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The production, purification and characterisation of two novel α-D-mannosidases from *Aspergillus phoenicis*

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Abstract—1,6-α-D-Mannosidase from *Aspergillus phoenicis* was purified by anion-exchange chromatography, chromatofocussing and size-exclusion chromatography. The apparent molecular weight was 74 kDa by SDS-PAGE and 81 kDa by native-PAGE. The isoelectric point was 4.6. 1,6-α-D-Mannosidase had a temperature optimum of 60 °C, a pH optimum of 4.0–4.5, a K_m of 14 mM with α-D-Man*p*-(1→6)-D-Man*p* as substrate. It was strongly inhibited by Mn^{2+} and did not need Ca^{2+} or any other metal cofactor of those tested. The enzyme cleaves specifically (1→6)-linked mannobiose and has no activity towards any other linkages, *p*-nitrophenyl-α-D-mannopyranoside or baker's yeast mannan. 1,3(1,6)-α-D-Mannosidase from *A. phoenicis* was purified by anion-exchange chromatography, chromatofocussing and size-exclusion chromatography. The apparent molecular weight was 97 kDa by SDS-PAGE and 110 kDa by native-PAGE. The 1,3(1,6)-α-D-mannosidase enzyme existed as two charge isomers or isoforms. The isoelectric points of these were 4.3 and 4.8 by isoelectric focussing. It cleaves α-D-Man*p*-(1→3)-D-Man*p* 10 times faster than α-D-Man*p*-(1→6)-D-Man*p*, has very low activity towards *p*-nitrophenyl-α-D-mannopyranoside and baker's yeast mannan, and no activity towards α-D-Man*p*-(1→2)-D-Man*p*. The activity towards (1→3)-linked mannobiose is strongly activated by 1 mM Ca^{2+} and inhibited by 10 mM EDTA, while (1→6)-activity is unaffected, indicating that the two activities may be associated with different polypeptides. It is also possible that one polypeptide may have two active sites catalysing distinct activities.

Keywords: 1,6-α-D-Mannosidase; Enzyme purification; Linkage specificity; Glycosidase

1. Introduction

Alpha-D-mannosidases are assigned the following five EC numbers by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology:[1] EC 3.2.1.24 to those that do not have strict linkage specificity, EC 3.2.1.77 to those acting on (1→2)- and (1→3)-linkages, EC 3.2.1.113 to those acting on (1→2)-only, EC 3.2.1.114 to those acting on (1→3)- and (1→6)-linkages and EC 3.2.1.137 to those acting on (1→2)- and (1→6)-linkages. There are no (1→6)- or (1→3)-linkage specific mannosidases included yet. There has, however, been one (1→6)-specific enzyme reported in the literature,[2] and is commercially available, but it has not been fully characterised. A (1→3)-linkage specific *exo*-mannosidase has not been discovered to date.

In vivo, mannosidases are involved in processing complex-type oligosaccharides in mammalian cells, and mannans in yeasts.[3,4] In vitro, mannosidases are useful tools for enzymatic analysis of high-mannose oligosaccharide structures,[5–7] and for oligosaccharide synthesis.[8–13]

The production of a novel 1,6-α-D-mannosidase from *Aspergillus phoenicis* ATCC 14332 (syn. *Aspergillus saitoi*)[14] has already been reported and the enzyme has

Abbreviations: AEC, anion-exchange chromatography; BCA, bicinchonic acid; BSA, bovine serum albumin; CHR, chromatofocussing chromatography; CV, column volumes; EDTA, ethylenediamine tetra acetic acid; *p*NPM, *p*-nitrophenyl-α-manno-D-pyranoside; RSD, relative standard deviation; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; U, unit.

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been employed in regioselective synthesis of manno-oligosaccharides.[13] It has not yet been purified and characterised. During the course of this work the effect of fermentation time on enzyme production was studied and the enzyme was purified and characterised. Unexpectedly, when *A. phoenicis* was grown on the commercial yeast mannan hydrolysate Bio-Mos® for 7 days in an attempt to maximise 1,6-α-D-mannosidase production, hydrolytic activity towards (1→3)-linked mannobiose was detected. It has been reported in the literature that, when grown on wheat bran, *A. phoenicis* produces a 1,2-α-D-mannosidase[15] and a 1,3(1,6–1,2)-α-D-mannosidase, which has high affinity for (1→3)-linkages but also hydrolyses (1→6)- and (1→2)-linkages.[16] Since there was no 1,2-α-D-mannosidase activity detected when *A. phoenicis* was grown on Bio-Mos®, and previously reported mannosidases from *A. phoenicis* did not possess (1→3)- or (1→3)/(1→6)-specific activities, we thought it to be desirable to purify and partially characterise this enzyme too.

2. Results

When grown on Bio-Mos®, the microorganism secreted a 1,6-α-D-mannosidase into the culture medium. The enzyme activity was highest after 7 days fermentation, although production of biomass reached a plateau on day 5 (data not presented). When the medium contained mannose, glucose or sucrose instead of Bio-Mos® the extract contained no detectable mannosidase activity, but the enzyme was produced in media lacking any single component other than Bio-Mos®.

