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PREPARATION AND APPLICATION OF A PENTAMANNOSYL MONOPHOSPHATE–BOVINE SERUM ALBUMIN CONJUGATE

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ABSTRACT

Pentamannosyl monophosphate, derived from *Hansenula holstii O*-phosphomannan, was conjugated to bovine serum albumin by reductive amination. The conjugate inhibited the binding of the porcine testis mannose 6-phosphate‡ receptor to the insoluble phosphomannan core. A mannose 6-phosphate receptor with a molecular weight of 200,000 was purified from porcine liver membranes, using an affinity matrix of the conjugate attached to Sepharose 4B. Rabbits were immunised with the conjugate, and the antisera were purified on a phosphomannan core–Sepharose 4B column in order to give an antibody which was specific for the 6-phosphate group and the equatorial HO-4 of D-mannose 6-phosphate. On Western blot analysis using the purified antibodies, ovalbumin, which contained a typical high-mannose type of oligosaccharide, was not recognised. However, a testicular glycoprotein fraction formed an immunostaining band. These results indicate the effectiveness of the conjugate as a ligand for mannose 6-phosphate receptors. The antibodies highly specific for mannose 6-phosphate may be used to detect or purify lysosomal enzymes.

INTRODUCTION

Various neoglycoproteins have been synthesised and used to study the role of carbohydrates in biological phenomena, to determine the specificities of lectins, and to purify carbohydrate-binding proteins[1–5]. The specificities of anti-carbohydrate antibodies raised by the neoglycoproteins have been also examined[6–8].

O-Phosphomannan, secreted by the yeast *Hansenula holstii,* is degraded by mild acid hydrolysis of the hemiacetal phosphodiester linkages. Pentamannosyl monophosphate (PMP), α-D-Man-6-phosphate-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→2)-D-Man, is abundant in the hydrolysate, whereas only a small quantity of the phosphorylated core fragment with a high molecular weight is

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‡Unless stated otherwise, all monosaccharides are D.

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obtained[9,10]. The phosphomannan core fragment attached to agarose gels has been applied in the purification of mannose 6-phosphate receptors (PMR) from various animal tissues and cell types[11-13]. The PMP are usually derivatised with (*p*-aminophenyl)ethylamine and coupled to albumins or cyanogen bromide-activated agarose gels[14,15]. The neoglycoprotein and substituted gels have been used successfully as a model compound of the uptake system for lysosomal enzymes and as an affinity matrix for PMR, respectively.

PMP has now been conjugated to bovine serum albumin (BSA) by the reductive amination method[16], and the conjugate applied to the purification of PMR from porcine liver membranous materials and to the preparation of antibody highly specific for mannose 6-phosphate.

EXPERIMENTAL

Materials. — *O*-Phosphomannan from *Hansenula holstii* NRRL Y-2448 was a generous gift from Dr. M. E. Slodki (U.S. Department of Agriculture Northern Regional Laboratory, Peoria, Illinois). Phospomannan core fragment and PMP were prepared[9] by mild acid hydrolysis of phosphomannan. BSA (Fraction V) and ovalbumin (crystallised and lyophilised powder, grade V) were purchased from Sigma, Sepharose 4B and Sephacryl S-200 from Pharmacia, complete and incomplete Freund's adjuvants from Difco Laboratories, and goat anti-rabbit IgG (H + L)–horseradish peroxidase conjugate from Bio-Rad Laboratories. Acetone powders from porcine liver and testis membranes were prepared by the method of Sahagian *et al.*[12] and stored at −20°. A PMR comprising a subunit of molecular weight 36,000 was purified from detergent-solubilised extracts of testis membranous materials, and iodinated[17] with $Na^{125}I$ (14 Ci/mg of iodine) in the presence of Iodogen (Pierce). A glycoprotein fraction, which retarded the binding of testicular β-D-galactosidase to the affinity column of *p*-aminophenyl 1-thio-β-D-galactopyranoside or lactose–BSA neoglycoprotein attached to Sepharose 4B, was prepared from the soluble extract from porcine testis. Briefly, the crude extract was precipitated by the addition of solid ammonium sulfate to 70% saturation. A solution of the precipitate in 10mM acetate buffer (pH 4.0) was dialysed against the same buffer, and then applied to a column (bed volume, 50 mL) of lactose–BSA–Sepharose 4B. The column was washed with the equilibrating buffer and the glycoprotein fraction was eluted with the same buffer containing 0.2M NaCl. Further purification was accomplished by gel filtration on a column of Sephacryl S-200. After concentration by ultrafiltration, the purified preparation was dialysed against 50mM Tris/HCl buffer (pH 7.5) and stored at −20°. Details of the purification procedures for the glycoprotein fraction and its characterisation will be reported elsewhere.

