic digestions

Samples in 50 m*M* sodium phosphate, pH 7.4 (1 ml, 1 mg/ml) were digested with trypsin by addition of 2 μl 1 m*M* trypsin and incubated for 6 h at room temperature. Before analysis by high-performance liquid chromatography (HPLC) the reaction was terminated by addition of TFA to 0.1%.

For digestion in situ of samples electroblotted to PVDF membrane pieces were first treated with 0.5% Brij-35 in 50 m*M* sodium phosphate, pH 7.4, and then incubated overnight at room temperature in 1 ml 50 m*M* sodium phosphate, pH 7.4 with 1 mg trypsin. The digestion mixture was then chromatographed directly on the HPLC after addition of TFA to 0.1%.

Alternatively, samples were eluted from PVDF membranes by 1 h incubation at room temperature with 5 ml 75% TFA. After addition of water to reduce the TFA concentration to 20%, the solution was freeze–dried, redissolved in a minimal volume 50 m*M* sodium phosphate, pH 7.4 and subjected to digestion with trypsin.

2.12. High-performance liquid chromatography

Two systems were used. (1) A model 130A HPLC

system (Applied Biosystems) with a 5-µm particle size RP-18 column (220×2.1 mm). Flow: 275 µl/min. A gradient from 0.1% TFA to 80% acetonitrile, 0.08% TFA over 60 min was used, and the absorbance was monitored at 215 nm. (2) A Waters system with a UK6 injector, two Model 501 pumps, a 486 absorbance detector and a Nova-Pak C_{18} column. The system was controlled by the Baseline 810 software using a NEC computer. Samples were injected into a 1-ml loop and chromatographed using a linear gradient from 0.1% TFA to 80% acetonitrile, 0.08% TFA over 30 min. The flow-rate was 1 ml/min and the absorbance was monitored at 220 nm. Fractions were collected manually.

2.13. Microsequencing

Preparation of SDS–PAGE gels for running samples for subsequent electroblotting to PVDF membrane and sequencing was performed as described [17].

N-Terminal sequencing was performed on an Applied Biosystems sequenator, Model 477A equipped with an on-line phenylthiohydantoin amino acid analyser, Model 120A, using chemicals and software supplied by the manufacturer.

2.14. Enzyme-linked immunosorbent assay (ELISA) for C3, C4 and α_2M

Samples were coated in microtitre plates (100 µl/well) overnight (4°C) at a dilution of 1:100 or 1:1000 in 50 mM sodium carbonate, pH 9.3. The wells were then washed three times with 50 mM Tris, pH 7.5, 0.9% NaCl (w/v), 0.05% Tween 20 (v/v). Subsequent incubations with rabbit immunoglobulins against C3, C4 or α_2M diluted 1:1000 in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20 were carried out for 1 h at room temperature on a shaking table and were followed by three washes in 50 mM Tris, pH 7.5, 0.9% NaCl (w/v), 0.05% Tween 20 (v/v). For detection of bound antibodies the plates were finally incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat immunoglobulins against rabbit immunoglobulins (1:1000) in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20. Bound alkaline phosphatase-conjugated antibodies were quantitated

using 1 mg/ml p-nitrophenylphosphate in 1 M diethanolamine, 0.5 mM MgCl$_2$, pH 9.8. The absorbance was read at 405 nm on a EAR 400 ATc ELISA reader from SLT Labinstruments (Salzburg, Austria) with background subtraction at 690 nm.

2.15. Binding of C3 and C4 to heat-aggregated human IgG

Human IgG (1 mg/ml) was incubated at 60°C for 1 h. The heat-aggregated IgG was then coated overnight at 4°C in microtitre plates (1:10 or greater dilution) with 50 mM sodium carbonate, pH 9.3 as buffer. The wells were blocked in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20 and incubated overnight at 4°C with human serum or eluate from affinity chromatography diluted 1:100 in 50 mM sodium phosphate, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20. After three washes the wells were incubated 1 h at room temperature with rabbit immunoglobulins against C3 or C4 diluted 1:1000 in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20. After three washes bound antibodies were detected as described above for ELISA.