To establish that production of the enzyme was induced by Bio-Mos®, the same strain of *Aspergillus* was also grown on wheat bran following the method published by Ichishima et al.[15] The microorganism grew as expected and produced 1,2-α-D-mannosidase activity, as well as activity towards *p*-nitrophenyl-α-D-mannopyranoside and baker's yeast mannan. After 7 days fermentation time, which resulted in maximum 1,6-α-D-mannosidase production, a second α-D-mannosidase activity was detected towards α-D-Man*p*-(1→3)-D-Man*p*. The extract did not exhibit any hydrolytic activity towards α-D-Man*p*-(1→2)-D-Man*p*.

The elution profiles of 1,6-α-D-mannosidase in each purification step are shown in Figures 1–3; the purification steps used are summarised in Table 1. In the first step (Fig. 1) fractions were assayed for (1→6)- and (1→3)-activities. In subsequent steps (Figs. 2 and 3) fractions were assayed for (1→6)-activity only. The apparent molecular weight of the 1,6-α-D-mannosidase was 74 kDa by SDS-PAGE (Fig. 4) and 81 kDa by native-PAGE (data not shown). The isoelectric point was 4.6 (data not shown). The enzyme had hydrolytic activity towards (1→6)-linked mannobiose only (Fig. 5).

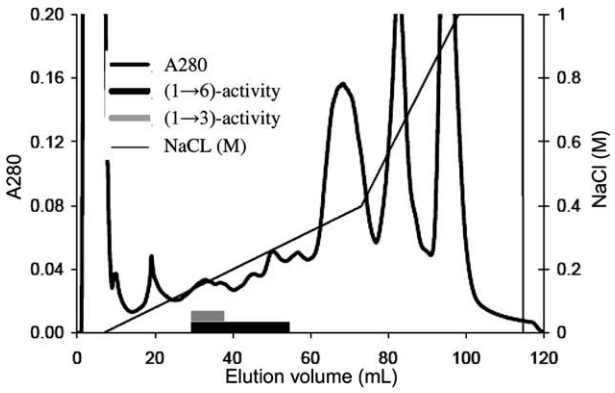

Figure 1. Anion-exchange chromatography of the ultrafiltration concentrated culture extract on a 4.6 mm × 100 mm column packed with POROS 20 HQ media. The column was eluted with a sodium chloride gradient 0–1 M. Fractions with enzyme activity are indicated by bars.

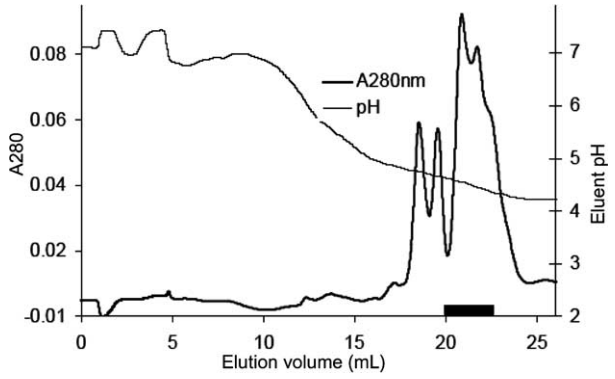

Figure 2. Chromatofocussing chromatography of the 1,6-α-D-mannosidase fraction from POROS 20 HQ column, onto a Pharmacia Biotech Mono P H/R 5/5 column. The column was eluted with Polybuffer 74, setting a pH gradient of 7.1–4.0. Fractions with enzyme activity are indicated by the bar.

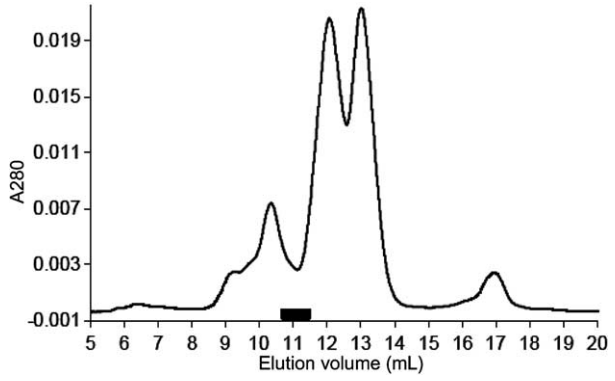

Figure 3. Size-exclusion chromatography of the 1,6-α-D-mannosidase fraction from Mono P H/R 5/5 column, onto a Pharmacia Biotech Superdex 200 H/R 10/30 column (fractionation range 10–600 kDa MW). The column was eluted with 0.01 M sodium acetate–0.15 M NaCI buffer, pH 4.5. The bar indicates fractions with enzyme activity that were pooled.

Table 1. Purification of 1,6-α-D-mannosidase from *A. phoenicis* based on assay with α-D-Man*p*-(1→6)-D-Man*p* as substrate

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Fold purification
Culture filtrate	1200	855	108	—	0.13	—
UF[a] concentrate	40	368	108	100	0.3	2
AEC[b]	6	14.4	51	47	3.5	27
CHR[c]	2	1.1	7	6.5	6.4	49
SEC[d]	1.2	0.10	4.8	4.4	48	370

[a] UF: ultrafiltration.
[b] AEC: anion-exchange chromatography.
[c] Chromatofocussing.
[d] Size-exclusion chromatography.

