# Identification of a Bovine Serum Mannan-Binding Protein Reactive with a Ra Chemotype Strain of *Salmonella* Serovar Typhimurium

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ABSTRACT. To study the relationship between a bovine serum mannan-binding protein (MBP) and a serum protein reactive with a Ra chemotype strain of *Salmonella* sorovar Typhimurium (Ra-reactive factor, RaRF), both proteins were isolated by use of their affinity for yeast mannan and the Salmonella cells followed by affinity chromatography on mannobiose-Sepharose 4B. Both purified proteins showed a major protein band with a molecular weight of 33,000 and a few faint bands in sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under reducing conditions. In Western blotting with rabbit anti-bovine MBP antibody, the major subunit of both proteins were found to be immunologically identical. Similar findings were also obtained with purified human MBP and RaRF. From these findings, bovine and human serum MBP are suggested to be electrophoretically and immunologically the same as their corresponding RaRF.—KEY WORDS: bovine, mannan-binding protein, RaRF, *Salmonella*.

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The serum mannan and/or mannose-binding protein is a C-type animal lectin which is supposed to play important role(s) in the nonimmune host defense system. It activates complement [7, 13, 17, 19, 20, 21] and opsonizes microorganisms bearing terminal mannose (Man) and/or N-acetylglucosamine (GlcNAc) [13, 15]. It also binds to the gp120 surface glycoprotein of the human immunodeficiency virus [4]. In bovine serum, at least three mannan and/or Man-binding proteins such as conglutinin (Kg), serum lectin (CL-43), and mannan-binding protein (MBP) have been identified and isolated [1, 5, 6, 12, 23]. The subunit size (42–43 KDa) of bovine serum MBP [12, 23], which is supposed to be the same as CL-43 [6], has been reported to be larger than those (30–32 KDa) of serum MBPs from other species [11, 14, 20]. In addition to Kg and CL-43, bovine serum MBP consisting of a subunit with a molecular mass of 28 KDa has been recently isolated [1, 5]. Human serum MBP has been reported to be identical to a carbohydrate-binding component of the human Ra-reactive factor (RaRF) [18]. However, it is not yet known at the present time whether the bovine MBP consisting of a subunit of 28 KDa is the same as bovine RaRF as reported previously [10]. To study the relationship between MBP and RaRF in bovine serum, therefore, the present study was undertaken to isolate both serum proteins and compare their immunological cross-reactivity.

## MATERIALS AND METHODS

*Chemicals*: Yeast mannan was purchased from Nacalai tesque Inc., Kyoto, Japan. Molecular markers for electrophoresis were obtained from Japan Bio-Rad Laboratories, Tokyo, Japan. CNBr-activated Sepharose 4B, DEAE-Sepharose CL-6B, and Sephacryl S-300 were the products of Pharmacia, Uppsala, Sweden. GlcNAc-Sepharose 4B, L-fucose (LFuc)-Sepharose 4B, maltotriose ($[Glc]_3$)-Sepharose 4B, and Protein A-Sepharose 4B were the products of Sigma Chemical Co., St. Louis, MO, USA. Mannobiose ($[Man]_2$)-Sepharose 4B and chitooligosaccharides ($[GlcNAc]_n$)-Sepharose 4B were purchased from Seikagaku Co., Ltd., Tokyo, Japan. Human serum was obtained by removal of coagulated substances from outdated human citrated plasma after recalcification with 25 mM $CaCl_2$. Bovine serum was purchased from Itoh Ham Food Inc., Ibaraki, Japan.

