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PURIFICATION OF THE α-L-RHAMNOSIDASE OF *Penicillium decumbens* AND CHARACTERISATION OF TWO GLYCOPEPTIDE COMPONENTS*

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ABSTRACT

The α-L-rhamnosidase component of naringinase from *Penicillium decumbens* was purified and characterised as a monomeric enzyme with a mol. wt. of ~90,000 and with a 50% content of carbohydrate. Digestion of the enzyme with pronase and fractionation of the products on Sephadex G-100 gave two glycopeptides. The larger glycopeptide contained galactofuranose and mannose residues together with lesser proportions of ethanolamine and glucose. Methylation analysis and ^{13}C-n.m.r. spectroscopy indicated its structure to be similar to that of the cell-wall peptido-galactomannan of *P. charlesii*. The smaller glycopeptide was of the *N*-linked type with 10–12 mannose residues per asparagine. The ^{1}H-n.m.r. spectrum indicated the structure to resemble those reported for *Saccharomyces cerevisiae* glycopeptides, with the addition of 1–2 galactofuranose residues and 3 ethanolamine substituents to the oligosaccharide unit.

INTRODUCTION

Naringinase is a commercial enzyme preparation that degrades naringin, a bitter-flavoured flavonoid constituent of citrus fruit that may contaminate citrus juices. The flavonoid contains the glycone α-L-Rha-(1→2)-β-D-Glc-(1→, and naringinase has both α-L-rhamnosidase and β-D-glucosidase activities. α-L-Rhamnosidases are not common but they may be useful in structural studies of bacterial polysaccharides[1–3]. Therefore, we have investigated this component of naringinase from *Penicillium decumbens*. The corresponding enzyme from *Aspergillus niger* has been studied by Pittner and co-workers[4,5] and by Kimaya *et al.*[6]. The *Penicillium* enzyme proved to have an unusual structure, being highly glycosylated with two types of glycopeptide, each of which contained ethanolamine substituents.

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EXPERIMENTAL

Naringinase from *P. decumbens* was obtained from Sigma Chemical Co. The α-L-rhamnosidase activity was measured using *p*-nitrophenyl α-L-rhamnopyranoside[7] (obtained from Sarsintex, Merignac, France), 1 unit of enzyme activity being the amount that released 1 μmol of *p*-nitrophenol/min at 57° and pH 3.5. The β-D-glucosidase activity was measured using *p*-nitrophenyl β-D-glucopyranoside (from Sigma Chemical Co.) at 30° in sodium acetate buffer (pH 5.0).

Molecular weights were estimated by (*a*) SDS-polyacrylamide gel electrophoresis, using a Pharmacia PHAST system with 12.5% acrylamide gels and silver staining; (*b*) gel-filtration on a column (1.6 × 80 cm) of Sephadex G-100 run in phosphate-buffered saline (pH 7.2) and calibrated with suitable standard proteins; and (*c*) size-exclusion h.p.l.c. using a Dupont Zorbax GF-250 column and 0.2M phosphate buffer (pH 7.0). The enzyme was de-glycosylated by treatment[8] with liquid HF at 0° for 2 h.

Ion-exchange chromatography was carried out with Pharmacia FPLC equipment and a Mono Q anion-exchange column eluted at 1 mL/min with 0.02M histidine buffer (pH 6.0) and a 30-min gradient 0 → 0.35M NaCl in the same buffer.

Glycopeptides were prepared by digestion with pronase of α-L-rhamnosidase (4 mg) that had been purified on Sephadex G-100. The conditions used were enzyme–substrate ratio of 1:50, 0.1M Tris buffer (pH 8.0), and overnight digestion at 37°, followed by addition of a second aliquot of pronase and digestion for 8 h. The products were separated on a column (1.6 × 80 cm) of Sephadex G-100, using 0.02M ammonium acetate buffer (pH 5.0), to give glycopeptide I, which was redigested and repurified on Sephadex G-100, and glycopeptide II, which was separated from other peptides by rechromatography on a column (1.6 × 85 cm) of Bio-Gel P-4 (−400 mesh) by elution with water.

