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PURIFICATION OF SOME GLYCOSIDE HYDROLASES BY AFFINITY CHROMATOGRAPHY*

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ABSTRACT

Two glycoproteins have been isolated from the cell walls of baker's yeast. One is a glucan–protein complex which has been partially characterised as having a branched carbohydrate structure composed of chains of (1→3)-linked β-D-glucosyl residues, some of which are attached by (1→6)-linkages to the main chain. Immobilization of this glycoprotein was achieved by covalent attachment to Sepharose, and the product was used to isolate a number of (1→3)-β-D-glucan hydrolases from *Helix pomatia*, malted barley, and *Basidiomycete* QM806. The second glycoprotein, a mannan–protein complex, after immobilization, has been used in the purification of an α-D-mannosidase from jack-bean meal.

INTRODUCTION

The purification of carbohydrate-metabolizing enzymes for use in structural analysis studies requires that the enzymes are free from other glycosidase activities. Conventional purification procedures are usually long and laborious, and often incomplete. Many reports have been made on the highly complex nature of the β-D-glucanase mixture present in snail digestive juice[1–4] and malted barley[5–6]. Attempts to purify these enzymes by affinity chromatography have either used such insoluble polysaccharides as pachyman or chitin[7], or ligands of small molecular weight covalently attached to Sepharose[8–14]. We now describe the preparation and use of glycoproteins immobilised on Sepharose 4B as an affinity support in the purification of some glycoside hydrolases.

EXPERIMENTAL

Enzymes. — Helicase, a crude mixture of snail digestive enzymes from *Helix pomatia*, was obtained from Industrie Bioloqique Francaise, S.A. Exo-(1→3)-β-D-glucanase from *Basidiomycete* QM806 was prepared by the method of Reese and Mandels[15].

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

Malted-barley enzyme extract was prepared from malted-barley flour (Var. Proctor, 1969 harvest) by stirring with acetate buffer (0.2M, pH 5.3; 3 l/kg of flour) for 3 h at 25°, followed by centrifugation (15,000 *g*, 30 min), and dialysis against running tap water (24 h). The extract was then treated with ammonium sulphate (to 50% saturation), and the precipitated material was redissolved in distilled water, and dialysed against distilled water (6 × 5 l; 60 h, 4°). After removal of insoluble material, the solution was freeze-dried.

Jack-bean meal (20 g, Sigma Chemical Company) was extracted (4°) for 45 h with distilled water (80 ml); the mixture was then filtered, and centrifuged (5,000 *g*) to give an opaque solution. Solid ammonium sulphate was added to 33% saturation, and the precipitate removed by centrifugation (30,000 *g*) and discarded. The supernatant was adjusted to 50% saturation with solid ammonium sulphate, and the precipitate was removed by centrifugation (30,000 *g*), dissolved in citrate–phosphate buffer (0.05M, pH 4.6; 5.0 ml), and dialysed against the same buffer (3 × 1 litre; 24 h, 4°). This material was purified further by chromatography on a column (1.9 × 22cm) of Sephadex G-75 equilibrated with M sodium chloride. The α-D-mannosidase travelled in the void volume of the column and was free from concanavalin A, which was eluted with 100mM D-glucose in M sodium chloride. Fractions containing the enzyme were dialysed against several changes of sodium acetate buffer (3 × 2 l; 0.01M, pH 4.6) and used for affinity chromatography on columns of Sepharose 4B conjugated mannan–protein.

Substrates. — Pachyman, from the fungus *Poria* (Pachyman) *cocus*, was obtained from the Sam-Ae Trading Company (Seoul, S. Korea). The inner portions of the sclerotia were ground, and the pigments were extracted into hot chloroform–methanol (2:1). The insoluble, dried residue (pachyman) was then treated in sequence with sodium periodate (0.02M) for 48 h, ethylene glycol (0.01M), sodium borohydride (0.05M) for 16 h, and hydrochloric acid (0.05M) for 1 h at 100°.

