Identification and Carbohydrate Specificity of a Chicken Serum Mannan-Binding Protein Reactive with a Ra Chemotype Strain of *Salmonella* Typhimurium

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ABSTRACT. To compare a chicken serum mannan-binding protein (MBP) with a chicken Ra-reactive factor (RaRF), both serum proteins were isolated by combination of different chromatography. In SDS-PAGE, both purified proteins were resolved into a major protein band with a molecular weight of 34,000 and a few minor protein bands. In Western blotting with both purified proteins, only a single band with a molecular weight of 34,000 was detected with anti-chicken serum MBP antibody, indicating that the major protein band of chicken serum MBP and RaRF are antigenically identical. The combining sites of these two proteins were most reactive with N-acetylmannosamine followed by mannose, N-acetylglucosamine, and L-fucose. These findings suggest that chicken serum MBP may be identical to its corresponding bactericidal factor RaRF in terms of antigenicity and monosaccharide specificity.—KEY WORDS: chicken, MBP, RaRF, *Salmonella* Typhimurium, serum protein.

Some of carbohydrate-binding serum proteins are known as acute-phase reactants such as C-reactive protein (CRP), serum amyloid P component (SAP), mannose and/or mannan-binding protein (MBP) [3, 16, 17, 23]. MBP is one of C-type lectins which are Ca^{2+}-dependent carbohydrate-binding proteins [2]. MBP activates complement [6, 14] and opsonizes microorganisms [10]. Human serum MBP has been reported to be antigenically identical to a carbohydrate-binding component of the human Ra-reactive factor (RaRF) which binds to a Ra chemotype strain of *Salmonella* Typhimurium [13]. Similar findings have been also obtained with bovine serum MBP and RaRF [18]. From these findings, the MBP is suggested to play an important role(s) against microbial infection in mammalian nonimmune defense system. In contrast to mammalian acute-phase reactants, it is less known about avian CRP, SAP, and MBP. Although chicken hepatic MBP has been isolated [15], chicken serum MBP has not yet been identified and isolated. To identify chicken serum MBP and to compare it with chicken RaRF, therefore, the present study was undertaken to isolate both chicken serum MBP and RaRF and to compare their antigenicity and carbohydrate specificities. The results obtained suggest that chicken serum MBP may be identical to its corresponding RaRF in terms of antigenicity and monosaccharide specificity.

MATERIALS AND METHODS

Serum and reagents: Pooled chicken sera were obtained from adult normal inbred chicken (line V: genotype $B^{15}B^{15}$) which had been maintained at the National Institute of Animal Health, Tsukuba, Japan. Yeast mannan, invertase, monosaccharides, and disaccharides were purchased from Nacalai Tesque Inc., Kyoto, Japan.

CNBr-Sepharose 4B, DEAE-Sepharose CL-6B, and Sephacryl S-300 HR were the products of Pharmacia, Uppsala, Sweden. Mannobiose-Sepharose 4B was purchased from Seikagaku Co., Ltd., Tokyo, Japan. Protein A-Sepharose 4B was the product of E. Y. Laboratories, San Mateo, CA, U.S.A.

Preparation of antisera and immunoadsorbents: Chicken IgM and IgG were isolated by the methods as described previously [20]. Antisera against chicken IgM and IgG and against purified chicken serum MBP were produced in rabbits as described previously [19]. Rabbit anti-chicken IgM and IgG antibodies were isolated by affinity chromatography on chicken IgM and IgG immunoglobulins-coupled Sepharose 4B [1]. Affinity-purified anti-chicken IgM and IgG antibodies were coupled to CNBr-Sepharose 4B as described previously [1]. Rabbit IgG antibody against purified chicken serum MBP was isolated by affinity chromatography on Protein A-Sepharose 4B. Further purification of the isolated IgG was performed by affinity chromatography on chicken IgM and IgG-coupled Sepharose 4B. Biotinylation of purified IgG was done by mixing 100 μg of NHS-LC-Biotin (Pierce, IL, U.S.A.) with 1 m*l* of purified IgG (1 mg/m*l*) as described previously [4].

