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Affinity purification of a mannose-binding protein, a sensitive tool in the diagnostics of IgM, via site-directed phosphorylated mannan bound to alumina

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Abstract

Ca^{2+}-dependent mannose-binding proteins (MPBs) belong to the family of animal lectins. They perform *in vivo* as defence molecules that act as opsonins by enhancing the clearance of mannose rich pathogens and have been used *in vitro* for the purification of IgM. MBPs have been previously isolated by methods based on binding the protein moiety of various mannan species to different matrices. However, the mannan–protein complexes did not have a constant protein content and the yield of the isolated MBPs was variable. In the present study we describe a new approach for the affinity purification of MBPs based on the main polysaccharide moiety of the complex. After removal of residual phosphate groups naturally occurring at the C-3 position of the sugar, which interfere with MBP recognition, the mannan was phosphorylated enzymatically at C-6, at which position the OH group is not required for lectin binding. The enzymatically phosphorylated mannan bound to an alumina column was used successfully for MBP separation from rabbit serum. The mannose-binding protein obtained was used in our study for diagnostic purposes in the identification and determination of very low concentrations of IgM.

1. Introduction

Mammalian mannose-binding proteins (MBPs) belong to the family of animal lectins, isolated from liver and serum of rabbits, humans and rodents [1–4]. They consist of homologous polypeptides (molecular mass of 26 or 32 kDa) which form polymers of 200 and 600 kDa in liver and serum, respectively. MBPs play an important role in the intracellular transport of glycoproteins and in several protective reactions including complementary activation and elimination of various pathogens [5]; MBPs are β-inhibitors of influenza A viruses [6]. *In vitro* MBP was used for isolation and purification of IgM molecules from ascitic fluids [7]; recent studies indicated that preincubation of HIV I with human mannose-binding protein results in almost 100% inhibition of HIV infection of CD_4H9 lymphoblasts [8]. These specific biological activities are all related to the binding to oligo-mannose units of the target proteins (IgM, the virus envelope or various pathogens).

In order to study these biological functions of MBP at the molecular level it was necessary to understand the fine oligosaccharide specificity of the carbohydrate binding regions of this lectin. Analysis of the encoded human MBP, like its two rat homologues [9], reveals that the protein is divided into three domains, a cysteine-rich

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NH_2 terminal domain which stabilizes the α-helix of the second collagen-like domain, and a third COOH terminal carbohydrate-binding region. The last region contained the Ca^{2+} dependent carbohydrate recognition domains (CRDs). MBP binds preferentially to mannose via a small binding site which is probably of a trough- rather than pocket-type [10]. The OH groups at the 3 and 4 positions of mannose are indispensable for binding to CRDs of MBP. Recent studies of MBP have defined the first three-dimensional structure of CRD domains. The mannose residue binds through hydroxyls 3 and 4 to the calcium ions and to an intricate network of co-ordination to H bonds which together stabilize the protein [10]. MBP has been isolated by methods based on the protein moiety of various mannan species [1–4]. Yeast mannan, commonly used in MBP separation, is a covalently linked polysaccharide–protein complex in which the protein constitutes 5–50% of the total weight, depending on the source of mannan and the extraction method. For instance, yeast mannan (60 kDa) from *S. cerevisiae* does not contain any protein [11]. Since the mannan–protein complex does not appear to contain a constant amount of protein, the yield of isolated MBP using such preparations was variable.

Based on the above considerations we developed an affinity purification procedure for MBP based on the main polysaccharide moiety of the mannan complex. After removal of residual phosphate groups naturally occurring at C-3 position of the sugar moiety, mannan was phosphorylated enzymatically at C-6, at which position the OH group is not required for MBP recognition. The phosphorylated mannan was immobilized on alumina, a specific matrix for binding of monosubstituted derivatives of phosphoric acids [12]. Alumina, which has the ability to strongly absorb organic molecules, is used in many analytical and preparative chromatographic procedures [12]. The isolated MBP was used in our study as a sensitive diagnostic tool for recognition and determination of very low levels of IgM in biological fluids.

Early detection of the appearance of minute amounts of IgM in biological fluids is of crucial importance in the diagnosis of many pathological cases.

2. Experimental

Yeast mannan, hexokinase-agarose, alkaline phosphatase, lactic dehydrogenase, pyruvate kinase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Alumina oxide 90 active, neutral form, was purchased from Merck (Darmstadt, Germany). Protein A was a product of Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and obtained from Sigma.

