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Isolation of human mannan binding lectin, serum amyloid P component and related factors from Cohn Fraction III

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SUMMARY. Cohn Fraction III, a waste product of plasma fractionation, is a source of several proteins involved in host defence as part of the innate immune system. Here a procedure is described for purifying mannan binding lectin and serum amyloid P component to virtual homogeneity from Cohn Fraction I + III, and their separation from other mannan-binding factors and from C-reactive protein. It is suggested that products derived from Cohn Fraction III could have therapeutic potential, either for specific substitution therapy or as a combined package of innate immune factors.

Key words: Cohn Fraction III, mannan binding lectin, plasma fractionation, serum amyloid P.

Human plasma contains collectins, pentraxins and other factors which might be important in innate immunity and therefore might have therapeutic potential as blood products (Kilpatrick, 1995). The significance of the pentraxins is difficult to assess in the absence of deficiency states, but the use of a transgenic mouse model has provided unequivocal evidence that C-reactive protein (CRP) makes an important contribution to host defence against pneumococcal infections (Szalai *et al*., 1995), while serum amyloid P component (SAP) also has the properties of an opsonin (Brown & Anderson, 1993; Ying *et al*., 1993). In contrast, mannan binding lectin (MBL: also called mannan or mannose binding protein), the major plasma collectin, is low or absent in some individuals, and this relative deficiency has been associated with various infections not only in infancy but also in older children and even adults (Sumiya *et al*., 1991; Garred *et al*., 1995, 1997; Nielsen *et al*., 1995; Summerfield *et al*., 1995, 1997; Hill *et al*., 1996). Indeed, awareness of the possible importance of MBL in first-line defence has increased greatly in the last few years, and already at least one patient has been treated with substitution therapy (Jensenius, 1995).

It was previously reported that MBL is significantly enriched relative to plasma in Scottish Cohn Fraction III (Kilpatrick *et al*., 1997), a waste product of IgG production by plasma fractionation (Fig. 1). Here a procedure is described for purifying MBL to virtual homogeneity from Cohn Fraction III and its separation from other mannan binding factors. This scheme also results in the isolation of SAP component, and the partial purification of another Ca^{2+}-dependent, N-acetylglucosamine (GlcNAc)-specific lectin of uncertain identity. The latter adds to the growing literature on GlcNAc-specific plasma proteins which includes a human analogue of bovine conglutinin (Baatrup *et al*., 1987; Thiel *et al*., 1987); P35, which has both collagen-like and fibrinogen-like domains (Matsushita *et al*., 1996); and the related proteins, S1 and S2, which differ in their detailed specificities for complex carbohydrates (Rapoport *et al*., 1996).

MATERIALS AND METHODS

Cohn Fraction III was obtained from the Scottish National Blood Transfusion Service Protein Fractionation Centre in the form of a Fraction I + III paste (Fig. 1). Murine monoclonal antibody to MBL was purchased from Serotec Ltd, Oxford, UK. Murine monoclonal antibodies to P35 (Matsushita *et al*., 1996) were a kind gift from Dr Matsushita. Chicken polyclonal antiserum to bovine conglutinin (Baatrup *et al*., 1987) was kindly given by Professor J. C. Jensenius, Aarhus, Denmark.

The affinity matrix, 3M Emphaze was purchased from Pierce & Warriner (Chester, UK) and coupled to yeast mannan (Sigma) based on manufacturer's guidelines. Briefly, Emphaze powder (7 g) was swollen in 112 mL of 0·5 M trisodium citrate/0·1 M-MOPS, pH 8·1, in which mannan (700 mg) had been dissolved. The reaction was allowed to proceed for 1 h at room temperature. After aspirating excess mannan solution, any remaining reactive groups in the Emphaze were blocked with 3M-ethanolamine, pH 9·0. After use, the gel was regenerated by elution with 2M-NaCl in 0·1 M glycine–HCl, pH 2·5.