Preparation of PMP–BSA conjugate. — Using the method of Schartz and Gray[16], a mixture of BSA (1 g), PMP (3 g), and sodium cyanoborohydride (1.5 g) in 0.2M potassium phosphate (74 mL, pH 9.0) was stored for 12 days at 37° and

then dialysed against several changes of water (6 L). The slight precipitate was removed by centrifugation and the supernatant solution was lyophilised. The neoglycoprotein was further purified by gel filtration on a column (2.2 × 85 cm) of Sephacryl S-200 previously equilibrated with 50mM Tris/HCl buffer (pH 7.5) containing 0.2M NaCl. Fractions containing the neoglycoprotein were combined, dialysed extensively against water, and then lyophilised. The purified PMP–BSA conjugate was stored at −20°.

Preparations of affinity gels. — The ligands were coupled to Sepharose 4B as described by Cuatrecasas[18]. Usually, the ligand (500 mg, BSA, PMP–BSA conjugate, or phosphomannan core) was mixed with 0.1M $NaHCO_3/Na_2CO_3$ buffer (100 mL, pH 9.2) containing Sepharose 4B (50 mL) previously activated for 12 min in M Na_2CO_3 (100 mL) with a solution of cyanogen bromide (5 g) in acetonitrile. After incubation overnight at 4°, the gels were washed with water, and the unreacted groups were blocked by stirring for 2 h at room temperature with M monoethanolamine in 0.1M $NaHCO_3/Na_2CO_3$ buffer (100 mL, pH 9.2). The substituted gels were washed with water and then stored at 4° in phosphate-buffered saline (PBS, pH 7.2) containing 0.02% of sodium azide.

Immunisation. — A solution of the conjugate (5 mg of powder) in PBS (1 mL) was emulsified by sonication with an equal volume of Freund's complete adjuvant, and injected intradermally at multiple sites into New Zealand White rabbits. The injection procedure was repeated twice at intervals of 2 weeks. The final injection was performed using Freund's incomplete adjuvant. Antisera were collected 2 weeks after the final injection and stored at −20°. The presence of antibody was checked by an Ouchterlony double immunodiffusion test.

Hapten inhibition assays of antibody. — The hapten inhibition assays were carried out according to the enzyme-linked immunosorbent assay (ELISA) procedure. The wells of a 96-well ELISA plate (EIA plate E, Sumitomobakelite Co., Tokyo) were coated with PMP–BSA solution (100 μL, 0.5 mg/mL in 0.1M carbonate buffer, pH 8.5) overnight at 4°, washed by filling three times with PBS, and then treated for 30 min at room temperature with PBS containing 1% of BSA. The wells were washed four times with PBS containing 0.05% of Tween 20 (PBST). Various concentrations of inhibitors (100 μL) were mixed with an equal volume of the diluted antibody. A portion (50 μL) of the mixture was added immediately to the wells and incubated for 1 h at room temperature. After washing five times with PBST, the wells were further incubated for 1 h with a 1:3,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (H + L) antibody in PBST (50 μL). The wells were washed five times with PBST and incubated for 1 h with 0.1M citrate buffer (100 μL, pH 5.0) containing 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.002% of hydrogen peroxide. The plates were then read on a Bio-Rad Model 2550 EIA Reader using a 405-nm filter. Each result was obtained in triplicate; control wells lacking PMP–BSA or the diluted antibody were included in each plate.

Immunostaining of Western blots. — Proteins were separated[19] by SDS–

PAGE and transferred electrophoretically to nitrocellulose sheets, using a Sartorius Semi-Dry Electroblotter. After blotting, the sheets were treated at 4° overnight with PBS containing 0.5% of BSA and 0.005% of thimerosal (Sigma). The sheets were washed with PBST and incubated for 1 h at room temperature with an appropriate dilution of antibody in the same buffer (100 mL). The sheets were washed well with PBST, treated with a 1:3,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate in PBST for 1 h, washed with PBST and PBS, and then incubated with a mixture of 12mM Tris/HCl buffer (50 mL, pH 8.0), 0.3% 4-chloro-1-naphthol in methanol (10 mL), and hydrogen peroxide (20 μL). The bands which reacted with the antibody were detected as purplish black ones and photographed immediately.

