RESEARCH COMMUNICATION

Improvements on the purification of mannan-binding lectin and demonstration of its Ca^{2+} -independent association with a C1s-like serine protease

Suet Mien TAN*, Maxey C. M. CHUNG*, Oi Lian KON*, Steffen THIEL‡, Szu Hee LEE† and Jinhua LU*§

*Departments of *Biochemistry and †Pathology, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Republic of Singapore, and ‡Department of Medical Microbiology and Immunology, University of Aarhus, Denmark

Mannan-binding lectin (MBL), previously called ‘mannan-binding protein’ or MBP, is a plasma C-type lectin which, upon binding to carbohydrate structures on micro-organisms, activates the classical pathway of complement. Purification of MBL relies on its Ca^{2+} -dependent affinity for carbohydrate, but existing methods are susceptible to contamination by anti-carbohydrate antibodies. In the present study a sequential-sugar-elution method has been developed which can achieve a preparation of virtually pure MBL and its associated serine protease (MBL-associated serine protease, MASP) by two steps of affinity chromatography. In further separation of MASP from MBL, it was found that activated MASP was associated with MBL

independent of Ca^{2+} . Since MBL was found to bind to underivatized Sepharose 4B, the MBL–MASP complex was purified using Sepharose 4B and protease inhibitors were included to purify the complex with MASP in its proenzyme form. Analysis of thus-purified MBL–MASP complex by gel filtration on a Sephacryl S-300 column at pH 7.8 showed that the proenzyme MASP was also associated with MBL independently of Ca^{2+} , but that the complex could be disrupted at a low pH (5.0). Therefore the mechanism of MBL–MASP-mediated complement activation appears to be significantly different from the C1-mediated classical pathway.

INTRODUCTION

C-type lectins are characterized by containing C-type carbohydrate-recognition domains (CRDs) and can be classified largely into six groups based on similarities in structures, functions and carbohydrate specificities [1]. The C-type lectins can also be grouped as mannose/glucose- or galactose-specific, binding properties which are largely determined by two of the five amino acid residues in the CRD involved in sugar binding [2]. A C-type CRD binds to two neighbouring hydroxy groups on a sugar residue, which are 3-OH and 4-OH in mannose [3]. A mannose/glucose-specific CRD can bind, with similar affinity, to mannose and a spectrum of other sugars [L-fucose, *N*-acetyl-D-glucosamine (GlcNAc), glucose, etc.], which contain two -OH groups in the same configuration as the 3-OH and 4-OH in mannose. The galactose-specific C-type lectins bind to two -OH groups in the opposite configuration. Compared with C-type lectins, which often bind to a spectrum of sugars, anti-carbohydrate antibodies are much more specific for individual sugars, i.e. anti-mannose antibodies normally do not bind to glucose. A fraction of these antibodies in plasma are Ca^{2+} -dependent and are difficult to separate from mannan-binding lectin (MBL). In previously described methods, affinity-purified MBL was heavily contaminated by anti-carbohydrate antibodies, and the removal of these immunoglobulins required extensive further purification by ion-exchange and gel-filtration chromatography [4,5].

MBL, a member of the collectin subfamily of C-type lectins, is a multimeric protein containing collagen-like sequences and has the overall ‘bundle-of-tulips’ structure first described for C1q [4,6,7]. It can activate the complement classical pathway after binding to carbohydrate structures on micro-organisms [8].

Studies using purified MBL and proenzyme C1s showed that MBL, like C1q, could interact with and activate the $\text{C1r}_2\text{C1s}_2$ serine protease complex [4,9]. However, a novel serine protease, different from C1r and C1s, has been found to co-purify with MBL at high salt concentrations (1 M) [10]. The protease shows sequence similarity to C1r and C1s and has been named the ‘MBL-associated serine protease’ (MASP) [10–12]. The importance of MBL-mediated complement activation has been demonstrated by the increased susceptibility of children who lack, or have low levels of, serum MBL to recurrent infections [13].

