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A general method for affinity purification of complement component C3b using factor H–Sepharose

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Complement component C3b has been purified from human, rabbit and bovine serum by affinity chromatography on human factor H–Sepharose after preliminary fractionation by poly(ethylene glycol) and DEAE-Sepharose. The yields are high (35–40%) and the whole process is rapid (3 days). Binding of C3b to factor H–Sepharose is equimolar, has a sharp optimum pH at 7.6 and is quite sensitive to ionic strength.

Complement component C3 is often considered to be the most important of the complement components because of its central position in the complement reaction pathways. It can be activated by either the classical or alternative pathways, and it contributes to the formation of the C5 convertase, thus activating the membrane-attack pathway [for reviews, see Fothergill & Anderson, (1978) and Reid & Porter (1981)].

The plasma form of C3 contains two disulphide-bridged polypeptide chains, α (M_r 116000) and β (M_r 70000), generated by proteolysis from a single chain precursor (Brade *et al.*, 1977). Activation of C3 is brought about by proteolytic cleavage of a single peptide link 77 residues from the *N*-terminus of the α-chain, liberating the anaphylatoxin C3a (Hugli & Müller-Eberhard, 1978) and leaving the α′-chain disulphide-linked to the unaltered β-chain. Concomitantly with activation an internal thioester bond in the α′-chain is cleaved, giving rise to a free thiol group and a reactive acyl group (Tack *et al.*, 1980; R. B. Sim *et al.*, 1981). In this respect C3 shows homologies with C4 (Campbell *et al.*, 1981; Harrison *et al.*, 1981) and to a lesser extent with α_2-macroglobulin (Swenson & Howard, 1980). There are also strong sequence homologies between C3a and the corresponding activation fragments of C4 (Moon *et al.*, 1981; Smith *et al.*, 1982) and C5 (Hugli & Müller-Eberhard, 1978).

After activation of C3, the large fragment, C3b, is capable of binding to a number of other complement components. It can bind to either of the C3 convertases that were responsible for its production via the classical or alternative pathways, thereby generating an enzyme of altered specificity that is now able to activate C5 and hence the membrane-attack pathway. C3b also binds factor B or its activated form Bb to generate the C3 convertase of the alternative pathway, thereby giving rise to the 'positive-feedback' aspect of the alternative pathway (Lachmann & Nicol, 1973). As part of the control mechanism of complement activation, C3b can also bind to factor H (previously known as β_1H or C3b inactivator accelerator), that is required for destruction of C3b by the proteolytic enzyme factor I (previously known as C3b inactivator or KAF) (Whaley & Ruddy, 1976) leading to the formation of fragments C3c and C3d (Harrison & Lachmann, 1980; E. Sim *et al.*, 1981). Binding of C3b to factor P (properdin) has the opposite effect, stabilizing the C3 convertase (Medicus *et al.*, 1976). Binding constants of C3b with its various ligands have been determined (Di Scipio, 1981), and factor H has one of the largest.

Because of its central importance, C3b is the subject of many investigations. We wished to develop a method of purification that would allow isolation of reasonably large quantities of C3b from a range of different species, without having to explore the details of non-specific methods of purification in each case. An affinity procedure, using factor H covalently linked to Sepharose, has been developed. Initial fractionation by poly(ethylene glycol) precipitation (Tack & Prahl, 1976) and a rapid ion-exchange step on DEAE-Sepharose is followed by binding to human factor H–Sepharose and elution with a salt gradient. Pure C3b in yields of 35–40% has been obtained in 3 days from human, bovine and rabbit serum.

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968, 1981). SDS, sodium dodecyl sulphate; PMSF, phenylmethanesulphonyl fluoride; PEG, poly(ethylene glycol) 4000.

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Materials and methods

Materials

Fresh human blood was collected from colleagues (with their informed consent) in the Department of Biochemistry by the North East of Scotland Blood Transfusion Service at Aberdeen Royal Infirmary, Aberdeen, U.K. Fresh bovine blood was obtained from W. Donald and Son, Portlethen, Kincardineshire, U.K. Fresh rabbit blood was removed from anaesthetized laboratory rabbits by cardiac puncture.

