Characterization of Truncated Human Mannan-Binding Protein (MBP) Expressed in Escherichia coli

Souji EDA,1 Yasuhiko SUZUKI,1 Takao KAWAI,2 Katsuki OHTANI,1 Tetsuo KASE,3 Takashi SAKAMOTO,4 and Nobutaka WAKAMIYA 4*

Department of 1Pathology, 2Food Microbiology, and 3Virology, Osaka Prefectural Institute of Public Health, Higashinari, Osaka 537-0025, Japan
4Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

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Mannan-binding protein (MBP) is a calcium-dependent mammalian serum lectin important in first-line host defense. MBP belongs to the collectin family, which is characterized by an NH2-terminal cysteine-rich domain, a collagen-like domain, a neck domain, and a carbohydrate recognition domain (CRD). We have expressed a recombinant human MBP, consisting of the short collagen region (two repeats of Gly-Xaa-Yaa amino acid sequences), the neck domain, and the CRD, in Escherichia coli. The truncated MBP was capable of forming trimers by association of the neck domain and could bind sugar with a specificity similar to that of the native form. Results of hemagglutination inhibition (HI) assay of influenza A virus showed that the truncated MBP inhibited hemagglutination less strongly, although the native MBP induced the HI phenomenon. These results suggest that an oligomeric structure is an advantage for MBP to have full biological activity against influenza A virus.

Key words: mannan-binding protein; c-type lectin; expression; influenza virus

Mannan-binding protein (MBP) is a calcium-dependent mammalian serum lectin. It is a member of the collectin family, which have similar structures characterized by a collagen-like domain and a carbohydrate recognition domain (CRD). The other collectins include surfactant protein A (SP-A), surfactant protein D (SP-D), CL-43, and conglutinin. MBP has a bouquet-like structure, which is assembled as hexamers of trimers consisting of three identical 32 kDa polypeptides. Collectins are believed to be important in immunoglobulin-independent host defense. They recognize the carbohydrate ligands on the cell surface of pathogens via CRDs. MBP can destroy bacteria by activating the complement pathway. MBP also might act as an opsonin by binding to the C1q receptor or collectin receptor on phagocytes. In addition, MBP inhibits the infectivity and hemagglutinating activity of influenza A virus as do conglutinin and SP-D.

We have reported the expression of truncated conglutinin and truncated human SP-D, lacking the N-terminal and collagen-like domain, in an Escherichia coli expression system. They could form the trimeric structure by association of the neck domains and had sugar binding and specificity similar to that of native forms. The trimeric structure of the truncated conglutinin as well as that of truncated SP-D is basically sufficient for agglutinating sheep red blood cells (SRBCs) or E. coli. Their hemagglutination inhibition (HI) tests of influenza A virus suggest that there are different mechanisms of HI between conglutinin and SP-D. To investigate the biological function of other collectins against pathogen, we produced a recombinant human MBP, consisting of the short collagen region (two repeats of Gly-Xaa-Yaa amino acid sequences), the neck domain, and the CRD in Escherichia coli and characterized this recombinant protein by comparing it with the native human MBP.

Materials and Methods

Buffers and Medium. Tris-buffer saline (TBS) contained 20 mM Tris-HCl and 140 mM NaCl, pH 7.4. TBS/C was TBS containing 5 mM CaCl2. TBS/TC was TBS containing 5 mM CaCl2 and 0.05% Tween 20. Denaturing buffer was TBS/C containing 6 M urea. Coating buffer contained 15 mM Na2CO3, 35 mM NaHCO3, and 0.05% (W/V) NaNO3, pH 9.6. SOB medium contained 2% (W/V) Bacto-tryptone, 0.5% (W/V) Bacto-yeast extract, 0.05% (W/V) NaCl, 2.5 mM KCl, 10 mM MgCl2, and 50 μg/ml ampicillin.

Purification of native human MBP. To purify MBP from human serum, the serum was first adjusted to 10 mM calcium with CaCl2 and was incubated overnight at 4°C with mannan-agarose (Sigma Chemical Co.) which had been equilibrated with TBS/C. The agarose gel was packed in a column and washed extensively with TBS/C. The columns were eluted with TBS containing 10 mM EDTA. Fractions were recalcified to 10 mM calcium and mixed again with mannan-agarose overnight at 4°C. The gel were packed in columns, washed as above, and then eluted with TBS/C containing 100 mM mannose. Crude MBP fractions were passed through a HiTrap

* To whom correspondence should be addressed. Tel: +81-6-879-8288; Fax: +81-6-875-3894; E-mail: wakamiya@biken.osaka-u.ac.jp

Abbreviations: SP-A, surfactant protein A; SP-D, surfactant protein D; MBP, mannan-binding protein; CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropylthiogalactoside; Bkg, bovine conglutinin.
column (Pharmacia Biotech) conjugated with anti-human immunoglobulins antibody, and that conjugated with protein G.