The composition of the enzyme and glycopeptides I and II were determined by amino acid analysis on a Durrum D-500 Analyzer of samples hydrolysed for 22 h in 6M HCl at 110°. Hexosamines and ethanolamine were determined on the D-500 after hydrolysis for 4 h in 4M HCl at 100°. The identification of ethanolamine was confirmed by g.l.c.–m.s. of an acetylated sample of the hydrolysate. Hexoses were determined[9] as alditol acetates with *myo*-inositol as the internal standard, or, for column fractions, by the phenol–sulfuric acid method[10].

The glycopeptides were characterised by ^{1}H- and ^{13}C-n.m.r. spectroscopy on solutions in D_2O containing 0.1% of acetone at 57°, using Bruker AM 500 and AM 200 spectrometers equipped with Aspect 3000 computers.

The larger glycopeptide (I) was subjected to methylation analysis (Hakomori procedure[11]) and subsequent g.l.c.–m.s. of the methylated alditol acetates.

RESULTS

Purification of α-L-rhamnosidase. —The commercial enzyme preparation con-

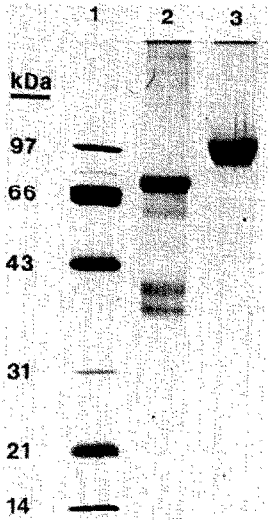


Fig. 1. SDS-PAGE of naringinase with silver staining: lane 1, protein standards, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; lane 2, HF-treated naringinase; and lane 3, naringinase.

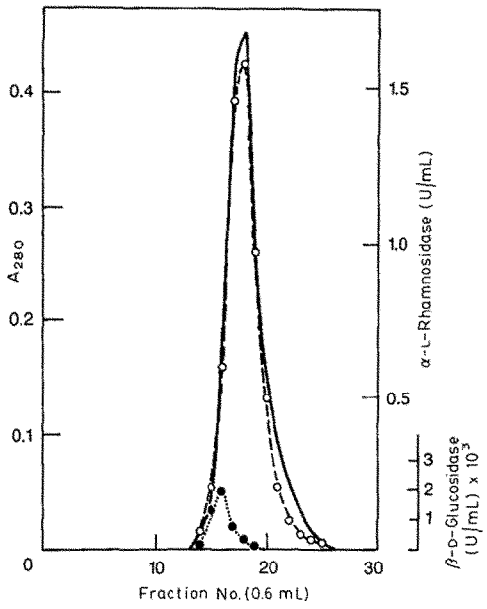


Fig. 2. Size-exclusion h.p.l.c. of naringinase on Zorbax GF-250: ——, A_{280}; --○--, α-L-rhamnosidase assay; and --●--, β-D-glucosidase assay.

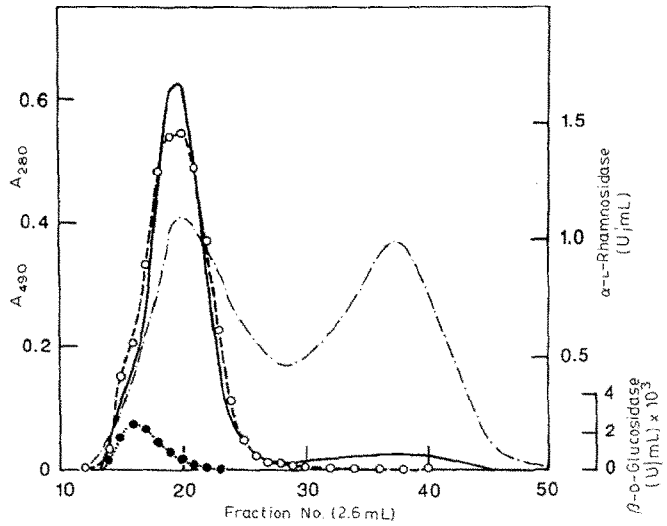


Fig. 3. Gel-filtration of naringinase on Sephadex G-100: ——, A_{280}; --○--, α-L-rhamnosidase assay; --●--, β-D-glucosidase assay; and –·–, hexose assay. Fractions 17–22 were combined and used for the preparation of glycopeptides.