Other sources of materials were as follows: DEAE-Sepharose CL-6B and Sepharose 4B from Pharmacia, Hounslow, Middx., U.K.; PMSF, 6-aminohexanoate and Coomassie Brilliant Blue R from Sigma, Poole, Dorset, U.K.; PEG 4000, EDTA, EGTA, $MgCl_2$, SDS, polyacrylamide and *NN'*-methylenebisacrylamide from BDH Chemicals Ltd., Poole, Dorset, U.K.; LSL agarose from Litex, Windsor Laboratories Ltd., Slough, Berks., U.K.; GelBond from F.M. Corp., Rockland, ME, USA.

Methods

SDS/polyacrylamide-gel electrophoresis. Slab 7.5% (w/v)-polyacrylamide gels in buffers containing SDS were run as described by Laemmli (1970). Protein samples were stained with Coomassie Blue and destained with methanol/acetic acid/water (2:3:35, by vol.). When antisera were not available, C3b was measured by gel scanning using bovine serum albumin as internal standard.

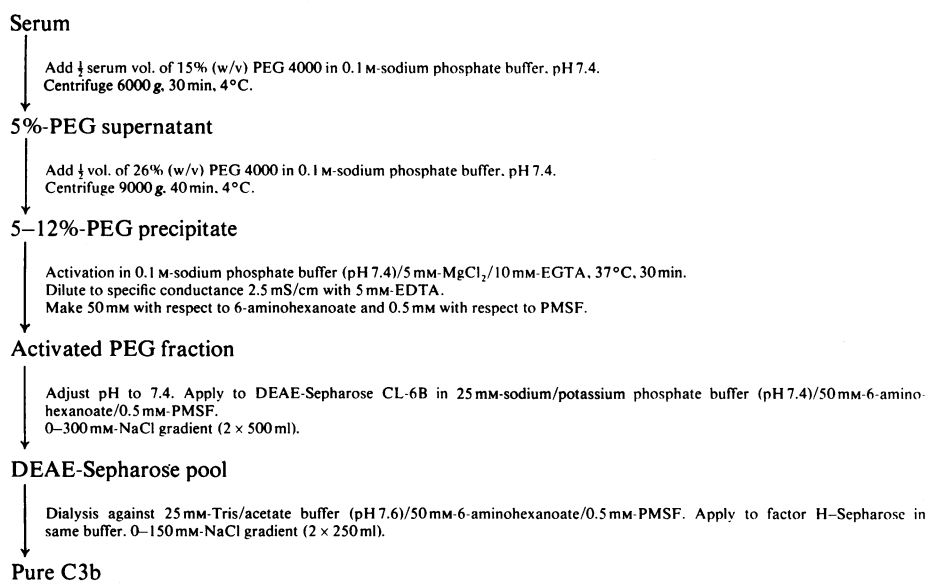
Protein determinations. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunological methods. Immunodiffusion analysis was carried out by the method of Ouchterlony (1958), as described by Campbell *et al.*, (1979). Rocket immunoelectrophoresis was carried out by a modification to the method of Laurell (1966) described by Weeke (1973). Agarose [1% (w/v)] in barbital buffer, pH 8.6, containing 2 mM-EDTA with 0.02% rabbit antiserum to human C3 was poured on to a 10 cm × 15 cm glass plate covered with 0.2 mm-thick GelBond. Electrophoresis was performed at 4–5 V/cm for 15 h. A standard calibration curve (0.05–0.5 μg) was produced from diluted human serum, assuming the C3 concentration to be 1300 μg/ml. An internal standard of purified C3b (0.5 μg) was run on each plate.

Human factor H. This was purified to homogeneity as described by Sim & Sim (1981); 150 mg were obtained from 2 litres of human serum.