In the present study a sequential-sugar-elution method has been developed which allowed the isolation of the MBL–MASP complex to almost homogeneity. Further study revealed that, unlike the C1 complex, the MBL–MASP complex is Ca^{2+} -independent and could be disrupted under non-dissociating conditions at low pH (5.0). The finding that MBL binds to Sepharose also suggested that a Sepharose pre-column should not be used in MBL purification.

MATERIALS AND METHODS

Materials

Sephacryl S-300, Q-Sepharose, 1,10-phenanthroline, divinyl sulphone, mannose, maltose and GlcNAc were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pefabloc SC [4-(2-aminoethyl)benzenesulphonyl fluoride], a water-soluble serine-protease inhibitor, was purchased from Pentapharm Ltd. (Basel, Switzerland). Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Mannose- and maltose-Sepharose were prepared by a previously described method [14]. Freshly

Abbreviations used: MBL, mannan-binding lectin; MASP, MBL-associated serine protease; PEG, poly(ethylene glycol); CRD, carbohydrate-recognition domain; GlcNAc, *N*-acetyl-D-glucosamine

§ To whom correspondence should be sent.

frozen plasma from apparently healthy blood donors were kindly provided by the National University Hospital, Singapore.

Purification of MBL

The purification process was carried out at 4 °C, except for FPLC® or where otherwise stated. A pool of freshly frozen human plasma (1 litre) was thawed from -20 °C and, after addition of poly(ethylene glycol) (PEG) 3500 to 7% (w/v), was stirred for 2 h. Precipitated proteins were pelleted by centrifugation for 15 min at 12000 *g*. The pellets were dissolved in 400 ml of ice-cold TBS-TCa²⁺ buffer (50 mM Tris/150 mM NaCl/0.05% Tween 20/20 mM CaCl₂, pH 7.8), and insoluble materials were removed by centrifugation for 5 min at 12000 *g*. The supernatant was mixed for 2 h with mannose-Sepharose (50 ml packed volume) which had been equilibrated with TBS-TCa²⁺. The resin was recovered by centrifugation for 5 min at 1000 *g* and packed into a column after washing with TBS-TCa²⁺ (500 ml). The column was further washed with TBS-TCa²⁺ (approx. 200 ml) and then eluted with TBS-TEDTA (TBS-TCa²⁺ where 20 mM CaCl₂ was replaced with 10 mM EDTA). The EDTA eluate was made 40 mM with respect to CaCl₂ and applied to a small maltose-Sepharose column (3 ml). After washing the column with TBS-TCa²⁺ (50 ml), bound proteins were eluted with 100 mM GlcNAc in TBS-TCa²⁺. MBL-containing fractions, as judged by SDS/PAGE, were pooled and further purified by FPLC® ion-exchange on a mono-Q (HR5/5) column (Pharmacia). The column was, after washing with buffer A (50 mM Tris/100 mM NaCl/2 mM EDTA/0.05% NaN₃, pH 7.8) (20 ml), eluted with a 100–600 mM NaCl gradient (40 ml). MBL-containing fractions were pooled and, after addition of CaCl₂ to 20 mM, applied to a small mannose-Sepharose column (0.4 ml packed volume). The column was washed with 5 ml of TBS-TCa²⁺ and eluted with TBS-TEDTA, 0.15 ml fractions being collected. The MBL-containing fractions were pooled (approx. 0.5 ml) and further purified by FPLC gel-filtration on a Superose 6 column (Pharmacia).

In another experiment, PEG-precipitated plasma proteins were mixed with equilibrated mannose-Sepharose and the resin was packed into a column after washing with TBS-TCa²⁺. The column was further washed with TBS-TCa²⁺ and eluted with 100 mM GlcNAc in TBS-TCa²⁺. Protein-containing fractions, as judged by SDS/PAGE, were pooled and, after being adjusted to 15 mM with respect to EDTA, applied to a Q-Sepharose column (1 cm × 10 cm) which had been equilibrated with buffer A. The column was washed with buffer A (50 ml) and eluted with a 100–600 mM NaCl gradient. Fractions were examined by SDS/PAGE.