Analytical procedures. — Protein concentrations were measured by the method of Hartree[20] or Bradford[21], using BSA as standard. The content of carbohydrate was determined by the phenol–sulfuric acid method[22] with mannose as the standard.

RESULTS

PMP–BSA conjugate was prepared by the reductive amination method[16]. Since the commercial BSA was not homogeneous, the conjugate was further purified by gel filtration on Sephacryl S-200 (data not shown) and a small amount of the contaminating proteins was removed. The purified conjugate averaged 13 mol of PMP per mol of BSA. On SDS–PAGE, the conjugate gave a single band with a molecular weight of 86,000 (Fig. 5, lane 3), which stained intensely with the periodic acid–Schiff reagent.

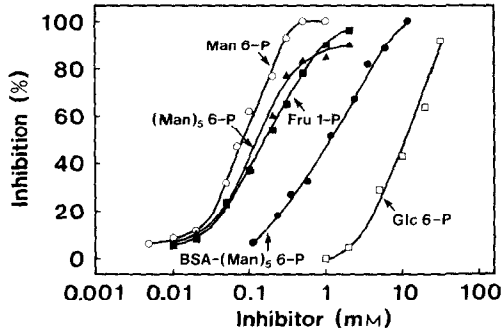

Fig. 1. Inhibition of binding of porcine testis PMR to insoluble phosphomannan core by various sugars and PMP–BSA. The binding activity of iodinated PMR was measured[17] by incubating the receptor with phosphomannan core–Sepharose 4B. The reaction mixtures (each 100 μL) consisted of 20,000 c.p.m. of iodinated PMR, various concentrations of inhibitors, and the insoluble phosphomannan core (35 μL of gel) in 50mM imidazole/HCl (pH 7.0), 10mM $MnCl_2$, 150mM NaCl, 0.05% Triton X-100, and 0.5 mg/mL BSA. The reaction was carried out for 90 min at room temperature. After centrifugation, a portion (50 μL) of the supernatant solution was measured for radioactivity. The inhibitors used were D-mannose 6-phosphate, (—○—), D-fructose 1-phosphate, (—■—), PMP, (—▲—), D-glucose 6-phosphate, (—□—), PMP–BSA, (—●—). The values shown are means of triplicate experiments.

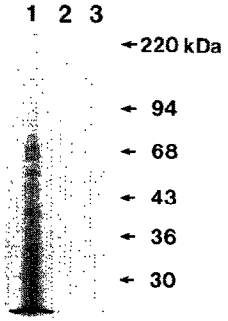

Fig. 2. Purification[12] of PMR from porcine liver membranes on PMP–BSA-substituted Sepharose 4B. A suspension of the acetone powder (50 g) in 1.4 L of 0.2M NaCl, 10mM EDTA, and 0.1M sodium acetate (pH 6.0) was stirred for 30 min. After centrifugation for 30 min at 16,000*g*, the pellet was resuspended in 1.4 L of water and recentrifuged. The washed, membranous material was suspended in 1.4 L of 50mM imidazole/HCl (pH 6.5), 0.4M KCl, and 1% Triton X-100, stirred for 3 h, and then centrifuged. The supernatant solution was applied to a column (bed volume, 20 mL) of PMP–BSA–Sepharose which had been equilibrated with the extraction buffer at 80 mL/h. The column was washed with 50mM imidazole/HCl (pH 6.5), 0.4M KCl, and 0.05% Triton X-100, and the PMR was eluted from the column with 5mM D-mannose 6-phosphate in the same buffer. In some experiments, $MnCl_2$ was added to the detergent-solubilised extract to give a final concentration of 10mM, and the affinity chromatography was performed using the same buffer system in the presence of 10mM $MnCl_2$. The solution of the purified proteins was concentrated to 2 mL by ultrafiltration and dialysed against 50mM imidazole/HCl (pH 7.0), 0.15M NaCl, and 0.05% Triton X-100 (2 L). Aliquots were then analysed by SDS–PAGE (7.5% acrylamide gel) and the protein bands were detected by Brilliant Blue staining. Crude detergent-solubilised extract (lane 1), and final preparation purified in the absence (lane 2) and presence of $MnCl_2$ (lane 3).