Factor H–Sepharose. This was prepared by a modification of the method of Cuatrecasas *et al.* (1968). Sepharose 4B (50 ml) was mixed with 50 ml of water and 5 g of CNBr. The solution was constantly stirred and the pH maintained at approx. 11 by the dropwise addition of 4 M-NaOH. When the reaction was completed (i.e. when the pH remained constant), the Sepharose was thoroughly washed with 0.1 M-$NaHCO_3/Na_2CO_3$ buffer, pH 9.0. The Sepharose was then resuspended in 0.1 M-$NaHCO_3/Na_2CO_3$, pH 9.0 (50 ml), and 34 mg of factor H in 6.8 ml of the same buffer was added quickly. This mixture was stirred gently at 4°C for 24 h, after which it was washed extensively with buffer and water before storage in 25 mM-Tris/acetate (pH 7.6)/50 mM-6-aminohexanoate/0.5 mM-PMSF/0.02% NaN_3.

Purification of C3b. The purification procedure is shown in Scheme 1. Fresh blood was allowed to clot for 1 h at 37°C and the clot was removed by centrifugation at 2250 ***g*** for 30 min at 4°C. Serum (100 ml) was immediately made 5% with respect to PEG 4000 by the addition of 50 ml of 15% (w/v) PEG 4000 in 0.1 M-sodium phosphate, pH 7.4. This solution was stirred slowly for 30 min at 4°C then centrifuged at 6000 ***g*** for 30 min. The supernatant (150 ml) was made 12% with respect to PEG 4000, by the addition of 75 ml of 26% (w/v) PEG 4000 in 0.1 M-sodium phosphate, pH 7.4. The solution was stirred slowly at 4°C for 30 min and the precipitate removed by centrifugation at 9000 ***g*** for 40 min at 4°C. The precipitate was immediately resuspended in 25 ml of 0.1 M-sodium phosphate, pH 7.4, at room temperature. Activation of the complement components was achieved by heating at 37°C for 30 min in the presence of 5 mM-$MgCl_2$ and 10 mM-EGTA, to favour the alternative pathway of activation (Lachmann & Hobart, 1978; Mann *et al.*, 1981). Activation was stopped by the addition of PMSF dissolved in propan-2-ol (20 mg/ml) to give a final concentration of 0.5 mM. The sample was diluted to a specific conductance of 2.5 mS/cm by the addition of cold 5 mM-EDTA. The solution was made 50 mM with respect to 6-aminohexanoate and 0.5 mM with respect to PMSF. The pH was adjusted to 7.4 by the addition of 1 M-NaOH. The diluted sample (approx. 400 ml) was loaded immediately and as quickly as possible (200 ml/h) on to a column of DEAE-Sepharose CL-6B (5 cm × 7 cm) equilibrated in 25 mM-sodium potassium phosphate (pH 7.4)/10 mM-EDTA / 50 mM-6-aminohexanoate / 0.5 mM-PMSF. After washing (90 ml/h) with starting buffer, a linear gradient (2 × 500 ml) of 0–300 mM-NaCl in the same buffer was applied (90 ml/h). Fractions containing C3b, located by SDS/polyacrylamide-gel electrophoresis and, for human C3b, by rocket immunoelectrophoresis were pooled and dialysed against 2 litres of 25 mM-Tris/acetate (pH 7.6)/50 mM-6-aminohexanoate/0.5 mM-PMSF for 12 h with three changes of buffer. After dialysis, the protein solution (190 ml) was applied (45 ml/h) to an affinity column (1.5 cm × 15 cm) of factor H–



Scheme 1. *Summary of the C3b purification procedure*

Sepharose 4B equilibrated with 25 mM-Tris/acetate (pH 7.6)/50 mM-6-aminohexanoate/0.5 mM-PMSF. After washing (90 ml/h) with starting buffer, a linear gradient (2 × 250 ml) of 0–150 mM-NaCl in the same buffer was then applied (90 ml/h). Fractions containing C3b were pooled, dialysed against 2 litres of 1% (w/v) NH_4HCO_3 and freeze-dried for storage.

