Purification of complement components by hydrophobic affinity chromatography on phenyl–Sepharose

Purification of human C5

A. Al Salihi, J. Ripoche, L. Pruvost and M. Fontaine

Inserm U-78, 543 Chemin de la Bretèque, F-76230 Bois-Guillaume, France

Received 7 October 1982

Human complement components C5 and C3 were purified with 41% and 20% yields, respectively, by euglobulin precipitation, DEAE–Sephacel ion-exchange chromatography and gel filtration. Phenyl–Sepharose chromatography allowed the complete separation of C3 and C5. C3 bound loosely on the resin whereas C5 bound firmly and was eluted with 50% glycerin solution. Gel filtration on Sephacryl S-300 allowed the depletion of C4bp and H that contaminated C5 preparations. Homogeneity of C5 and C3 preparations was demonstrated by SDS–PAGE and immunochemical analysis. C5 and C3 consisted of two chains (α, 110000; β, 75000) linked by disulfide bridges.

Complement components C5 and C3 *Purification* *Affinity chromatography, hydrophobic*
Phenyl–Sepharose *Native C3* *Separation of C5*

1. INTRODUCTION

Purification of human complement components C3 and C5 is now well established [1–5], but the recent knowledge of some structure features of C3 molecule (i.e., presence of thiolester bond that could be easily hydrolyzed, leading to inactivated form of the molecule [6]) has pointed out the necessity to introduce further purification steps to eliminate inactivated products [6,7]. These new steps of purification decrease the recovery of the native protein and are time consuming. Here, we present fast procedures for the purification of human C5 and C3, using hydrophobic chromatography. Phenyl–Sepharose affinity chromatography permitted the complete separation of C3 and C5 and the recovery of pure native C3 thus eliminating further purification steps. Phenyl–Sepharose replaces advantageously the hydroxylapatite chromatography generally used to perform the separation of C5 from C3 preparations [1,5].

Abbreviations: EDTA, ethylene-diamine-tetraacetic acid; ϵACA, ϵ-amino-caproïc acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Nomenclature: components of complement are those recommended by World Health Organization (1982) Eur. J. Immunol. 668–669.

2. MATERIALS AND METHODS

All the chemicals were of analytical grade. Fresh human serum was obtained from the Centre Régional de Transfusion Sanguine (Bois-Guillaume) and was added with EDTA and ϵACA at 0.02 M and 0.05 M final concentrations (pH 7.2), respectively, and was immediately used or stored for a short period (1 month) at -80°C.

Haemolytic assays of human C3 and C5 were performed as in [8, 9].

Immuno-electrophoresis and immunodiffusion were done on microscopic slides covered with 1.3% Agar Noble (Difco) in 0.05 M veronal buffer (pH

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

8.6), using the following antisera: anti-whole human serum (Organon Teknika), anti-C5 (Meloy), anti-I (Kent), anti-C4bp (Dr Colomb, Grenoble), anti-C3c and anti-H (prepared in our laboratory).

Presence of H and I in purified fractions was further checked by haemagglutination and inhibition of immune adherence respectively, using EA $C\overline{1423}$ cells and O^+ human red cells.

Homogeneity of purified proteins was assessed by SDS–PAGE [10] using 5% (w/v) acrylamide gel for non-reduced samples and 7.5% (w/v) acrylamide gel for reduced samples. Reduction of samples was made by 5% (v/v) β-mercaptoethanol.

C3 and C5 were quantified by radial immunodiffusion using C3c partigen (Institut Behring) and anti-C5 immunodiffusion plates (Meloy) according to the manufacturer's instructions.

3. RESULTS

3.1. *Purification of C3/C5*

3.1.1. Euglobulin precipitation

Human serum treated with inhibitors (500 ml) was dialyzed twice against 10 l of 8 mM EDTA, pH 5.3 at 4°C. The precipitate was recovered by centrifugation at 8000 rev./min and washed twice by cold 8 mM EDTA (pH 5.3). The precipitate was redissolved in 1/5 of the starting volume of 0.15 M NaCl, 0.02 M EDTA, 0.05 M ϵACA (pH 7.2).

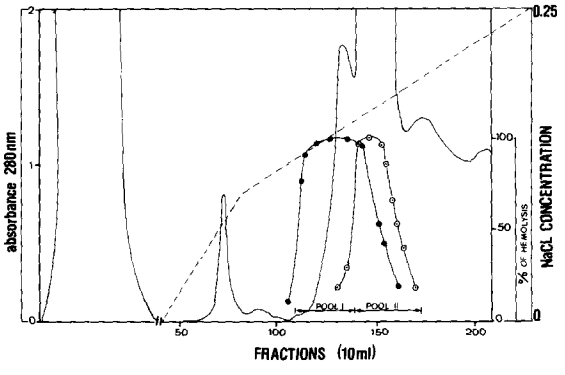

Fig. 1. Elution of complement components C3 and C5 from DEAE-cellulose: (●—●) C5 haemolytic activity; (⊙—⊙) C3 haemolytic activity; (———) A_{280}; (---) [NaCl].

