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Purification and Characterization of a Low Molecular Weight of β-Mannanase from *Penicillium occitanis* Pol6

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Abstract The highest β-mannanase activity was produced by *Penicillium occitanis* Pol6 on flour of carob seed, whereas starch-containing medium gave lower enzymes titles. The low molecular weight enzyme was purified to homogeneity by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography procedures. The purified β-mannanase (ManIII) has been identified as a glycoprotein (carbohydrate content 5%) with an apparent molecular mass of 18 kDa. It was active at 40 °C and pH 4.0. It was stable for 30 min at 70 °C and has a broad pH stability (2.0–12.0). ManIII showed K_m, V_{max}, and K_{cat} values of 17.94 mg/ml, 93.52 U/mg, and 28.13 s^{-1} with locust bean gum as substrate, respectively. It was inhibited by mannose with a K_I of 0.610^{-3} mg/ml. ManIII was activated by $CuSO_4$ and $CaCl_2$ (2.5 mM). However, in presence of 2.5 mM Co^{2+}, its activity dropped to 60% of the initial activity. Both N-terminal and internal amino acid sequences of ManIII presented no homology with mannanases of glycosides hydrolases. During incubation with locust bean gum and Ivory nut mannan, the enzyme released mainly mannotetraose, mannotriose, and mannobiose.

Keywords β-Mannanase · *Penicillium occitanis* · Flour of carob seed · Inhibition · Mannose

Introduction

Mannans are the major polysaccharide of softwood hemicelluloses, accounting for 15–20% (dry basis) in softwoods but only 5% in hardwoods [1]. The basic molecular structure of a

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mannan is a linear backbone composed of β-1,4 linked D-mannopyranosyl residues which, depending on the origin and method of extraction, may be substituted with branches containing mainly acetyl and galactosyl residues [2–4].

The complete cleavage of the complex structure of β-1,4-mannan requires the combined action of β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), and β-glucosidase (EC 3.2.1.21) with the debranching enzymes such as α-galactosidase (EC 3.2.1.22) and acetyl esterase (EC.3.1.1.6) [3, 5, 6]. All endo-1,4-β-mannanases cleave the main chain of mannans, yielding mainly mannotriose and mannobiose [7–9], while only a few β-mannanases also release mannose from mannans [10].

Mannanases have been used in several applications in food technology. In fact, they can be used for the hydrolysis of high molecular weight mannans, e.g., in coffee pulp for the production of soluble coffee [11] or in fruit as well as in vegetables for the manufacture and clarification of juice or the extraction of oil [12, 13].

Mannanases can be used also for the production of mannooligosaccharides from cheap agricultural by-products such as copra or konjac mannan. These oligosaccharides are reported to be excellent prebiotics stimulating growth of beneficial intestinal microorganisms and hence could be used in pharmaceutical or food stuffs [14, 15]. In the pulp and paper industry, mannanases can act synergistically with xylanases as biological prebleaching agents, allowing a significant reduction of chlorine bleaching chemicals and thereby lowering levels of hazardous halogenated organics released into the environment [16–18].

β-Mannanases are classified in family 5 and family 26 of glycosyl hydrolases GH-A according to the classification by Henrissat and Bairoch [19]. GH-A enzymes share the α/β barrel fold and a retaining reaction mechanism [20]. Frequently, β-mannanases are modular enzymes carrying additional modules such as carbohydrate-binding modules [21, 22].

Many mannanases were purified and characterized from fungi such as *Penicillium purpurogenum* [23], *Aspergillus niger* [24], *Trichoderma reesei* [25], *Trichoderma harzianum* [26], and *Sclerotium rolfsii* [27].

Regarding the natural abundance and complexity of hemicellulose, many microorganisms, including *Penicillium occitanis* Pol6 have a variety of enzyme systems able to hydrolyze specific polysaccharides completely into simpler sugars that can be used as a source of energy [28]. Production of enzymes by this strain was studied and optimized [29]. Also, cellulases (endoglucanases, β-glucosidases, and exoglucanases) of Pol6 were purified and characterized [30–32]. In this work, we present the β-mannanase production, purification, and characterization from the strain of *P. occitanis* Pol6.

Material and Methods

Chemicals

Locust bean gum (LBG), oat spelt xylan, carboxymethylcellulose, starch, pectin, *p*-nitrophenyl β-D-mannopyranoside, and mannose were obtained from Sigma (St. Louis, Mo, USA).

Ivory nut mannan was isolated from ivory nuts as described by Hägglund and coworkers [33]. Mannobiose was a kind gift from Dr. W. Nerinckx (Ghent University, Belgium).