Isolation of MBP: Approximately 200 m*l* of pooled chicken sera was centrifuged at 100,000 × *g* 4°C for 30 min to remove insoluble substances. The supernatant was directly subjected to a mannan-Sepharose 4B column (2 × 15 cm) which was equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 3 mM $CaCl_2$ (Buffer I). Adsorbed proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 10 mM EDTA (Buffer II). The eluate was pooled, recalcified with 25 mM $CaCl_2$, and again subjected to another mannan-Sepharose 4B column equilibrated with Buffer I. The adsorbed proteins were eluted with 0.3 M mannose in Buffer I. The eluate was concentrated by ultrafiltration in Amicon cell with a diaflo

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YM-30 membrane (Amicon Co., Denver, MA, U.S.A.). The concentrate was recalcified and then subjected to a mannobiose-Sepharose 4B column (1 × 10 cm) equilibrated with Buffer I. Adsorbed proteins were eluted with 50 mM mannose in Buffer I. The effluents were concentrated by ultrafiltration. Chicken IgM and IgG in the concentrate were absorbed with anti-chicken IgM and IgG antibodies-coupled Sepharose 4B. The MBP-enriched fraction was further fractionated by DEAE-Sepharose CL-6B chromatography equilibrated with 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA. The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the starting buffer. Then, further purification was performed by gel filtration on Sephacryl S-300 HR (1 × 90 cm) equilibrated with Buffer II.

Preparation of formalin-killed bacterial cells and isolation of RaRF: Formalin-killed bacterial cells of *Salmonella* serovar Typhimurium strain rfb 388, which was cultured on nutrient agar plates, were prepared by treating with 5% formalin as reported previously [7]. For isolation of RaRF, chicken pooled sera were centrifuged at 100,000 × g 4°C for 30 min to remove insoluble substances. The supernatant was directly mixed with formalin-treated bacterial cells (2 mg/ml) and kept stirring at 4°C for 4 hr as reported previously [7]. After the bacterial cells were collected by centrifugation at 25,000 × g for 20 min at 4°C, the cells were extensively washed in Buffer I. Crude RaRF was eluted with 10% N-acetylglucosamine in Buffer I as reported previously [7]. Immunoglobulins in the crude RaRF were removed by absorption with anti-chicken IgM and IgG antibodies-coupled Sepharose 4B. Then, purification of crude RaRF was performed by affinity chromatography on mannobiose-Sepharose 4B followed by gel filtration on Sephacryl S-300 HR as described above.

Enzyme-linked immunosorbent assay (ELISA): Sandwich ELISA for studying the carbohydrate specificity of chicken serum MBP and RaRF was done by the methods described previously [24]. Each well of ELISA plates (Corning Inc., NY, U.S.A.) was coated at 4°C overnight with 100 μl of yeast mannan or invertase (50 μg/ml) diluted in 0.1 M carbonate buffer, pH 9.5. After washing the plates 4 times with 0.05% Tween 20 in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1 mM CaCl₂ and 1 mM NaN₃ (Buffer I), 200 μl of 0.1% gelatin in Buffer I (Buffer II) was added to each well. The plates were incubated at 37°C for 1 hr for blocking. After the plates were similarly washed, 100 μl of sugar inhibitor and 100 μl of purified MBP (1 μg/ml) diluted in Buffer II were added to each well. After similarly washing the plates, 100 μl of biotinylated anti-MBP antibody (diluted to 1:500) in Buffer II was added to each well. After incubation at 37°C for 1 hr, the plates were similarly washed. After then, 100 μl of ExtrAvidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, U.S.A., diluted to 1:4,000) in Buffer II was added to each well. After incubation at 37°C for 1 hr, the plates were similarly washed. One hundred μl of the substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, U.S.A.) in 10%

diethanolamine, pH 9.8, containing 0.5 mM MgCl₂ and 1 mM NaN₃ was finally added to each well and allowed to develop color at room temperature for 45 min. To stop the reaction, 50 μl of 5 N NaOH was added to each well. The enzyme-substrate reaction was determined spectrophotometrically with ImmunoReader NJ-2000 (Japan InterMed Co., Ltd., Tokyo) at 405 nm. The formula used to calculate percentage inhibition is: $100 \times (1 -$

$$\frac{\text{absorbance without inhibitor} - \text{absorbance with inhibitor}}{\text{absorbance without inhibitor}} \Bigg)$$

Other methods: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the methods described previously [11]. Western blotting was also carried out by the methods described previously [18, 25]. Protein content was determined by the methods of Lowry et al. [12] using bovine serum albumin as the standard. Immunogel double diffusion test was carried out by the methods described previously [19]. Ca^{2+}-dependent agarose- and phosphorylcholine-binding proteins in chicken serum were isolated by the methods described previously [21, 22].