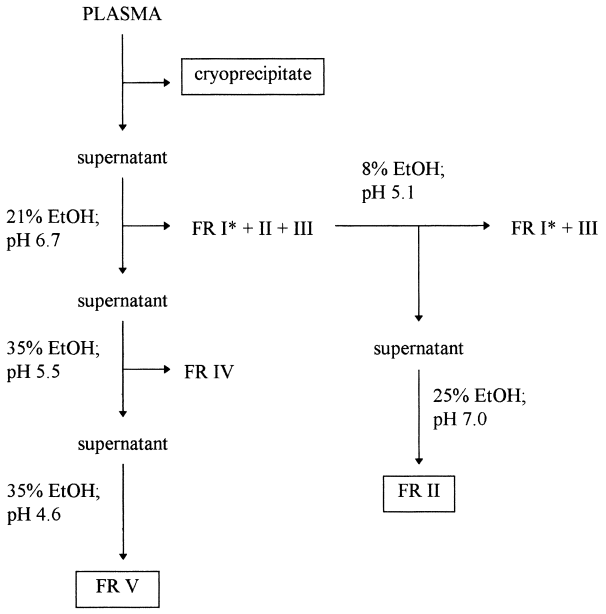

Fig. 1. Simplified outline of protein fractionation. Cohn fractionation of plasma (Cohn *et al*., 1946) depends on ionic strength, pH, temperature and ethanol (EtOH) concentration. This outline illustrates the two pathways followed to obtain end-products albumin (Fraction V) and immunoglobulin G (Fraction II). A third therapeutic product, cryoprecipitate, is obtained before Cohn fractionation and contains proteins that would otherwise form part of Fraction I; the latter has been given an asterisk to denote that distinction.

Purification of MBL

Frozen Cohn Fraction I + III paste (500 g) was allowed to thaw overnight in 2 L of 0·02 M Tris/0·1 M NaCl, pH 8·0, containing 0·02% sodium azide. The suspension was clarified by centrifugation before ammonium sulphate was added to 42% saturation. The resulting precipitate was collected by centrifugation and redissolved in 0·02 M Tris/0·5 M NaCl/0·01 M $CaCl_2$, pH 8·0, containing 0·02% sodium azide (Column Buffer) and dialysed against the same. Insoluble material was removed by centrifugation and the clear solution (900 mL) applied to a column (18 cm × 1·6 cm diameter) of Emphaze-mannan. After extensive washing with Column Buffer, the calcium-dependent binding proteins were eluted with 10 mM EDTA in Ca^{2+}-free Column Buffer. Fractions (10 mL) forming a peak of A_{280} absorbing material were pooled. This first EDTA eluate was recalcified by the addition of 50 mM $CaCl_2$ and was extensively dialysed against Column Buffer. It was then applied to a second, smaller (7 cm × 1·6 cm diameter) column of Emphaze–mannan, prepared after the first column had been regenerated and the acid eluate retained for analysis. The column was sequentially eluted with 50 mL of (1) 100 mM mannose, (2) 100 mM N-acetylglucosamine (GlcNAc) and (3) 10 mM EDTA, with washes of Column Buffer between each elution. All operations were carried out at room temperature.

Assays

Mannan binding lectin was measured by ELISA as previously described (Kilpatrick *et al*., 1995). This ELISA was modified as follows to measure relative activity towards human P35 and bovine conglutinin: by diluting samples initially into the ELISA buffer adjusted to pH 8·0; by substituting mouse anti-P35 at 1/700 dilution and chicken anticonglutinin at 1/500 dilution, respectively, instead of anti-MBL; and by substituting alkaline phosphatase-conjugated antimouse IgG and antichicken IgG, respectively, both at 1/100 dilution. Protein was estimated by absorbance at 280 nm or by the Folin method of Lowry *et al*. (1951). Total IgG, IgM, IgA and C-reactive protein were measured by rate nephelometry (Price *et al*., 1983) on a Beckman Array Protein System using Beckman antisera.

Electrophoresis and double diffusion

Electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS–PAGE) and Ouchterlony double diffusion were carried out as previously described (Kilpatrick *et al*., 1984), using commercial rabbit antiserum to C-reactive protein and serum amyloid P component (Calbiochem-Novabiochem UK, Nottingham, UK) and sheep antisera to human immunoglobulins G, A and M obtained from the Scottish Antibody Production Unit, Carluke, UK.

RESULTS

Purification of MBL

A necessary preliminary step is ammonium sulphate precipitation after reconstitution of the Fraction I + III paste in a Ca^{2+}-free buffer. This avoids the formation of an unmanageable gel and has the additional benefit of decreasing the volume. When the scheme described in the Methods section is followed, the MBL is eluted with mannose from the second (smaller) affinity column. A representative set of purification data is given in Table 1; typically a purification ratio of around 10 000 is obtained with a recovery of around 60%. By this means, 5 mg of MBL can be obtained from 500 g of Fraction I + III.

