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PROPERTIES OF A β-D-MANNOSIDASE FROM *ASPERGILLUS NIGER*

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Summary

The β-D-mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) from culture filtrate of *Aspergillus niger* has been purified in large amounts by fractionation with $(NH_4)_2SO_4$ and DEAE-cellulose chromatography. The removal of traces of α-D-galactosidase was performed on a Sepharose-ε-aminocaproyl-galactosylamine column. The final enzyme preparation (specific activity 188 units) has no other glycosidase activity and is judged homogeneous.

The enzyme has a molecular weight of 130 000 ± 5000 and an isoelectric point of 4.7.

The amino acid composition of the enzyme is characterized by high proportion of acidic amino acids and no cysteine residues and a single chain structure of the enzyme is suggested.

The enzyme shows maximum activity on *p*-nitrophenyl-β-D-mannopyranoside at pH 3.5 and at 55°C. The presence of 80% of β-sheet structure in the protein and 20.8% of monosaccharides (Gal : 1.3; Man : 7; GlcNAc : 1) could explain this relative high heat stability (up to 2 h at 55°C). Enzyme activity is inhibited by mannose (K_i = 7.85 mM) and the specificity is examined.

Introduction

β-Man-(1 → 4)-β-GlcNAc-(1 → 4)-β-GlcNAc-(1 →)-Asn is a common sequence found in numerous glycoproteins (for review see ref. 1). Characterization of a β-D-mannosyl linkage in this sequence was first established by Sukeno et al. [2] and confirmed by the use of partially purified β-mannosidase from snail [3,4], hen oviduct [5] and pineapple [6]. A highly purified form of β-mannosidase from the mushroom *Polyporus sulfureus* was described by Wan et al. [7] and more recently some properties of a β-mannosidase from *Aspergillus niger* were published [8]. In this paper, we report more details of the preparation, physico-chemical and enzymatical properties of the latter enzyme.

Materials and Methods

Materials. β-D-mannosidase was prepared from a culture filtrate of *Asp. niger* which was a generous gift of 'Société RAPIDASE', Sèclin, France. *p*-nitrophenyl-α-D-mannopyranoside (*p*-NP-α-D-Man); *p*-nitrophenyl-β-D-mannopyranoside (*p*-NP-β-D-Man); *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide (*p*-NP-α-D-GlcNAc); *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*-NP-β-D-GlcNAc); *p*-nitrophenyl-α-D-galactopyranoside (*p*-NP-α-D-Gal); *p*-nitrophenyl-β-D-galactoside (*p*-NP-β-D-Gal); *p*-nitrophenyl-α-D-glucopyranoside (*p*-NP-α-D-Glc); *p*-nitrophenyl-β-D-glucopyranoside (*p*-NP-β-D-Glc) and *p*-nitrophenyl-α-L-fucoside (*p*-NP-α-L-Fuc) were purchased from Koch Light, Ltd Colnbrook, U.K.

The following substrates were generous gifts: *p*-NP-β-D-Man, synthetized by Blanc-Muesser et al. [9]; *O*-β-D-mannopyranosyl-(1 → 6)-2 acetamido-2 deoxy-α-D-glucopyranose; *O*-β-D-mannopyranosyl-(1 → 3)-2-acetamido-2-deoxy-α-D-glucopyranose; *O*-β-D-mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose from Dr. R.W. Jeanloz (Mass. General Hospital, Boston); *O*-α-D-mannopyranosyl-(1 → 3)-*O*-β-D-mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose isolated from the urine of a mannosidosis patient by Strecker et al. [10]; core glycopeptides were obtained from human serotransferrin [11] and lactotransferrin [12] asialo-glycopeptides after a stepwise digestion with Jackbean exoglycosidases. DEAE-cellulose 22 was obtained from Whatman, Sepharose 4B from Pharmacia and Ultrogel AcA-34 from LKB.

For affinity chromatography : ε-aminocaproyl-galactosylamine prepared by Lotan et al. [13] was coupled to Sepharose 4B by the method of Cuatrecasas [14].

Enzyme assays. With *p*-nitrophenyl-β-D-mannoside as substrate, one unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at 37°C. Enzyme assays were carried out in 0.05 M citric acid/0.10 M disodium phosphate, pH 3.5; no cation was added.