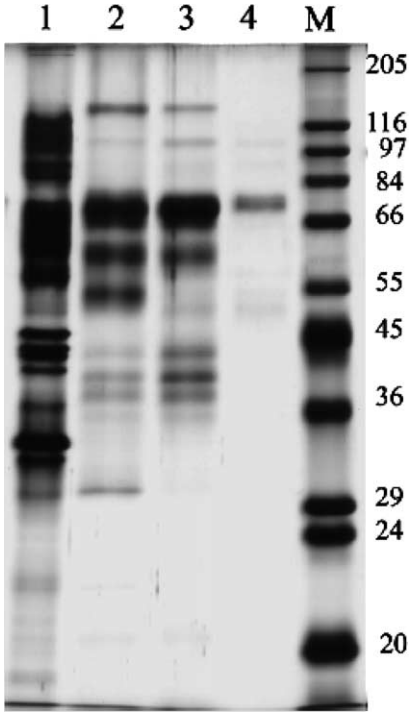

Figure 4. Silver-stained SDS-PAGE of the purification steps of the 1,6-α-D-mannosidase. Lane numbers correspond to: 1: concentrated extract, 2: extract after anion-exchange chromatography, 3: extract after chromatofocussing, 4: extract after size-exclusion chromatography, M: molecular weight standard markers, Sigma wide-range. On the right: molecular weights (kDa) of the standards.

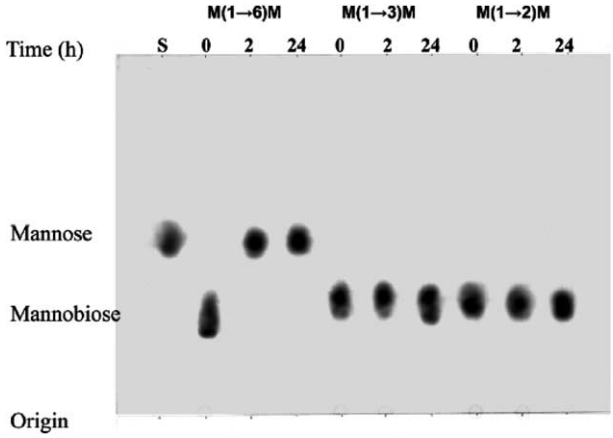

Figure 5. TLC plates showing the hydrolytic activity of the 1,6-α-D-mannosidase towards differently linked disaccharides, monitored over 24 h incubation time at 30 °C, pH 4.5, using two ascents of butanol–ethanol–acetic acid–water 9:6:3:1 v/v/v/v. The plates were dipped in 5 mL of 0.1 M $Ce(SO_4)_2$ diluted in 100 mL of 15% v/v H_2SO_4 and the sugar spots visualised at 100 °C for 15 min. M(1→6)M: α-D-Man*p*-(1→6)-D-Man*p* etc., S: mannose 2% w/v standard.

It did not hydrolyse the other two mannobioses, *p*NPM or baker's yeast mannan, even after prolonged incubation. It was free from β-galactosidase, β-mannosidase and β-*N*-acetylglucosaminidase activities.

The effect of temperature on activity and stability of 1,6-α-D-mannosidase is presented in Figures 6 and 7. The temperature optimum was 60 °C and more than 95% of activity retained after storage at 4 °C for 3 months or at ambient temperature (~23 °C) for 7 days (data not presented). It was also stable at 30 °C for 24 h but was inactivated rapidly above that temperature. Freeze and thaw cycles and freeze drying had a detrimental effect with ~60% activity lost (data not presented).

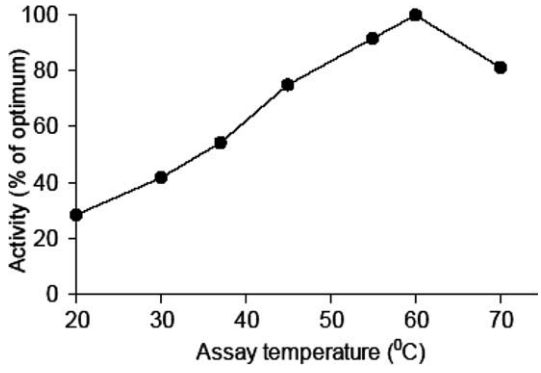

Figure 6. Effect of temperature on activity of 1,6-α-D-mannosidase. The enzyme activity towards or α-D-Man*p*-(1→6)-D-Man*p* was measured at different temperatures (20–70 °C). Values are means of duplicate determinations.

The optimum pH of 1,6-α-D-mannosidase was 4.0–4.5 (Fig. 8). Although activity fell sharply at pH values greater than 5.0, the enzyme retained ~36% of its maximum activity at pH 3.0. The 1,6-α-D-mannosidase was strongly inhibited by 1 mM Mn^{2+} and partially inhibited

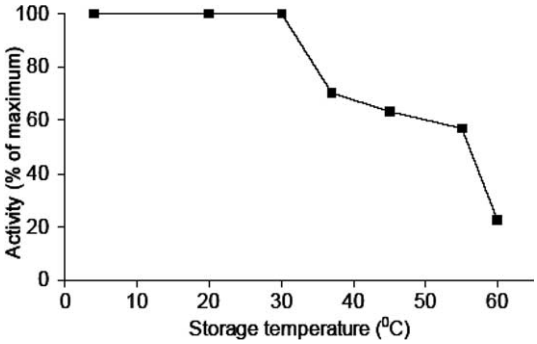

Figure 7. Thermal stability of 1,6-α-D-mannosidase. Aliquots of the enzyme were incubated for 24 h at different temperatures and the residual activity determined at 30 °C as described in Section 4.4. Values are means of duplicate determinations.