tained ~35% of non-dialysable material. The retained protein fraction gave a single, somewhat diffuse band on SDS-PAGE with an apparent molecular weight of 97,000 (Fig. 1). Gel-filtration on Sephadex G-100 indicated a molecular weight of 88,000, but size-exclusion h.p.l.c. on Zorbax GF-250 gave a value of 52,000, suggesting that some adsorption had taken place. Although the peak from the GF-250 column was relatively sharp (Fig. 2), the α-L-rhamnosidase activity was distributed uniformly while the β-D-glucosidase activity was restricted to the leading edge of the peak. The purified enzyme had a reduced content of glucose, suggesting the loss of a glucan. Similar profiles were obtained in large-scale separations on Sephadex G-100 (Fig. 3), and a second large carbohydrate peak was eluted after the enzyme. The yield of the glycoprotein fraction was 43% of the applied (dialysed) enzyme sample.

Ion-exchange chromatography on a high-resolution column, Mono Q, partially separated the two enzyme activities (Fig. 4). Some enzyme activity was also eluted early together with a large amount of carbohydrate (Glc:Man:Gal ratios of 1:0.27:0.22). The enzyme activities were as follows: initially, 0.48 U/mg (dry weight); after dialysis, 1.1 U/mg, and after elution from Sephadex G-100, 2.04 U/mg.

The composition of the Mono Q product is given in Tables I and II. The chromatogram from the amino-acid analyser showed an additional peak between those for lysine and ammonia in the position for ethanolamine. G.l.c.–m.s. of the acetylated products of hydrolysis revealed the *N,O*-diacetyl derivative of

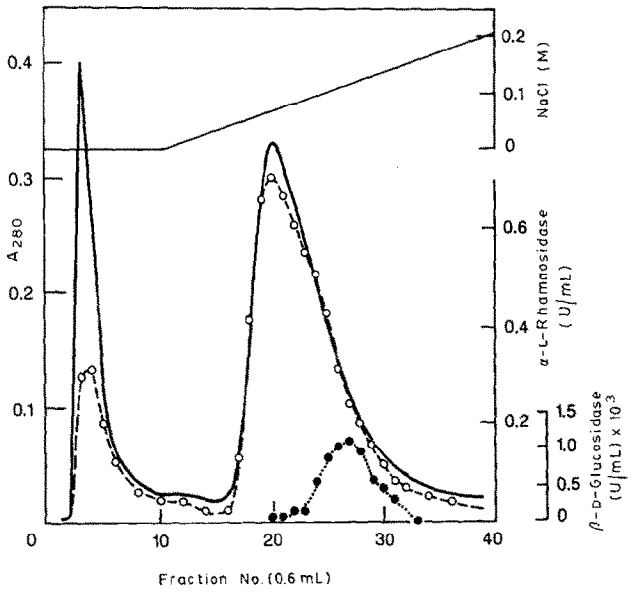


Fig. 4. Anion-exchange chromatography of naringinase on Mono Q: ——, A_{280}; --O--, α-L-rhamnosidase assay; and --●--, β-D-glucosidase assay. The NaCl gradient is shown at the top.

ethanolamine. The major sugar components were Man and Gal, and the total carbohydrate content was 50%. Phosphorus was also present (~0.7 mol/mol of ethanolamine).

Treatment of the Mono Q product with liquid HF removed the carbohydrate and gave material of molecular weight ~41,000 (Fig. 1); a second component at ~71,000 was also present. Neither of these bands stained with Coomassie Blue, unlike the native protein.