The interaction of PMP–BSA and the porcine testis PMR was examined by measurement of the inhibition of PMR binding to the insoluble phosphomannan core (Fig. 1). The ligand-binding activity of the PMR was selectively stimulated by $MnCl_2$, whereas EDTA had no effect on the binding activity[17]. The binding was most effectively inhibited by D-mannose 6-phosphate, D-fructose 1-phosphate, and PMP. However, the inhibitory effect of D-glucose 6-phosphate was much poorer[17]. PMP–BSA was also an effective inhibitor, but was one order of magnitude less effective than the three effective inhibitors.

The PMP–BSA neoglycoprotein was coupled to Sepharose 4B and the product was used to purify PMR from the 1% Triton X-100-solubilised extract of porcine liver membranes. The proteins retained on the affinity column were eluted with 5mM D-mannose 6-phosphate. As shown in Fig. 2, the purified preparation contained one major protein band which migrated on SDS–PAGE with an apparent molecular weight of 200,000. When the affinity chromatography was carried out in the presence of 10mM $MnCl_2$, a similar result was obtained (Fig. 2, lane 3). The yield of the purified protein was 0.7 mg from 50 g of the original acetone powder from porcine liver.

Double immunodiffusion tests revealed that the antisera produced to the PMP–BSA conjugate formed a precipitin band either with the neoglycoprotein or

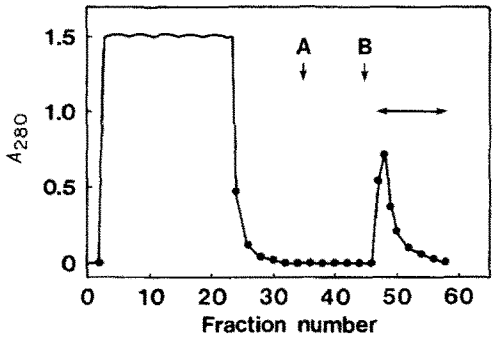

Fig. 3. Purification of antibodies specific for D-mannose 6-phosphate on phosphomannan core–Sepharose 4B. Antisera (30 mL) were diluted with an equal volume of PBS and fractionated by precipitation with ammonium sulfate at 40% saturation. The antibodies were passed through a column (10-mL bed volume) of BSA-substituted Sepharose equilibrated with PBS, and the column was washed with the same buffer. The effluent and washings (~50 mL) were combined and applied to a 5-mL affinity column of phosphomannan core attached to Sepharose 4B. The column was washed with PBS, and the retained antibodies were eluted with 0.1M mannose in PBS (A) followed by 5mM D-mannose 6-phosphate (B). Fractions (3.5 mL) were collected at 30 mL/h and the absorbance at 280 nm (A_{280}, —●—) was measured.

with the original BSA (data not shown), indicating the presence of anti-BSA antibodies. The antisera (30 mL) were diluted with an equal volume of PBS, and ammonium sulfate was added to 40% saturation. The precipitate was collected by centrifugation at 10,000*g* for 10 min, dissolved with PBS, and dialysed overnight against PBS (2 L). The dialysed solution was passed through a column of BSA–Sepharose 4B (10 mL) previously equilibrated with PBS. The column was washed with PBS and the eluate (~50 mL) was applied to a column of phosphomannan core–Sepharose 4B (5 mL), as shown in Fig. 3. After washing the column with PBS, the retained antibodies were eluted with 0.1M D-mannose in PBS followed by 5mM D-mannose 6-phosphate. The last eluate was concentrated by ultrafiltration

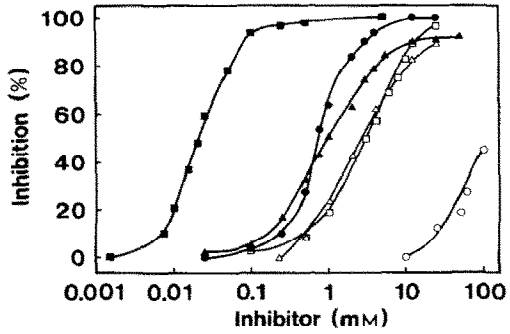

Fig. 4. Inhibition of the binding of purified antibodies to PMP–BSA in the solid phase by D-galactose 6-phosphate (—○—), D-fructose 1-phosphate (—□—), 2-amino-2-deoxy-D-glucose 6-phosphate (—△—), D-glucose 6-phosphate (—▲—), D-mannose 6-phosphate (—●—), and PMP (—■—). The values shown are means of triplicate experiments. For details see Experimental.