An aliquot of enzyme solution (10—50 μl, approx. 1 mU) was added to 400 μl of 4 mM *p*-NP-β-D-Man in assay buffer. After 10—15 min, the reaction was stopped by the addition of 1 ml 1 M Na_2CO_3 and the liberated *p*-nitrophenol was measured spectrophotometrically.

Substrate specificity studies were performed on 0.275 μmol of disaccharides or 0.5 μmol of core glycopeptides solubilized in 100 μl of phosphate/citrate buffer (pH 3.5) containing 2 or 4 mU of β-D-mannosidase. The mixture was incubated for 1 h at 37°C and the reaction was stopped by heating for 3 min at 100°C. Liberated monosaccharides were estimated by gas liquid chromatography on 3% silicon QF 1, Chromosorb WHMDS (100—200 mesh) column after trimethylsilylation according to the method of Fournet [15].

When compounds (in assay buffer) were tested for possible inhibition, they were preincubated for 30 min at 37°C with the enzyme.

Activation energy was determined from the formula

$$E_a = \frac{4.56 \cdot T_1 T_2 (\log V_2 - \log V_1)}{T_2 - T_1}$$

where V_1 and V_2 represent, respectively, hydrolysis rate at absolute temperatures T_1 and T_2. E_a is expressed in cal/mol.

Protein determination. Protein concentration was determined by the method of Lowry et al. [16] with crystalline bovine serum albumin as a standard.

Chemical composition. Amino acid analyses were carried out on a Beckman Multichrom auto-analyzer [17]. Proteins were hydrolysed in 5.6 M HCl at 110°C in evacuated, sealed tubes for 24, 48 and 72 h. The quantitative determination of hexosamines and neutral sugars was determined by classical colorimetric method [18] and by gas-liquid chromatography of the trimethylsilyl derivatives [15].

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Davis [19] using pH 8.3 Tris/glycine buffer and that of Neville [20] after 6 h treatment of the enzyme at 37°C in 5% SDS/10% β-mercaptoethanol.

Isoelectrofocusing. Isoelectric point determinations were performed on an LKB 110 ml column using Ampholine of p*I* 3.5—10 and 3.5—5 according to the method of Vesterberg and Svensson [21].

Molecular weight determination. The molecular weight was determined by gel filtration on a Ultrogel AcA-34 column (1.2 × 82 cm) equilibrated in 0.2 M sodium phosphate buffer (pH 6.7) and by sedimentation equilibrium in a Spinco E ultracentrifuge. Experiments were performed according to the method of Chervenka [22] at 20°C with 1.5% protein in 0.1 M Tris · HCl (pH 7.3). Ultracentrifugations were performed in double sector cells, with column height of 3 mm, at 44 000 rev./min for 10 h. Sedimentation velocity experiments were achieved as described in the legend of Fig. 3. The partial specific volume of the enzyme was calculated from the amino acid composition according to the method of McKeen and Marshall [23].

Circular dichroism spectrum. The circular dichroism spectrum was recorded with a Jobin-Yvon Dichrograph R.J. Mark III with 0.1 mm pathlength cells at an absorbance less than 1.5. The ellipticity was expressed as mean residual molar ellipticity $[\theta]$ in degrees $cm^{-2} \cdot dmol^{-1}$.

Immunological studies. Rabbit antiserum specific for *Asp. niger* extract was prepared according to the procedure of Vaitukaitis et al. [24]. For immunoelectrophoresis under standard conditions 2 μl of 5% antigen were run at 4V/cm for 2 h; 20 μl volume of antiserum reagent was added to the troughs.

Results

Isolation of enzyme

Samples were routinely centrifuged in Beckman JA 21 refrigerated centrifuge for 30 min at the following speeds: 9000 rev./min for the JA-10 (large capacity rotor) and 13 000 rev./min for the JA 14 rotor. Small samples were centrifuged in a JA 20 rotor at 18 000 rev./min. All purification procedures were performed at 0—4°C.