Biogel P-100 and Mono-Q Sepharose were purchased respectively from Bio-Rad and Pharmacia chemicals (GE Healthcare, Suede).

Mannan- and nonmannan-based carbon sources such as the carob seed flour from *Ceratonia siliqua*, the flour of tomato seed, coconut meal, corn flour, and barley bran were obtained locally.

Strain

The *P. occitanis* mutant Pol6 was supplied by Cayla Co. (Toulouse, France). The Pol6 strain is a hypercellulolytic mutant selected by Jain et al. [34] after eight rounds of mutagenesis from the CL100 wild-type strain.

Growth Procedure

For the production of β-mannan-degrading activity, *P. occitanis* Pol6 was cultivated at 30 °C for 7 days in a liquid-state medium containing carob seed flour as a carbon source. Erlenmeyer flasks of 1 l containing the substrate (2%) and 200 ml of Mandel's medium in (g/l): KH_2PO_4, 2; $NaNO_3$, 5; $MgSO_4$, $7H_2O$, 0.3; $CaCl_2$, 0.3; yeast extract, 1 g; trace elements solution, 1 ml/l, and tween 80, 1 ml/l were inoculated with a mycelium suspension from a routine subculture. The trace elements solution contained (in g/l): $CoCl_2$, 2; $MnSO_4$. H_2O 1.6; $ZnSO_4$. H_2O, 1.4; and $FeSO_4$. 7 H_2O, 5.0. The pH value was adjusted to 5.5 with NaOH (10 N) [29]. After the growth procedure, the culture supernatant was separated from the mycelium by centrifugation at 8,000 rpm for 30 min, filtered, and stored at 4 °C for subsequent use as a source of β-mannanase activity.

Assays

β-Mannanase was assayed by using a 5-g/l solution of locust bean gum galactomannan in 50 mM sodium acetate buffer pH 5.5 (0.5 ml) to which 0.5 ml of the appropriately diluted enzyme solution was added. The release of reducing sugars for 30 min at 50 °C was measured as mannose equivalents by the dinitrosalicylic acid method [35]. The cellulase (CMCase), β xylanase, pectinase, and α-amylase activities were measured in a similar way using a 10-g/l solution of carboxymethylcellulose, xylan, pectin, and starch, respectively. β-Mannosidase activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl mannopyranoside (5 mM final concentration) as described by Rättö and Poutanen [36].

All enzymatic activities were expressed as micromole reducing sugar formed per minute and per milliliter enzyme solution, i.e., as international units per milliliter. The protein concentration was measured by the method of Bradford [37] using bovine serum albumin as a standard. The presence of glycan chains in the purified mannanase was checked by anthrone–sulfuric acid method using glucose as a standard [38].

Kinetic Properties

For the kinetic experiments, locust bean gum was used as substrate in a concentration range of 0.1–5.0 mg ml^{-1} and incubated with the purified mannanase at 40 °C for 30 min. K_m and V_{max} values were estimated from Lineweaver–Burk equation. For the inhibition studies, the activity with 4 mM of mannose was studied. The K_I was determined by incubating the enzyme at different mannose concentrations (0.5, 1, 2, 4 mM) with substrate concentrations ranging from 1 to 5 mg/ml of LBG in 50 mM citrate buffer, pH 4.0 at 40 °C for 30 min.

Effect of pH, Temperature, and Some Chemical Ions on Enzyme Activity

The determination of the optimum temperature of the mannanase was carried out in the temperature range of 20–60 °C. The optimum pH of ManIII was determined by measuring

the activity at 40 °C for 30 min at various pH values from 2.0 to 9.0 using 50 Mm of citrate-phosphate buffer (pH 2 to 6) and Tris–HCl buffer (pH 7 to 9).

The thermal stability was determined by assaying for residual β-mannanase activity after incubation of β-mannanase in 50 mM phosphate citrate buffer, pH 4.0 at various temperatures (4–80 °C) for 30 min. Following incubation, the remaining activity was determined under standard enzyme assay conditions. The pH stability was determined by incubating the enzyme solutions at different pH values at 4 °C for 24 h. The activities were measured for as described above.

The effect of some chemical ions at 2.5 mM ($CuSO_4$, $FeSO_4$, $COSO_4$, $ZnCl_2$, and $CaCl_2$) and ethylenediaminetetraacetic acid (EDTA) at 5 mM final concentrations on the ManIII activity was determined by performing the assay under the same conditions as described above. Also, the effect of EDTA at 5 mM with different concentrations of $CaCl_2$ on mannanase activity was studied.