2.1. Preparation of dephosphorylated mannan

Mannan (10 mg dissolved in 2 ml of 100 m*M* glycine buffer pH 10.4 containing 1 m*M* $MgCl_2$) was incubated at 37°C for various periods of time (1–3 h) with alkaline phosphatase 10 U/ml, bound to Protein A via anti-mouse IgG (0.3 ml), according to Ey *et al.* [13]. Control samples were incubated identically, except that alkaline phosphatase was omitted from Protein A–anti-mouse IgG complex. The effect of alkaline phosphatase was monitored by determination of the ability of the dephosphorylated mannan to bind to alumina, as described below.

2.2. Site-directed phosphorylation of mannan

Dephosphorylated mannan was phosphorylated at the 6-OH position of the mannose moiety of the mannan by hexokinase bound to agarose beads in the presence of ATP, as follows: mannan (10 mg in 1 ml buffer containing 0.05 *M* Tris-HCl pH 7.4 and 13.3 m*M* $MgCl_2$) was incubated by gentle shaking with 1 mg hexokinase agarose beads (~0.015 enzyme units) previously washed and equilibrated in the same buffer, in the presence of 20 m*M* ATP. The reaction was carried out overnight at room temperature. After centrifugation the supernatant was removed and dialysed against water at 4°C. The phosphorylation reaction was followed

by determination of released ADP according to the following reactions:

$$\text{D-mannose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{D-mannose-6-phosphate} + \text{ADP}$$

$$\text{Phosphoenol pyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{NAD}$$

The oxidation of NADH to NAD was followed by a decrease in absorbance at 340 nm.

2.3. Preparation of alumina–mannan conjugates

Various mannan preparations were incubated with alumina and their binding efficiency was followed by the disappearance of the mannan in the supernatants, as determined by the phenol test [14]. Alumina oxide (1 g) prewashed, equilibrated with 0.1 *M* Tris buffer (pH 7.0) was collected by centrifugation. The various mannan preparations (10 mg in 1 ml 0.1 *M* Tris buffer pH 7.0) were added to alumina and each reaction mixture was gently shaken at room temperature for 1 h.

2.4. Affinity purification of mannose-binding protein from rabbit serum

The columns (9 × 0.5 cm) were packed with various alumina–mannan preparations and equilibrated in 0.02 *M* imidazole-HCl buffer (pH 7.8) containing 0.02 *M* calcium chloride and 2.5 *M* NaCl. The columns were built using the naturally phosphorylated mannan of 3-OH and from 6-OH phosphorylated mannan–alumina preparations. Frozen rabbit serum (50 ml) was thawed and diluted with an equal volume of the above buffer containing 0.04 *M* $CaCl_2$. The serum samples were applied on the pre-equilibrated columns. The columns were washed with the loading buffer until no absorbance at 280 nm could be detected. Elution was performed with a buffer containing 0.02 *M* imidazole-HCl (pH 7.8), 1.25 *M* NaCl and 2 m*M* EDTA. MBP was retained only by the column containing 6-OH phosphorylated mannan. The peak was collected, recalcified and passed over a second mannan–alumina column and eluted with 50 mmol of D-mannose. C-reactive protein and other serum proteins were retained on the column and only MBP was eluted. Subsequent elution of the second column with buffer containing EDTA released other Ca^{2+}-dependent proteins.

2.5. Labelling of MBP

MBP was labelled with horse-radish peroxidase HRP according to Tijssen and Kurstak [15] using periodate treatment of the enzyme prior to binding of amino groups of MBP.

2.6. Enzyme immunoassay for the detection of IgM using labelled MBP

ELISA detection of IgM

A 96-well plate was coated with increasing amount of human IgM (0–100 ng) and human IgG (0–100 ng) in 100 μl of PBS for 2 h at 37°C. After blocking with 0.1% gelatin solution containing 0.1% PEG, 0.1% Triton and 20 m*M* $CaCl_2$ overnight at 4°C, MBP–HRP and goat α-HIgM–HRP at the same concentration were added to the washed wells. The amount of HRP bound was detected using 1,2-phenylene-diamine dihydrochloride according to manufacturer's instructions.