3.1.2. DEAE-cellulose ion-exchange chromatography

The euglobulin fraction was diluted by adding distilled water to lower the conductivity to 3 mΩ (20°C) and applied onto a DEAE–Sephacel (Pharmacia) column chromatography (70 cm × 2.6 cm) equilibrated with 0.005 M Tris, 0.005 M EDTA, 0.01 M ϵACA (pH 7.3). The column was washed with the equilibrating buffer until A_{280} was null. Then, elution was carried out with a gradient of NaCl (0–0.25 M) presenting two linear steps (gradient Former, Gilson). In a typical experiment, 2 l of NaCl gradient were run through the column. Fractions of 10 ml were collected. Elution of proteins was monitored by the measure of A_{280} and elution of C3 and C5 was observed by haemolytic assays of C3 and C5 in the fractions (fig. 1). C5 eluted at a conductivity of 5 mΩ before C3 which appeared at a conductivity of 7 mΩ, but the two proteins partially overlapped. C5-rich, C3-poor fractions were pooled as indicated (pool 1) and were immediately used for the purification of C5. C3-rich, C5-poor fractions were pooled (pool 2), concentrated and stored at −80°C until used for C3 purification.

3.2. *Purification of C5*

3.2.1. Hydrophobic affinity chromatography

The C5-rich, C3-poor fraction (pool 1) was directly loaded on a phenyl–Sepharose (Pharmacia) column chromatography (30 cm × 2.6 cm) equilibrated with 0.15 M NaCl, 0.02 M EDTA and 0.05 M ϵACA (pH 7.2). C5 bound firmly to the column whereas the major part of the contaminant was eliminated on the breakthrough peak of the column. C5 was recovered by elution with a 50% glycerin solution in distilled water. The eluate was dialyzed intensively against 0.15 M NaCl, 0.02 M EDTA, 0.05 M ϵACA (pH 7.2) and concentrated by ultrafiltration on PM 30 (Amicon). At this point of the purification scheme, C5 represented 90% of the total protein and was contaminated with H, C4bp and albumin.

3.2.2. Gel filtration

C5 was separated from the impurities cited above by gel filtration on a Sephacryl S-300 (Pharmacia) column chromatography (1.5 cm × 100 cm) equilibrated with 0.15 M NaCl, 0.02 M EDTA, 0.05 M ϵACA (pH 7.2). C4bp and H were

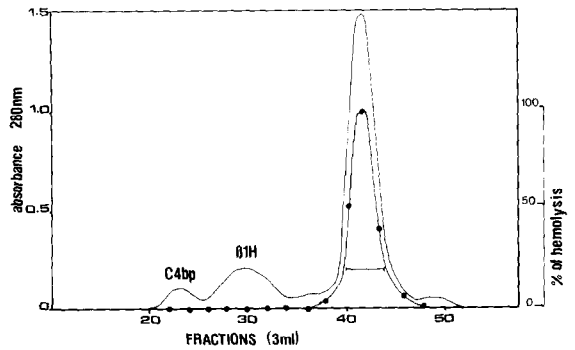

Fig. 2. Gel filtration of the post-phenyl–Sepharose C5-rich, C3-poor fraction (pool 1): (●—●) C5 haemolytic activity.

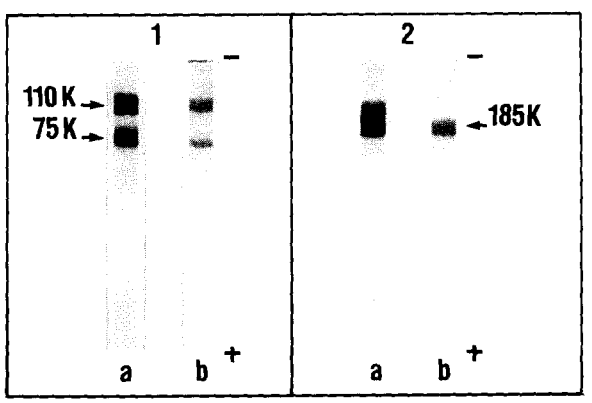

Fig. 3. SDS–PAGE of purified C5 and C3: (1) 7.5% acrylamide with 5% β-mercaptoethanol, (a) purified C3; (b) purified C5; (2) 5% acrylamide without β-mercaptoethanol, (a) purified C3, (b) purified C5.

eluted before C5 whereas albumin was eluted later (fig. 2). This procedure allowed the recovery of 25 mg pure C5 giving a 34% recovery of initial C5. The haemolytic activity per mg of C5 was constant throughout the purification procedure (2×10^5 CH 50 units/mg), indicating negligible inactivation of C5 during the course of purification. A typical C5 purification is illustrated by table 1. C5 was free of C3 (and of its degradation products), of H and C4bp, as demonstrated by haemolytic assay, haemagglutination assays and immunodiffusion. C5 exhibited a single band in SDS–PAGE of M_r 185000 and two bands under reducing conditions, α M_r 110000 and β M_r 75000 (fig. 3).