Determination of binding capacity of factor H–Sepharose. Sepharose (7.5 ml) coupled to factor H (0.59 mg of factor H/ml of Sepharose gel) was equilibrated in 25 mM-Tris/acetate (pH 7.6)/0.5 mM-PMSF and packed into a small column (1 cm × 9.5 cm). Human C3b (10 mg) in 5 ml of the same buffer was applied and the column washed until the A_{280} returned to zero. The protein was eluted with 150 mM-NaCl in the same buffer. The amount of C3b eluted was determined by rocket immunoelectrophoresis.

Optimum pH of C3b/factor H–Sepharose interaction. Samples of human C3b (10 mg in 5 ml) were loaded on to the small column of factor H–Sepharose. The samples and columns were equilibrated in 25 mM-Tris/acetate buffers over a pH range from 4 to 9. The elution and measurement of C3b were as described above.

Results

Purification of human C3b

A summary of the purification of human C3b is given in Table 1. After precipitation with PEG 4000, 80% of the C3 (as determined by rocket immunoelectrophoresis) was found in the 5–12%-PEG fraction. Activation of C3 was carried out initially in whole serum, but low final yields (10%) were obtained, owing to enzymic degradation of C3b. Activation after the PEG fractionation gave much higher yields. Chromatography on DEAE-Sepharose separates C3b from factor I, thus minimizing enzymic degradation. Elution of C3 from the ion-exchange column (Fig. 1) was monitored by SDS/polyacrylamide-gel electrophoresis and immunodiffusion. The recovery of C3 measured by rocket immunoelectrophoresis was 57%. C3c is eluted earlier in the salt gradient at a conductance of 4.3 mS/cm and could be detected immunochemically and electrophoretically.

When the C3-containing pool was applied to the factor H–Sepharose column, any unactivated C3 did not bind and could be detected in the eluate after one column volume. When the gradient was applied, a single major peak of protein containing only C3b (Fig. 2) was eluted at a conductance of 8.5 mS/cm.

Purification of rabbit and bovine C3b

A summary of the purification of rabbit and bovine C3b is shown in Table 2. Antisera to these proteins were not available, so characterization was solely by SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions. The results were similar to those obtained with human C3b at all stages of the purification, indicating that human factor H is a good ligand for both rabbit and bovine C3b.

Table 1. *Purification of human C3b*

Purification step	Volume (ml)	Total protein (mg)	C3b (mg)	Yield (%)	Purification factor
Serum	100	10300	122	100	1
5–12%-PEG ppt.	385	2190	97	80	3.7
DEAE-Sepharose	190	186	69	57	31
Factor H–Sepharose	250	51	51	42	84