Purification Procedure

All the purification steps were carried out at 4 °C and the eluted proteins were monitored at 280 nm. The proteins in culture filtrates were precipitated using 60% (*w*/*v*) ammonium sulfate. The precipitates were dissolved in 20 mM Tris–HCl buffer (pH 8.0).

Aliquots (6 ml) of the concentrate were fractionated by gel filtration on Biogel P-100 (2.2×87 cm) columns pre-equilibrated with 20 mM Tris–HCl buffer containing 20 mM NaCl, pH 8.0. Fractions of 3 ml were collected at a flow rate of 16 ml/h.

Fractions with β-mannanase activity (16.5 ml) were pooled and loaded onto a MonoQ-Sepharose column, equilibrated with 20 mM Tris–HCl buffer, pH 8.0. Fractions of 5 ml were collected at a flow rate of 45 ml/h by washing the column with buffer followed by a stepwise with 0.1, 0.15, 0.20, 0.25, 0.3, and 0.4 mol l^{-1} of NaCl. Fractions corresponding to β-mannanase activity were pooled and stored for later use at 4 °C or at −20 °C in glycerol.

Electrophoresis

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [39] using a 12% of acrylamide gel. The proteins in the gel were stained with Coomassie brilliant blue. A native PAGE was done similarly but without SDS. The electrophoresis was carried out at 4 °C in a gelcasting apparatus (Bio-Rad) using the TBE buffer (89 mM Tris, 2 mM EDTA, and 89 Mm boric acid). A replicate native PAGE containing 0.5% locust bean gum was stained for protein and β-mannanase activity using Congo red [7]. After the electrophoresis, the replicate of polyacrylamide gel was stained in a Congo red solution (2 mg ml^{-1}) for 30 min at 50 °C. It was destained with 1 M NaCl. Clear areas in a dark red background indicated β-mannanase activity.

Molecular Mass Determination

The molecular mass of ManIII was estimated by electrophoresis (SDS-PAGE) using low molecular weight standards for calibration from Bio-Rad (Hercules, CA, USA). It was also estimated by gel filtration on Biogel P100.

Limited Proteolysis of ManIII and Purification of Peptides

Limited proteolysis of ManIII was performed with trypsin. Purified enzyme (1 mg ml^{-1}) was incubated with proteolytic enzyme in various conditions: two temperatures of 4 °C and 37 °C, incubation times varying from 1 to 24 h and trypsin/mannanase molar ratio varying from 0.05 to 0.1. The reaction was stopped by addition of acetic acid (20% final concentration). The resulting peptides from enzymatic cleavage were then separated by chromatography on a C-8 reverse-phase column (250×4.6 mm). Elution was carried out with a gradient from 0% to 80% of acetonitrile for 30 min at a flow rate of 0.6 ml/min.

Sequence of N-Terminal Amino Acids

The N-terminal sequence was determined by automated Edman's degradation, using an applied Biosystems Protein Sequencer Procise 492 cLC [40]. N-terminal sequence homology was analyzed using the BLAST database.

Hydrolysis Experiments

The hydrolysis of Ivory nut mannan and locust bean gum was carried out in 50 mM sodium acetate buffer, pH 4.0 at 40 °C in a shaken water bath (150 rpm) using respectively substrate concentration of 2.5 and 5 g/l. The purified mannanase was added at an activity of 40 U/g of substrate. After 6 and 24 h, samples were removed, boiled to inactivate the enzyme activity, centrifuged, and lyophilized.

The released oligosaccharides were analyzed by high performance anion exchange chromatography with pulsed amperometric detection on an ICS-3000 (Dionex Sunnyvale), using a CarboPac™ PA1 (2×250 mm) analytical column. NaOH (100 mM) and NaOAc (200 mM, in 100 mM NaOH) were used as eluents.

The oligosaccharides were eluted in a linear gradient of 10 to 170 mM NaOAc in 27 min at a flow rate of 0.25 ml/min. The retention times of the samples were compared to the standards (mannose and mannobiose) and to the hydrolysis products of ivory mannan by Cel5A (previously known as EGII) from *T. reesei* (a gift from R. Fagerström, Röhm Enzyme Finland, Rajamäki), which produces mainly mannobiose, mannotriose, mannotetraose, and mannopentaose [41].

Results and Discussion

Production of Mannanases

In order to choose the best substrate for β-mannanase secretion by *P. occitanis* Pol6, the fungus was grown on different carbon sources (carob seed flour, tomato seed flour, coconut meal, barley bran, and corn flour), and the supernatant after various times of cultivation (5, 7, and 10 days) was assayed for β-mannanase activity (Fig. 1).