$(NH_4)_2SO_4$ precipitation. Culture filtrate was concentrated 30-fold by ultrafiltration on an Amicon XM 50 membrane. After addition of powdered $(NH_4)_2SO_4$ to 80% saturation, the precipitate was collected, dissolved in water, $(NH_4)_2SO_4$ and cellulase activity were eliminated by filtration on Amicon H1 DX 50 membrane. The filtrate was further dialyzed against 0.01 M sodium phosphate buffer (pH 6.7).

DEAE-cellulose column chromatography. The dialyzed solution was loaded on a DEAE-cellulose column (3 × 60 cm) previously equilibrated with 0.01 M sodium phosphate buffer (pH 6.7). A stepwise increase in phosphate buffer concentration under the conditions described in the legend of Fig. 1 led to the isolation of β-D-mannosidase, α-D-galactosidase and *N*-acetyl-β-D-hexosaminidase in the peak I, II and III respectively. The β-D-mannosidase fractions were pooled, dialyzed against distilled water and then lyophilised. When the lyophilised β-D-mannosidase fraction was tested against *p*-NP-α-D-gal; *p*-NP-β-D-Gal; *p*-NP-α-D-GlcNAc; *p*-NP-β-D-GlcNAc; *p*-NP-α-D-Glc; *p*-NP-β-D-Glc; *p*-NP-α-D-Man and *p*-NP-α-L-Fuc, only 5% contamination by α-D-galactosidase activity was found.

Affinity chromatography. The contaminating α-galactosidase activity was retained on a Sepharose 4B-ε-aminocaproyl-galactosylamine column (1.5 × 15 cm) equilibrated with 0.01 M sodium phosphate (pH 6.15). The β-D-mannosidase was eluted with the starting buffer, the elution of α-D-galactosidase occurred with 0.2 M sodium phosphate (pH 6.7). The β-D-mannosidase fractions were pooled, dialyzed against distilled water and lyophilised.

A summary of the purification is shown in Table I. After the affinity chromatography a purification rate of 21 408 was obtained and the specific activity was 188 units/mg. In this connection it is interesting to note the remarkable efficiency of the last step of enzyme purification by affinity chromatography which realizes a 13.6-fold purification despite the fact that α-galactosidase is only a minor (5%) contaminant of the preparation.

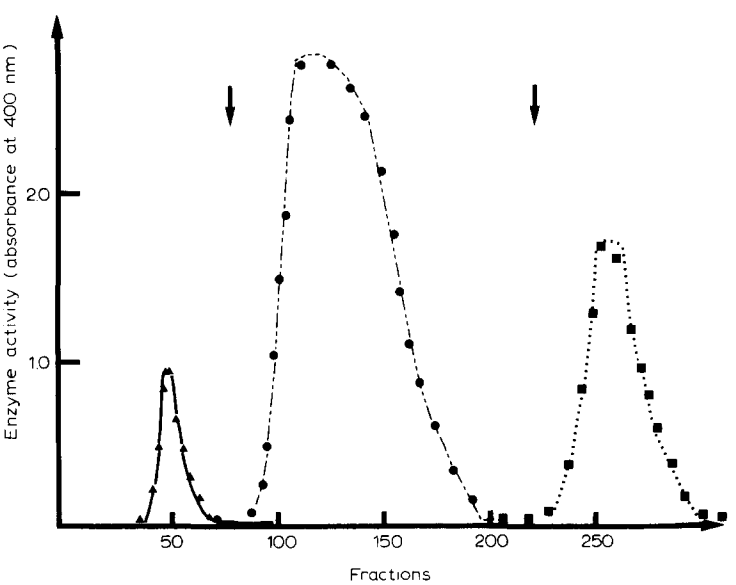


Fig. 1. DEAE-cellulose (DE22) chromatography of 3.5 g of the preparation of β-D-mannosidase. Enzymatic activities were eluted by stepwise increase in phosphate buffer concentration/elution rate: 28 ml/h, fractions collected: 15 ml. Arrows indicate changes in concentration. ▲———▲, β-D-mannosidase activity eluted at 0.021 M sodium phosphate buffer (pH 6.7). ●———●, α-D-galactosidase activity eluted at 0.066 M sodium phosphate buffer (pH 6.7). ■———■, *N*-acetyl-β-D-hexosaminidase activity eluted at 0.2 M sodium phosphate buffer (pH 6.7).