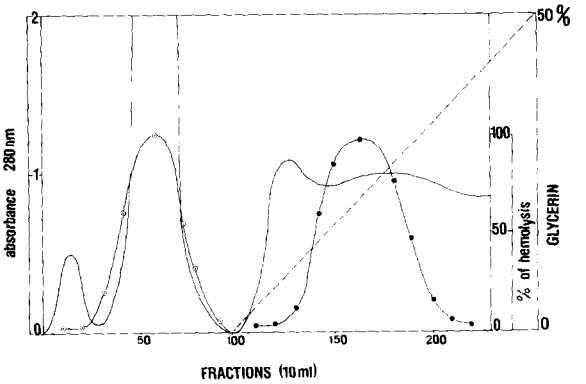

Fig. 4. Elution of complement component C3 from phenyl–Sepharose column chromatography: (———) A_{280}; (⊙—⊙) C3 haemolytic activity; (●—●) C5 haemolytic activity; (---) [glycerin] (%, v/v).

3.3. *Purification of C3*

The C3-rich, C5-poor fraction (pool 2) could be further purified by phenyl–Sepharose affinity chromatography using the above conditions. A representative elution pattern is depicted in fig. 4. The first peak, corresponding to the void volume of the column, contained the majority of impurities. C3 was retarded and appeared later. Material that bound firmly on phenyl–Sepharose was recovered by applying a glycerin gradient from 0–50% (v/v) in distilled water. Contaminant C5 appeared late in the gradient as shown by haemolytic monitoring of the column.

C3 recovered in the retarded peak was free of C5, H, I and C4bp. Furthermore, C3 was free of inactivated form such as C3b-like [11], as judged by immuno-electrophoresis in agar, that showed an homogeneous precipitin line, and by haemolytic criteria. C3b-like was recovered in the glycerin gradient since a protein of M_r 185000 built of two chains M_r 110000 and 75000, that reacted with the anti-C3c immuneserum, was recovered in the first peak of the gradient. This C3 fraction was devoided of haemolytic activity. This procedure allowed the recovery of ~20% of initial C3 (table 2).

The remaining C5 obtained in the glycerin eluate could be purified by gel filtration as in section 3.2.2. followed by a chromatography on a Sepharose anti-C3c to eliminate completely C3b-like contaminants. This procedure increased the recovery of C5 of 5–7% giving an overall yield of 40–42% for this protein.

Table 1

Summary of C5 and C3 purifications

	C5 mg	Yield	C5 CH50	Yield	C3 mg	Yield	C3 CH50	Yield
Serum	80.0	100.00%	1.70×10^6	100.00%	675	100.00%	5.40×10^6	100.00%
Euglobulin	60.0	75.00%	1.26×10^6	74.00%	386	57.00%	1.72×10^6	32.00%
DEAE–Sephacel								
pool 1	35.4	44.25%	0.71×10^6	41.70%	6.7	1.00%	n.d.	n.d.
pool 2	7.6	9.50%	1.02×10^5	6.00%	214	31.70%	1.40×10^6	26.00%
Pool 1								
Phenyl–Sepharose								
glycerin eluate	28.0	35.00%	0.58×10^6	34.30%	–	–	–	–
Pool 2								
Phenyl–Sepharose								
retarded fraction	–	–	–	–	130	19.20%	1.05×10^6	19.40%
glycerin eluate	4.5	5.60%	0.11×10^6	6.40%	n.d.	n.d.	0	–
Total recovery	32.5	40.60%	0.69×10^6	40.50%	130	19.20%	1.05×10^6	19.40%

[a]One unit CH50 was the reciprocal of C-dilution that would lyse 2.5×10^8 cells

4. DISCUSSION

One of the major difficulties encountered during the purification of complement component C5 is its separation from C3. C3 and C5 have the same M_r and similar charge. C3 is at least 10-fold more concentrated than C5 and therefore is a major contaminant in C5 purification. Generally, C3 and C5 are separated by chromatography on hydroxylapatite [2, 5]. This material is not easy to manipulate.

In [12–14] about the hydrophobicity of C3, we demonstrated the usefulness of phenyl–Sepharose to prepare some fragments of C3. Here, we showed that phenyl–Sepharose is a powerful tool for the purification of C5 and of its major contaminant C3. Hydrophobic chromatography was first used in complement technology in [5] with hexylamino-agarose resin. This resin was able to bind C3 and C5 but failed to separate the two components.

