Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Polymers 57 (2004) 23–29
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Purification and characterization of a β-mannanase from *Trichoderma harzianum* strain T4

Humberto Malheiros Ferreira, Edivaldo Ximenes Ferreira Filho*

Laboratório de Enzimologia, Departamento de Biologia Celular, Universidade de Brasília, Campus Universitario, Asa Norte, CEP 70910-900, Brasília, DF, Brazil

Received 29 April 2003; revised 26 December 2003; accepted 26 February 2004

Available online 9 June 2004

Abstract

An extracellular β-mannanase was isolated from crude extract samples of the mesophilic fungus *Trichoderma harzianum* strain T4 when grown on wheat bran as the carbon source. The induction profile showed that the β-mannanase activity was detected from the sixth day of cultivation period. The enzyme was purified to apparent homogeneity by ultrafiltration, gel filtration and ion-exchange chromatography procedures. The purified β-mannanase (Man I) had molecular masses of 32.5 and 36.5 kDa, as determined by SDS-PAGE and gel filtration chromatography, respectively. It was most active at 55 °C and pH 3.0 and showed thermal stability with half-lives approx. of 5 h at 55 °C and 4 h at 60 °C and at 70 °C, Man I showed half-life of 1 h. Man I showed Km and Vmax values of 1.3 mg/ml and 3.6 IU/ml with β-galactomannan as the substrate, respectively. The purified enzyme was not active against cellulose and xylan. Man I was highly activated by β-mercaptoethanol, cysteine, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT). On the other hand, *N*-bromosuccinimide (NBS) was a strong inhibitor of Man I activity. The thermal stability of the purified Man I make this enzyme attractive for use in industrial applications.

Keywords: *Trichoderma harzianum* strain T4; β-Mannanase; Wheat bran

1. Introduction

Mannan is one of the major constituents of hemicellulosic materials in softwoods (Ademark et al., 1998; Filho, 1998; Stalbrand, Siika-aho, Tenkanen, & Viikari, 1993). The basic molecular structure of mannan is a linear backbone comprised of β-1,4-linked D-mannopyranose residues which, depending on the origin and method of extraction, may be substituted with branches containing mainly acetyl and galactosyl residues (Filho, 1998; Singh, Madlala, & Prior, 2003; Xu, Hägglund, Stalbrand, & Janson, 2002a). Galactoglucomannan has a hetero-β-1,4-linked backbone of mannose and glucose residues. 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 debranching enzymes such as α-galactosidase (EC 3.2.1.22) and acetyl esterase (EC 3.1.1.6) (Duffaud, McCutchen, Leduc, Parker, & Kelly, 1997; Gübitz, Hayn, Sommerauer, & Steiner, 1996; Singh et al., 2003).

Traditionally, enzymatic saccharification of lignocellulosic biomass has potential applications in the transportation fuel production, solid waste disposal, animal feed, and the paper/textile industry (Filho, 1994; Gúbitz et al., 1996; Hägglund et al., 2003; Wong & Saddler, 1993). Hemicellulases, including β-mannanase, play an important role in the bioconversion of lignocellulose material. The coconut residue is a source of highly concentrated mannan which can be hydrolyzed by mannan-degrading enzyme system to produce single-cell protein (Hossain, Abe, & Hizukuri, 1996). Besides, mannooligosaccharides produced by the enzymatic hydrolysis of β-mannan are used in food and feed-processing industries (Hossain et al., 1996). β-Mannanases can reduce the viscosity of instant coffee (Wong & Saddler, 1993). They also have applications in the fruit-juices industry (Gübitz et al., 1996). Given the natural abundance and complexity of hemicellulose, many microorganisms, including *Trichoderma harzianum*, have

* Corresponding author. Tel.: +55-61-3072152; fax: +55-61-2734608.
E-mail address: eximenes@unb.br (E.X.F. Filho).

0144-8617/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2004.02.010

a variety of enzyme systems to hydrolyze specific polysaccharides completely into simpler sugars that can be used as energy source (Filho, 1994, 1998; Brummer, Sims, & Sinnott, 1999).