Expression and purification of recombinant protein. The recombinant human MBP was expressed in an E. coli expression system as previously described. A cDNA fragment, encoding the short collagen region, the neck domain, and the carbohydrate recognition domain of human MBP, was amplified using the polymerase chain reaction (PCR) method with two primers (5'-GGGCTCGAGGAGACCCCTGGAAGAAAGTCCG-3' and 5'-GGGGAAATTCTACGATAGGGAACCTCAGAC-3'). The PCR was done in a Zymoreactor (Atto Co.) for 35 cycles consisting of denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The PCR product was digested with EcoRI and Xhol and inserted into a bacterial expression vector pRSET-A (Invitrogen Co.) and used to transform E. coli JM109 cells. E. coli cells were grown to OD600 of around 0.3 in 200 ml of SOB medium. After the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM, the cells were grown for an additional 1 h and infected with an M13 phage that contained the T7 RNA polymerase gene driven by the E. coli lac promoter, at a multiplicity of infection of 5 plaque forming units per cell. After incubation for another 3 h, the cells were harvested and suspended in 10 ml of denaturing buffer. After sonication, the lysates were centrifuged at 9000 × g for 15 min. The supernatant was incubated with nickel-nitriotriacetic acid-agarose (Qiagen) for 60 min. The nickel-agarose was packed in a column and washed with the denaturing buffer. The histidine-tagged protein was eluted with the denaturing buffer containing 0.25 M imidazole. The eluate was dialyzed against TBS/C with 1 M urea and 0.5 M urea and TBS/C. The recombinant protein was purified by affinity chromatography on a mannann-agarose column. The purity of the recombinant human MBP was confirmed by SDS-PAGE and Western blotting analysis, using rabbit anti-(human MBP) serum.

Gel-filtration chromatography. Gel-filtration chromatography was done on a Superose 12 HR 10/30 column (Pharmacia Biotech) at a flow rate of 0.5 ml/min with TBS containing 2 mM EDTA at pH 8.0. After reduced treatment with TBS containing 10 mM dithiothreitol, recombinant MBP (20 μg) was put on the column. Calibration was done using a Gel Filtration Standard (Bio-Rad) containing thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12.

Chemical cross-linking study. The recombinant protein was dissolved at a concentration of 70 μg/ml in 0.1 M HEPES buffer, pH 7.5, containing 10 mM CaCl2. Samples were treated with bis (sulfosuccinimidyl) suberate (Pierce Chemical Co.) at 37°C for 1 h. They were boiled in SDS-PAGE sample buffer for 10 min. The polypeptides fractionated in the SDS-PAGE were silver stained with a Silver Stain II Kit Wako (Wako Pure Chemical Ind.).

Binding of human MBP to mannan. Microtiter plates were coated with 10 μg/ml of mannan in the coating buffer at 37°C for 1 h and blocked with Block Ace (Dainippon Pharmaceuticals Co.). The plates were washed with TBS/TC after each step. The plates were incubated with dilutions of lectins (recombinant or native human MBP) at 37°C for 1 h. After washing, biotinylated rabbit anti-(human MBP) serum, avidin D (Vector), and biotinylated horseshadish peroxidase (Vector) were added successively and incubated at 37°C for 30 min. The horseradish peroxidase activity was detected with TMB-peroxidase substrate (Kirkegaard & Perry Laboratories, USA). The reaction was stopped with 1 M phosphoric acid and the absorbance at 450 nm was measured. A sugar inhibition assay was used for this ELISA system. The recombinant human MBP was incubated with an increasing concentration of sugars on the mannan coated microtiter plates. IgG was defined as the concentration of sugar that inhibited 50% of the binding between MBP and mannan. 119

Hemagglutinating inhibition (HI) Test. HI tests were done using a standard microtiter assay method as previously described. 118 Influenza virus suspension (16 hemagglutinin units) was incubated with serial dilutions of MBPs in TBS/C or TBS containing 10 mM EDTA at 37°C for 1 h in 96-well microtiter plates. After incubation, 0.5% (v/v) chicken erythrocytes were added. The HI activity of MBPs was decided after a 1 h of incubation at 4°C.

Lectin blotting of viral proteins. After ultracentrifugation of A/Baraki/1/90 at 74000 × g for 120 min, virions (10 mg) were dissolved with SDS sample buffer, separated by SDS-PAGE, and transferred to a BioBlot NC membrane (Coaster Co.). After the membranes were blocked with TBS/TC containing 5% bovine serum albumin (BSA), the membranes were incubated with recombinant MBP for 60 min, washed in TBS/TC, and then incubated with the rabbit anti-(human MBP) serum in TBS/TC for 60 min. After being washed in TBS/TC, the membranes were incubated with goat anti-rabbit IgG-conjugated biotin (Vector) and alkaline phosphatase-conjugated streptavidine (Gibco BRL), and stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP-NBT system, Gibco BRL).