*Purification of MBP and Kg*: To isolate Kg and MBP, either heat-treated or untreated whole serum was directly subjected to a mannan-Sepharose 4B column (2 × 15 cm), which was prepared by the methods described previously [2], equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 3 mM $CaCl_2$ (Buffer I) by the methods reported previously [23]. Adsorbed proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 10 mM EDTA (Buffer II). After recalcification of the eluate with 25 mM $CaCl_2$, affinity chromatography on the mannan-Sepharose 4B column was again carried out. Adsorbed proteins were eluted with 0.3 M Man in Buffer I. Immunoglobulins (mainly IgG) in the eluate were removed by either Protein A-Sepharose 4B (1 × 5 cm) or DEAE-Sepharose CL-6B (1 × 5 cm) chromatography at pH 8.0. To obtain the Kg-enriched fraction, the crude mannan-binding proteins were subjected to a GlcNAc-Sepharose 4B column (1 × 5 cm) equilibrated with Buffer I after recalcification. The adsorbed proteins were eluted with 50 mM GlcNAc in Buffer I (Kg-enriched fraction). On the other hand, unadsorbed proteins were subjected to a $[Man]_2$-Sepharose 4B column (1.5 × 5 cm) equilibrated with Buffer I. Adsorbed proteins were eluted with 50 mM Man in Buffer I (MBP-enriched fraction). After concentration by ultrafiltration in an Amicon cell (Amicon Co., MA, U.S.A.) with a YM-30 membrane, the concentrates of the Kg- and MBP-enriched fractions were separately further purified by gel filtration on a Sephacryl S-300

column (1 × 90 cm) equilibrated with Buffer II.

*Preparation of formalin-killed Salmonella and isolation of RaRF*: Formalin-killed bacterial cells of *Salmonella* serovar Typhimurium chemotype strain rfb 388 cultured on nutrient agar plates at 37°C for 18 hr, were prepared by treating with 5% formalin as reported previously [8]. To isolate RaRF, human and bovine whole sera were directly mixed with the formalin-killed bacterial cells (2 mg/m*l*) and kept stirring at 4°C for 4 hr as reported previously [8]. After collecting the bacterial cells by centrifugation at 25,000 ×*g* at 4°C for 20 min, the cells were extensively washed in Buffer I. Crude RaRF was eluted with 10% GlcNAc in Buffer I as reported previously [8]. Immunoglobulins in the crude RaRF were removed by either affinity chromatography on Protein A-Sepharose 4B or by ion-exchange chromatography on DEAE-Sepharose CL-6B as described above. The crude RaRF was further purified by affinity chromatography on the $[Man]_2$-Sepharose 4B column equilibrated with Buffer I as described above. After concentration by ultrafiltration, the concentrate was purified by gel filtration on the Sephacryl S-300 column equilibrated with Buffer II.

*Electrophoresis and Western blotting*: Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and Western blotting were carried out by the methods described previously [16, 24]. The specific antibody for Western blotting was prepared as described below. Two hundred μg of purified MBP in 0.5 m*l* were mixed with 0.5 m*l* of Freund's complete adjuvant (Nacalai tesque, Inc., Kyoto, Japan). The emulsion was subcutaneously injected into a female albino rabbit (2.0 kg) once a week for three weeks. The rabbit was bled one week after the final injection. Specific antibodies were isolated by affinity chromatography on Kg- and MBP-coupled Sepharose 4B as described previously [3, 22]. For Western blotting, the purified serum protein was run in SDS-PAGE and transferred to a nitrocellulose paper (Atto Co., Ltd., Tokyo, Japan). After the nitrocellulose was treated with purified antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Capel Laboratories, Organon Teknika Corp., West Chester, PA, U.S.A.) (diluted to 1:4,000) was used. After washing the nitrocellulose 5 times in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20, the specific antigen was detected by use of 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, U.S.A.).

*Other method*: Ouchterlony tests were perfomed as described previously [22].

## RESULTS AND DISCUSSION

*Reactivities of Kg and MBP with different affinity gels*: With heat-treated bovine serum, $Ca^{2+}$-dependent mannan-binding proteins were found to mainly contain IgM, IgG, Kg, and CL-43 by analysis on SDS-PAGE under reducing conditions as reported previously [23]. With untreated bovine serum, on the other hand, $Ca^{2+}$-dependent mannan-binding proteins contained IgM, IgG, Kg, CL-43, and MBP consisting of a subunit with a molecular weight of 33,000. To study the carbohydrate specificities of Kg containing CL-43 and MBP, bindings of bovine MBP, CL-43, and Kg in crude mannan-binding proteins to different affinity gels were compared. After elution of the adsorbed proteins with Buffer II, the eluates were analyzed by SDS-PAGE under reducing conditions. As presented in Table 1, both $[Man]_2$-Sepharose 4B and LFuc-Sepharose 4B were found to bind to both Kg containing CL-43 and MBP. GlcNAc-Sepharose 4B bound to Kg containing CL-43 more than MBP, whereas $[GlcNAc]_n$-Sepharose 4B and $[Glc]_3$-Sepharose 4B bound to MBP more than Kg containing CL-43. These findings support the previous findings [1, 5, 6] that GlcNAc-Sepharose 4B was useful to isolate Kg from MBP. On the other hand, human serum MBP was found to effectively bind to $[Man]_2$-Sepharose 4B (data not shown). This is consistent with the previous finding [2] that human MBP was reactive with $[Man]_2$.