Dot blot assay

Spots of human IgM (0.01–1 μg) were applied on nitrocellulose membrane disks of 6 mm diameter. After drying, the paper was blocked using a solution of 0.1% gelatin containing 0.1% Triton, 0.1% PEG and 20 m*M* $CaCl_2$ during 16 h at 4°C. The detection of IgM was performed in parallel with MBP–HRP and α-IgM–HRP at the same working concentrations. The spots were visualised using an insoluble substrate of 4,0-chloronaphtol according to manufacturer's instructions.

3. Results and discussion

One of the major results of the present work has been the confirmation of the importance of specific positions in the ligand molecule for MBP recognition, which had previously been postulated and demonstrated by structural studies [9,10]. The most important OH group in the ligand molecule is at the 3 position, which must be equatorial for high-affinity binding to MBP; the absence or substitution of this OH group abolishes binding [10]. The studies reported in the literature suggest that the OH groups at C-3 and C-4 of the ligand sugar residues are probably in close contact with the binding site of MBP. The OH group in the 6 position does not interact with any of the lectine binding sites. For these reasons, our preliminary attempts to oxidize the sugar moiety by periodate treatment, prior to binding to an amino-containing insoluble matrix [16], led to immobilized mannan which was not able to bind and isolate MBP from rabbit serum. Moreover, binding of mannan to alumina via the naturally occurring phosphate moiety resulted in a matrix ineffective of separating MBP. In both cases the positions of the ligand necessary for MBP recognition were affected; in the mannan molecule the phosphate groups are attached to α-1,3-mannobiose units [11].

The above considerations led us to develop a new approach for the preparation of immobilized mannan via its sugar moiety based on the preservation of the key positions of the mannose available for MBP binding. In order to keep the OH of the C-3 and C-4 of mannose available for recognition of the carbohydrate domains of MBP, we removed the naturally occurring phosphate groups of the mannan using an alkaline phosphatase–protein A system. The dephosphorylation process was followed by determination of the ability of treated mannan to bind onto alumina, known to be specific only for immobilization of phosphoproteins [12]. As shown in Table 1, more than 90% of the native mannan was bound to alumina, while after dephosphorylation for 3 h at 37°, 95% of the mannan was found in the supernatant.

The dephosphorylated mannan exposed to hexokinase agarose beads was specifically phosphorylated at the C-6 position of mannose. The phosphorylation reaction was followed by measurement of ADP released in the reaction mixture by a lactate dehydrogenase coupled assay system. The oxidation of NADH was monitored by the decrease in adsorbance at 340 nm. According to the calibration curve constructed with known amounts of ADP only 0.4 m*M* of ATP was bound to the 6-OH position in the mannan molecule (Fig. 1).

As shown in Table 1 more than 60% of the

Table 1
Binding of various mannan preparations to alumina

Mannan preparations	Coupling yield (%)	A_{495} [a]	
		Before immobil.	After immobil.
Mannan without any modification	94	1.9	0.1
Dephosphorylated mannan	5	1.9	1.8
6-OH phosphorylated mannan	61	1.8	0.7

[a] The presence of mannan in supernatant was determined by the phenol test as follows: to a 1-ml mannan sample 2.5 ml, H_2SO_4 99% + 25 μl phenol 80% were added. The reaction mixture was gently vortex-mixed. A calibration curve of known amounts of mannan was prepared and the amounts of mannan before and after immobilization measured by change in the absorbance at 495 nm.

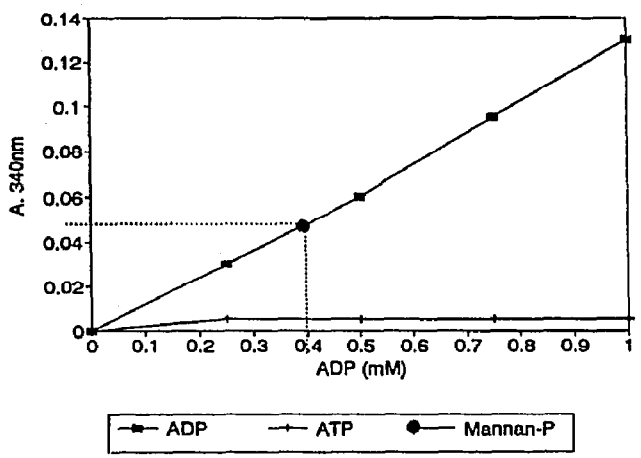

Fig. 1. Determination of the concentration of released ADP from the phosphorylation reaction mixture of mannan. Aliquots of the supernatant of the phosphorylation reaction were incubated with 6.6 m*M* NADH (100 μl), 45 m*M* phosphoenolpyruvate (100 μl), 10 μl of lactic dehydrogenase (1.3 units) in a total volume of 3 ml of buffer containing 0.05 *M* imidazole-HCl (pH 7.6), 0.1 *M* KCl and 0.062 *M* $MgSO_4$ for 5 min at room temperature. Pyruvate kinase (10 μl) containing 2.5 units was added to start the reaction which was followed by measuring the decrease of absorbance at 340 nm. Calibration curves with known amounts of ADP and ATP (for comparison) were made under the same experimental conditions.