Results

Expression of recombinant human MBP in E. coli

The recombinant human MBP, composed of synthetic peptides with a histidine tag and two Gly-Xaa-Yaa repeats of the collagen domain, the neck domain, and the CRD, was expressed in E. coli. Figure 1 shows the construction of a plasmid vector for expression of the recombinant human truncated MBP. The recombinant protein with a histidine tag was purified by affinity chromatography on the nickel column. After dialysis, the recombinant protein was further purified by affinity
Fig. 1. Construction of the Expression Vector pRSSET-A/hMBP CRD.
The partial human MBP DNA, consisting of a short collagen region (two repeats of Gly-Xaa-Yaa amino acid sequences), the neck domain and the carbohydrate recognition domain (CRD), was inserted within a multiple cloning site of pRSSET-A, downstream from the T7 promoter and histidine tag.

![Diagram](image)

Fig. 2. SDS/PAGE Analysis of Human MBPs.
SDS-PAGE analysis of human MBPs in 10-20% gel under reducing conditions or 4-20% gel under non-reducing conditions. Samples were stained with Coomassie Blue. Native human MBP (lanes 1 and 3) and purified recombinant protein (lanes 2 and 4) are shown. Molecular weight markers are shown at the left.

<table>
<thead>
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<th>kDa</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>16.5</td>
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Conditions, the native MBP formed several disulfide-dependent oligomeric structures (lane 3), and the recombinant MBP showed a monomeric form only (lane 4). The recombinant protein was identified as MBP by Western blotting analysis using rabbit anti-(human MBP) serum (data not shown).

Structural characterization of recombinant human MBP
The non-covalent oligomerization of recombinant protein was investigated by gel filtration chromatography and chemical cross-linking. The recombinant MBP showed a major peak of 75.6 kDa on gel-filtration analysis, which corresponded to the size of a trimer (Fig. 3). Furthermore a chemical cross-linking study confirmed that the recombinant protein had a trimeric form (Fig. 4). These results indicate that this recombinant protein is able to assemble in trimers by association of its neck domain. These results are consistent with previous findings on truncated conglutinin and SP-D.

Carbohydrate binding activities of recombinant human MBP
The binding of recombinant MBP to mannose was examined. Figure 5 showed that the binding of recombinant MBP to mannose was dose- and calcium-dependent as was that of native MBP. In addition, the binding was inhibited by the presence of 100 mM mannose and
10 mM EDTA. The sugar specificities of recombinant protein was identified using an ELISA system. The results showed that the sugar specificity of recombinant MBP was similar to that of native MBP (Table 1). Taken together, these results indicate that the carbohydrate recognition domain of recombinant MBP was correctly folded to have its biochemical activity. Sugar inhibition study also showed that MBPs could recognize broad varieties of sugars in comparison with concanavalin A.

**Hemagglutinating inhibition (HI) activity of recombinant human MBP**

MBP inhibits influenza virus-induced agglutination of erythrocytes in the presence of Ca\(^{2+}\) ions.\(^{16,19}\) Figure 6 showed that native MBP inhibited the virus-induced agglutination of erythrocytes but recombinant MBP inhibited agglutination less strongly, in the presence of Ca\(^{2+}\) ions. Both MBPs had no inhibition in the presence of EDTA. The lowest concentration of the recombinant MBP and native MBP that inhibited hemagglutination was 12.5 \(\mu\)g/ml and 0.1 \(\mu\)g/ml, respectively. In comparison with native MBP, the recombinant MBP showed no inhibition at the average concentration of human MBP in serum (about 1–2 \(\mu\)g/ml).\(^{20}\)

**Table 1. Sugar Specificities of Recombinant MBP and Native MBP**

<table>
<thead>
<tr>
<th>Sugar Inhibitors</th>
<th>L(_50) (mM)</th>
<th>Native MBP</th>
<th>Recombinant MBP</th>
<th>Native Concanavalin A</th>
<th>Recombinant Concanavalin A</th>
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<tr>
<td>L-Fucose</td>
<td>17</td>
<td>28</td>
<td>NI</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>D-Fucose</td>
<td>65</td>
<td>&gt;100</td>
<td>NI</td>
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<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>13.5</td>
<td>20</td>
<td>6</td>
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<tr>
<td>Lactose</td>
<td>&gt;100</td>
<td>49</td>
<td>NI</td>
<td></td>
<td></td>
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<tr>
<td>Glucose</td>
<td>30.5</td>
<td>34</td>
<td>30</td>
<td></td>
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<tr>
<td>Maltose</td>
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<td>32.5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>12.5</td>
<td>17</td>
<td>76</td>
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<tr>
<td>N-Acetyl-d-mannosamine</td>
<td>21.5</td>
<td>41</td>
<td>&gt;100</td>
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<td></td>
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<tr>
<td>Galactose</td>
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<td>NI</td>
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<td></td>
</tr>
<tr>
<td>N-Acetyl-d-galactosamine</td>
<td>NI</td>
<td>NI</td>
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**Recombinant human MBP can bind both influenza viral glycoproteins, HA and NA**