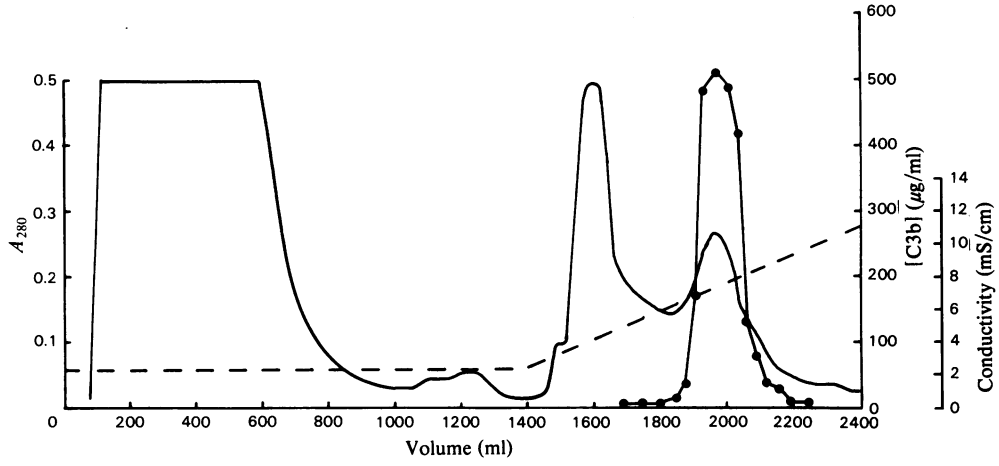


Fig. 1. *Preliminary fractionation of 5–12%-PEG precipitate by gradient elution from DEAE-Sepharose CL-6B*
Experimental details are given in the text. C3b, detected by rocket immunoelectrophoresis or SDS/polyacrylamide-gel electrophoresis (●) was eluted by a salt gradient (– – –). The A_{280} (——) was off-scale initially, but was brought close to zero by washing with starting buffer before the gradient was applied.

Table 2. *Purification of rabbit and bovine C3b*
Abbreviation used: ND, not determined.

Purification step	Volume (ml)	Total protein (mg)	C3b (mg)	Yield (%)	Purification factor
Rabbit					
Serum	65	5850	85	100	1
5–12%-PEG ppt.	325	1440	ND	ND	ND
DEAE-Sepharose	120	375	ND	ND	ND
Factor H–Sepharose	210	31	29	34	79
Bovine					
Serum	100	11400	130	100	1
5–12%-PEG ppt.	400	2670	ND	ND	ND
DEAE-Sepharose	165	147	ND	ND	ND
Factor H–Sepharose	235	48	45	35	82

Characterization of C3b

SDS/polyacrylamide-gel electrophoresis of C3b from human, rabbit and bovine serum showed a single band ($M_r \sim 180000$) under non-reducing conditions and two bands ($M_r \sim 105000$ and 70000) under reducing conditions (Fig. 3). No other components were detectable. By Ouchterlony immunodiffusion, human C3b showed only a single line against anti-(whole human serum): neither rabbit

nor bovine C3b showed any reaction with anti-(human C3).

Determination of binding capacity of factor H–Sepharose

When excess human C3b (10mg) was passed through the factor H–Sepharose column containing 4.4mg of factor H, the amount of C3b bound was 4.0mg. The binding capacity of the factor H–Sepharose is thus 0.91mg of C3b per mg of factor H, a molar ratio of C3b to factor H of 0.77.

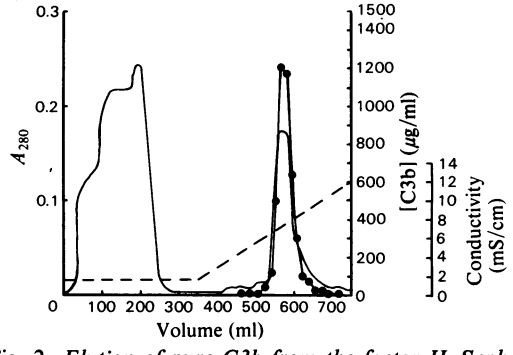


Fig. 2. *Elution of pure C3b from the factor H–Sepharose column*

Experimental details are given in the text. After the sample was applied, the column was eluted with starting buffer until A_{280} (——) returned to zero. A salt gradient (– – – –) then eluted C3b, detected by rocket immunoelectrophoresis or SDS/polyacrylamide-gel electrophoresis (●).

Optimum pH for C3b/factor H–Sepharose interaction

Fig. 4 shows the variation with pH of the binding of human C3b to factor H–Sepharose. The optimum pH is 7.6, where 0.54mg of C3b bound per ml of factor H–Sepharose. Binding of C3b decreases quite rapidly on each side of the maximum. Because of the marked effect of ionic strength on binding, the conductivity of the buffers was kept reasonably constant over the pH range and was well below that required for elution at pH 7.6.

Discussion

This method produces good yields of C3b in a short time. Once the factor H–Sepharose column has been prepared, 50mg of C3b can be readily obtained from 100ml serum in 3 days. The factor H–Sepharose column can be used repeatedly, and has shown no degeneration over several months' use when kept at 4°C in the pH 7.6 buffer.