Sodium carboxymethylcellulose (CMC, Cellofas B, medium viscosity) was obtained from I.C.I. Ltd. Sodium carboxymethylpachyman (CMP) was prepared by the method of Clarke and Stone[16]. Barley glucan was prepared from Ymer barley by the method of Preece and MacKenzie[17]. Laminarin from *Laminaria hypoborea* was the sample examined by Fleming[18], and has a glucose content of 94% and d.p. 21. *p*-Nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranosiduronic acid, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, D-glucose oxidase (type II), peroxidase, and *o*-dianisidine hydrochloride were commercial materials (Sigma).

Analytical methods. — Protein was determined by the method of Lowry *et al.*[19] with bovine serum albumin as standard. Total nitrogen was measured by a modified Kjeldahl procedure[20]. Total carbohydrate was estimated by the phenol–sulphuric acid method[21] with D-glucose as standard. Reducing sugars were measured by a modified Nelson method[22]. D-Glucose was determined specifically either with D-glucose oxidase[23] or by the hexokinase:ATP method[24]. D-Mannose was measured in conjunction with D-glucose by the hexokinase:ATP method[25].

Descending paper chromatography was performed on Whatman No. 1 paper with ethyl acetate–pyridine–water (10:4:3) and propan-1-ol–ethyl acetate–water (14:2:7). Reducing sugars were detected with alkaline silver nitrate[26].

Gel electrophoresis was performed by the procedure of Ornstein and Davis[27,28] with 7.5% polyacrylamide gels in Tris-glycine buffer (0.01M, pH 8.0). Protein was located in the gels by staining with Coomassie Blue, and enzyme activities were located after incubation of 1-mm slices of the gels with substrate in the standard procedures. Consumption of periodate ions was measured by the method of Aspinall and Ferrier[29]. Methylation of glycoproteins was effected by the Hakomori procedure, as described by Bjorndal *et al.*[30], and identification of the derived methylated alditol acetates was by g.l.c.–m.s. on columns of 3% of OV 225, as described by Bjorndal *et al.*[31].

Column chromatography. — Both molecular-sieve and affinity chromatographic separations were carried out at 4°. Protein was monitored by measurement of the absorbance at 280 nm.

Ultracentrifugation. — Samples were dissolved in methyl sulphoxide, and the sedimentation behaviour was examined in a Beckman Model E analytical ultracentrifuge equipped with a standard 12-mm cell and an AN-D rotor.

Enzyme assays. — During purification of the exo-(1→3)-β-D-glucanase activity (EC 3.2.1.58), the enzyme was determined by measurement of either the reducing sugars or D-glucose produced. In a final volume of 1.0 ml, digests contained substrate (laminarin, 2.5 mg), acetate buffer (0.05M, pH 4.8), and gelatin (0.02% w/v) for stabilization of enzymic activity; the incubation time was 15 min at 37°. The reaction was stopped by heating at 100° for 5 min, and the liberated D-glucose was measured by the D-glucose oxidase method, or by addition of the Nelson–Somogyi reagent when reducing power was measured. Specific activities are expressed as μmol of D-glucose produced per min per mg of protein.

For the measurement of either endo-(1→3)-β-D-glucanase (EC 3.2.1.39) or endo-(1→4)-β-D-glucanase (EC 3.2.1.4) activities, the decrease in viscosity of their respective substrates (CMC and CMP) was measured in a No. 1 B.S.S. Ostwald viscometer. Digests containing 2 ml of substrate (CMP, 7 mg/ml; CMC, 3 mg/ml) and buffer (acetate, 0.05M, pH 4.8; 0.5 ml) were mixed with enzyme solution (0.5 ml) at 37°. The digest (2 ml) was added to the viscometer in a constant-temperature water bath. Enzyme activity was expressed as the rate of increase of the reciprocal of specific viscosity with time: $d/dt(1/\eta_{sp})$.

The release of *p*-nitrophenol from *p*-nitrophenyl glycosides was measured in digests containing substrate (0.01M), acetate buffer (0.05M, pH 4.6) containing zinc sulphate (2mM) in a total volume of 0.5 ml, and an appropriate quantity of enzyme, with an incubation time of 15 min at 37°. The reaction was stopped by the addition of 2.5 ml of glycine–NaOH buffer (0.04M, pH 10.5), and the released *p*-nitrophenol was measured at 405 nm.