TABLE I

PURIFICATION OF β-D-MANNOSIDASE FROM A CULTURE FILTRATE OF *ASP. NIGER*

Specific activity is expressed in units per mg of protein; one unit is defined as the amount of enzyme which hydrolyzes 1 μmol of substrate (*p*-NP-β-D-Man) per min at 37°C.

Purification steps	Protein (mg)	Total units	Specific activity	Recovery	Purification rate
Crude extract	885 714	7440	0.008	100	1
$(NH_4)_2SO_4$ fractionation	9 660	5916	0.61	79.5	76
DEAE-cellulose chromatography	280	3864	13.8	51.9	1 568
Affinity chromatography	17	3204	188	43	21 408

Properties of the β-D-mannosidase

Enzyme homogeneity. Purified β-D-mannosidase examined by polyacrylamide gel electrophoresis showed a single band and by immunoelectrophoresis only a single line of precipitate was formed (Fig. 2).

An ultracentrifugal analysis of the purified enzyme preparation showed a single protein peak with a $s^0_{20,w}$ value of 6.58 (Fig. 3).

Isoelectrofocusing. A value of 4.7 was found for the isoelectric point of the purified β-D-mannosidase.

Molecular weight. The purified β-mannosidase and the markers proteins were applied to the Ultrogel AcA-34 and a molecular weight of 140 000 was calculated, in a good agreement with that obtained by sedimentation equilibrium (130 000 ± 5000).

No modification of the molecular weight was noticed by gel electrophoresis after reduction of the enzyme by β-mercaptoethanol in the presence of SDS. This result suggests a single chain structure for β-D-mannosidase.

Carbohydrate composition. The neutral sugar content of the enzyme was 17.8% as measured by the orcinol/H_2SO_4 method and the *N*-acetylhexosamine content was 2.6% as determined by the Elson-Morgan method. The results obtained by gas liquid chromatography indicate the presence of galactose, mannose and *N*-acetylglucosamine in a molar ratio of 1.3 : 7 : 1 (Table II).

Amino acid composition. The amino acid composition of β-D-mannosidase

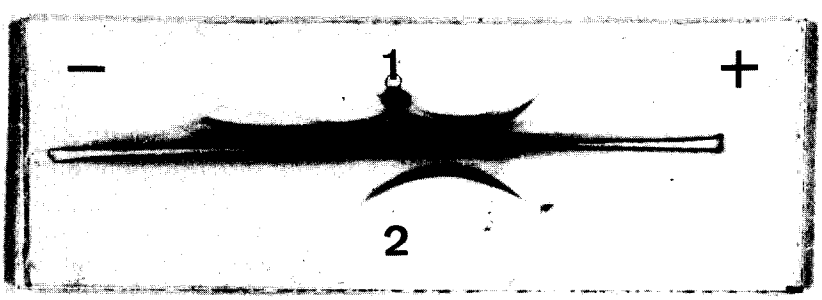
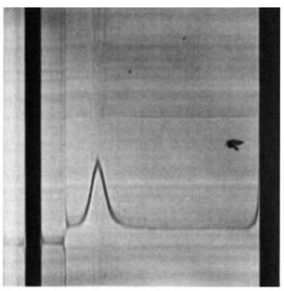


Fig. 2. Immunoelectrophoresis of *Asp. niger* extract (well 1) and purified β-D-mannosidase (well 2) against rabbit antiserum specific for *Asp. niger* extract.



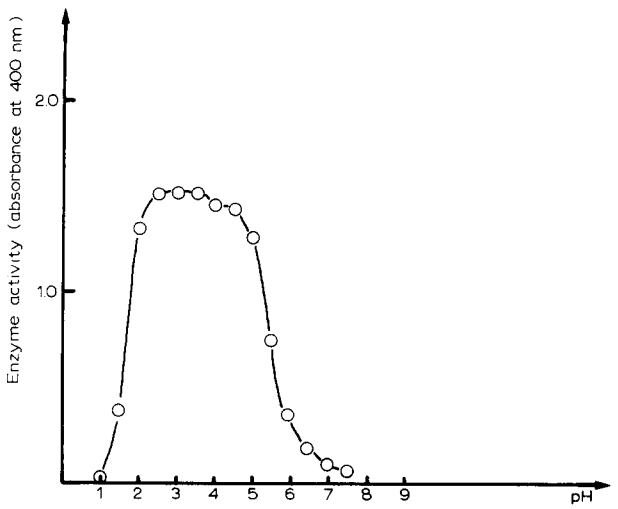


Fig. 3. Sedimentation-velocity. The protein concentration was 15 mg/ml in a solvent of 0.1 M Tris · HCl buffer (pH 7.3). The temperature was 20°C. The photograph was taken 32 min after reaching 44 000 rev./min. The direction of sedimentation is from left to right.