2.16. Binding of α_2M to immobilized trypsin

Trypsin was coated overnight at 4°C in microtitre plates (100 µl/well) at a concentration of 0.01 mg/ml in 50 mM sodium carbonate, pH 9.3. The plates were then washed three times in 50 mM Tris, pH 7.5, 0.05% Tween 20 and incubated for 1 h at room temperature with human serum or eluate from affinity chromatography diluted 1:100 in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20. After three washes in 50 mM Tris, pH 7.5, 0.05% Tween 20 the plates were incubated 1 h at room temperature with rabbit immunoglobulins against α_2M diluted 1:1000 in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20. Bound antibodies were then detected as described in Section 2.14.

2.17. Incorporation of biotinhydrazide in C3, C4 and α_2M

ELISA plates were coated overnight at 4°C with rabbit immunoglobulins against C3, C4 or α_2M at a concentration of 1 µg/ml in 50 mM sodium carbon-

ate, pH 9.3. The plates were then washed three times in 50 mM Tris, pH 7.5, 0.05% Tween 20 and incubated overnight with normal human serum or eluates from affinity chromatography, which had been preincubated with biotinhydrazide, diluted 1:100 or more in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20 [pretreatment with biotinhydrazide was performed by incubating 10 µl serum or eluate with 40 µl 50 mM sodium phosphate, pH 7.2 and 2 µl biotinhydrazide (1 mg/ml water) for 30 min at 60°C]. The plates were then incubated for 1 h at room temperature with alkaline phosphatase-conjugated avidin 1:1000 in 50 mM Tris, pH 7.5, 0.9% NaCl, 1% BSA, 1% Tween 20 and bound avidin quantitated as described for ELISA in Section 2.14.

2.18. Determination of hemolytic activity

Determination of hemolytic activity was performed essentially as described by Garvey et al. [18]. Sheep red blood cells were isolated from sheep blood by centrifugation and washed three times in 5 mM 5,5-diethylbarbituric acid, 0.15 M NaCl, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$, pH 7.2 (barbital-buffer). A 1% suspension of erythrocytes was then sensibilized for 1 h at 37°C with rabbit immunoglobulins against sheep erythrocytes diluted 1:1000. The erythrocytes were again washed three times in barbital-buffer. For determination of complement hemolysis 100 µl of 1% sensibilized erythrocytes were incubated 2 h at 37°C with 100 µl normal human serum or eluates from affinity chromatography diluted 1:10 in barbital-buffer. After incubation the plates were centrifuged and the absorbance of the supernatants read at 550 nm.

3. Results

As a result of affinity chromatography of human pregnancy serum on AH-Sepharose we observed that several fractions contained large amounts of a single essentially pure protein, and SDS–PAGE, peptide mapping and microsequencing identified this protein as C4 (Fig. 1). The binding of C4 to the affinity matrix could be the result of a purely ionic interaction with the positively charged AH-Sepharose
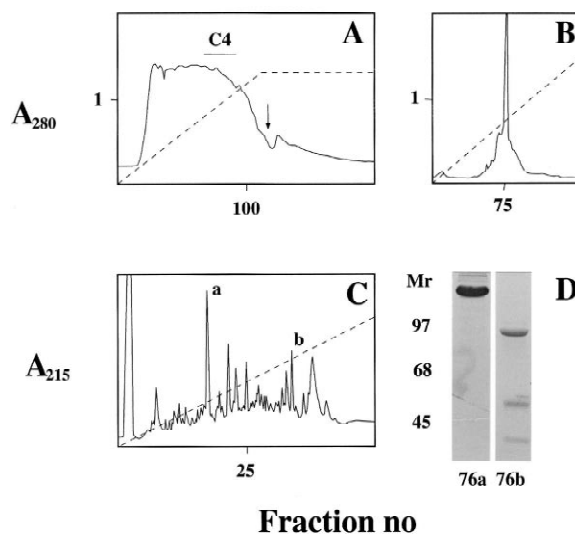

Fig. 1. Identification of C4 in the eluate from affinity chromatography of human pregnancy serum on an AH-Sepharose column. (A) Chromatography on AH-Sepharose. The column was eluted with a linear gradient to 0.2 M PB as shown and elution with 10 mM octylamine in 0.2 M PB was started at the arrow. Five ml fractions were collected. The position of C4 is indicated by the straight bar. (B) Ion-exchange chromatography of fraction 85 from the AH-Sepharose column on a Mono Q column. The gradient to 1.5 M NaCl is shown. (C) Peptide mapping of fraction 76 from B after cleavage with trypsin. The gradient of increasing acetonitrile is shown. (D) SDS–PAGE analysis of fraction 76 from B (a, without reduction; b, with reduction).