Based on preliminary studies, we showed that mannan carbon sources were added in concentrations not exceeding 2% (*w*/*v*; data not shown), since the viscosity of solutions of higher concentrations limits oxygen transfer, growth of organisms, and production of mannanase. In fact, only the mannan carbon source (carob seed flour, tomato seed flour, and coconut meal) induce a mannanase formation while starch carbon sources (corn flour

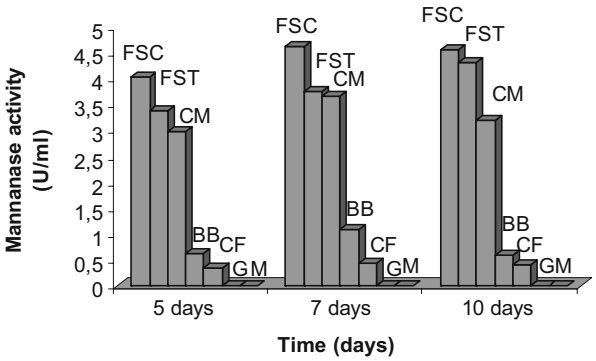

Fig. 1 Time course of β-mannanase production by *P. occitanis* in the presence of different carbon sources (2%): carob seed flour (*FSC*), tomato seed flour (*FST*), coconut meal (*CM*), corn flour (*CF*), barley bran (*BB*), glucose (*G*), and mannose (*M*)

and barley bran) produced only very low mannanase activities. No mannanase activity was detected in media containing glucose and mannose as substrates. This suggested that mannanase was subjected to a catabolic repression whereas *S. rolfsii* was able to produce endoβ-1, 4 mannanase when grown in media containing glucose [42].

Interestingly, the highest mannanase activity was obtained on the carob seed flour as a carbon source (about 4.5±0.15 U/ml). This production level of mannanase activity by *P. occitanis* Pol6 on carob seed flour was higher than *Thermomyces lanuginosus* (0.18 U/ml) and *S. rolfsii* (3.24 U/ml) on commercial LBG [43], whereas it was lower than *Aspergillus fumigatus* (19.9 U/ml) [44].

The maximum level of β-mannanase activity was reached after 7 days for all tested carbon sources. Beyond this period, the loss of activity could be attributed to denaturation–proteolysis.

For the degradation of the carob seed flour, *P. occitanis* produces multi-enzyme systems. Using the zymogram technique with locust bean gum as substrate after a separation by the native PAGE gel, three distinct mannanase proteins (ManI, ManII, and ManIII) were detected in the culture filtrate on the carob seed flour and tomato seed, while only two mannanases were induced on coconut meal (Fig. 2). These data suggested that there was no common coordinated regulatory mechanism that controlled the synthesis of Man I and both ManII and ManIII.

Similarly, *T. reesei*, *T. harzianum*, *A. fumigatus*, and *S. rolfsii* [7, 42, 44, 45] were also shown to produce several mannanases.

Our attention has been focused on small enzymes. Because of their low molecular weight, these enzymes penetrate easily within the lignocelluloses systems and then cleaved the mannan efficiently. In this paper, we are interested in mannanase ManIII from *P. occitanis* with a low molecular weight. For production of mannanase, we used carob seed flour because it induced the highest mannanase activity. So ManIII was purified to electrophoretical homogeneity out of three β-mannanases detected in the culture filtrate on the carob seed flour.

Purification of ManIII

For the purification of mannanase ManIII to homogeneity, a combination of an ammonium sulfate precipitation (60%) and a chromatographic procedure came to an end. For further purification, the precipitate was subjected to gel filtration chromatography on Biogel P100.

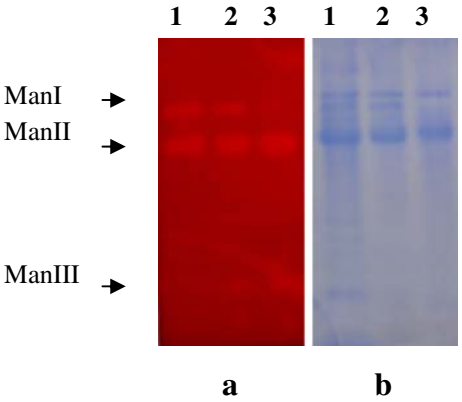

Fig. 2 Native electrophoresis of β-mannanase from *P. occitanis* Pol 6. **a** Mannanase activity zymogram with LBG as substrate. *Lane 1* enzyme culture on carob seed flour (20 μg of protein, 0.12 U of mannanase activity); *lane 2* enzyme culture on tomato seed flour (20 μg of protein, 0.1 U of mannanase activity); *lane 3* enzyme culture on coconut meal (20 μg of protein, 0.1 U of mannanase activity). **b** Stained for protein by Coomassie brilliant blue G-250

The sample elution resulted in the separation of two peaks of β-mannanase activity named respectively (a) and (b) (Fig. 3a).