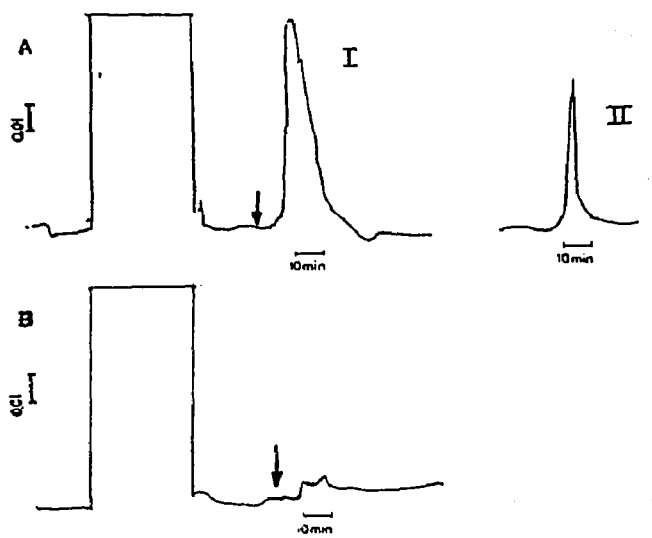

Fig. 2. Chromatographic pattern of MBP separation using alumina columns of: (A) mannan phosphorylated at 6 position. The first peak was eluted with EDTA (I) and the second peak (II) was obtained by elution with D-mannose. (B) naturally phosphorylated mannan. The arrows indicate the start of elution.

6-OH phosphorylated mannan was bound on the alumina. Aluminas are known to strongly interact with phosphoric and phosphonic acids, as well as with their monoesters yielding insoluble complexes stable in aqueous solutions at high ionic strength over a wide range of pH values [12]. Alumina beads display many of the properties required for an ideal biosupport in terms of loading capacity and operational stability. The capability of the two types of mannan–alumina preparations to purify the MBP from rabbit serum is demonstrated in Fig. 2. The naturally phosphorylated mannan did not bind MBP. Only with the 6-OH phosphorylated mannan alumina column was it possible to isolate MBP (1 mg protein from 50 ml serum). Binding of MBP to mannan is a calcium-dependent reaction. The modulation of the affinity of the carbohydrate recognition domains of MBP for Ca^{2+} and for mannose emphasizes the importance of the interactions between distinct parts of these domains with the hydrophobic core of the lectin [17].

Mannose-binding protein was used in our study for identification and determination of IgM in biological fluids. Binding of IgM to free or immobilized MBP is a calcium dependent process and bound IgM can be dissociated by treatment with EDTA. MBP served as a sensitive and specific reagent for the detection of the IgM due to its specificity to the mannose moiety found only in IgM carbohydrate regions.

MBP labelled with HRP proved to be a sensitive diagnostic tool for detection of the human IgM in a direct ELISA assay (Fig. 3) or dot blot assays (Fig. 4). In both cases the sensitivity and specificity of MBP *versus* anti-IgM were higher. MBP was hence better suited for detection of picograms concentrations of human IgM. Early detection of appearance of IgM in biological fluids is of great significance in the diagnostics of many pathological cases.

Mannose-binding protein is of crucial importance *in vivo* as a defence molecule that can act as an opsonin by enhancing the clearance of mannose rich pathogens and it can be used as a sensitive and important diagnostic tool, exhibiting good reproducibility for identification and

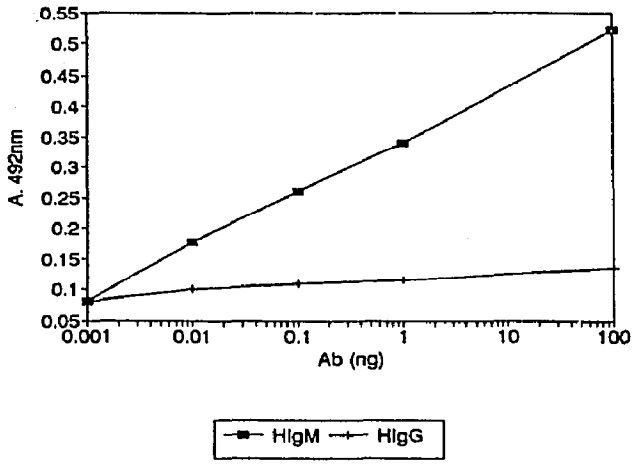

Fig. 3. Determination of human IgM and IgG using MBP–HRP in the ELISA system. For details see Experimental section.