Characterisation of the glycopeptides. — Gel-filtration of the products of

TABLE I

AMINO ACID ANALYSIS OF α-L-RHAMNOSIDASE PURIFIED BY MONO Q CHROMATOGRAPHY

Amino acid	*Residue (%)*	*Amino acid*	*Residue (%)*
Asp	8.9	Ile	4.7
Thr	10.2	Leu	8.7
Ser	14.2	Tyr	4.5
Glu	6.8	Phe	3.4
Pro	5.0	His	1.8
Gly	10.7	Lys	3.3
Ala	10.8	Arg	2.3
Val	4.7	Ethanolamine	11.8[a]

[a]Mol/100 mol of amino acids.

TABLE II

CARBOHYDRATE ANALYSES OF α-L-RHAMNOSIDASE AND DERIVED GLYCOPEPTIDES[a]

	Man	*Glc*	*Gal*	*GlcNAc*	*Ethanolamine*
Enzyme	1.89	0.35	1	0.03	0.20
Glycopeptide I	1.30	0.2	1	0	0.17
Glycopeptide II	8.4	tr	1	0.60	2.03

[a]Expressed relative to Gal; tr, trace.

digestion of the α-L-rhamnosidase with pronase (Fig. 5) revealed two glycopeptides. The higher-molecular-weight glycopeptide (I) was redigested and the products were repurified. The yield was 22% of the Sephadex-purified glycoprotein. Amino-acid analysis of glycopeptide I (Table III) revealed <7% of amino acids, including serine and threonine, but a significant amount of ethanolamine. The major hexoses (Table II) were Man and Gal with minor amounts of Glc, and no significant amounts of HexN. The ^{1}H-n.m.r. spectrum of glycopeptide I indicated a complex structure, with broad multicomponent peaks in the region for anomeric protons (Fig. 6a). The ^{13}C-n.m.r. spectrum (Fig. 7) contained a major peak at 108.0 p.p.m. consistent with the presence of β-Gal*f* constituents[12]. The spectrum resembled that reported for the galactomannan of *P. charlesii*[13]. Methylation analysis indicated the presence of Gal*f* and Man*p* end-groups together with 2- and 6-substituted Man*p* and 5-substituted Gal*f* residues.

The lower-molecular-weight glycopeptide (II), after rechromatography on Bio-Gel P-4, was obtained in ~8% yield and also contained Man and Gal together with GlcN, ethanolamine (Table II), aspartic acid, and hydroxyamino acids (Table

TABLE III

AMINO ACID ANALYSES OF THE GLYCOPEPTIDES

	Glycopeptide II[a]	*Glycopeptide I*[b]	P. charlesii *galactomannan*[b]
Asp	1.0	0.39	0.13
Thr	0.78	0.89	0.73
Ser	0.74	1.0	1.0
Glu	0.30		
Pro	0.32		
Gly	0.49	0.91	0.32
Ala	0.38	0.73	0.47
Val		1.08	0.17
Leu		0.52	0.07
Ethanolamine[c]	1.9	4.6	

[a]Relative to Asp. [b]Relative to Ser; the *P. charlesii* data are for the native galactomannan[13]. [c]Recoveries of ethanolamine were higher after hydrolysis under milder conditions.

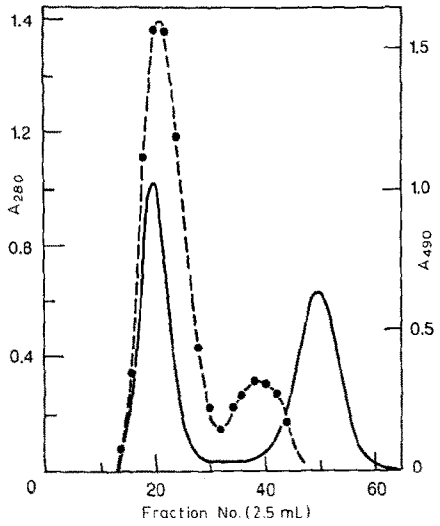


Fig. 5. Gel-filtration on Sephadex G-100 of the products of digestion of α-L-rhamnosidase with pronase: ——, A_{280}; —●—●—, hexose assay. Fractions 16–28 were combined as glycopeptide I, and fractions 34–44 as glycopeptide II.