The zymogram technique after the separation by the native PAGE gel of peaks (a) and (b) showed two bands (Man I and ManII) in peak (a) and one band (ManIII) in peak (b) (Fig. 3b). Thus, peak (b) was pooled and further purified using an anion exchange chromatography on Mono-Q Sepharose column (Fig. 4). The mannanase ManIII was eluted at a concentration of 150 mM. The purification steps were summarized in Table 1.

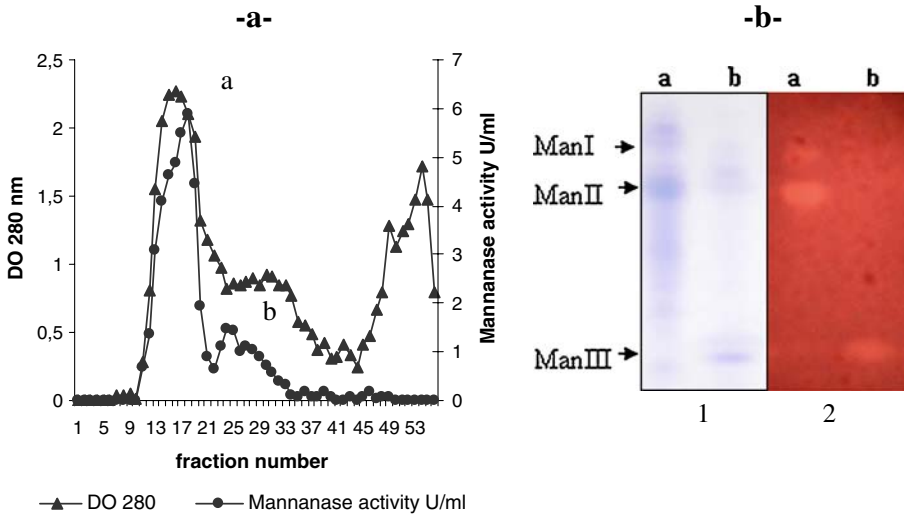

Fig. 3 **a** Elution profile of β-mannanase on Biogel P100 chromatography. **b** Native PAGE of fractions from the major peaks resulting after the first separation step. *Panel 1*: *lane 1* peak *a* (30 μg of protein, 0.5 U of mannanase activity); *lane 2* peak *b* (30 μg of protein, 0.44 U of mannanase activity). Protein was detected with Coomassie brilliant blue. *Panel 2* mannanase activity zymogram with LBG as substrate

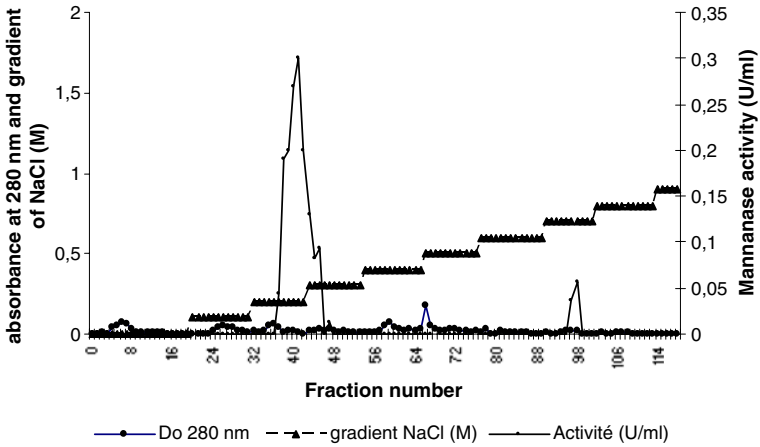

Fig. 4 Elution profile of β-mannanase on Mono Q sepharose chromatography

The recovery of ManIII activity was very low (1.87%). Since these enzymes acted synergistically for the complete hydrolysis of mannans, the yield and fold values were probably underestimated. This protein migrated as 18 kDa on SDS-PAGE (Fig. 5a). ManIII was a glycoprotein. The carbohydrate content measured with the anthrone–sulfuric acid method was determined to be 5%. In support of SDS-PAGE result, native PAGE of ManIII also revealed isoenzyme band coincident with that staining for the mannanase activity (Fig. 5b). A clear hydrolysis activity zone was formed against a dark background.