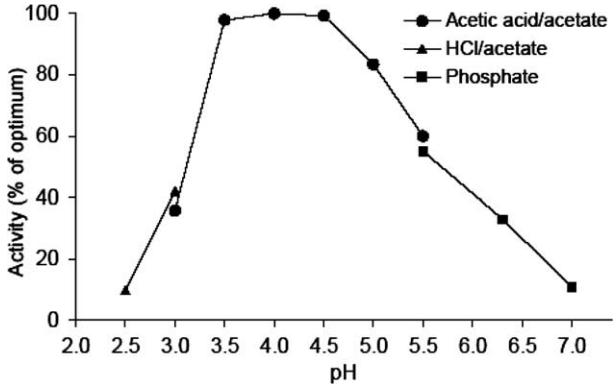

Figure 8. Effect of pH on the activity of the 1,6-α-D-mannosidase. The enzyme assay was performed at 30 °C in 100 mM of: CH_3COOH + HCl/CH_3COONa ▲, CH_3COOH/CH_3COONa ● and K_2HPO_4/KH_2PO_4 ■. Values are means of duplicate determinations.

by Mg^{2+} but EDTA did not affect activity of the enzyme even at a concentration of 10 mM (Table 2). The 1,6-α-D-mannosidase had a K_m, calculated from a Michaelis–Menten plot by nonlinear regression, of 14 mM with α-D-Man*p*-(1→6)-D-Man*p* as substrate.

The 1,3(1,6)-α-D-mannosidase enzyme was also purified and characterised. The elution profiles in each purification step are presented in Figures 1, 9 and 10, and the steps of purification are summarised in Table 3. In the first step (Fig. 1) fractions were assayed for (1→3)- and (1→6)-activity. In subsequent steps (Figs. 9 and 10) fractions were assayed for (1→3)-activity only. The apparent molecular weight of the 1,3(1,6)-α-D-mannosidase was 97 kDa by SDS-PAGE (Fig. 11) and 110 kDa by native-PAGE (data not shown). The enzyme appears to exist as two charge isomers (isoforms) as evidenced by the appearance of two bands on an isoelectric focussing gel with pI values of 4.3 and 4.8 (data not shown). The enzyme hydrolysed (1→3)- and (1→6)-linked mannobioses only, although at a different rate (Fig. 12). It also hydrolysed *p*NPM but at a very slow rate (0.7 U/mg of the purified enzyme), and baker's yeast mannan only after prolonged incubation (the activity was not quantified). It was free from β-galactosidase, β-mannosidase and β-*N*-acetylglucosaminidase activities.

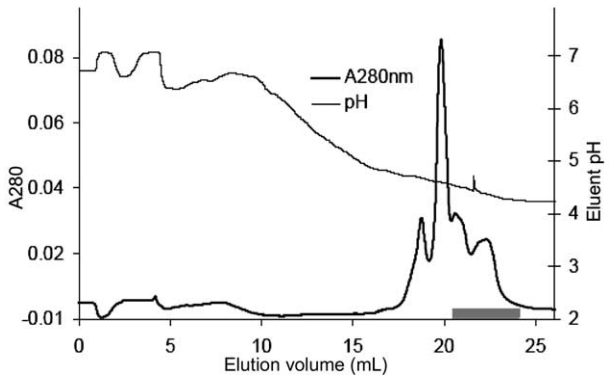

Figure 9. Chromatofocussing chromatography of the 1,3(1,6)-α-D-mannosidase fraction from POROS 20 HQ column, onto a Pharmacia Biotech Mono P H/R 5/5 column. The column was eluted with Polybuffer 74, setting a pH gradient of 7.1–4.0. Fractions with enzyme activity towards α-D-Man*p*-(1→3)-D-Man*p* are indicated by the bar.

Table 2. Effect of ethylenediamine tetra acetic acid (EDTA) and various metal ions in the form of chloride salts on the activity of the 1,6-α-D-mannosidase

Metal ion (1 mM)	Activity (% of blank)
Blank	100
EDTA (10 mM)	107
Zn^{2+}	100
Cu^{2+}	100
Ca^{2+}	98
Fe^{3+}	97
Co^{2+}	96
Li^{+}	96
Fe^{2+}	94
Mg^{2+}	60
Mn^{2+}	18

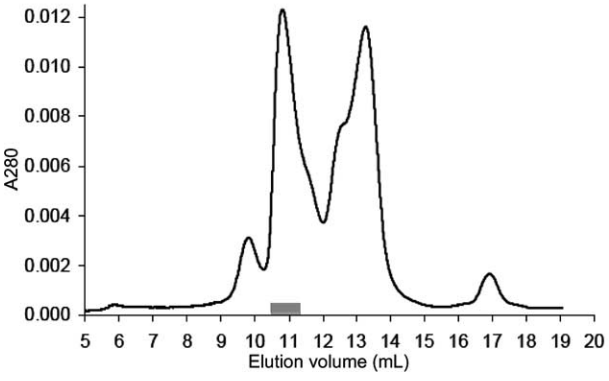

Figure 10. Size-exclusion chromatography of the 1,3(1,6)-α-D-mannosidase fraction from Mono P H/R 5/5 column, onto a Pharmacia Biotech Superdex 200 H/R 10/30 column (fractionation range 10–600 kDa MW). The column was eluted with 0.01 M sodium acetate–0.15 M NaCI buffer, pH 4.5. Fractions with enzyme activity towards α-D-Man*p*-(1→3)-D-Man*p* are indicated by the bar.