column, and this might actually be expected, as the protein was eluted from the column by a gradient of increasing phosphate concentration. However, the same behaviour of C4 was not observed when the pregnancy serum had been subjected to ammonium sulfate precipitation and dialysis of the precipitate prior to chromatography on AH-Sepharose (not shown). Since C4 was precipitated by ammonium sulfate under the conditions used but apparently did not bind to the AH-Sepharose column after this treatment, we hypothesized that C4 may have a strong affinity for amine compounds, and that this might be diminished by ammonium sulfate treatment due to the inevitable presence of ammonia. Furthermore, the affinity might be related to the thiol ester group present in C4 and two other plasma proteins (C3, α_2M), as these proteins are rapidly inactivated by primary amines.

In order to investigate these possibilities we

synthesized several affinity matrices with a primary amino group separated from the matrix by varying lengths of spacer arm, varying from 2 to 10 carbon atoms, and analysed these for their ability to bind thiol ester proteins from human pregnancy serum.

Fig. 2 shows the amount of α_2M remaining in solution after incubation with the various matrices. It can be seen that relative to the control matrix, the dodecylamine substituted matrix did not remove more α_2M from the solution. All the diamine-substituted matrices removed some α_2M, and the matrix with the longest spacer arm was most effective. Furthermore, all of the matrices removed C4 from the serum, while varying amounts of C3c and C3d were observed in the serum after incubation (not shown). The 1,10-diaminododecyl–DVS-agarose was most effective in binding the thiol ester proteins. In contrast to the thiol ester proteins the amount of factor B was not diminished relative to the control matrix except for the dodecylamino–DVS-agarose matrix, which removed about 60% of the factor B. All in all the results show removal of thiol ester proteins from the serum by the primary amine containing matrices. The experiment described in Fig. 2 was conducted in the absence of EDTA and protease inhibitors, but similar results were obtained in the presence of EDTA, PMSF and aprotinin (not shown).
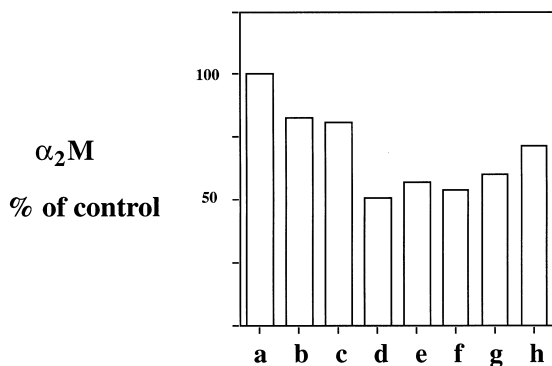
Fig. 3. Time course for removal of α_2M, C4 and factor B from human pregnancy serum upon incubation with 1,10-diaminododecyl–DVS-agarose.

Fig. 3 shows time course experiments for incubation of pregnancy serum with 1,10-diaminododecyl–DVS-agarose and removal of α_2M, C4 and factor B. It may be seen that C4 was removed fastest from the serum followed by α_2M, whereas factor B was only removed to a modest degree.

Two types of matrices and two ligands were chosen for further investigation: 1,4-diaminobutyl- and 1,10-diaminododecyl-derivatized CNBr-Sepharose and DVS-agarose. When these were used for chromatography of human pregnancy serum and placenta extract, it was again found that the binding of thiol ester proteins was dependent on the length of the spacer arm. Fig. 4 shows SDS–PAGE and Western blot analyses of eluates from 1,4-diaminobutyl- and 1,10-diaminododecyl-derivatized CNBr-Sepharose and DVS-agarose. The pregnancy serum or placenta extract was incubated with the matrices, which were then washed extensively and finally incubated with 50 mM ethanolamine as eluting agent. From the SDS–PAGE analysis of the eluates it can be seen that the 1,10-diaminododecyl–DVS-agarose bound most protein, whereas the corresponding CNBr-Sepharose matrix bound considerably less protein (Fig. 4A,B). When the eluates were analysed for thiol ester proteins by immunoblotting, it was found that C3 and factor B was present in the eluates from the 1,10-diaminododecyl–DVS-agarose column but absent from the other eluates (Fig. 4C,E). C4 was present in largest amounts in the same eluate but was also present in significant amounts in other eluates except for the eluate from 1,4-diaminobutyl-derivatized CNBr-Sepharose,