TABLE I

INHIBITION OF BINDING OF PURIFIED ANTIBODIES TO PMP–BSA IN THE SOLID PHASE BY VARIOUS SUGARS

Inhibitor (25mM)	*Inhibition of the binding*[a] *(%)*
D-Mannose	2.8
D-Glucose	3.2
D-Galactose	2.0
D-Fucose	6.4
L-Rhamnose	1.2
D-Mannitol	7.6
Methyl α-D-mannopyranoside	2.8
2-Amino-2-deoxy-D-mannose	3.6
2-Amino-2-deoxy-D-glucose	6.0
D-Mannose 6-phosphate	100.0
D-Glucose 6-phosphate	90.5
D-Galactose 6-phosphate	12.8
D-Fructose 6-phosphate	8.8
2-Amino-2-deoxy-D-glucose 6-phosphate	88.7
D-Fructose 1-phosphate	96.2
D-Ribose 5-phosphate	20.4
PMP	100.0

[a]All binding assays for the purified antibodies to PMP–BSA were carried out as described in Experimental. The results shown are the mean of triplicate experiments.

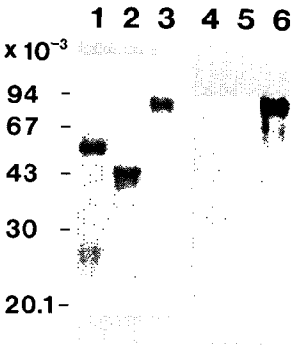

Fig. 5. Western blot analysis of glycoproteins and purified antibodies. Aliquots of testicular glycoprotein fraction (lanes 1 and 4), ovalbumin (lanes 2 and 5), and PMP–BSA (lanes 3 and 6) were treated at 100° for 3 min in 10mM Tris/HCl buffer (pH 6.8) containing 1% of SDS and 1% of 2-mercaptoethanol (final concentrations), and subjected to SDS–PAGE (10% acrylamide gel). After the electrophoresis at 20mA, the gel was cut into two portions. The protein staining (lanes 1–3) with Coomassie Brilliant Blue and the immunostaining (lanes 4–6) with the purified antibodies were carried out as described in Experimental. Lanes 1 and 4 contained 13 and 114 μg of testicular glycoprotein, respectively, lanes 2 and 5 contained 11 and 98 μg of ovalbumin, respectively, and lanes 3 and 6 contained 24 μg each of PMP–BSA.

and dialysed overnight against PBS. The original antisera yielded 6.6 mg of the purified antibodies as protein.

The binding of purified antibody to immobilised PMP–BSA was not inhibited significantly by D-mannose, D-glucose, D-galactose, D-mannitol, methyl α-D-mannopyranoside, D-fructose 6-phosphate, 2-amino-2-deoxy-D-mannose, 2-amino-2-deoxy-D-glucose, L-rhamnose, and D-fucose (25mM each, see Table I). D-Galactose 6-phosphate and D-ribose 5-phosphate caused 12.8 and 20.4% inhibition at 25mM, respectively. D-Mannose 6-phosphate, D-glucose 6-phosphate, D-fructose 1-phosphate, 2-amino-2-deoxy-D-glucose 6-phosphate, and PMP gave 50% inhibition at 0.7, 1.0, 3.0, 3.0, and 0.02mM, respectively (Fig. 4).

The carbohydrate contents, determined by the phenol–sulfuric acid method[22] using mannose as standard, of PMP–BSA conjugate, testicular glycoprotein fraction, and ovalbumin were 14.0, 1.3, and 5.1%, respectively. These glycoproteins were separated by SDS–PAGE (10% acrylamide gel), transferred electrophoretically to a nitrocellulose sheet, and reacted with the purified antibodies (Fig. 5). The original conjugate reacted well. The immunostaining band was in the same position as the protein band visualised by Coomassie Brilliant Blue (86 kDa, lanes 3 and 6). When ~100 μg of ovalbumin with 9 mol of D-mannose per mol was run, no visible immonostaining band was observed (lane 5). The testicular glycoprotein (114 μg/lane), which consisted of a major (55 kDa) and a minor (26 kDa) fragment, formed weak immunostaining bands with the purified antibodies (lane 4).