This work describes the purification and characterization of a β-mannanase (Man I) from *T. harzianum* strain T4 grown on wheat bran as the substrate. To our knowledge, this is the first report on the β-mannanase production, purification and characterization from a new strain (T4) of *T. harzianum*.

2. Material and methods

2.1. Chemicals

Locust bean gum, oat spelt xylan, carboxymethyl cellulose, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitropheyl-α-D-galactopyranoside, L-cysteine, β-mercaptoethanol, dithiothreitol (DTT), *N*-bromosuccinimide (NBS), diethyl pyrocarbonate (DEPC), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) and 2,2-dithiodipyridine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sephacryl S-100, Sephacryl S-300, Sephadex G-50 and DEAE-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Growth procedure

The aerobic fungus *T. harzianum* strain T4 was kindly provided by Itamar S. Melo (Embrapa/CNPMA, Brasil) and have been maintained in culture collection at the Laboratory of Enzymology from University of Brasília (Silveira, Melo, & Filho, 1997). For production of β-mannan-degrading activity, *T. harzianum* strain T4 was cultured at 28 °C for 11 days in a liquid-state medium containing wheat bran as carbon source. Flasks of 500 ml containing the substrate (0.5%) and supplemented medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.1% $(NH_4)_2SO_4$ and 0.06% yeast extract at pH 7.0) were inoculated with spore suspension (1.0×10^6 ml^{-1}) from a routine subculture. After the growth procedure, the crude extract was centrifuged for 30 min at 10,400*g* and 5 °C. The resulting supernatant was filtered and stored at 5 °C for subsequent use as source of β-mannanase activity. For β-mannanase induction, aliquots were harvested every 6 h during 11 days, and used to estimate the enzyme activity.

2.3. Assays

β-Mannanase activity was determined by mixing 50 μl of enzyme solution with 100 μl of locust bean gum (1%) in distilled water for 30 min at 50 °C. The release of reducing sugar was measured using the dinitrosalicylic reagent method (Miller, 1959). Mannose was used as the standard. β-Mannanase activity was expressed as μmol reducing sugar formed min^{-1} ml^{-1} enzyme solution, i.e. as IU ml^{-1}. β-Mannosidase, cellulase (CMCase and Fpase), β-xylanase and α-galactosidase activities were determined as reported elsewhere (Silveira et al., 1997; Ximenes et al., 1997). Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. For the kinetic experiments, locust bean gum was used as substrate in a concentration range of 0.1–7.0 mg/ml. K_m and V_{max} Values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program (Leatherbarrow, 1987). The determination of optimum temperature of Man I was carried out in the temperature range of 30–80 °C. The optimum pH of Man I was determined by measuring the activity at 50 °C at various pH values between 2.2 and 7.5. All buffers were adjusted to the same ionic strength with NaCl. The stability of Man I was carried out by preincubating the enzyme solution at 55, 60 and 70 °C and removing aliquots at intervals to measure its activity as described above. The effect of some reagents at 10 mM (NBS, iodoacetamide, EDC, DEPC, NEM, 2,2-dithiodipyridine and DTNB) and 20 mM (L-cystein, β-mercaptoethanol and DTT) final concentrations in the Man I activity was determined by performing the assay as the same conditions as described above. Each experiment above was repeated at least three times. The standard deviation values were less than ±20% of the mean.

2.4. Purification procedure

All the purification steps were carried out at 4 °C unless otherwise specified. The crude extract was concentrated by ultrafiltration using an Amicon system (Amicon Inc., Beverly, MA 01915, USA) with a 30 kDa cut-off point membrane (PM 30). Aliquots (4 ml) of the concentrate were fractionated by gel filtration on Sephacryl S-100 (2.9×91 cm^2) and Sephadex G-50 (2.3×42 cm^2) columns pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0 and 50 mM sodium phosphate buffer, pH 7.0, respectively. Fractions of 5.0 ml were collected at a flow rate of 20 ml/h. Fractions with β-mannanase activity were pooled, and loaded onto a DEAE-Sepharose column, equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Fractions of 5.0 ml were collected at a flow rate of 20 ml/h by washing the column with buffer followed by a linear gradient of NaCl (0–1 M), pH 7.0. Fractions corresponding to β-mannanase activity were pooled, concentrated by freeze-drying and stored for later use at 4 °C.