On the phenyl–Sepharose resin in physiological conditions of ionic strength and pH, C3 was only retarded but C5 bound firmly to the resin.

As an initial step for C5 purification, we chose the precipitation of the euglobulins. This precipitation allowed a good recovery of C5 (75%) but was less efficient for C3 ($\simeq$50%).

Using DEAE–Sephacel for ion-exchange chromatographic resin allowed a nearly complete separation of C3 and C5, as found in [3] as well. The two linear-step gradient permitted us to obtain a considerable enrichment of C3 and C5, but H, that eluted before C5, trailed at a low level all along the elution of C5 and C3, and was a potent contaminant for the two proteins.

After phenyl–Sepharose chromatography, C5 was contaminated by C4bp, H and albumin only. These proteins were easily removed by gel filtration on Sephacryl S-300. C4bp eluted in the void volume and appeared very pure. H eluted between C4bp and C5 exhibiting M_r 300000, as found in [15].

This four-step procedure allowed the recovery of $\simeq$35% of the initial C5 with a complete integrity of the haemolytic activity. In some cases, we observed the constant presence of a minute amount of albumin (detectable only by SDS–PAGE with β-mercaptoethanol) even after a chromatography on con A–Sepharose. In view of C5 structural data [16] showing the presence of a free thiol group, it was postulated that albumin might complex C5 through a disulfide bond as well as α-1-antitrypsin and IgA. This oxydation would be favoured by the concentration of C5 fractions during the isolating process.

This paper revealed that phenyl–Sepharose was very efficient for the purification of C3. C3 was purified in 3 steps only but with a rather low yield compared to the method in [3]. The initial step of purification was the major cause of this fact. As it

was expected, phenyl–Sepharose was able to discriminate between C3- and C3b-like molecules. We showed the possibility to separate C3 from C3b by phenyl–Sepharose chromatography [13]. C3b-like acquired the functional properties of C3b [11] and exhibited an enhancement of hydrophobicity [17]. These results indicated that C5 was certainly more hydrophobic than C3. This point is under investigation.

This procedure allowed the rapid recovery of C3 and C5 at a very high degree of purity and free of other complement components. We checked the presence of H, I and C4bp. Moreover, we obtained some mg pure C4bp. C3 and C5 consisted of two polypeptide disulfide-linked chains of M_r 110000 and 75000, as shown [2, 5].

ACKNOWLEDGEMENTS

This work was supported by the University of Rouen and INSERM (CRL no. 82.10.40). A. Al Salihi received financial support from the French Foreign office. We thank Drs M.G. Colomb and M.B. Villiers for generous gift of anti-C4bp and Mrs A. Chaube and Mrs M.M. Fontaine for the preparation of the manuscript.

REFERENCES

[1] Tack, B.F. and Prahl, J.W. (1976) Biochemistry 15, 4515–4521.
[2] Tack, B.F., Morris, S.C. and Prahl, J.W. (1979) Biochemistry 18, 1490–1497.
[3] Hammer, C.H., Wirtz, G.H., Renfer, L., Gresham, H.D. and Tack, B.F. (1981) J. Biol. Chem. 256, 3995–4006.
[4] Harrison, R.A. and Lachmann, P.J. (1979) Mol. Immunol. 16, 767–776.
[5] Kunkel, S.L., Kreutzer, D.L., Goralnick, S. and Ward, P.A. (1980) J. Immunol. Meth. 35, 337–351.
[6] Janatova, J., Tack, B.F. and Prahl, J.W. (1980) Biochemistry 19, 4479–4485.
[7] Parkes, C., Discipio, R., Kerr, M.A. and Prohaska, A. (1981) Biochem. J. 193, 963–970.
[8] Fontaine, M., Joisel, F. and Dumouchel, L. (1980) J. Immunol. Methods 33, 145–148.
[9] Al Salihi, A., Joisel, F. and Fontaine, M. (1982) Ann. Immunol. (Inst. Pasteur) 133C, 299–307.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1981) J. Exp. Med. 154, 856–867.
[12] Fontaine, M., Joisel, F. and Lebreton, J.P. (1980) FEBS Lett. 111, 148–151.
[13] Fontaine, M., Daveau, M. and Lebreton, J.P. (1980) Mol. Immunol. 17, 1075–1078.
[14] Fontaine, M., Aubert, J.P., Joisel, F. and Lebreton, J.P. (1982) Mol. Immunol. 19, 27–37.
[15] Whaley, K. and Ruddy, S. (1976) J. Exp. Med. 144, 1147–1163.
[16] Discipio, R. (1981) Biochem. J. 199, 497–504.
[17] Isenman, D.E., Kells, D.I.C., Cooper, N.R. H.J. and Pangburn, M.K. (1981) Biochemistry 20, 4458–4467.