Table 3. Purification of 1,3(1,6)-α-D-mannosidase from *A. phoenicis* based on assay with α-D-Man*p*-(1→3)-D-Man*p* as substrate

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Fold purification
Culture filtrate	1200	855	—	—	Very low	—
UF conc.	40	368	4.1	100	0.011	—
AEC	6	2.9	3.3	81	1.1	100
CHR	2	0.31	1.9	46	6.0	545
SEC	1.2	0.07	1.3	32	19.7	1770

A single determination was carried out for each purification step.

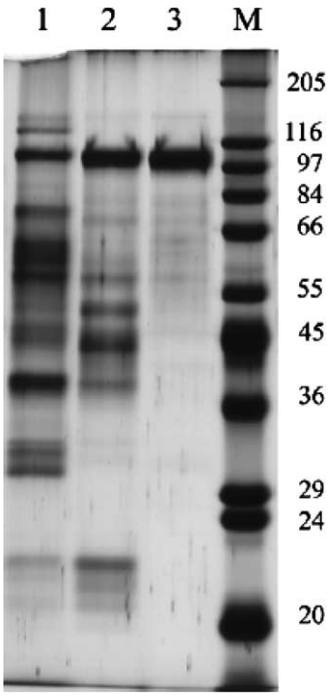

Figure 11. Silver-stained SDS-PAGE of the purification steps of the 1,3(1,6)-α-D-mannosidase. Lane numbers correspond to: 1: anion-exchange chromatography, 2: chromatofocussing, 3: size-exclusion chromatography, M: molecular weight standard markers, Sigma wide-range. On the right: molecular weights (kDa) of the standards.

Time-course assays of the two activities of 1,3(1,6)-α-D-mannosidase, in the absence as well as in the presence of calcium ion showed that Ca^{2+} stimulated (1→3)-activity by 70% but (1→6)-activity was enhanced by less than 4%, a very low value which is within the RSD of the assay. The effects of EDTA and Ca^{2+} on each activity are presented in Table 4. (1→3)-Activity is stimulated by Ca^{2+} and inhibited by EDTA, while 1,6 activity is unaffected. The rate of hydrolysis of α-D-Man*p*-(1→3)-D-Man*p* was more than 11 times higher than that of α-D-Man*p*-(1→6)-D-Man*p* when 1 mM Ca^{2+} was present.

3. Discussion

Various carbon sources supported growth but none of them could induce production of 1,6-α-D-mannosidase except Bio-Mos®. The result was the same when the mineral solution composition was altered. Additionally, the same strain of *Aspergillus* produced 1,2-α-D-mannosidase when grown on wheat bran according to the method of Ichishima et al.[15] These results indicate that we are not dealing with a different strain of microorganism and that Bio-Mos® induces production of the enzymes.

A fraction of 1,6-α-D-mannosidase free of (1→3)-activity could be obtained by anion-exchange chromatography (AEC). However, (1→3)-activity co-eluted

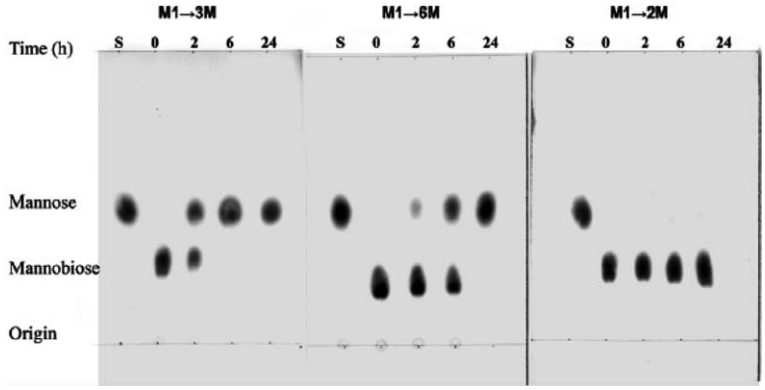

Figure 12. TLC plates showing the hydrolytic activity of the 1,3(1,6)-α-D-mannosidase towards differently linked disaccharides, monitored over 24 h incubation time at 30 °C, pH 5.0, using two ascents of butanol–ethanol–acetic acid–water 9:6:3:1 v/v/v/v. The plates were dipped in 5 mL of 0.1 M $Ce(SO_4)_2$ diluted in 100 mL of 15% v/v H_2SO_4 and the sugar spots visualised at 100 °C for 15 min. M(1→6)M: α-D-Man*p*-(1→6)-D-Man*p* etc., S: mannose 2% w/v standard.