Fig. 4. Effect of pH on β-D-mannosidase activity. Enzyme was preincubated for 30 min in the buffer before assay with synthetic substrate.

given in Table II is characterized by high values of acidic amino acids and the lack of cysteine residues.

Circular dichroism. The intrinsic dichroic spectrum was performed from 180-250 nm. A minimum was observed at 210 nm. Application of the equation of Chen, Yang and Martinez [25] shows that the percentage of α-helix, β-sheet and random coil are 13%, 84% and 3%, respectively. These data show a particularly high level of the β-sheet structure.

Effect of pH. The activity of the β-D-mannosidase studied with *p*-nitrophenyl-β-D-mannopyranoside in a series of phosphate/citrate McIlvaine buffers [26] demonstrates that the optimum pH was 3.5 (Fig. 4). The enzyme was stable over the pH range of 2.5—7.5 after 2 h preincubation at 37°C.

Effect of temperature. At the optimal pH of 3.5 the maximum activity for the *p*-nitrophenyl-β-D-mannopyranoside was obtained at 55°C (Fig. 5). No loss of activity was noticed after 2 h preincubation under these conditions. Activation energy was 8441 cal · mol^{-1}. This value is in agreement with the activity of the β-D-mannosidase.

Some kinetic properties. Some kinetic properties of purified β-D-mannosidase were determined using *p*-nitrophenyl-β-D-mannoside and the *O*-β-D-mannopyranosyl-(1 → 4)-2 acetamido-2 deoxy-α-D-glucopyranose as substrates, whereas the amounts of the other disaccharides were too low to be examined. Apparent K_m values determined from the Lineweaver-Burk plot for *p*-nitrophenyl-β-D-mannoside and for the disaccharide were, respectively, 0.46 mM ± 0.04 and 8.4 mM ± 0.08. It is seen that β-D-mannosidase possesses a higher affinity for the *p*-NP-β-D-Man, however the maximal velocity (V) for the disaccharide (6 μmol/min) is higher than for the *p*-NP-β-D-Man (3 μmol/min).

Inhibition studies. The effects of various inhibitors of the β-D-mannosidase

TABLE II

COMPOSITION OF β-D-MANNOSIDASE FROM *ASP. NIGER*

Composition	Mol% *	Residues per molecule **	Nearest integral numbers of residues
Amino acids ***			
Asp	9,35	105	105
Thr	5.44	69.9	70
Ser	6.80	101.48	102
Glu	7.34	73.94	74
Pro	5.35	71.57	72
Gly	6.69	150.07	150
Ala	7.34	134.38	134
Val	4.46	58.52	58
Ile	2.91	33.43	33
Leu	7.16	82.22	82
Tyr	4.49	35.80	36
Phe	3.74	33.02	33
Lys	1.97	20.03	20
His	2.27	21.53	22
Arg	2.88	23.97	24
Carbohydrates			
Gal	2.8	21.27	21
Man	15.4	111.15	111
GlcNAc	2.6	15.92	16
Total	98.99		1162

* Molar ratio of each component relative to total.