The isolated MBL yields a single Coomassie Blue staining band of apparent M_r 28 000 when analysed by SDS–PAGE at low to medium loadings. At heavier loadings, less intense bands of higher M_r are apparent,

Table 1. Purification of MBL

Step	Total activity ($\times 10^{-6}$ units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
1. Crude extract	2·8	94×10^3	29·8	–	
2. Ammonium sulphate precipitate	3·0	90×10^3	33·3	1·1	107
3. First EDTA eluate	2·1	20·5	$1{\cdot}02 \times 10^5$	3423	75
4. Mannose eluate	1·8	5·2	$3{\cdot}46 \times 10^5$	11610	64

Activity refers to reactivity by ELISA in arbitrary units as described in Kilpatrick *et al*. (1995). Each unit corresponds to 0·14 μg of purified, native MBL.

but these are variable in intensity on each occasion and may represent incomplete detergent solubilization (Figs 2 and 3).

Purification of SAP

The major protein eluted from the affinity column with EDTA, which tends to form a precipitate during dialysis, has an apparent M_r of 23 000 on SDS–PAGE. After isolation of MBL with mannose, the material subsequently eluted with EDTA consists mainly of this 23k band with a few minor extra bands of which a 66k band is most prominent. By eluting the column with N-acetylglucosamine after mannose, subsequent elution with EDTA yields a virtually pure preparation of the 23k protein, which reacts strongly with antiserum to SAP (Table 2). This procedure yields $\approx$ 15 mg SAP per 500 g of Fraction I + III, although a variable but significant amount is lost through precipitation.

Other mannan-binding factors

The starting Fraction I + III extract contained IgG (13 g L^{-1}), IgA (5 g L^{-1}) and IgM (3·7 g L^{-1}). Although most of the immunoglobulin content of the Fraction I + III extract does not bind to the immobilized mannan column, traces of IgG and IgM can be detected serologically after elution with EDTA, and indeed IgM contaminates the purified SAP preparation (Table 2).

A separate immunoglobulin preparation is recovered by washing the EDTA-eluted affinity column with 2 M NaCl in glycine–HCl buffer, pH 2·5. This appears rich in IgM, since the most prominent bands on SDS–PAGE analysis correspond to M_r values of 75k and 24k (Fig. 3) and there is a strong reaction with anti-IgM serum. Approximately 1·5 mg protein is obtained per 500 g of Fraction I + III. This preparation also contains IgG.

The protein eluted from the second affinity column with N-acetylglucosamine after MBL had previously

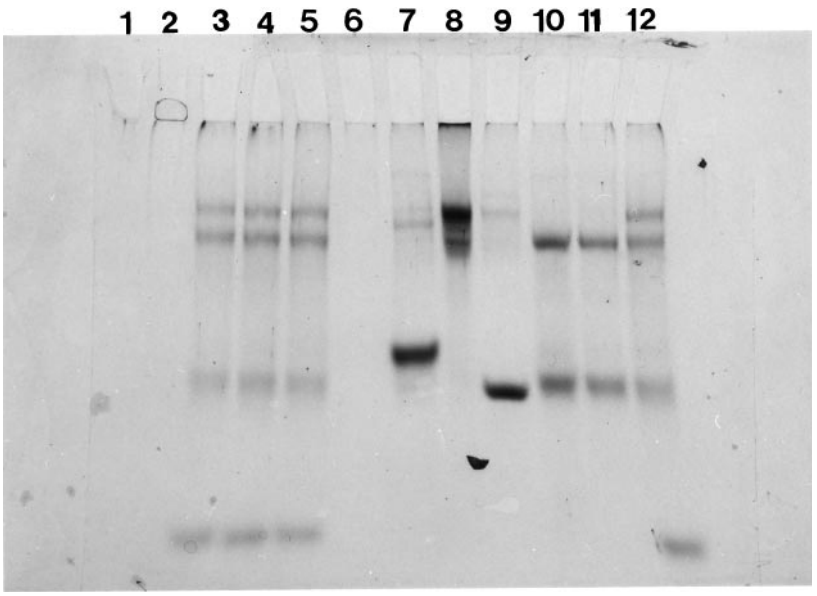

Fig. 2. SDS–PAGE of purified mannan-binding proteins. Proteins sequentially eluted from the affinity column with mannose (lane 7), N-acetylglucosamine (lane 8) and EDTA (lane 9) are compared to molecular weight markers consisting of IgG (50k & 24k; lanes 10 & 11) or a mixture of BSA (66k), IgG and cytochrome c (12k) in lanes 3, 4, 5 and 12.