Table 4. Effect of ethylenediamine tetra acetic acid (EDTA) and calcium chloride on the activity of the 1,3(1,6)-α-D-mannosidase towards α-D-Man*p*-(1→3)-D-Man*p* and α-D-Man*p*-(1→6)-D-Man*p*

Reagent and concentration	Activity on α-D-Man*p*-(1→3)-D-Man*p* (% of blank)	Activity on α-D-Man*p*-(1→6)-D-Man*p* (% of blank)
Blank	100	100
Ca^{2+} 1 mM	170	103
EDTA 10 mM	77	110

with a part of the (1→6)-activity in this and every subsequent purification step. It is not clear whether the two activities are properties of the same polypeptide or are present on two distinct polypeptides.

The 1,6-α-D-mannosidase, purified 370-fold over the culture crude extract, was obtained in 4.4% yield with a specific activity of 48 U/mg of protein. The final yield is low, partly due to a significant yield sacrifice in the AEC step (in order to avoid (1→3)-activity contamination), and partly to low recovery in the chromatofocussing step. The apparent molecular weight of the 1,6-α-D-mannosidase under denaturing and nondenaturing conditions was 74 and 81 kDa, respectively, indicating that the enzyme is a monomer. The isoelectric point determined by IEF, was 4.6 which is in agreement with the elution position of the enzyme in the chromatofocussing purification step.

The 1,3(1,6)-α-D-mannosidase, purified 1770-fold over the culture crude extract, was obtained in a reasonably high yield (32%) with a specific activity of 20 U/mg of protein. The 1,3(1,6)-α-D-mannosidase appears to be a monomer with molecular weight 97 kDa by SDS-PAGE and 110 kDa by native-PAGE. Two distinct bands appeared in the IEF at 4.3 and 4.8 indicating the presence of charge isomers or isoforms. This is consistent with two peaks of (1→3)-activity being seen in the chromatofocussing step.

A. phoenicis is reported to produce a specific 1,2-α-D-mannosidase[15] and a nonspecific α-D-mannosidase.[16] The 1,6-α-D-mannosidase has not been reported previously from this species although it is the second enzyme with such specificity to de described, the first produced by *Xanthomonas manihotis*.[2]

The second enzyme reported here cleaves only (1→3)- and (1→6)-mannobiose linkages. The rate of (1→3)-cleavage is 11 times higher than the rate of (1→6)-cleavage. It is activated by calcium (increase 2-fold compared to EDTA treated sample), but this is observed only with (1→3)-activity. (1→6)-Activity was not affected by either reagent. This may possibly indicate that the two activities are associated with different polypeptide chains. It is also possible that a single polypeptide may have two distinct active sites, the conformation and/or activity of one being influenced by the presence of calcium ions. A similar enzyme from *A. phoenicis*, reported by Amano and Kobata,[16] has equally high affinity for (1→3)-linkage compared to (1→6)-linkage, but it is reported to cleave (1→2)-linkages too, and is completely inactive towards *p*-nitrophenyl-α-D-mannopyranoside. It is strongly (13 times) activated by addition of 1 mM Ca^{2+}. Whether calcium has the same effect on the other two activities has not been investigated. Since the above enzyme has been purified from an extract of a culture that also produces 1,2-α-D-mannosidase, it can be surmised that the low activity towards (1→2)-linkages was contaminating activity and the enzyme is actually the same as the one we report here. Unfortunately, no other detail is available to compare with our findings. Regardless, we did not observe as strong activation by Ca^{2+} as reported in the above publication. Additionally, the 1,3(1,6)-α-D-mannosidase reported here, cleaves *p*-nitrophenyl-α-D-mannopyranoside. It is therefore concluded that this is a different enzyme.

The pH optimum of the 1,6-α-D-mannosidase is 4.0–4.5, which is quite low, like the *Aspergillus niger*[17,18] the *Vigna umbellata*[19] and the watermelon[20] mannosidases. All other mannosidases have optimum pH between 5.0 and 7.0.[7,15,16,20–23] Like some mannosidases reported earlier,[16,19] this enzyme retained most of its activity even at a pH of 3.5.

The thermal stability of 1,6-α-D-mannosidase was studied after 24 h incubation. Most published reports determine thermal stability over a very short incubation time, usually 10 min,[7,15,21] but a longer duration stability study gives more meaningful results, especially useful when lengthy concentration/purification steps have to be employed (ultrafiltration, gel filtration), or in cases where the enzymes have to be transported. The 1,6-α-D-mannosidase was found to be very stable at ambient temperature. It also exhibited a high temperature optimum, ~60 °C. Other microbial mannosidases have optima ranging from 30 °C[15,16] to 50–55 °C[20,21] but a plant mannosidase has an optimum of 60 °C.[19]

The 1,6-α-D-mannosidase did not need Ca^{2+} or any other metal ion for activity. The 1,6-α-D-mannosidase from *X. manihotis* is also Ca^{2+} independent[2] and so appear to be all linkage specific 1,2-α-D-mannosidases.[17,21,23,24] In a very recent study it was found that *A. phoenicis* 1,2-α-mannosidase has a calcium-binding consensus sequence and binds Ca^{2+} (1 mol/mol of enzyme).[25] Calcium, when bound, increases thermal stability of the enzyme but is not required for enzymatic activity. The only exception reported is the 1,2-α-D-mannosidase from *Trichoderma reesei*, which is inactivated by EDTA and its activity restored by Ca^{2+}, although no calcium-binding consensus sequence was identified.[26] The 1,3(1,6)-α-D-mannosidase was activated by Ca^{2+}, like most,[2,16,22,27] but not all[18,20] nonlinkage specific mannosidases.