Characterization of ManIII

ManIII exhibited no detectable activity toward carboxymethylcellulose, oat spelt xylan, starch, pectin, and *p*-nitrophenyl-β-D-mannopyranoside. Thus, the purified mannanase was a specific endo-β-mannanase. The K_m, V_{max}, and K_{cat} values for locust bean gum as the substrate were 17.94 mg/ml, 93.52 U/mg, and 28.13 s^{-1}, respectively (Fig. 6). ManIII exhibited a higher apparent K_m value than β-mannanase from *T. reesei* [25], *T. harzianum* [26], and *A. fumigatus* [44]. β-Mannanase was inhibited by 4 mM of mannose (competitive inhibitor). However, mannose in concentrations of up to 150 mM showed no effect on the mannanase activity of *S. rolfsii* [46]. The K_I was 0.6 10^{-3} mg/ml.

Table 1 Summary of the purification of ManIII.

Purification step	Total activity (UT)	Total protein (mg)	Specific activity (UI/mg)	Yield (%)	Purification (*x*-fold)
Culture supernatant	441.71	85.71	5.15	100	1
Sulfate ammonium precipitation	439.068	36.6	12	99.4	2.33
Biogel P100	15.812	1.062	14.9	3.58	2.9
Mono Q	8.28	0.064	129.37	1.87	25.17

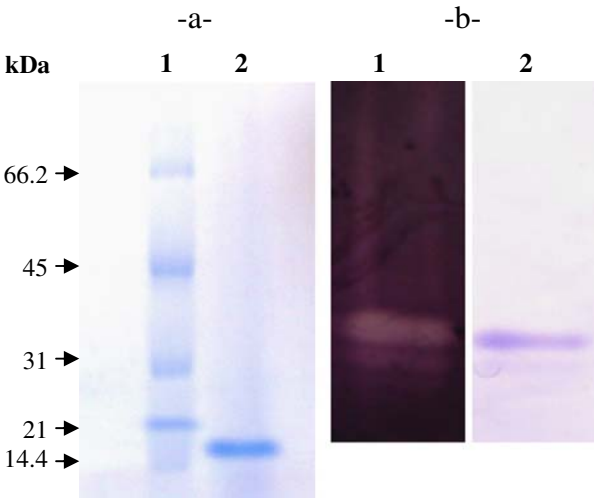

Fig. 5 SDS-PAGE of the purified mannanase with zymogram activity. **a** SDS-PAGE *lane1* molecular weight markers standards of indicated size; *lane 2* purified ManIII after Mono-Q chromatography (5 μg of protein). **b** Native electrophoresis *lane1* purified mannanase activity zymogram (5 μg of protein corresponding to 0.64 U of mannanase activity); *lane 2* purified β-mannanase fraction stained by Coomassie brilliant blue

The enzyme was most active at pH 4.0 (Fig. 7a). About the same pH result was found for β-mannanase from *T. reesei* [7] and *A. fumigatus* [44]. Greater than 80% of the maximal activity was observed between pH 3.0 and 5.0.

The optimal temperature was 40 °C (Fig. 7b) while β-mannanase from *P. purpurogenum* displayed a higher activity at 70 °C [23]. ManIII retained more than 80% of its maximal activity at 50 °C. Temperature above 50 °C resulted in a more rapid inactivation of ManIII. The enzyme demonstrated a broad pH stability, as more than 70% of the maximal activity remained following a 24-h incubation in buffers within a pH range of 4.0–10.0 (Fig. 7c). β-Mannanase from *P. purpurogenum* was stable in the pH range of 4.5 to 8 [23]. However, the enzyme from *S. rolfsii* was not stable under alkaline conditions [6]. The enzyme retained more than 50% of its maximal activity for 30 min at 70 °C (Fig. 7d). It was more stable than β-mannanase from *T. reesei*, *Aspergillus aculeatis* which only retained stability at 50 °C and below [25, 47].