III). The proportions of the constituents indicated an average composition of $Man_{10-12}GlcNAc_2Asn_1Gal_{1-2}ethanolamine_3$, consistent with an *N*-linked glycopeptide structure. The region (Fig. 6b) for anomeric protons in the ^{1}H-n.m.r. spectrum resembled that from *Saccharomyces cerevisiae N*-linked glycopeptides[14,15] and also included signals for NAc at 2.02 and 2.07 p.p.m. and for CH_2NH_2 of the ethanolamine at 3.31 p.p.m. The chemical shift data are compared to literature values in Table IV. The galactose was readily released by mild acid hydrolysis (50mM HCl, 100°, 30 min), indicating the Gal*f* form.

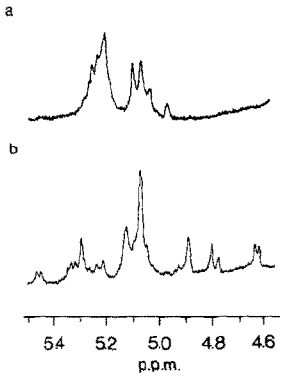


Fig. 6. ^{1}H-N.m.r. spectra in the region for anomeric protons of (a) glycopeptide I and (b) glycopeptide II.

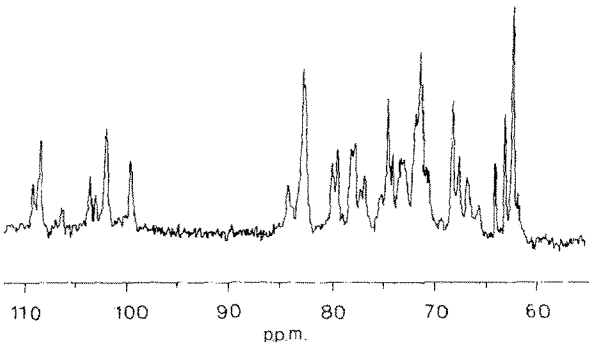
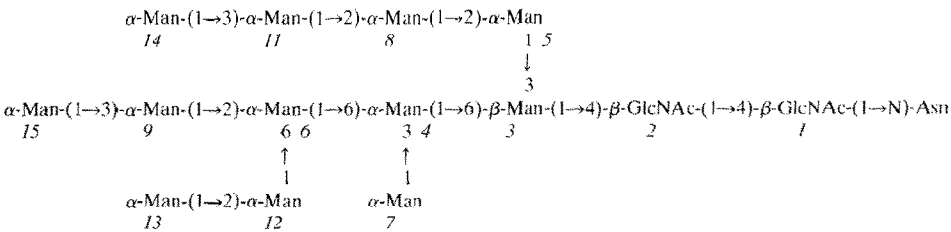


Fig. 7. ^{13}C-N.m.r. spectrum of glycopeptide I.

TABLE IV

CORRESPONDENCE OF THE ^{1}H-N.M.R. CHEMICAL SHIFTS OF THE RESONANCES OF GLYCOPEPTIDE II H-1 AND NAc GROUPS TO THOSE OF *S. cerevisiae* AND OTHER HIGH-MANNOSE GLYCOPEPTIDES[a]



Glycopeptide II	*Reference data*		
Chemical shift	*Chemical shift*	*Assignment*	*Residue*
5.32, 5.33	5.33	→2)-α-Man-(1→3)	5
5.29	5.29	→2)-α-Man-(1→2)	8
5.11	5.14	→2,6)-α-Man-(1→6)	6
		→2)-α-Man-(1→6)	12
		α-Man-(1→3)	7,14,15
5.04–5.09	5.02	→4)-β-GlcNAc-(1→N)	1
	5.04	→3)-α-Man-(1→2)	9,11
		α-Man-(1→2)	13
4.88	4.87	→3,6)-α-Man-(1→6)	4
4.76, 4.79	4.77	→3,6)-β-Man-(1→4)	3
4.62	4.61	→4)-β-GlcNAc-(1→4)	2
2.07	2.05–2.07	NAc	1
2.02	2.00–2.01	NAc	2

[a]The data for mannose H-1 are from Trimble and Atkinson[15] and Tsai *et al.*[14]. The residue numbering scheme is from Trimble and Atkinson[15]. The data for GlcNAc H-1 and NAc are from Vliegenthart *et al.*[16], Table XXVI.