Isolation of the glycoproteins from yeast cell-walls. — The method was essentially that developed by Korn and Northcote[32], by extraction of the cell walls of

baker's yeast *Saccharomyces cerevisiae* (Distillers Company Ltd.) with anhydrous ethylenediamine for 3 days at 37°. The glucomannan glycoprotein (GP_2B), which was insoluble in water and ethylenediamine, contained 5.9% of protein and 85% of carbohydrate. Total acid hydrolysis of a sample of this preparation indicated[25] the presence of D-glucose and D-mannose in the ratio of 10:1. Glycoprotein (GP_2A) containing 10% of protein and 80% of carbohydrate was soluble in both water and ethylenediamine, and contained D-mannose as the only carbohydrate component.

Coupling to Sepharose 4B. — Glycoprotein (GP_2A or GP_2B) (20 mg) was suspended in sodium hydrogen carbonate (0.1M, pH 9.0; 1.0 ml), and sodium hydroxide (4M) was added dropwise until the glycoprotein dissolved. The pH was readjusted to 9.0 with acetic acid (2M) before its addition to cyanogen bromide-activated Sepharose 4B (Pharmacia, 2 g) suspended in 20 ml of sodium hydrogen carbonate (0.1M, pH 9.0) and sodium chloride (0.5M). The reaction mixture was agitated gently at 4° for 24 h, followed by extensive washing with sodium hydrogen carbonate (0.1M, pH 9.0), sodium chloride (0.5M), and distilled water. Any unreacted, cyanogen bromide-active sites on the Sepharose molecules were reacted by incubation of the reaction product with M ethanolamine for 1 h at room temperature, followed by washing with sodium hydrogen carbonate (0.1M, pH 9.0) and distilled water. Hydrolysis of samples of the resulting gels and analysis of the liberated D-glucose and D-mannose indicated the binding of 300 μg of glycoprotein/ml of swollen gel. This gave sufficient material for the preparation of columns (6 × 1 cm) which retained their abilities to bind enzymes for numerous chromatographic runs.

RESULTS AND DISCUSSION

Structure of the carbohydrate portion of the glucan–protein complex. — The glucan–protein complex (GP_2B) isolated from the yeast cell-wall was soluble in borate buffer (pH 9.5) and also in methyl sulphoxide, and sedimented in the ultracentrifuge as one symmetrical, homogeneous peak. From the total carbohydrate and protein contents of 85 and 5.9%, respectively, it appears to be similar to a glycoprotein isolated by Korn and Northcote[32]. The analysis of the methylated product from GP_2B indicated the presence of 79% of 2,4,6-tri-*O*-methyl-D-glucose and 3% of 2,3,4-tri-*O*-methyl-D-glucose, whilst the results of periodate oxidation indicated that 89% of the D-glucose residues were resistant to oxidation by the periodate ion. Thus, a substantial part of the carbohydrate moiety is in the form of linear chains of (1→3)-linked D-glucosyl residues with a small (3%) number of (1→6)-linked D-glucosyl residues. The formation of 2,4-di-*O*-methyl-D-glucose (2%) indicated the presence of interchain linkages in the molecule. The anomeric configuration of the D-glucose residues was shown to be β by virtue of the isolation and characterization of a homologous series of laminarisaccharides (up to laminariheptaose), on partial hydrolysis with acid, together with gentiobiose. In addition, D-glucose was the only product when GP_2B was hydrolysed with the exo-(1→3)-β-D-glucanase preparation from *Basidiomycete* QM806. Thus, the carbohydrate moiety of this glycoprotein

shows major structural similarities to the alkali-insoluble glucan isolated previously by Manners *et al.*[33].