*Isolation of Kg and MBP*: According to the results obtained above, GlcNAc-Sepharose 4B was used to obtain the Kg-enriched fraction. MBP in the unadsorbed fraction was then obtained by affinity chromatography on $[Man]_2$-Sepharose 4B. After concentration by ultrafiltration, both fractions were further purified by gel filtration on Sepharcyl S-300. Both proteins were eluted in a symmetrical peak at the position of the void volume. SDS-PAGE under reducing conditions showed that the Kg-enriched fraction migrated as two protein bands with molecular weights of 44,000 (Kg) and 42,000 (CL-43), whereas the MBP-enriched fraction migrated as a major protein band with a molecular weight of 33,000 and a few faint protein bands (Fig. 1). On the other hand, human MBP obtained by affinity chromatography on $[Man]_2$-

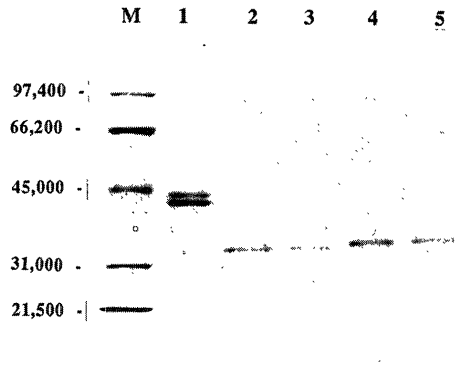


Fig. 1. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under reducing conditions. Samples used were molecular markers (M), Kg-enriched fraction (1), bovine serum MBP-enriched fraction (2), purified bovine RaRF (3), purified human MBP (4), and purified human RaRF (5). Molecular markers contained rabbit muscle phosphorylase (97,400), bovine serum albumin (66,200), egg white albumin (45,000), bovine carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

Sepharose 4B similarly migrated as a major protein band with a molecular weight of 34,000 and faint bands on SDS-PAGE under reducing conditions (Fig. 1). Thus, the subunits of bovine MBP-enriched fraction are electrophoretically similar to those of human MBP but different from the previous findings [5, 6] that bovine MBP was made up of a single subunit of 28 KDa. Although the difference between the present and previous findings [5, 6] is not yet accounted for at the present time, it may be due to the different methods used for purification of bovine MBP. Because ion-exchange chromatography on Mono Q column has been used at the final step of purification in the previous studies [5, 6], whereas it was not used in the present study.

*Isolation of RaRF*: To isolate bovine and human RaRFs, serum proteins reactive with formalin-killed Salmonella bacterial cells were eluted with 10% GlcNAc. The GlcNAc-eluate migrated as several protein bands by analysis on SDS-PAGE under reducing conditions, indicating that the RaRF in the eluate was still impure. Thus, further purification was performed by affinity chromatography on either Protein A-Sepharose 4B or DEAE-Sepharose CL-6B, affinity chromatography on $[Man]_2$-Sepharose 4B followed by gel filtration on Sephacryl S-300. In SDS-PAGE under reducing conditions, purified human RaRF migrated as a major protein band with a molecular weight of 34,000 and a few faint protein bands (Fig. 1) as reported previously [18]. On the other hand, purified bovine RaRF showed a major protein band with a molecular weight of 33,000 and three minor bands with molecular weights of 66,200, 44,000, and 23,000. These findings suggest that RaRF, Kg, and CL-43 may bind to $[Man]_2$-Sepharose 4B since both Kg and CL-43 could bind to the same affinity gels as presented in Table 1. Thus, Kg and CL-43 are suggested to be also weakly reactive with a Ra chemotype strain of *Salmonella* serovar Typhimurium. After removal of Kg and CL-43 by affinity chromatography on GlcNAc-Sepharose 4B, purified bovine RaRF migrated as a major protein band with a molecular weight of 33,000 and a few faint protein bands on SDS-PAGE analysis (Fig. 1) as found for bovine MBP as described above. These findings suggest that bovine RaRF may be composed of at least two distinct subunits as found for mouse, rat, and human RaRFs [8–10, 18].