Fig. 2. Amount of remaining α_2M in human pregnancy serum, as determined by rocket immunoelectrophoresis, after incubation with aminoalkyl–divinylsulfonyl-agarose matrices using the following ligands. (a) Control without matrix, (b) control matrix without ligand, (c) dodecylamine, (d) 1,10-diaminododecane, (e) 1,8-diaminooctane, (f) 1,6-diaminohexane, (g) 1,4-diaminobutane, (h) ethylenediamine.
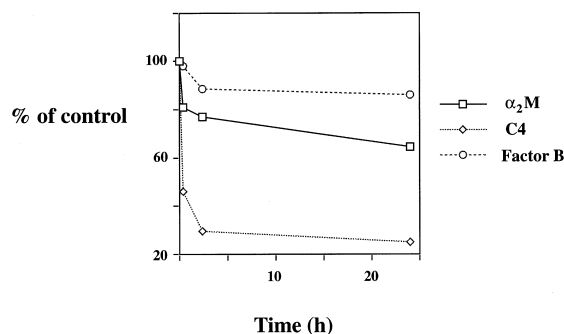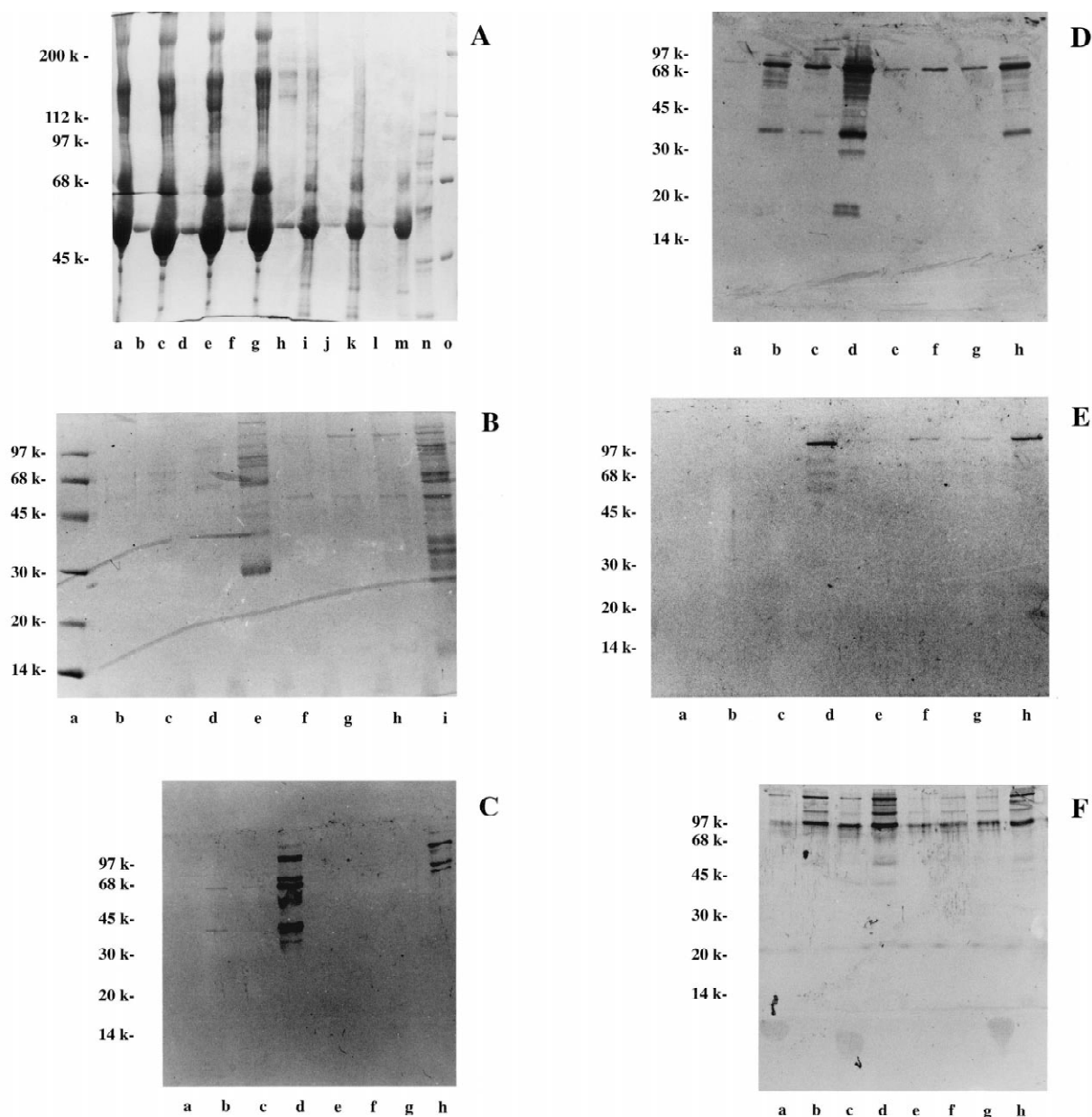
Fig. 4. SDS–PAGE and Western blot analyses of human pregnancy serum and placenta proteins eluted from 1,4-diaminobutyl- and 1,10-diaminododecyl-derivatised CNBr-Sepharose and DVS-agarose. (A) SDS–PAGE using non-reducing sample buffer of supernatants (a, c, e, g, i, k, m) and eluates (b, d, f, h, j, l, n) from incubation of pregnancy serum (a–h) or placenta extract (i–n) with 1,4-diaminobutyl-derivatised CNBr Sepharose (a, b, i, j), 1,10-diaminododecyl-derivatised CNBr Sepharose (c, d), 1,4-diaminobutyl-derivatised DVS-agarose (e, f, k, l) or 1,10-diaminododecyl-derivatised DVS-agarose (g, h, m, n). Lane o contains molecular mass standard proteins. (B) SDS–PAGE of eluates using reducing sample buffer. a, Molecular mass standard proteins; b–e, pregnancy serum; f–l, placenta extract. Matrices: b, f, 1,4-diaminobutyl-derivatised CNBr Sepharose; c, g, 1,10-diaminododecyl-derivatised CNBr Sepharose; d, h, 1,4-diaminobutyl-derivatised DVS-agarose; e, i, 1,10-diaminododecyl-derivatised DVS-agarose. (C–F) Western blot analysis of eluates (a–d, pregnancy serum; e–h, placenta extract) from the following matrices: a, e, 1,4-diaminobutylderivatised CNBr Sepharose; b, f, 1,10-diaminododecyl-derivatised CNBr Sepharose; c, g, 1,4-diaminobutyl-derivatised DVS-agarose; d, h, 1,10-diaminododecyl-derivatised DVS-agarose. The blots were made using antisera to: (C) C3, (D) C4, (E) Factor B and (F) α_2M.