The above-described method was further modified for use with underivatized Sepharose 4B. Buffers, except those used in large quantity in the washing of Sepharose resins, were adjusted to 0.1 mM with respect to Pefabloc SC and 40 mM with 1,10-phenanthroline. PEG-precipitated plasma proteins were dissolved in TBS-TCa²⁺, and insoluble materials were removed by centrifugation for 15 min at 12000 *g*. The supernatant was mixed with Sepharose 4B (50 ml packed volume) for 2 h and the resin was recovered by centrifugation for 5 min at 1000 *g*. After washing with TBS-TCa²⁺ (500 ml) by centrifugation, the resin was packed into a column. The column was further washed with TBS-TCa²⁺ (200 ml) and then eluted with TBS-TEDTA. Protein-containing fractions, as judged by SDS/PAGE, were pooled and, after addition of CaCl₂ to 40 mM, applied to a smaller Sepharose 4B column (3 ml). The column was washed with TBS-TCa²⁺ (50 ml), and bound proteins were eluted with 200 mM mannose in TBS-TCa²⁺. Protein-containing fractions, as judged by SDS/

PAGE, were adjusted to 15 mM with respect to EDTA. A fraction of the eluate (0.3 ml) was applied to a Sephacryl S-300 column (1 cm × 45 cm) which had been equilibrated with TBS-TEDTA and the column was eluted with the same buffer at a flow rate of 0.3 ml/min. Gel filtration was also similarly carried out using an acetate buffer (100 mM sodium acetate/200 mM NaCl/5 mM EDTA, pH 5.0). Fractions (1 ml) were analysed by SDS/PAGE, and proteins were detected by silver staining. No protease inhibitor was added to the gel-filtration buffer.

SDS/PAGE, Western blotting and protein sequencing

SDS/PAGE was carried out as described by Laemmli [15]. To examine proteins under reducing conditions, samples were heated for 5 min at 90–100 °C in the presence of 50 mM dithiothreitol. For Western blotting, proteins separated by SDS/PAGE were electrotransferred on to nitrocellulose membranes. The blots were detected with goat anti-human immunoglobulins (IgM, IgA and IgG) conjugated with alkaline phosphatase (Sigma). For sequence analysis, the proteins separated by SDS/PAGE were electroblotted on to Problot membrane (Applied Biosystems, Foster City, CA, U.S.A) and excised for automated sequencing on an Applied Biosystems 477A/120A sequencer using a blot cartridge.

RESULTS

Purification of MBP by sequential sugar elution

The sequential use of different sugars, either immobilized or in elution buffers, in MBL purification can theoretically remove all contaminating anti-carbohydrate antibodies from MBL. As seen in Figure 1, the EDTA eluate was shown, by SDS/PAGE, to contain the 32 kDa MBL polypeptide, a 65 kDa polypeptide shown to be the heavy chain of MASP by N-terminal sequencing (HTVELNNMFGQIQSP; [12]) and a number of contaminants which have been identified by Western-blot analysis to include IgM, IgG and serum amyloid P component among others

Figure 1 Purification of MBL by sequential sugar elution

Human plasma (lane 1) was precipitated with 7% (w/v) PEG 3500 and the re-solubilized proteins (lane 2) were mixed with mannose-Sepharose. The mannose-Sepharose was, after washing with TBS-TCa²⁺, eluted with EDTA. The EDTA eluate (lane 3) was, upon addition of CaCl₂, applied to a small maltose-Sepharose column. Bound proteins were eluted with GlcNAc. The GlcNAc eluate (lane 4) was adjusted to 15 mM with respect to EDTA and subjected to FPLC® ion-exchange on a mono-Q column. Lane 5 is a pool of MBL-containing fractions eluted from the mono-Q column in a 100–800 mM NaCl gradient. The samples collected at different steps of the purification procedure were subjected to SDS/PAGE on a 10% (w/v) gel under reducing conditions, and proteins were revealed by silver staining. IgM, IgG and serum amyloid P component were detected in lane 3 by Western blotting (results not shown). At the extreme left are shown molecular masses in kDa ('Kd').