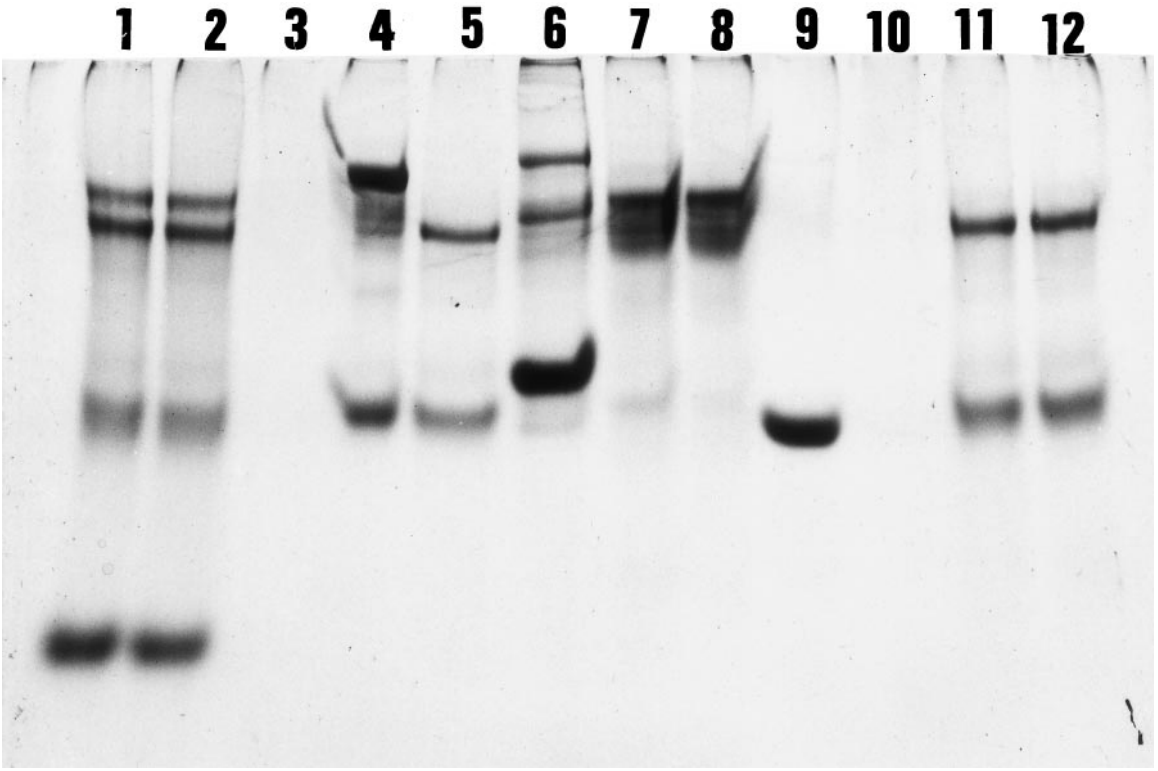

Fig. 3. SDS–PAGE of mannan binding proteins-2. Proteins sequentially eluted from the second affinity column with mannose (lane 6), galactose (lane 7), N-acetylglucosamine (lane 8) and EDTA (lane 9) are shown with the acid (pH 2·5) eluate from the first affinity column (lane 4). The molecular weight markers used were a mixture of BSA, IgG and cytochrome c (lanes 1 & 2) or just IgG (lanes 5, 11 & 12).

been removed with mannose yields at least two or three bands on SDS–PAGE analysis, of which the most intense migrates with an apparent M_r of 66 000 (Fig. 2). This eluate possessed only traces of MBL activity by ELISA, and did not react with antisera to SAP, IgA, IgG or IgM (Table 2). In a variation of the purification procedure, on one occasion a galactose (100 mM) elution step was included between the mannose and N-acetylglucosamine elutions. The galactose elution gave a product with an apparently identical pattern on SDS–PAGE (Fig. 3).

ELISA methods are under development to detect P35 (Matsushita *et al*., 1996) and the human conglutinin analogue (Thiel *et al*., 1987). Preliminary results indicate that both activities are present in the Fraction I + III extract; possibly some conglutinin activity, but no P35 activity, was detected in the fraction eluted from the affinity column with N-acetylglucosamine.