RESULTS AND DISCUSSION

Isolation of chicken serum MBP: To isolate chicken serum MBP, Ca^{2+}-dependent chicken serum proteins reactive with mannan were isolated by affinity chromatography on mannobiose-Sepharose 4B using 50 mM mannose in the presence of Ca^{2+} as an eluant. On SDS-PAGE under reducing conditions, chicken IgM and IgG were detected in the eluate. Affinity chromatography on rabbit anti-chicken IgM and IgG-coupled Sepharose 4B followed by gel filtration on Sephacryl S-300 HR was carried out to remove chicken immunoglobulins. The main protein peak appeared at the void volume and was concentrated by ultrafiltration. As shown in Fig. 1, the main protein peak migrated as one major and two minor protein bands on SDS-PAGE under reducing conditions. The molecular weight of the major protein band was estimated to be 34,000, whereas those of the minor ones 80,000 and 36,000. The largest one was found to correspond to the heavy chain of chicken IgM, indicating that chicken IgM is supposed to still remain in the purified serum protein (Fig. 1). The major protein band was different from those of chicken Ca^{2+}-dependent agarose- and phosphorylcholine-binding proteins as shown in Fig. 1. Approximately 50 to 100 μg of chicken serum MBP was obtained from 200 ml of pooled chicken sera.

Isolation of chicken RaRF: Chicken serum proteins reactive with Salmonella bacterial cells were eluted with 10% GlcNAc as reported previously [7, 8]. The eluate showed several protein bands on SDS-PAGE analysis under reducing conditions, indicating that chicken RaRF in the eluate was still impure. Further purification was performed by affinity chromatography on mannobiose-Sepharose 4B followed by gel filtration on Sephacryl S-300 HR. The purified protein showed a major protein band

M   1   2   3   4   5   6
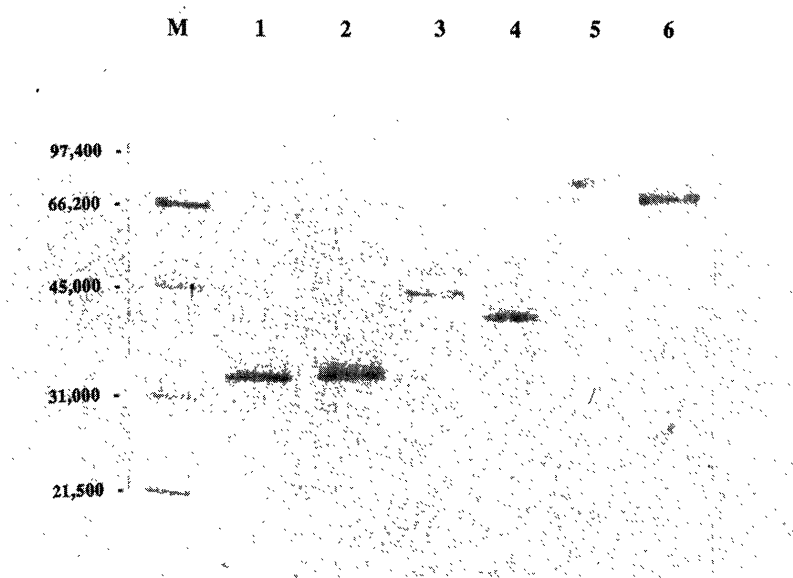
97,400 -

66,200 -

45,000 -

31,000 -

21,500 -

Fig. 1. SDS-PAGE under reducing conditions. Samples were molecular markers (M), purified chicken serum MBP (1), purified chicken RaRF (2), chicken phosphorylcholine-binding protein (3), chicken agarose-binding protein (4), chicken IgM (5), and chicken IgG (6). 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).

1   **2**

97,400 -
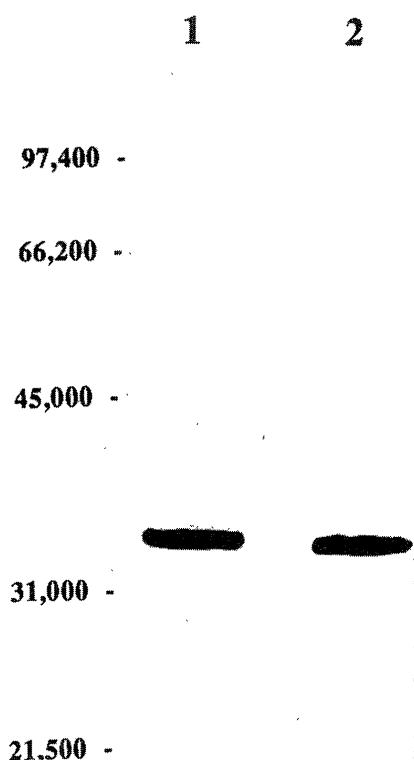
66,200 -

45,000 -

31,000 -

21,500 -

Fig. 2. Western blotting analysis. Purified chicken serum MBP (1) and RaRF (2) were run in SDS-PAGE under reducing conditions and transferred to a nitrocellulose paper. The specific antigen was detected with rabbit antiserum against purified chicken serum MBP. Molecular weights indicate migration of the same molecular markers described in the legend of Fig. 1.

with a molecular weight of 34,000 and at least two minor protein bands with molecular weights of 70,000 and 36,000 (Fig. 1). As shown in Fig. 1, the molecular weight of the major protein band of chicken RaRF was found to be identical to that of chicken serum MBP. These findings suggest that chicken MBP may be a candidate for the carbohydrate-binding component of chicken RaRF as found for bovine and human MBP and RaRF [13, 18]. However, the larger subunit of chicken RaRF was not fully detectable in Fig. 1 although human, mouse, and rat RaRF have been composed of two distinct subunits with molecular weights of 70,000 (as a minor band) and 31,000 (as a major band) [7, 8, 13].