The 1,3(1,6)-α-D-mannosidase belongs to the EC 3.2.1.114 mannosidases. A new EC number is needed for the 1,6-α-D-mannosidase.

4. Experimental

4.1. Materials

Mannose was purchased from Sigma (Poole, Dorset, UK). Mannose containing disaccharides linked α-D-Man*p*-(1→2)-D-Man*p*, α-D-Man*p*-(1→3)-D-Man*p* and α-D-Man*p*-(1→6)-D-Man*p* were purchased from Dextra Laboratories Ltd., Reading, UK. α-D-Man*p*-(1→6)-D-Man*p* in large quantities was prepared according to the procedure described by Athanasopoulos et al.[13] Bio-Mos® was a gift from Alltech (UK) Ltd., Stamford, UK. All other chemicals used were purchased from Sigma or BDH-Merck. The TLC plates used were 10 cm Silica Gel 60 by Merck, Darmstadt, Germany.

4.2. HPLC

Mannose released from the enzyme action on disaccharides was quantified by HPLC as described by Athanasopoulos et al.[13]

4.3. Protein assay

For determination of specific activities, protein content was determined by the BCA assay[28] using bovine serum albumin (BSA) as a standard.

4.4. Enzyme activity assays

A qualitative enzyme detection in the fractions collected after the chromatographic separations was performed as follows. A volume (2 μL) of each fraction was incubated with 2 μL of 2% (w/w) α-D-Man*p*-(1→6)-D-Man*p* or α-D-Man*p*-(1→3)-D-Man*p*, at pH 4.5, 30 °C for 2–24 h (depending on the expected enzyme dilution after each chromatographic technique), and the presence of monosaccharide was detected on Silica Gel 60 TLC plates as described by Athanasopoulos et al.[13]

Enzyme activity was quantified as follows. An appropriate enzyme dilution was incubated with 1% (w/w) α-D-Man*p*-(1→6)-D-Man*p*, total volume 240 μL, at pH 4.5, 30 °C for 20 min. The reaction was stopped by immersion in boiling water for 2 min followed by cooling in ice bath. The mannose released was determined by HPLC. The progress of the reactions was always <15% substrate conversion. One unit is defined as the amount of enzyme that releases 2 μmol of mannose per minute at 30 °C. Determinations were carried out in triplicate and relative standard deviations (RSD) were calculated. A similar procedure was followed using α-D-Man*p*-(1→3)-D-Man*p* but the incubation time was 60 min at pH 5.0 and single determinations were carried out.

The activity of α-mannosidase on *p*-nitrophenyl-α-D-mannopyranoside (*p*NPM) was determined as follows: 100 μL substrate (5.5 mM *p*NPM in 0.01 M sodium acetate buffer pH 5.0) was incubated with 20 μL of the enzyme solution at 30 °C for 2 h. The enzyme reaction was stopped by the addition of 1.1 mL of 0.1 M sodium carbonate, and the absorbance was measured at 420 nm against the blank. The concentration of *p*-nitrophenol released was determined from a standard curve. One unit of enzyme is defined as the amount of enzyme which releases 1 μmol *p*-nitrophenol per minute at 30 °C. Other glycosidase activities (β-galactosidase, β-mannosidase and β-*N*-acetylglucosaminidase) were determined likewise on the corresponding *p*-nitrophenyl-glycosides.

The activity on baker's yeast mannan was determined by the Nelson–Somogyi reducing sugar assay by incubating a volume of a 2% w/v yeast mannan solution in 0.01 M sodium acetate buffer pH 5.0 with an equal volume of the purified enzyme preparations at 30 °C for 2 h. The presence or absence of enzyme activity on this substrate was merely checked qualitatively.

4.5. Fermentation, extraction and concentration of mannosidase

The procedure employed to produce the enzymes was essentially the same as the one described by Athanasopoulos et al.[13] except that the fermentation time was extended to 7 days (instead of three), and the concentration by ultrafiltration was 24-fold (instead of 10-fold).

4.6. Purification of enzymes

The following chromatographic separations were carried out using a PE Biosystems BioCAD/SPRINT Perfusion Chromatography System with a semi-preparative flow cell (PE Biosystems, Birchwood Science Park North, Warrington, Cheshire, UK). All separation procedures were carried out at ambient temperature (~23 °C) and the pooled fractions were concentrated and stored at 4 °C prior to each subsequent purification step. Before pooling, the fractions were assayed for enzyme activity qualitatively, using mannobioses, as described above.

Anion-exchange chromatography (AEC) was carried out using 4.6 mm × 100 mm (1.66 mL) column packed with POROS 20 HQ media (surface functional group was quaternised polyethyleneimine, 10 μm particle size). The buffers used were, A: 0.025 M bis-Tris (pH 6.3 with HCl) and B: 0.025 M bis-Tris–1 M NaCI (pH 6.3 with HCl). A 5 mL volume of the sample was injected into the column, which was eluted for 5 column volumes (CV) with buffer A followed by a linear gradient over 40 CV to 40% buffer B, followed by a linear gradient over 20 CV to 100% buffer B. The flow rate was 4 mL/min and the column effluent was monitored at 280 nm.