Figure 2 Ion-exchange chromatography of plasma proteins that bound to mannan-Sepharose and were eluted with GlcNAc

PEG-precipitated plasma proteins were solubilized and mixed with mannan-Sepharose. Bound proteins were eluted with 100 mM GlcNAc and, upon addition of EDTA to 15 mM, applied to a Q-Sepharose column (1 cm $\times$ 10 cm). The column was eluted with a 100–800 mM NaCl gradient, and ten fractions between approx. 250 and 400 mM of the gradient were examined by SDS/PAGE on a 10% (w/v) gel under reducing conditions (lanes 1–10). The gel was stained with Coomassie Blue. 'H-chain' denotes the heavy chain of activated MASP. The 76 kDa band eluted in front of MBL–MASP was the heavy chain of IgM as determined by Western blotting (results not shown). At the extreme left are shown molecular masses in kDa ('Kd').

Figure 3 Purification of the MBL–MASP complex with MASP being in its proenzyme form

The sequential-sugar-elution method was modified to mix plasma proteins with Sepharose 4B. EDTA eluate from the first Sepharose 4B column (50 ml) was, upon addition of CaCl_2 , applied to a smaller Sepharose 4B (3 ml) column from which bound proteins were eluted with mannose. Fractions 6–10 (lanes 1–5) of the EDTA eluate from the first Sepharose column and fractions 1–5 (lanes 6–10) of the mannose eluate from the second column were examined by SDS/PAGE on a 10% (w/v) gel and the gel was stained with Coomassie Blue. At the extreme left are shown molecular masses in kDa ('Kd').

(lane 3) (results not shown). The only other major protein besides MBL after mannose elution from the maltose-Sepharose column, which was detectable by Coomassie Blue staining, was the 65 kDa MASP heavy chain (results not shown), though other contaminants were detected by silver staining (Figure 1, lane 4). Proenzyme MASP consists of a single polypeptide of approx. 90 kDa which can be activated by a single cleavage [10]. The activated MASP thus contains two polypeptides linked by a disulphide bond: a 65 kDa heavy chain and a 25 kDa light chain fragment. The light chain was shown to co-migrate with MBL on SDS/PAGE under reducing conditions (results not shown).

To separate MBL from the co-purified MASP, the GlcNAc

Figure 4 Separation of MBL from MASP

The mannose eluate from Sepharose (Figure 3, lane 8) (0.3 ml) was subjected to gel filtration on a Sepharacryl S-300 column (1 cm $\times$ 45 cm). The column was eluted with either TBS-EDTA (A) or 0.1 M sodium acetate, pH 5.0, containing 200 mM NaCl (B). The column was eluted at 0.3 ml/min, and 1 ml fractions were collected. Alternate fractions (13, 15, 17, 19, 21, 23 and 25) from each elution were examined by SDS/PAGE on 10% (w/v) gels.

eluate from maltose-Sepharose was, after addition of EDTA, subjected to FPLC[®] ion-exchange on a mono-Q column. It was surprising to find that the activated MASP was co-eluted with MBL in the NaCl gradient, even in the presence of 5 mM EDTA (Figure 1, lane 5). MASP eluted from mono-Q was concentrated to a small volume (0.5 ml) and further analysed by FPLC[®] gel filtration on a Superose 6 column in the presence of 5 mM EDTA. It was again co-eluted with MBL (results not shown). To investigate whether MASP, in proenzyme form, is also associated with MBL in a Ca^{2+} -independent manner, the sequential-sugar-elution method was simplified to one step by direct elution of MBL from the mannose-Sepharose column with GlcNAc and the eluate was, after being adjusted to 15 mM with respect to EDTA, quickly applied to a Q-Sepharose column (1 cm $\times$ 10 cm) (Pharmacia) at 4 °C. As seen in Figure 2, a fraction of MASP

eluted from the Q-Sepharose column migrated as the 90 kDa proenzyme under reducing conditions, but both the activated and proenzyme MASPs were co-eluted with MBL in every fraction (lanes 4–9). This implied that the Ca^{2+} -independent interaction between MASP and MBL was probably not due to newly exposed site(s) on MASP as the result of its activation. The identity of MASP in Figure 2 was confirmed by N-terminal sequencing (results not shown).