which has the shortest spacer arm (Fig. 4D). The amount of α_2M in the eluates followed the pattern of C4 (Fig. 4F). From these results we conclude that the chromatographic process is not a simple ion-exchange chromatography, as this would be essentially independent of the length of the spacer arm. Furthermore since the proteins could be eluted by 50 mM ethanolamine, the process is not a purely hydrophobic interaction chromatography, but rather an affinity chromatography.

If C3 is bound to the matrix, it might be expected to be ''activated'' and bind factor B, and this was actually observed. Small amounts of factor B were observed but mainly in the eluate from the more effective matrix, 1,10-diaminododecyl–DVS-agarose (Fig. 4).

When chicken and trout serum were incubated with the 1,10-diaminododecyl–DVS-agarose matrix, it was observed that several proteins bound specifically and could be eluted by ethanolamine. Fig. 5 shows SDS–PAGE analysis of these eluates. By

Western blotting analysis with antisera to the corresponding human proteins and by amino acid sequencing of peptides released from the M_r 200 000 chicken band by tryptic digestion, some of the bands were identified as chicken C3, chicken α_2M and trout α_2M, which seemed to be present in three forms.

In order to test the biological activity of the eluted proteins the eluates from human serum were analysed for binding of C3 and C4 to heat-aggregated human IgG, for hemolytic activity towards sensibilized sheep erythrocytes and for the ability of α_2M to bind immobilised trypsin [19]. In contrast to normal human serum, which exhibited hemolytic activity and binding of C3 to heat-aggregated IgG, the eluates showed neither hemolytic activity nor binding of C3 and C4 to heat-aggregated human IgG (Fig. 6). However, the ability of α_2M to bind immobilised trypsin seemed to be intact as the α_2M-containing eluates showed binding in this assay. This shows that the biological activity of C3 and C4 has been disturbed by the chromatographic process, presumably by the same mechanism responsible for the inactivation of C3 in plasma by methylamine [20,21].