2.5. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) using a 12% gel. After electrophoresis, the protein bands were silver stained by the method of Blum, Beier, and Gross (1987). Replicate native PAGE containing 1% locust

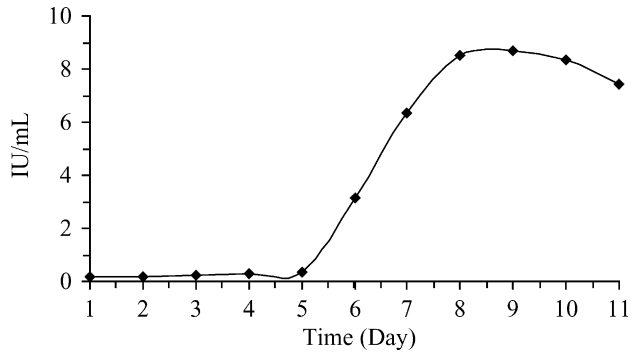

Fig. 1. Time course of β-mannanase production by *T. harzianum* strain T4 in the presence of wheat bran.

bean gum was stained for protein and β-mannanase activity using Congo Red (Biely, Markovic, & Mislovicova, 1985). After the electrophoresis, the polyacrylamide gel was stained in a Congo red solution (1 mg/ml) for 1 h at room temperature. It was destained with 1 M NaCl and fixed with 0.5% acetic acid. Clear areas in a dark blue background indicated β-mannanase activity.

2.6. Molecular mass determination

The molecular mass of Man I was estimated by electrophoresis (SDS-PAGE and native PAGE) and gel filtration using molecular mass marker kits from Sigma Chemical Co. and Amersham Pharmacia Biotech (USA), respectively. The gel filtration chromatography was performed in a Sephacryl S-100 ($2.6 \times 64\ cm^2$) column equilibrated with 20 mM sodium phosphate buffer, pH 7.0 at a flow rate of 16 ml/h and fraction size of 4.0 ml.

2.7. Incubation of Man I with glycosidases

The incubation of Man I with some glycosidases (HEXase I, GALase III, PNPGase F, NANase II and O-glycosidase DS) was carried out was described in the Deglycosylation Enhancement Kit (Bio-Rad Laboratories, CA, USA). HEXase I and GALase III release non-reducing terminal β-linked *N*-acetyl-glucosamine and galactose, respectively. These enzymes together with PNPGase F, NANase II and O-glycosidase DS will be required for the complete O-linked deglycosylation. The treated and untreated samples were run in separate lanes in PAGE under denaturing and non-denaturing conditions.

3. Results and discussion

3.1. Induction of β-mannanase activity

The induction profile after growth of *T. harzianum* strain T4 on wheat bran showed that β-mannanase was detected from the sixth day to the end of cultivation period (Fig. 1). The growth profile was accompanied by more than one peak of mannan-degrading enzyme activity, including β-mannosidase and α-galactosidase (result not shown). The multiplicity of forms is commonly described for hemicellulases from fungi and bacteria as result of differential mRNA processing and posttranslational modifications (Filho, 1994; Wong, Tan, & Saddler, 1988). SDS-PAGE of the crude extracts from inducing media showed a strong protein band of 32.5 kDa at the incubation range of 5–11 days, subsequently identified as β-mannanase (result not shown).