Separation of MASP from MBL

In order to investigate further the interaction between proenzyme MASP and MBL, and to establish a method for the purification of proenzyme MASP without using monoclonal antibodies against MBL [10], the sequential-sugar-elution method was modified to include protease inhibitors. Since MBL was found to bind to underivatized Sepharose 4B (J. Lu, unpublished work), the method was further modified to use Sepharose 4B as an affinity resin. As seen in Figure 3, MASP in the EDTA eluate from the first Sepharose 4B column migrated as the 90 kDa proenzyme under reducing conditions (Figure 3, lanes 2–5) and it was still in proenzyme form after being eluted from the second, small Sepharose 4B column with mannose (Figure 3, lanes 7–9). MASP in the thus-purified MBL–MASP complex was identified by N-terminal sequencing (results not shown). Since the MBL–MASP complex can be purified to near homogeneity simply by the above affinity-chromatographic method, the focus of improving the method for the purification of MASP would be the separation of MASP from MBL. To search for conditions capable of completely disrupting the MBL–MASP interaction, a gel-filtration step was attempted on a Sephacryl S-300 column, using a low-pH sodium acetate buffer. As seen in Figure 4, whereas MASP co-purified with MBL on Sephacryl S-300 at neutral pH (7.8) even in the presence of 5 mM EDTA (Figure 4A), MBL was clearly separated from MASP at pH 5.0 on the same column (Figure 4B). When the MBL–MASP complex was disrupted by dialysis against the acetate buffer, pH 5.0, and subsequently dialysed back into the TBS-TEDTA buffer (pH 7.8), MBL and MASP were shown to be in similar complexes (the results, not shown, were similar to those shown in Figure 4). Therefore the low-pH dissociation of the MBL–MASP complex is reversible.

DISCUSSION

The sequential-sugar-elution method developed in the present study yields approx. 300–600 μg of virtually pure MBL–MASP complex from 1 litre of plasma. Minor contamination with IgM was sometimes detected, and this could be easily removed by passing the affinity-purified MBL–MASP complex through a small anti-(human IgM) column if only the MBL–MASP complex is required (results not shown). The sequential-sugar-elution method may be modified for the purification of other lectins, especially those present in animal tissues or body fluids that often contain high levels of immunoglobulins.

Structural and functional studies on the collectins have been, so far, largely inspired by their structural similarity to C1q. Most collectins have been reported to bind to the same receptor to which C1q binds [16]. The MBL-mediated activation of the classical complement pathway was also suggested by its structural

similarity to C1q [8]. But the individual structural and functional properties of the collectins should be recognized. For example, the collectin lung surfactant protein D (SP-D) has been shown not to bind to C1q receptor(s) on alveolar macrophages [17], and MBL is the only collectin shown to activate complement (J. Lu and K. B. M. Reid, unpublished work). The MBL–MASP complex is known, from this study, to be distinct from the C1 complex in several respects: (1) Ca^{2+} is required to form the C1 or $\text{C1r}_2\text{C1s}_2$ complex, but is apparently not required for the formation of the MBL–MASP complex. (2) Unlike the C1 complex, which is not stable at high salt concentration [18], MASP co-purified with MBL at 1 M NaCl [10]. (3) The C1 or $\text{C1r}_2\text{C1s}_2$ complex appeared to be not disrupted at low pH (5.5) in the presence of Ca^{2+} [19], but the Ca^{2+} -independent MBL–MASP complex can be disrupted at similar pH (5.0) (Figure 4B). Therefore care should be taken when the C1 complex is referred to when interpreting data obtained with the MBL–MASP complex. For example, if $\text{C1r}_2\text{C1s}_2$ is not associated with C1q at 1 M NaCl, it may not co-purify with MBL at similar NaCl concentrations, although $\text{C1r}_2\text{C1s}_2$ has indeed been shown to interact with MBL at physiological salt concentrations [9,20]. The Ca^{2+} -independent interaction between MBL and MASP shown in the present study implies that purified MBL used in previous studies was probably contaminated with MASP. Therefore, caution should be exercised in interpreting the relevant data.

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