The involvement of some bivalents ions on ManIII was investigated. ManIII was activated by $CuSO_4$ and $CaCl_2$. The assay of Man III at 40 °C showed 109% and 110% of activation in the presence of $CuSO_4$ and $CaCl_2$ at 2.5 mM, respectively. However, in the presence of 2.5 mM Co^{2+}, the enzyme activity dropped to 60% of its initial activity. When mannanase was preincubated with 5 mM EDTA, an inhibition was observed (22%

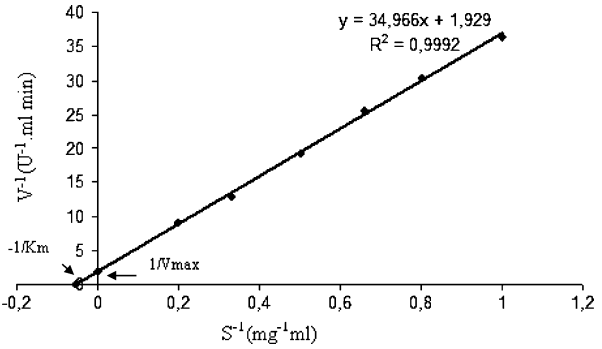

Fig. 6 Lineweaver–Burk analysis of locust bean gum. β-Mannanase was incubated in 50 mM citrate-phosphate buffer (pH 4) at 40 °C with 0.1–5.0 mg ml^{-1} locust bean gum

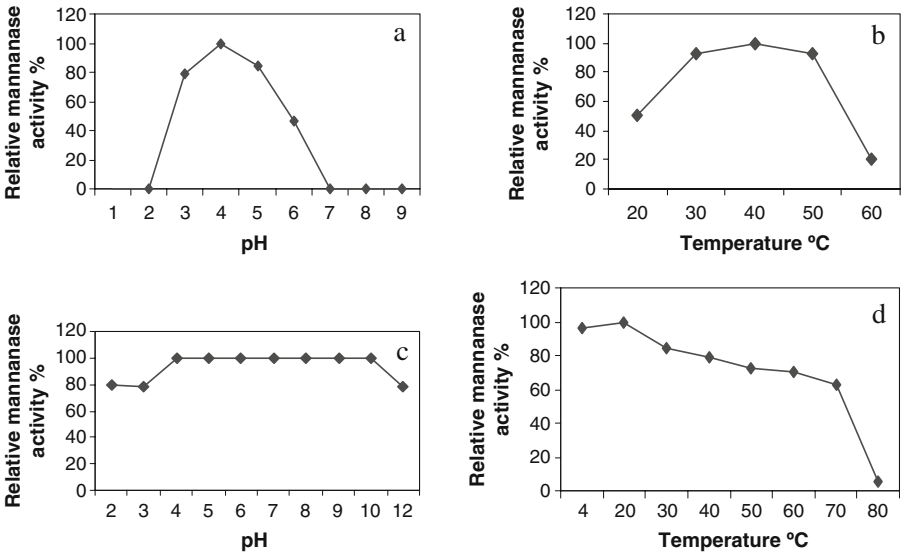

Fig. 7 Influence of pH and temperature on activity and stability of purified *P. occitanis* ManIII. **a** pH optimum determined in the following 50 mM buffers: citrate phosphate (3.0–7.0); Tris/HCl (8.0) and glycine/NaOH (9.0–10.0). **b** Optimum temperature determined in 50 mM citrate phosphate buffer, pH 4.0. **c** Residual enzyme activity after 24 h incubation at 40 °C in 50 mM buffers described in **a**. **d** Stability of enzyme in 50 mM citrate phosphate, pH 4.0, at temperatures indicated

inhibition). The enzyme recovered its original activity by the addition of 5 mM Ca^{2+} after preincubation with 5 mM EDTA (Table 2).

Determination of Internal Peptide Sequences of Mannanase Man III by Limited Proteolysis

To gain insights to the primary structure, we performed a limited proteolysis experiment on purified mannanase (ManIII). The procedure used for this experiment included the

Table 2 Effect of metal ions on the activity of the purified mannanase ManII.

Treatment	Relative mannanase activity (%)
None	100
Metal ions (2.5 mM)	
$CaCl_2$	110
$CuSO_4$	109
Zn Cl_2	86
Fe SO_4	71
Co SO_4	42
EDTA (5 mM)	77
EDTA (5 mM) +	
$CaCl_2$ (2 mM)	64
$CaCl_2$ (5 mM)	111
$CaCl_2$ (7 mM)	143
$CaCl_2$ (9 mM)	124
$CaCl_2$ (12 mM)	72