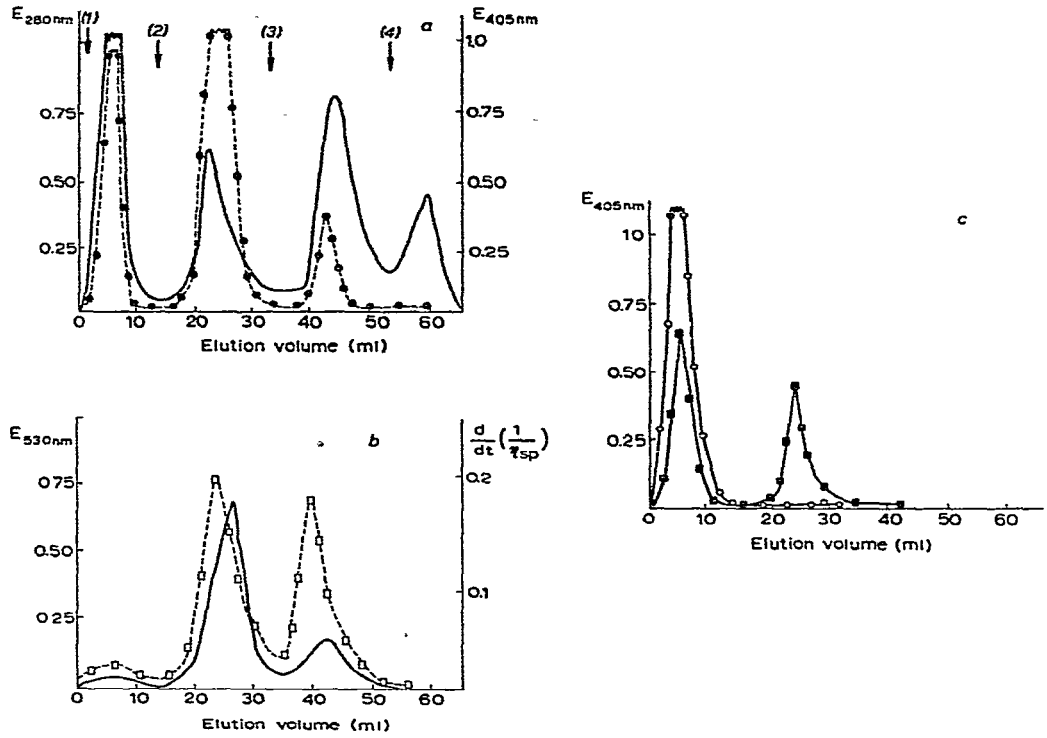

Fig. 1. Fractionation of *Helix pomatia* enzymes on GP_2B-Sepharose columns: (*a*) ————, protein; – – ●– – –, β-D-glucosidase; (*b*) ————, endo-(1→3)-β-D-glucanase; ——□——, exo-(1→3)-β-D-glucanase; (*c*) ——○——, β-D-glucuronidase; ——■——, *N*-acetyl-β-D-glucosaminidase activities; elution with (*1*) water; (*2*) acetate buffer (0.05M, pH 4.8); (*3*) borate buffer (0.05M, pH 8.2); and (*4*) buffer (*3*) containing M sodium chloride.

Purification of β-D-glucanases on GP_2B-Sepharose. — When the glycoprotein was attached covalently to Sepharose 4B, binding of proteins from the digestive juice of *Helix pomatia* occurred, whereas it was shown that Sepharose alone fails to retain any of the enzyme protein. The protein bound to the column was recovered by a sequence of elutions with water, 0.05M acetate buffer (pH 4.8), 0.05M borate buffer (pH 8.2), and 0.05M borate buffer (pH 8.2) containing M sodium chloride (Fig. 1). The material emerging at pH 4.8 contained endo-(1→3)-β-D-glucanase, exo-(1→3)-β-D-glucanase (Fig. 1b), β-D-glucosidase (Fig. 1a), and *N*-acetyl-β-D-glucosaminidase (Fig. 1c) activities, whilst the material eluted in the first borate buffer showed the same enzyme activities, except that the hexosaminidase activity was absent. The endo-(1→3)-β-D-glucanase was present as a minor component in this fraction; on re-chromatography of the fraction through a column (8 × 1 cm) of pachyman using

identical elution procedures, only exo-(1→3)-β-D-glucanase activity was detected. On gel electrophoresis, this sample appeared to be the major (90%) protein when stained with Coomassie Blue; two minor impurities were present. The enzyme was capable of hydrolysing a number of different substrates, all containing (1→3)-linked β-D-glucosyl residues (Table I). In all cases, with the exception of the mixed-linkage barley β-D-glucan, D-glucose was the only sugar component identified by paper chromatography of the digests. The barley glucan released substantial amounts of oligosaccharides and only traces of D-glucose. The exo-nature of the enzyme was also demonstrated by the fact that negligible quantities of D-glucose were released on