Furthermore, as a test for the thiol ester status the eluates were tested for the ability to incorporate biotinhydrazide in C3. In contrast to normal human serum, which readily incorporated biotinhydrazide in C3 at 60°C, only a slight incorporation was observed for the eluates, despite the presence of large amounts of C3.
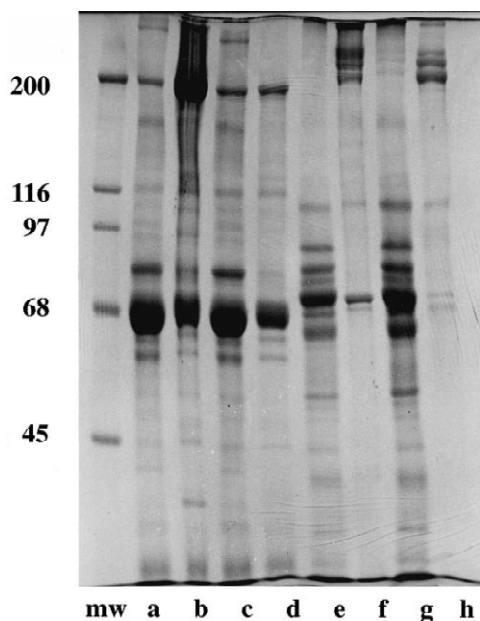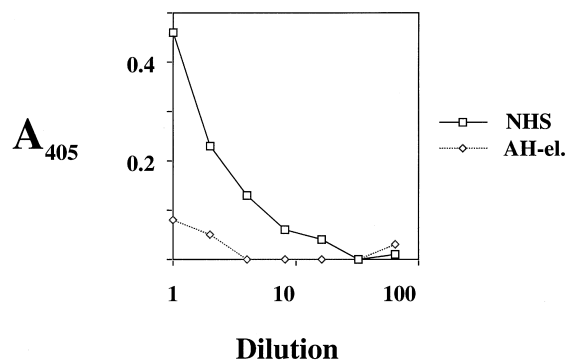

Fig. 5. SDS–PAGE (reducing sample buffer) of supernatants (a, c, e, g) and eluates (b, d, f, h) from incubation of chicken (a–d) and trout serum (e–h) with 1,10-diaminododecyl–DVS-agarose. In a, b, e and f the experiment was conducted in the absence of protease inhibitors whereas in c, d, g and h protease inhibitors were present. Mw, molecular mass standards.

Fig. 6. Binding of C3 in human serum to heat-aggregated IgG. NHS: normal human serum. AH-eluate: eluate from aminohexyl–agarose column.

4. Discussion

Judging from the results presented here it appears that thiol ester containing proteins can be isolated by affinity chromatography on aminoalkyl-derivatised columns, provided that the spacer arm is sufficiently long.

C4 was isolated in pure form as a result of direct chromatography of human pregnancy serum on an AH-Sepharose column. The protein could be eluted by 0.2 *M* sodium phosphate and must therefore have been bound either by ionic interactions or by its affinity for amino groups. With longer spacer arms a more stable binding to the matrices was obtained, and both C4 and $\alpha_2 M$ showed a time dependent binding to 1,10-diaminododecyl–DVS-agarose. The bound proteins could be eluted by ethanolamine, and the binding was found to depend on the length of the spacer arm.

The mechanism of the chromatography is not known, but may be a result of ionic interactions or a result of an affinity of thiol ester proteins for primary amino groups. Observations in favor of the latter are that the chromatography is only applicable to serum, which has not been ammonium sulfate-precipitated, that the yield is dependent on the length of the spacer arm used, and that the eluted C3 is devoid of biological activity. It seems reasonable to assume that the thiol ester in the unreacted thiol ester proteins is protected from the amino groups present in the environment, and that it is exposed upon binding to substrates. Small aliphatic amines, however, are capable of reacting with the thiol ester, as are possibly also primary amino groups on a sufficiently long spacer arm. The binding/covalent reaction, observed by us during the affinity chromatography, is reversible since the protein can be eluted by ethanolamine.