** Calculated base on a molecular weight for the enzyme of 130 000.

*** Tryptophan not determined.

activity has shown that the most potent inhibitor of the enzyme activity was the mannose (Table III). Its inhibition constant (K_i) determined by Dixon plot when *p*-nitrophenyl-β-D-mannopyranoside was used as substrate was 7.85 ±

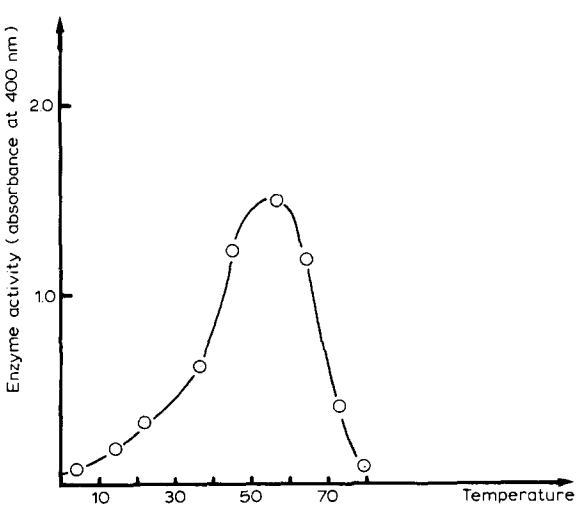


Fig. 5. Effect of temperature on β-D-mannosidase activity. The conditions of incubation are described in Materials and Methods.

TABLE III

EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF β-D-MANNOSIDASE FROM *ASP. NIGER*

The concentration of the inhibitors was 20 mM, only *p*-chloromercuribenzoic acid was used with 1 mM concentration.

Inhibitors	Relative activity
Ca^{2+}	100
Mn^{2+}	100
Mg^{2+}	100
Cu^{2+}	78
Zn^{2+}	100
Mannose	49
Mannitol	98
N-acetylglucosamine	109
EDTA	100
p-chloromercuribenzoic acid	89

TABLE IV

SUBSTRATE SPECIFICITY OF β-D-MANNOSIDASE FROM *ASP. NIGER* ON SACCHARIDES AND GLYCOPEPTIDES

Enzyme activities were assayed as described in Materials and Methods.

Substrates	Relative activity (%)
β-D-Man-(1 → 4)-D-GlcNAc	100
β-D-Man-(1 → 3)-D-GlcNAc	0.04
β-D-Man-(1 → 6)-D-GlcNAc	0.1
β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-β-D-GlcNAc-Asn-Lys	100
β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-[α-L-Fuc-(1 → 6)]-β-D-GlcNAc-Asn-peptide	80

0.05 mM. Metal ions do not seem to participate in the enzyme activity, as no loss of β-D-mannosidase activity was noticed in the presence of EDTA in increasing concentration.

Substrate specificity. As shown in Table IV the β-D-mannosidase appears to be relatively specific for the mannosyl β-1,4 linkages since the β-1,3 and β-1,6 linkages are hydrolyzed very slowly. The β-1,4 linked mannose present in the compound: β-Man-(1 → 4)-β-GlcNAc-(1 → 4)-β-GlcNAc-Asn isolated from human serotransferrin and lactotransferrin was completely liberated in the conditions described previously. Cheron et al. [27] have obtained after β-D-mannosidase digestion, 80% liberation of free mannose from a tetrasaccharide-asparaginyl peptide in which fucose residue is α-1,6 linked to the first *N*-acetylglucosamine of the above trisaccharide.

Discussion

The β-D-mannosidase obtained in large amount from the culture filtrate of *Asp. niger* was purified to complete homogeneity 21 408-fold with a recovery of 43%. Its specific activity is 13 times higher than that reported by Elbein et

al. [8]. The value of the molecular weight (130 000 ± 5000) is similar to the value (120 000) previously reported [8]. The purified enzyme is a glycoprotein with interesting characteristics: 80% of β-sheet structure and 21% of carbohydrates (galactose, mannose and *N*-acetylglucosamine in a ratio of 1.3 : 7 : 1) and these results differ from those of ref. 8. The structure of the carbohydrate units has not been studied but it can be assumed that similarities exist with the structure of oligomannosidic type glycoprotein [1]. It is interesting to notice that an acid phosphatase isolated recently from *Asp. niger* [28] contains mannose, galactose and *N*-acetylglucosamine in a ratio similar to that reported here. The numerous carbohydrate units may play a role in the protection of the protein from heat denaturation. The enzyme does not seem to be a metalloenzyme as it is resistant to EDTA inhibition. The β-D-mannosidase has a good activity on *p*-nitrophenyl-β-D-mannoside, its relatively high specificity towards β-1,4 linkages allows one to use this enzyme as a very interesting tool for structural studies of glycopeptides which are known to contain this type of linkage [1].

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