DISCUSSION

The data now reported show that the α-L-rhamnosidase from *P. decumbens* is a highly glycosylated, monomeric enzyme. Neither ion-exchange nor gel-filtration chromatography separated the β-D-glucosidase activity from the α-L-rhamnosidase completely. The higher-molecular-weight band in SDS-PAGE of the deglycosylated protein may represent the β-D-glucosidase and its native molecular weight may be slightly larger than that of the α-L-rhamnosidase. However, the formation of a complex between the two enzymes[4] is possible. In view of the glycosylation, the molecular weight data must be viewed as approximate since the techniques used are reliable only for globular non-glycosylated proteins. Also, the diffuseness of the band in SDS-PAGE suggests some heterogeneity in size, attributable to variation in carbohydrate content. The molecular weight of the deglycosylated component (41,000) taken with the carbohydrate content indicates a native molecular weight of ~82,000, compared to 88,000–97,000 measured by gel-filtration chromatography and SDS-PAGE.

For structural work on oligo- and poly-saccharides containing L-rhamnose residues, this enzyme has the merits of being inexpensive and easily purified. Ion-exchange chromatography is the best purification method, removing more of the β-D-glucosidase activity as well as extraneous polysaccharide. It is worth noting that the activity of the β-D-glucosidase towards other substrates may well be higher than that observed with *p*-nitrophenyl β-D-glucopyranoside.

A full determination of the structure of glycopeptides I and II was not attempted. The data are consistent with two types of structure previously reported for glycoproteins from moulds or yeasts. Glycopeptide I is a galactomannan with phosphoethanolamine constituents, and the galactose is probably in the furanose form. These characteristics, the linkage data from methylation analysis, and the ^{13}C-n.m.r. data indicate that glycopeptide I has a structure similar to that of a cell-wall glycopeptide[13,17] from *P. charlesii*. This galactomannan has a core of (1→2)- and (1→6)-linked α-Man residues with outer chains that contain 8–10 (1→5)-linked β-Gal*f* residues, and phosphoethanolamine units are attached to the Man portion. The phosphoethanolamine–mannose ratio (1:10) is close to the value for the *P. decumbens* enzyme. The galactose–mannose ratios cannot be compared because the values for *P. charlesii* glycopeptide are dependent upon culture conditions and molecular weight[17]. The *P. charlesii* galactomannan is attached to a short polypeptide chain through the hydroxyl group of serine or threonine. The peptide portion of glycopeptide I was also rich in serine and threonine, and, given the absence of GlcN, an *O*-linkage to peptide is probable. Similar mannose–serine linkages are also found in *A. niger* glucoamylase[18], although its glycon consists of short manno-oligosaccharides.

Glycopeptide II is considerably smaller than glycopeptide I and has the constituents of an *N*-linked type of glycopeptide, including aspartic acid as the major amino acid and the presence of two GlcNAc residues. High-mannose glycopeptide

structures and their biosynthesis have been studied extensively in yeast[19], and ^{1}H-n.m.r. data for glycopeptides from *Saccharomyces cerevisiae* with 8–14 mannose residues have been reported in detail[14,15]. Comparison of these spectra with that of glycopeptide II is made difficult by the presence of the Gal*f* and ethanolamine residues in the latter. However, many of the signals for anomeric protons are clearly analogous from the partial assignment given in Table IV.

The glycopeptides of the α-L-rhamnosidase therefore consist of one type that is homologous to the cell-wall galactomannan of *P. charlesii* and a second type that is basically an *N*-linked glycopeptide of the type found in *S. cerevisiae* with the addition of galactose and ethanolamine, perhaps attached by the same enzymes that synthesised the cell-wall homologue. Glycopeptides from such trypanosomatids as *Leptomonas samueli* and *Crithidia fasciculata* contain 1–3 Gal*f* units on a $Man_9GlcNAc_2$ core, 2-linked to the terminal Man units[20,21]. It is possible that the *P. decumbens* glycopeptide I has a similar structure with the added feature of ethanolamine.

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