Chromatofocussing chromatography (CHR) was carried out using a Pharmacia Biotech Mono P H/R 5/5 column. One millilitre of the sample was injected into the column. A 5 min elution with 0.025 M bis-Tris buffer (pH 7.1 with iminodiacetic acid) was followed by a 20 min elution with Polybuffer 74 (diluted 1/10, pH 4.0 with iminodiacetic acid), setting a pH gradient of 7.1–4.0 for 20 min. The flow rate was 1 mL/min and the column effluent's pH and absorbance at 280 nm was monitored.

Size-exclusion chromatography (SEC) was carried out using a Pharmacia Biotech Superdex 200 H/R 10/30 column (fractionation range 10–600 kDa MW). The column was eluted with 0.01 M sodium acetate–0.15 M NaCI buffer (pH 4.5 with acetic acid). A 0.3 mL sample volume was injected into the column. The flow rate was set at 0.3 mL/min and the effluent monitored at 280 nm. The sample was eluted with the above sample buffer for 80 min.

4.7. Characterisation of hydrolytic specificity

The hydrolytic specificity was determined by incubating 5 μL of the purified enzyme preparation with 5 μL of 2% w/w of α-D-Man*p*-(1→2)-D-Man*p*, α-D-Man*p*-(1→3)-D-Man*p* or α-D-Man*p*-(1→6)-D-Man*p*, at pH 4.5 in 0.01 M sodium acetate buffer at 30 °C for up to 24 h. Samples (1 μL) were removed at regular time intervals and applied to TLC plates to monitor the hydrolysis of the differently linked disaccharides. The TLC plates were run and developed as described by.[13]

4.8. Estimation of molecular weights and isoelectric points

The purity and apparent molecular weight of the purified enzymes were estimated by a discontinuous SDS-PAGE system[29] on a 10 × 7 cm slab gel (3.5% stacking gel and 10% resolving gel), using molecular weight markers (Sigma wide-range Marker Kit, Sigma, Dorset, UK). Proteins in the gel were silver stained using the method of Chrambach.[31] The gel images were acquired and analysed using the SynGene system of Synoptics Ltd. and the Gene-Tools software.

The native molecular weight of the purified enzymes was estimated by native-PAGE. The standards used were: bovine serum albumin (BSA) monomer (66 kDa), BSA dimer (128 kDa) and Jack bean urease trimer (272 kDa), all contained in Sigma nondenatured protein molecular weight marker kit. The standards and the purified enzymes were electrophoresed on four different separating gel acrylamide concentrations (7%, 8%, 9% and 10%), with a stacking gel acrylamide concentration of 3.5% and Bromophenol Blue as a tracking dye. The molecular weights of the two enzymes determined according to Hedrick and coworkers.[30,31]

The isoelectric point (pI) was determined by agarose isoelectric focussing (IEF) on IsoGel® plates, pH 3–10, (Cambrex Bioscience, Berkshire, UK) according to the manufacturer's instructions, using IEF-Mix 3.6–9.3 markers from Sigma.

4.9. Effects of metal ions on activity

The effect of EDTA and various metal ions in the form of chloride salts on the activity of the 1,6-α-D-mannosidase was studied by incubating the enzyme with 1 mM of each metal ion (10 mM for EDTA) and estimating the activity as described in Materials and methods. The results were compared with a reagent-blank enzyme assay.

4.10. Temperature optimum and thermal stability

The 1,6-α-D-mannosidase activity towards α-D-Man*p*-(1→6)-D-Man*p* was measured at different temperatures (20–70 °C in 0.01 M sodium acetate buffer, pH 4.5) to determine the temperature optimum. In order to determine the thermal stability of 1,6-α-D-mannosidase, aliquots of the enzyme were incubated for 24 h at different temperatures and the residual activity determined at 30 °C as described in Section 4.4.

4.11. pH Optimum

The effect of pH on the activity of the 1,6-α-D-mannosidase was determined by assaying the enzyme activity in the pH range: 2.5–7.0, in 100 mM buffers (CH_3COOH + HCl/CH_3COONa, CH_3COOH/CH_3COONa and K_2HPO_4/KH_2PO_4) at 30 °C.

4.12. Kinetic studies

The 1,6-α-D-mannosidase was assayed in 0.1 M sodium acetate buffer, pH 4.5, at 30 °C with a range of concentrations of α-D-Man*p*-(1→6)-D-Man*p* (1, 4, 8, 10, 20, 40 and 50 mM) in a final volume of 240 μL for 10 min with appropriate blanks. The mannose released was determined by HPLC and the Michaelis–Menten kinetic parameters were estimated by nonlinear regression using BioChem Lab Assistant software.

4.13. 1,3(1,6)-α-D-Mannosidase: ratio of activities and effect of Ca^{2+} and EDTA

The ratio of the two activities was determined by assaying the purified enzyme preparation with the two substrates using a time-course assay. The mannose concentrations in two reaction mixtures were determined and the values were plotted against the incubation time to indicate the susceptibility of each linkage to the enzyme action in the absence as well as in the

presence of calcium ion. The fixed time assay was also used but with longer incubation times, to determine the effect of EDTA and calcium chloride on the two activities, and the results were expressed as percentages of the blanks.

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