TABLE I

SUBSTRATE SPECIFICITY OF *Helix pomatia* EXO-(1→3)-β-D-GLUCANASE

Substrate	*Predominant linkages present (all β types)*	*Product(s)*
Laminaribiose	1→3	Glucose
Laminaritriose	1→3	Glucose
Laminaritetraose	1→3	Glucose
Laminarin	1→3	Glucose
Pachyman	1→3	Glucose
Yeast glucan	1→3	Glucose
Barley glucan	1→3; 1→4	Oligosaccharides
Luteose	1→6	—
CM-Cellulose	1→4	Glucose (trace)

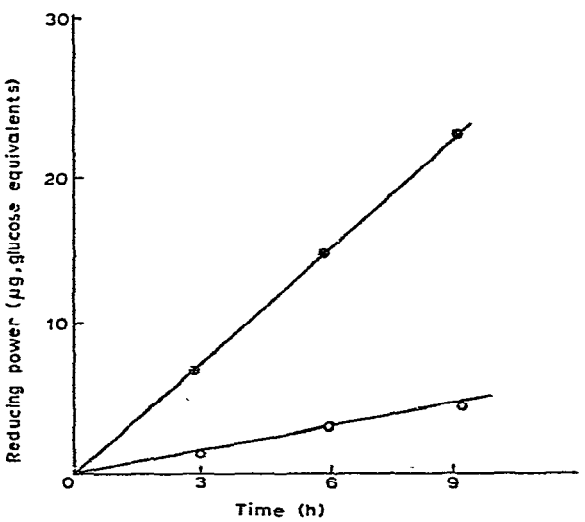

Fig. 2. Reducing power released by exo-(1→3)-β-D-glucanase of *H. pomatia* from laminarin, ——●——; and periodate-oxidised laminarin, ——○——.

incubation of the preparation with periodate-oxidised laminarin[34] (Fig. 2). The pH optimum of the purified enzyme was 4.8, and it showed a temperature optimum of 55°. Thus, this enzyme appears to have similar properties in terms of pH, temperature optima, and substrate specificity to one purified (by conventional procedures) from *H. pomatia* by Marshall and Grand[3]. The enzyme appears to have a less-rigid specificity than most of the other exo-β-D-glucanases. For instance, the corresponding enzyme[35] from *Basidiomycete* QM806 and those from various yeasts[36–38] do not degrade the mixed-linkage (1→3),(1→4)-β-D-glucans, and that from *Euglena gracilis*[39] releases only small amounts of glucose from such substrates.

It has not been possible to calculate an overall purification factor for the enzyme, because of the multiplicity of (1→3)-β-D-glucanases present in the starting material.

Samples of malted-barley extract[6] and a preparation of the exo-(1→3)-β-D-glucanase[34,35] from *Basidiomycete* QM806 were also chromatographed on the GP_2B-Sepharose column. In each case, protein was adsorbed and eluted with 0.05M borate buffer (pH 8.2), and enzymic activity was demonstrated against (1→3)-β-D-glucans (Table II). The malted-barley preparation, in addition to containing exo-(1→3)-β-D-glucanase, exhibited considerable contamination with an endo-(1→4)-β-D-glucanase.

Thus, a complex system of β-D-glucoside hydrolases having various specificities toward (1→3)-linked β-D-glucose molecules have been isolated by using this immobilized β-D-glucan–protein complex. This finding is in contrast to the results obtained when such simple carbohydrate derivatives as *p*-aminophenyl glycosides[14], *p*-aminophenyl 1-thioglycosides[8], and saccharo-1,4-lactone[10] are used as immobilised supports, and simple exo-glycosidases are usually adsorbed on the support.