The lectin blot showed that recombinant MBP could bind to both HA and NA of glycoproteins (Fig. 7, lane 1). HA molecule was presented as monomer, dimer, and trimer under non-reducing conditions. EDTA interfered with these bindings (Fig. 7, lane 2).
Fig. 6. Inhibition of Hemagglutination of Influenza A Virus by Incubation with Recombinant MBP or Native MBP in the Presence of Ca\(^{2+}\) or EDTA.

The hemagglutination inhibition test was done with a 2-fold dilution of recombinant MBP (A, B) or native MBP (C, D) in 96-well V plates to which 16 hemagglutinin units (final concentration) of the influenza A virus (A/Baraki/) were then added. The mixture of MBPs was incubated with 0.5% chicken erythrocytes in TBS/C with or without Ca\(^{2+}\) or 10 mM EDTA (B, D) at 4°C for 1 h.

Fig. 7. The lectin Blotting of Influenza A Virus A/Baraki/1/90 (H3N2).

The viral proteins transferred on the membrane were treated with recombinant MBP. Lane 1: lectin blotting with recombinant MBP. Lane 2: lectin blotting with recombinant MBP and EDTA.

Discussion

In this report, we showed the expression of human MBP composed of a histidine tag, two Gly-Xaa-Yaa repeats of a collagen domain, a neck domain, and a carbohydrate recognition domain (CRD). The crystallization analysis showed that the truncated recombinant MBP could form a trimeric structure by association of the neck domain.\(^{16,20}\) The sugar inhibition assay showed that the sugar specificity of truncated MBP was almost the same as that of native MBP. It was also shown that the sugar binding of truncated MBP was calcium-dependent as was that of native MBP. These results suggested that the CRD in the truncated MBP was correctly folded to have its lectin activity.

Several studies on truncated human MBP have been reported.\(^{22,24}\) Truncated MBP lacking the collagen domain could bind to mannose-rich Salmonella montevideo\(^{20}\) but not increase complement activation.\(^{21,24}\) These results indicated that the collagen domain is essential for MBP to activate complement. The truncated MBP that we used here also failed to activate complement (data not shown).

Collectins inhibit the hemagglutination activity of influenza A virus by binding to a high-mannose oligosaccharide near the sialic acid binding site of viral hemagglutinin (HA).\(^{18}\) We have reported that truncated conglutinin, consisting only of the trimeric form, inhibited the hemagglutination by binding to the critical site on HA. In this case, the oligomeric structure was not essential for the hemagglutination inhibition.\(^{19}\) Our recent study on truncated human SP-D indicated that SP-D could bind to both hemagglutinin and neuraminidase (NA), which is another viral envelope protein of influenza virus, and also inhibited the hemagglutination.\(^{16}\) It is considered that the steric interference between RBC receptor and virion, due to the oligomeric structure of collectin which binds to both HA and NA, should induce the hemagglutination. In a different influenza A virus, human MBP and SP-A were able to inhibit the hemagglutination and NA activity.\(^{20}\) These inhibitions resulted from direct binding of human MBP and SP-A to NA. In our experiment, truncated MBP failed to inhibit the virus-induced agglutination of erythrocytes at physiological concentration in human sera. A lectin blotting study indicated that truncated MBP could bind to both NA and HA of influenza A virus as native MBP did,\(^{19}\) although conglutinin could bind only to HA.\(^{16}\) These results suggest that MBP might bind to another oligosaccharide of HA which is not as critical to the hemagglutination, although the saccharide specificities are almost the same between human MBP and conglutinin.\(^{16}\) Therefore it is considered that MBP might induce the HI reaction by binding to both NA and HA like SP-D, so that an oligomeric structure has advantage for MBP to have its full biological activities (neutralizing and viral growth inhibition activities) against influenza A virus.

Truncated recombinant MBP appears to be useful as an affinity ligand. Compared with concanavalin A, truncated MBP can bind to broad varieties of sugars (N-acetyl-d-glucosamine, d-mannose, l-fucose, maltose and glucose) (Table 1).

In this study, we showed the expression of recombinant MBP lacking the collagen domain and characterized its biochemical and biological activities. This recombinant MBP will be useful for further structural and functional studies.

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References