There are two main reasons why factor H was chosen as the affinity ligand. It has one of the highest affinities for C3b (Di Scipio, 1981) and can be obtained in reasonably large quantity without difficulty (serum concentration ~300μg/ml). It also retains biological activity after chemical linking to Sepharose, and is remarkably stable in this form. One disadvantage is that C3b is susceptible to proteolysis by factor I when bound to factor H. To avoid this problem we took simple steps to separate factor I from C3b before using the factor H column. However, this property of the factor H column could

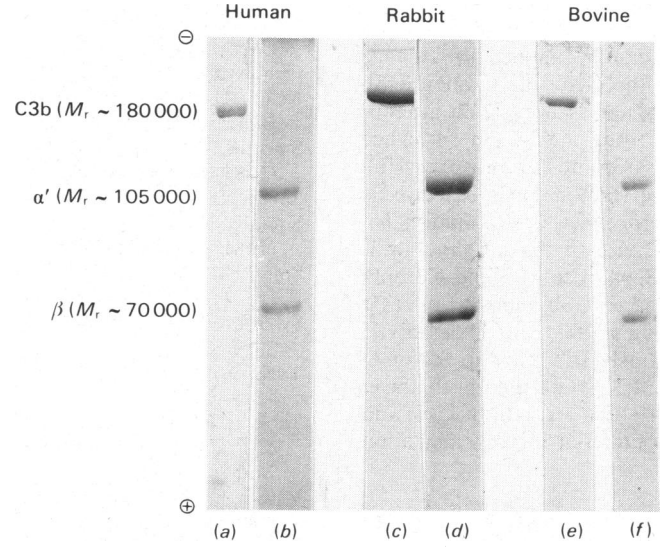


Fig. 3. *SDS/polyacrylamide-gel electrophoresis of purified C3b*

Gels (a) and (b) show human C3b, (c) and (d) rabbit C3b, (e) and (f) bovine C3b. Samples (b), (d) and (f) were run after reduction; samples (a), (c) and (e) were not reduced.

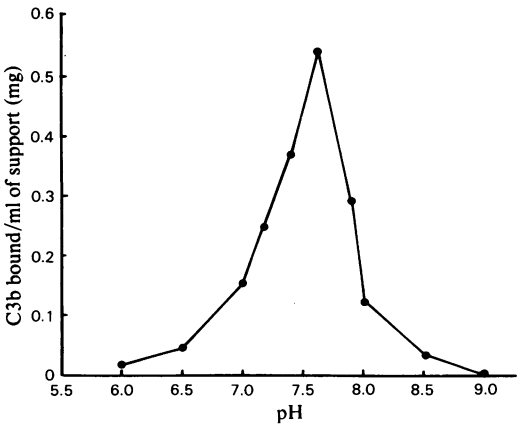


Fig. 4. *Dependence on pH of binding of human C3b to the factor H–Sepharose column*
Experimental details are given in the text. C3b was detected by rocket immunoelectrophoresis.

be useful in an investigation of the action of factor I on C3b when bound to factor H.

The molar binding of C3b to factor H–Sepharose was found to be 0.77. This is sufficiently close to unity to suggest that C3b and factor H normally interact in equimolar ratio. The pH-dependence of the binding shows a sharp maximum at physiological pH values, with marked decreases on each side. The interaction is also quite sensitive to ionic strength, showing complete binding at a conductance of 2.0 mS/cm, but complete loss of binding at 8.5 mS/cm.

One of the major advantages of this purification method is that, by using a single column of human factor H–Sepharose, it can be applied to a range of species. The yield with bovine and rabbit C3b was only marginally reduced. However, an attempt at purification of C3b from the serum of plaice (*Pleuronectes platessa*) was completely unsuccessful, suggesting that the C3b–factor H interaction is limited in its species coverage, perhaps to mammals.

Another important feature of the affinity purification is that factor H–Sepharose will bind C3 only after it has been activated to C3b. This means that the purified C3b is unsuitable for haemolytic studies, although it obviously still binds to factor H and is sensitive to factor I. C3b purified in this way provides adequate material for structural work and could produce sufficient material for crystallization studies.

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