Purification of an α-D-mannosidase by affinity chromatography on GP_2A Sepharose. — The D-mannan of the cell wall of *S. cerevisiae*, which has been studied by both chemical[40] and enzymic techniques[41,42], has been found to be a highly branched polysaccharide composed of (1→2)-α- and (1→3)-α-linked side-chains attached to an (1→6)-α-linked backbone of ~50 mannose units, in a mannan subunit containing ~150 "anhydromannose" units. Some of the mannose is attached to the polypeptide chain as short oligosaccharides, glycosidically linked to serine and threonine, but the majority is attached as polysaccharide chains of mannose residues linked *via* 2-acetamido-2-deoxy-D-glucose to asparagine[43]. α-D-Mannosidase (EC 3.2.1.24), a metallo-enzyme, isolated from jack-bean meal can hydrolyse (1→6)-α-, (1→2)-α-, and (1→3)-α-linked oligosaccharides of D-mannose, although only 5% of the total mannose present in yeast mannan is liberated by this enzyme. The enzyme preparations from this source bind to the GP_2A-Sepharose columns, and the enzyme could be recovered by use of increasing concentrations of salt. However, no separation of the α-D-mannosidase from β-D-galactosidase or *N*-acetyl-β-D-glucosaminidase was achieved. In addition, the columns had very low enzyme-binding capacities. The separations achieved were probably affected by the presence of large amounts of concanavalin A in the extracts. That concanavalin A will bind to yeast mannan has

TABLE II

RELATIVE SPECIFIC ACTIVITIES AND PRODUCTS OF HYDROLYSIS OF β-D-GLUCANASE PREPARATIONS FROM DIFFERENT SOURCES[a]

Substrate	*Malted barley*		*Basidiomycete QM806*		Helix pomatia	
	Activity[b]	*Products*[c]	*Activity*[b]	*Products*[c]	*Activity*[b]	*Products*[c]
p-Nitrophenyl β-D-glucopyranoside	2	N.d.	0.01	N.d.	8.8	N.d.
Laminarin	100	Glucose	100	Glucose	100	Glucose
Barley β-D-glucan	167	Glucose + oligosaccharides	3.8	Glucose	500	Glucose (trace) + oligosaccharide
O-(Carboxymethyl)cellulose	410	Glucose + oligosaccharides	0	nil	0	
O-(Carboxymethyl)pachyman	92	Glucose + oligosaccharides	0	nil	0	

[a]In each case, the enzyme fraction was that adsorbed on GP_2B-Sepharose and eluted with 0.05M borate buffer (pH 8.2). [b]Activities relative to laminarin, taken as 100. [c]Products were determined after digestion of substrates for 20 h. N.d. = not determined.

been shown by So and Goldstein[45]. Concanavalin A was removed from the enzyme extract by adsorption on columns of Sephadex G-75. When the resulting lectin-free α-D-mannosidase preparation was chromatographed on GP_2A-Sepharose, the enzyme eluted with 0.5M sodium chloride showed an 80-fold enrichment in activity when compared to the aqueous extract, and was free from contaminating β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase activities (Table III), thus comparing favourably with the preparations of Snaith[46]. Gel electrophoresis of this fraction on polyacrylamide showed the presence of one major protein-staining zone and two minor

TABLE III

SPECIFIC ACTIVITIES DURING PURIFICATION OF GLYCOSIDASES FROM JACK-BEAN MEAL

Purification step	*α-D-Mannosidase*	*β-D-Glucosaminidase*	*β-D-Galactosidase*
Aqueous extract	0.04	0.01	0.01
50% Ammonium sulphate	0.16	0.06	0.05
Sephadex G75	0.98	0.13	0.04
GP_2A Sepharose	3.2 (0.28[a])	0.00 (0.08[a])	0.00 (0.05[a])

[a]Values obtained from chromatography of the 50% ammonium sulphate extract on GP_2A-Sepharose.