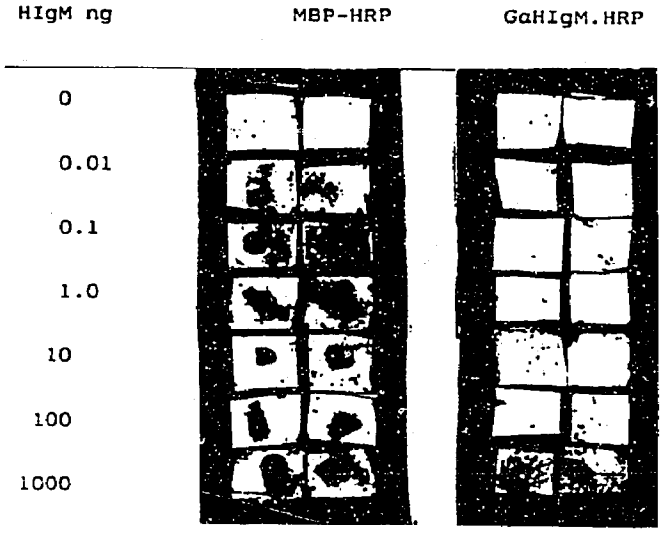

Fig. 4. Dot-blot assay for detection of minute amounts of IgM using MBP–HRP (left side) and αIgM–HRP (right side).

quantitation of the IgM in the early stages of its appearance in biological fluids. As shown in the present study it can serve as a sensitive diagnostic alternative to anti-IgM at least when very low concentrations of IgM should be detected.

References

[1] T. Kawasaki, R. Eton and I. Yamashura, *Biochem. Biophys. Res. Commun.*, 81 (1978) 1018.
[2] Y. Kozutsumi, T. Kawasaki and Y. Yamashina, *Biochem. Biophys. Res. Commun.*, 95 (1980) 658.
[3] R. Townsend and P. Stahl, *Biochem. J.*, 194 (1981) 209.
[4] J. Wild, D. Robinson and B. Winchester, *Biochem. J.*, 210 (1983) 167.
[5] H. Lis and N. Sharon, *Curr. Opin. Struct. Biol.*, 1 (1991) 741.
[6] E.M. Anders, C.A. Hartley and D.C. Jackson, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 4485.
[7] J. Nevens, A.K. Mallia, M.W. Wendt and P. Smith, *J. Chromatogr.*, 597 (1992) 247.
[8] R.A.B. Ezekowitz, M. Kuhlman, J.E. Groopman and R.A. Byrn, *J. Exp. Med.*, 169 (1989) 185.
[9] K. Drickamer, M.S. Dordal and L. Reynolds, *J. Biol. Chem.*, 261 (1986) 6578.
[10] R.T. Lee, Y. Ichikawa, M. Fay, K. Drickamer, M.C. Shao and Y.C. Lee, *J. Biol. Chem.*, 266 (1991) 4810.
[11] C.E. Ballou in A. Meister (Editor), *Advances in Enzymology, Vol. 40*, Wiley, New York, 1977, p. 239.
[12] M.A. Coletti-Previero and A. Previero, *Anal. Biochem.*, 180 (1989) 1.
[13] P.L. Ey, S.J. Prowse and C.R. Jenkin, *Immunochemistry*, 15 (1978) 429.
[14] G. Ashwell, *Methods Enzymol.*, 8 (1966) 85.
[15] P. Tijssen and E. Kurstak, *Anal. Biochem.*, 136 (1984) 451.
[16] G. Fleminger, E. Hadas, T. Wolf and B. Solomon, *Appl. Biochem. Biotechnol.*, 23 (1990) 123.
[17] R.A. Childs, T. Feizi, C.T. Yuen, K. Drickamer and M.S. Quensenberry, *J. Biol. Chem.*, 265 (1990) 20770.