C-reactive protein

C-reactive protein was present in the crude extract at a concentration of $2\,\mu g\,mL^{-1}$. (The sensitivity limit of the nephelometric assay is $0{\cdot}5\,\mu g\,mL^{-1}$.) As far as could be detected serologically, all the CRP passed directly through the affinity column (Table 2). Consequently, none of the proteins isolated from the affinity column with mannose (MBL), N-acetylglucosamine or EDTA (SAP) had any CRP detectable by nephelometry.

Table 2. Double diffusion analysis

Fraction	αSAP	αCRP	αIgG	αIgA	αIgM
Crude extract	++	++	+++	++	++
Ammonium sulphate ppt.	++	++	+++	++	++
Affinity column unbound	–	++	+++	++	++
1st EDTA eluate	++	–	+	+	+
Acid (pH 2·5) eluate	–	–	+	–	+++
Mannose eluate	–	–	–	–	–
GlcNAc eluate	–	–	–	–	–
2 mL EDTA eluate	++	–	+	–	++

α = antiserum to.

Table 3. Other known lectins from human blood

Name	Source	M_r	Reference
Conglutinin analogue	plasma (not serum)	330k (NR) 66k (R)	Thiel *et al*. (1987)
P35	recalcified plasma	320k (NR) 35k (R)	Matsushita *et al*. (1996)
SL-1 & SL-2	serum	67k	Rapoport *et al*. (1996)
Galactose-binding lectin	plasma	29·5k	Hamazaki (1986)

The apparent M_r refers to determinations made with polyacrylamide gels in the presence of SDS either under reducing (R) or nonreducing (NR) conditions.

DISCUSSION

Cohn Fraction III is currently an unused by-product of immunoglobulin G production, with over 50 kg discarded from our Scottish protein fractionation plant every week. Yet it is clearly a rich source of a number of factors involved in the innate immune system including MBL, SAP, CRP, P35, the human conglutinin analogue and 'natural' antibodies.

At the very least, this plasma fraction is a useful alternative to plasma or serum for isolation of MBL for biochemical studies. The median plasma MBL concentration is $\approx 1\ \mu g\ mL^{-1}$, so a litre is required to obtain less than a milligram. In contrast, 10 mg of MBL can readily be obtained from less than 1 kg of Fraction I + III, which has a specific MBL activity seven-fold greater than that of pooled plasma (Kilpatrick *et al*., 1997).

The availability of large quantities of Fraction I + III also facilitates the investigation of factors which may be present in plasma in trace quantities. In this work, evidence is presented for both Ca^{2+}-independent and (unexpectedly) Ca^{2+}-dependent IgM antibodies to yeast mannan, since IgM was eluted from the immobilized mannan column first with EDTA and then with low pH buffer. Both presumably represent natural antibodies with a first-line defense function against micro-organisms. An unidentified plasma lectin was also found with an apparent M_r of 66 000. Some possible candidates for this unknown GlcNAc-binding protein are listed in Table 3. From preliminary experiments, it appears unlikely to be P35 (Matsushita *et al*., 1996), but its properties are consistent with the bovine conglutinin analogue (Baatrup *et al*., 1987; Thiel *et al*., 1987) and the galactose-binding lectin described by Rapoport *et al*. (1996). Indeed the latter two factors may be the same. The protein described by Hamazaki (1986) may be SAP, as indeed might be the 'MBP-2' described by Taylor & Summerfield (1987). Further investigation is required to resolve these questions.

As well as an advantageous starting material for laboratory studies, it is possible Fraction I + III could be developed as a therapeutic/prophylatic blood product. MBL purified in a similar way has already been used therapeutically (Jensenius, 1995), and a highly purified preparation would be required in clinical studies intending to demonstrate that any benefit is specifically due to MBL and not to other components. On the other hand, unfractionated Fraction I + III with its complex mixture of opsonins might be a far more potent antimicrobial product and could be valuable in selected clinical settings. Indeed, there is evidence that an IgM-rich immunoglobulin preparation can be clinically effective in Gram-negative sepsis (Schedel *et al*., 1991). It is noteworthy that a process-scale purification of IgM from Cohn Fraction III has been described using caprylic acid (Ng *et al*., 1993). In preliminary investigations, conditions have been found which permit the coretention of MBL and IgM after precipitation of lipoproteins by caprylic acid (unpublished data) and could form the basis of the development of a therapeutic product.

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