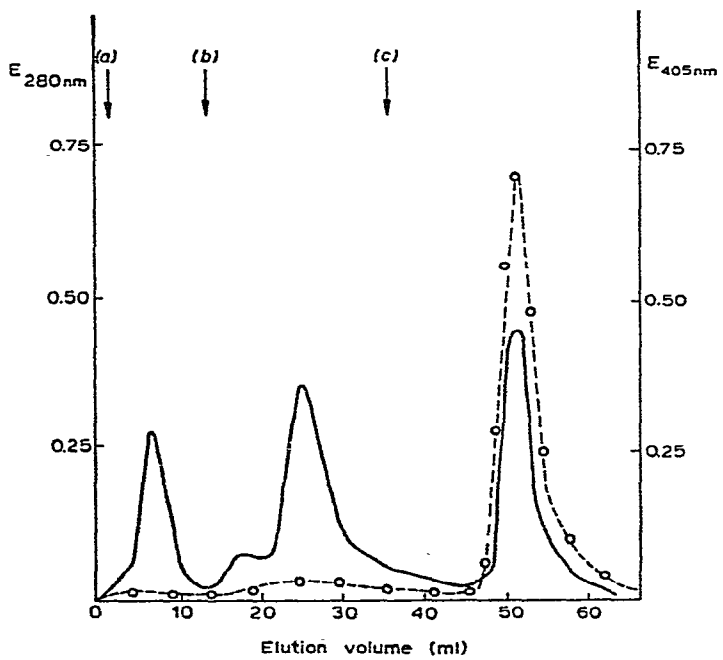

Fig. 3. Fractionation of α-D-mannosidase from jack-bean meal on GP_2A-Sepharose columns: ————, protein; ——○——, α-D-mannosidase activity; elution with (*a*) sodium acetate buffer (0.01M, pH 7.0) containing 2mM zinc sulphate, (*b*) buffer (*a*) containing 0.05M sodium chloride, (*c*) buffer (*a*) containing 0.5M sodium chloride.

ones. When slices of a duplicate gel were incubated with substrate, enzymic activity was found to be associated with the major protein zone. Difficulties experienced in early attempts to purify α-D-mannosidase from jack-bean meal have been shown to result from working at slightly acid pH values[47]. Addition of Zn^{2+} appeared to overcome these difficulties. When partially purified α-D-mannosidase, free from concanavalin A, was chromatographed on GP_2A-Sepharose, the enzyme was eluted from the column by addition of 0.03M sodium chloride. However, the enzyme proved to be relatively unstable, with all activity being lost on storage for two days at 4°. When chromatography of the same enzyme extract was performed in 2mM Zn^{2+}, it was necessary to use 0.5M sodium chloride to elute the enzyme (Fig. 3). Thus, the metallo-enzyme complex appeared to have a much stronger affinity for the immobilized mannan–protein complex in the presence of Zn^{2+} than in the absence of these ions.

Naturally occurring glycoproteins or glycopeptides have not been widely used as ligands for affinity chromatographic supports. Wheat-germ agglutinin, which is a glycoprotein, has been immobilized on agarose and used in the isolation of lysosomal β-D-galactosidases[13], the binding of the enzymes to the lectin being attributed to the presence of α-D-mannopyranosyl and 2-acetamido-2-deoxy-D-glucosyl residues in the enzymes. A glycopeptide containing 2-acetamido-2-deoxy-D-galactosyl, D-glucosyluronic acid, and D-galactosyl residues has been isolated from the mucopolysaccharide of bovine nasal septum. The glycopeptide has been immobilised and used in the purification of hexosaminidase from human-skin fibroblasts and liver[48]. Difficulties experienced in obtaining the hexosaminidase free from contaminating β-D-galactosidase activity could be due to the presence of D-galactosyl residues in the glycopeptide or to the presence of a small proportion of galactose-containing keratan sulphate in the glycopeptide fraction. Such problems could be expected using the *S. cerevisiae* mannan–protein complex, as it is known to contain 2-acetamido-2-deoxy-D-glucose residues[32,43]. However, it is probable that this sugar is located at the region of protein–carbohydrate linkage, and is unlikely to be available for affinity binding. The *S. cerevisiae* glycoprotein is thus effectively composed of only one type of hexosyl residue. In a recent report, an insoluble (1→3)-β-D-glucan was isolated from purified cell-walls of *Candida utilis* and used in a study similar to our own in the isolation of a (1→3)-β-D-glucanase preparation from the culture fluid of the same organism[49]. Since immobilization of the glucan to a support was not attempted, the application of their method will be restricted to the use of highly insoluble polysaccharides.

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