DISCUSSION

The neoglycoprotein conjugate prepared contained 13 mol of PMP per mol of BSA. Brown and Farquhar[15] have reported the synthesis of a PMP–rabbit serum albumin conjugate, *via* the (*p*-aminophenyl)ethylamine derivative of PMP, which averaged 38 mol of PMP per mol of the albumin. The amount of the conjugated PMP was almost three times as much as that of PMP–BSA. However, the direct coupling of PMP to BSA provides an easy route to the neoglycoprotein, in spite of the long reaction time.

The PMP–BSA conjugate inhibited the binding of porcine testis 36-kDa-PMR to insoluble phosphomannan core (Fig. 1), indicating the effectiveness of the conjugate as the ligand of PMR, although the inhibition is an order of magnitude poorer than that of mannose 6-phosphate.

When purification of the PMR from a detergent-solubilised extract of porcine liver membranes was attempted using PMP–BSA–Sepharose 4B, a PMR with a subunit molecular weight of 200,000 was isolated by elution with 5mM D-mannose 6-phosphate (Fig. 2). The mass was similar to that (215,000) of PMRs isolated from various tissues and cells[11–13,15,23–26]. Kornfeld *et al.*[25,26] isolated a cation-dependent PMR composed of 46-kDa subunits from bovine liver and P388D_1 macrophage. The 46-kDa PMR was distinguishable from the 215-kDa receptor by its requirement for divalent cations such as Mn^{2+} and its low affinity for methyl

phosphodiesters[25]. In the present work, only the 200-kDa PMR was purified by affinity chromatography either in the presence or the absence of 10mM $MnCl_2$ (Fig. 2), which may reflect the difference of the solubilisation method for membranous materials. Only Triton X-100 was used to solubilise porcine liver membranes, whereas the cation-dependent 46-kDa PMR was solubilised with Triton X-100 and sodium deoxycholate[25]. No attempt was made to purify the low-molecular-weight PMR according to the procedure described by Hoflack and Kornfeld[25].

Antisera raised to PMP–BSA were purified by affinity chromatography on phosphomannan core–Sepharose (Fig. 3), which yielded antibodies highly specific for D-mannose 6-phosphate, D-fructopyranose 1-phosphate, 2-amino-2-deoxy-D-glucose 6-phosphate, and D-glucose 6-phosphate (Table I and Fig. 4). These sugars are structurally similar. When the $^2C_5$ conformer of D-fructopyranose 1-phosphate is rotated 180° around an axis through C-4 and O-6, the phosphate group and HO-3 are superimposable on the 6- and 4-substituents, respectively, of D-mannose 6-phosphate. The poor inhibitory effect was demonstrated by the replacement of the equatorial HO-4 of D-glucose 6-phosphate by an axial group as in D-galactose 6-phosphate (Fig. 4). Thus, the affinity-purified antibodies have high specificities for the equatorial HO-4 and the 6-phosphate group of D-mannose 6-phosphate, but have a poor specificity for the stereochemical orientation of the HO-2.

Unfortunately, the inhibition of D-mannose 6-phosphate for binding of purified antibodies to PMP–BSA conjugate was one order of magnitude lower than that of PMP (Fig. 4). This finding suggests that the purified antibodies may well recognise the α-(1→3)-linked structure of PMP, in addition to HO-4 and the 6-phosphate group of the non-reducing mannose 6-phosphate end-unit. Therefore, further studies are necessary to obtain an antibody more highly specific for mannose 6-phosphate.

The purified antibodies did not react with ovalbumin, a typical glycoprotein having a high-mannose type oligosaccharide, whereas the testicular glycoprotein fraction composed of 55- and 26-kDa subunits was recognised (Fig. 5). This result suggests that the testicular glycoprotein contains oligosaccharides with mannose 6-phosphate units. Distler *et al.*[27,28] isolated a glycoprotein fraction from bovine testis, which, as well as β-D-galactosidase, bound to an affinity column of phenyl 1-thio-β-D-galactopyranoside-substituted agarose. The testicular glycoprotein fraction strongly inhibited an assimilation of testicular β-D-galactosidase by human skin fibroblasts, implying that the glycoprotein had the common recognition marker for PMR. The presence of D-mannose 6-phosphate in the testicular glycoprotein was confirmed by the same workers[27]. Although the bovine testicular glycoprotein is not characterised well, it seems likely that it is identical to the glycoprotein fraction isolated in this study. Further characterisation of the porcine testis glycoprotein fraction is in progress.

When this study was complete, von Figura and co-workers described[29] a PMP–BSA conjugate, with an apparent molecular weight of 100,000 and a carbohydrate content of 30%, which was prepared by the reductive amination method[16].

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