Table 1. Binding of Kg and MBP in bovine crude mannan-binding proteins to different affinity gels

| Affinity gel | Kg* | MBP |
|---|---|---|
| GlcNAc-Sepharose 4B | + | ± |
| $[GlcNAc]_n$-Sepharose 4B | − | + |
| $[Glc]_3$-Sepharose 4B | ± | + |
| $[Man]_2$-Sepharose 4B | + | + |
| LFuc-Sepharose 4B | + | + |
| Mannan-Sepharose 4B | + | + |

+: binding, ±: much less binding, −: no binding. Kg*: Kg containing CL-43

*Immunological cross-reactivity between MBP and RaRF*: Since the results obtained above suggest that there may be structural similarity between bovine MBP and RaRF, immunological cross-reactivity between both proteins was studied. In Ouchterlony tests with rabbit anti-bovine MBP antibody, bovine MBP and RaRF formed precipitin lines which were found to be immunologically identical (data not shown). Similar results were obtained with human MBP and RaRF in Ouchterlony tests with anti-human MBP antibody. In Western blotting, only the major subunit of bovine MBP with a molecular weight of 33,000 was detected with rabbit anti-MBP antibody (Fig. 2). Similarly, only the major subunit of purified bovine RaRF was detected with anti-bovine MBP antibody (Fig. 2). With rabbit anti-human MBP, on the other hand, only the major subunits of both human MBP and RaRF with a molecular weight of 34,000 were found to be similarly detected (Fig. 2). Human serum MBP has been reported to be antigenically identical to a carbohydrate-binding component of human RaRF [18]. This is supported by the present studies that the major subunits of bovine and human serum MBP may be antigenically identical to those of their corresponding RaRF.

From the present findings, bovine serum MBP is electrophoretically and immunologically identical to bovine RaRF. To study the role of MBP in the nonimmune host defense against microbial infection, further investigation on complement-activating, bactericidal, and

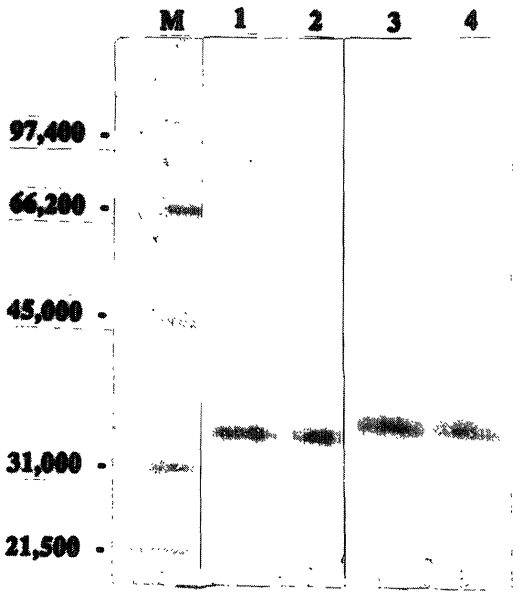


Fig. 2. Western blotting analysis. Bovine MBP-enriched fraction (1), purified bovine RaRF (2), purified human MBP (3), and purified human RaRF (4) were run in SDS-PAGE under reducing conditions and transferred to a nitrocellulose paper. The specific antigen was detected with rabbit anti-bovine MBP antibody for (1) and (2) and with rabbit anti-human MBP antibody for (3) and (4), respectively. M: molecular markers were described in the legend of Fig. 1.

opsonic activities of bovine serum MBP will be needed in future.

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