Immunological cross-reaction between chicken serum MBP and RaRF: Since the results obtained above suggest that there may be structural similarity between chicken serum MBP and RaRF, immunological cross-reaction between both proteins was studied. Before use, rabbit anti-chicken MBP antibody was extensively absorbed with chicken IgM and IgG-coupled Sepharose 4B. In Ouchterlony tests with the absorbed anti-chicken MBP antibody, purified MBP and RaRF formed a antigenically identical precipitin line (data not shown). In Western blotting with purified chicken serum MBP, only a single band with a molecular weight of 34,000 was detected with the absorbed antibody as shown in Fig. 2. Similar results were obtained with purified chicken RaRF. These findings indicate that chicken serum MBP is antigenically identical to its corresponding RaRF as found for human and bovine serum MBP [13, 18].

Carbohydrate specificities of purified chicken MBP and RaRF: To study carbohydrate specificities of the combin-
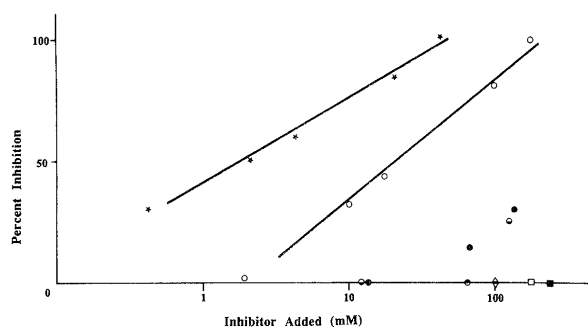
Fig. 3. Competitive binding assays by different sugar inhibitors of the binding of purified chicken serum MBP to invertase. For competitive binding assays, 100 μl (1 μg/ml) of purified chicken serum MBP and 100 μl of invertase (50 μg/ml) coated in each well of the ELISA plate were mixed for 1 hr at 37°C with different amounts of inhibitors. Inhibitors used were mannose (O), L-fucose (●), galactose (□), glucose (■), N-acetylmannosamine (*), N-acetylgalactosamine (◓), N-acetylglucosamine (◑), melibiose (▽), and lactose (△).

Table 1. Carbohydrate specificity of chicken serum MBP with that of chicken RaRF

Inhibitors	mM required to 50% inhibition[a]	
	MBP	RaRF
Mannose	20	13
L-Fucose	>134 (30%)[b]	>134 (20%)
Galactose	>177 (0%)	>134 (0%)
Glucose	>240 (0%)	>240 (0%)
N-acetylmannosamine	1.8	8.5
N-acetylglucosamine	>125 (25%)	>125 (33%)
N-acetylgalactosamine	>63 (0%)	>63 (0%)
Lactose	>100 (0%)	>100 (0%)
Melibiose	>100 (0%)	>100 (0%)

a) mM required to 50% inhibition of 100 μl (1 μg/ml) of either purified chicken serum MBP or RaRF to 100 μl of invertase (50 μg/ml) coated in each well of the ELISA plate.

b) Numbers in parentheses indicate maximum inhibition obtained by the inhibitors at the highest concentrations tested.

ing sites of both chicken serum MBP and RaRF, competitive binding assays were performed using sandwich ELISA. Since both purified chicken serum MBP and RaRF were found to bind invertase better than mannan in the presence of Ca^{2+}, inhibition of different sugars of the binding of both chicken serum MBP and RaRF to invertase was compared. As shown in Fig. 3 and Table 1, a most potent inhibitor to the binding of purified chicken serum MBP to invertase was N-acetylmannosamine. Mannose was a less potent inhibitor. Both N-acetylglucosamine and L-fucose showed 25–30% inhibition at the highest concentrations used. Other inhibitors were not active at the highest concentrations tested (Fig. 3 and Table 1). Similar results were also obtained with purified chicken RaRF. From these findings, the carbohydrate

specificity of chicken serum MBP is similar to that of chicken RaRF. Their carbohydrate specificities are also similar to that of bovine serum MBP as reported previously [5, 9].

From the present findings, chicken serum MBP is concluded to be identical to its corresponding RaRF in terms of antigenicity and monosaccharide specificity, suggesting that chicken serum MBP may play important roles against microbial infection in chicken nonimmune defense system. To elucidate this, more detailed studies will be needed in future.

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