The molecular basis for the transamination and transesterification reactions in the thiol ester proteins has recently been described in detail [22], and it was found that the reactions are facilitated by nucleophilic groups adjacent to the thiol ester. These groups will presumably also facilitate release of covalently bound protein by transamination. In agreement with this, it has previously been found that covalently bound C3 can be released from zymosan by hydroxylamine or ammonia treatment [23,24]. Moreover, in agreement with the results reported here, which show a lack of biological activity of the eluted C3, treatment of plasma with methylamine has been found to inactivate C3 [20,21].

The method presented here should be generally applicable to the study of thiol ester proteins, and it was found, that several proteins bound specifically, when chicken and rainbow trout serum were chromatographed on the matrix. These proteins, when analysed by SDS–PAGE under reducing conditions and by immunoblotting were found to comprise chicken $\alpha_2 M$, C3 and C4 and rainbow trout $\alpha_2 M$. Rainbow trout C3 and C4 might also have been present but did not cross-react with the antisera used.

In summary, the method may be useful for studying known thiol ester proteins and for identifying new members of this group of proteins.

References

[1] L. Sottrup-Jensen, in W.F. Putnam (Editor), The Plasma Proteins, Vol. V, Academic Press, New York, 1987, p. 191.
[2] T.C. Faries, J.P. Atkinson, Immunol. Today 12 (1991) 295.
[3] A.W. Dodds and A.J. Day, in K. Whaley, M. Loos and J.M. Weiler, (Editors), Complement in Health and Disease, Kluwer, Dordrecht, 1993, p. 39.
[4] B.F. Tack, R.A. Harrison, J. Janatova, M.L. Thomas, J.W. Prahl, Proc. Natl. Acad. Sci. USA 77 (1980) 5764.
[5] R.D. Campbell, J. Gagnon, R.R. Porter, Biochem. J. 199 (1981) 359.
[6] D.E. Isenman, J.R. Young, J. Immunol. 132 (1984) 3019.
[7] S.K.A. Law, A.W. Dodds, R.R. Porter, EMBO J. 3 (1984) 1819.
[8] M.K. Pangburn, H.J. Müeller-Eberhard, J. Exp. Med. 152 (1980) 1102.
[9] D.E. Isenman, D.I.C. Kells, N.R. Cooper, H.J. Müller-Eberhard, M.K. Pangburn, Biochemistry 20 (1981) 4458.
[10] M.K. Hostetter, M.L. Thomas, F.S. Rosen, B.F. Tack, Nature 298 (1982) 72.
[11] D.E. Isenman, D.I.C. Kells, Biochemistry 21 (1982) 1109.
[12] L. Sottrup-Jensen, J. Biol. Chem. 264 (1989) 11539.
[13] T. Kinoshita, Immunol. Today 12 (1991) 291.
[14] K.B.M. Reid, Immunologist 3 (1995) 206.
[15] U.K. Laemmli, Nature 227 (1970) 680.
[16] N.H. Axelsen, E. Bock, P. Larsen, S. Blirup-Jensen, P.J. Svendsen, K.J. Pluzek, O.J. Bjerrum, T.C. Bøg-Hansen and J. Ramlau, in N.H. Axelsen (Editor), Handbook of Immuno-precipitation-in-Gel Techniques, Blackwell, Oxford, 1983, p. 87.
[17] G. Houen, Methods Mol. Cell. Biol. 4 (1993) 105.

[18] J.S. Garvey, N.E. Cremer and D.H. Sussdorf, Methods in Immunology, Benjamin/Cummings, Reading, MA, 1977, p. 379.

[19] W. Barth, Biochim. Biophys. Acta 838 (1985) 361.

[20] T.E. Jessen, V. Barkholt, K.G. Welinder, J. Immunol. Methods 60 (1983) 89.

[21] M. Ploug, T.E. Jessen, K.G. Welinder, V. Barkholt, Anal. Biochem. 146 (1985) 411.

[22] A.W. Dodds, X.-D. Ren, A.C. Willis, S.K. Law, Nature 379 (1996) 117.

[23] S.K. Law, R.P. Levine, Proc. Natl. Acad. Sci. USA 74 (1977) 2701.

[24] S.K. Law, N.A. Lichtenberg, R.P. Levine, J. Immunol. 123 (1979) 1388.