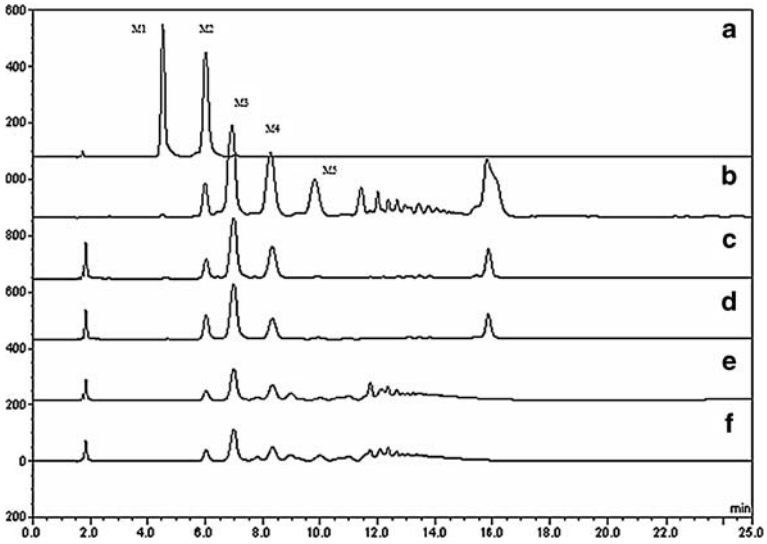

Fig. 8 End products analysis of Ivory nut mannan and locust bean gum hydrolysis at 40 °C by Dionex Chromatography. *A* Standards: *M1* and *M2* indicate mannose and mannobiose respectively. *B* Hydrolysis products of Ivory nut mannan by *T. reesei* Cel5A: *M3*, *M4*, and *M5* indicate respectively mannotriose, mannotetraose, and mannopentaose. Chromatogram obtained after incubation of Ivory nut mannan with purified mannanase for 6 (*C*) and 24 h (*D*). Chromatogram obtained after incubation of locust bean gum with purified mannanase for 6 (*E*) and 24 h (*F*)

hydrolysis of trypsin, the separation and isolation of the resulting peptides by chromatography on a C8 reverse-phase column, and finally sequencing of the proteolytic fragment by automated Edman degradation.

Purified enzyme (1 mg/ml) was incubated with proteolytic enzymes in various conditions: temperatures of 4 °C and 37 °C, incubation times varying from 1 to 24 h. The best conditions for the proteolysis appeared to be 2 h at 37 °C with a protease–mannanase ratio of 0.05.

The NH_2-terminal sequencing of two purified fragments (F1 and F2) allowed unambiguously the identification of 11 residues for F1: AQNGIDSNDQY and nine residues for F2: ANAVESEND. No similarity between the N-terminal amino acid residues of the internal fragments (F1 and F2) was found with those of known other β-mannanase of glycosides hydrolases.

Amino Acid Sequencing

Edman degradation of ManIII yielded a single N-terminal amino acid sequencing (12 residues): ADRGSETVSGIG. This sequence showed no homology with mannanase of glycoside hydrolase. Its isoform had also the same N-terminal sequence.

Hydrolysis Products

The main hydrolysis product of Ivory nut mannan and locust bean gum was mannotetraose, mannotriose, and mannobiose (Fig. 8). This indicates that the purified mannanase was an endomannanase. In comparison to the hydrolysis products of Ivory nut mannan by *T. reese*i Cel5A, we observed also an unidentified mannooligosaccharide with a retention time of 16 min (Fig. 7). A similar result was obtained by the mannanase of *Bacillus subtilis* WY34

in the hydrolysis of copra mannan and locust bean gum [48]. It has been shown that mannobiose and mannotriose, but not mannotetraose, were the major hydrolysis products of ivory nut mannan by the mannanase of *S. rolfsii* [46], *T. reesei* [7], *A. niger* [24], and *P. purpurogenum* [49]. Thus, the ManIII could be used to produce mannooligosaccharide, which are used as functional food additives for selective growth of human-beneficial intestinal microflora [50].

In conclusion, we purified one of hemicellulolytic enzymes produced by *P. occitanis* by describing the properties of its β-mannanase. ManIII exhibited higher thermal and broader pH stability, which is significant in view of their potential industrial application. The activity of this enzyme can be enhanced by incubation with $CuSO_4$ and $CaCl_2$. ManIII also showed no activity against xylan, which is encouraging to use it in pulp bleaching. That made it possible to increase brightness like that from *T. reesei.* Also, mannanase from *P. occitanis* efficiently hydrolyzed ivory nut mannan to mannooligosaccharides used as prebiotic. To our knowledge, ManIII was the first low molecular weight mannanase described in the literature. So, these enzymes penetrated easily into the lignocellulose system and then efficiently cleaved the mannan. Its N-terminal sequence and internal peptide sequences showed no homology with other β-mannanase fungi. This is why family assignment is still not possible. This sequence will be further used for preparing the corresponding oligonucleotides and then cloning the cDNA.

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