3.2. Purification of Man I

A β-mannanase was isolated from the mannan-degrading system of *T. harzianum* strain T4, and purified to apparent homogeneity by a combination of ultrafiltration and chromatographic procedures. The ultrafiltration experiment indicated that the β-mannanase activity could only be found in the concentrate. For further purification, the concentrate

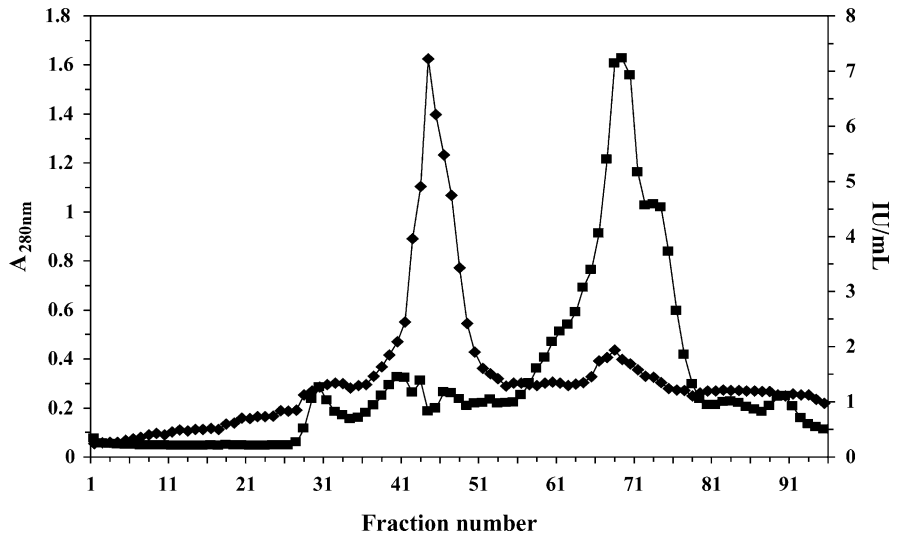

Fig. 2. Elution profile of β-mannanase on Sephacryl S-100 chromatography. (◆) Fractions containing β-mannanase activity; (■) Protein profile at 280 nm.

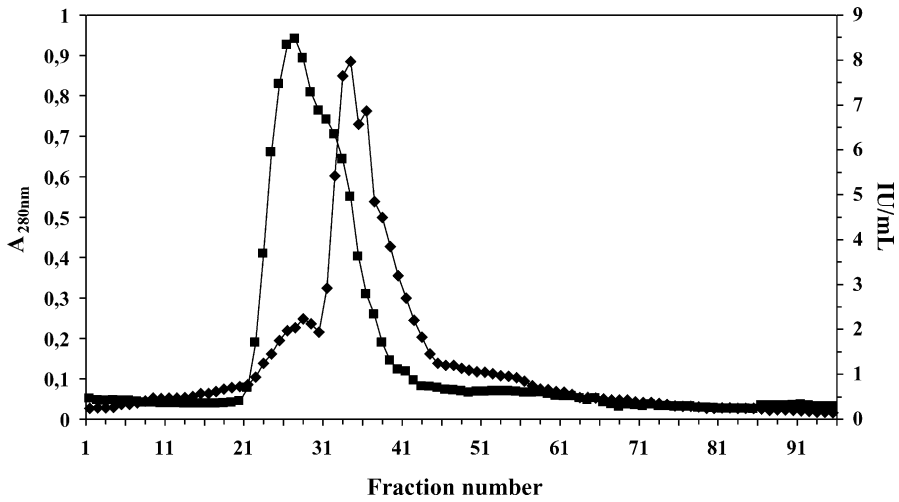

Fig. 3. Elution profile of β-mannanase on Sephadex G-50 chromatography. (◆) Fractions containing β-mannanase activity; (■) Protein profile at 280 nm.

was subjected to gel filtration chromatography on Sephacryl S-100 (Fig. 2). The sample elution resulted in the separation of two peaks of β-mannanase activity. The first peak, designated Man I, was used for further purification by Sephadex G-50 chromatography (Fig. 3). The profile exhibited two peaks of β-mannanase activity, being the second peak purified by anion-exchange chromatography on DEAE-Sepharose column (Fig. 4). In this purification procedure, Man I was eluted in the pre-gradient wash fractions. The purification steps are summarized in Table 1. The recovery of Man I activity was very low (3.18%). The low yield value was mainly due to loss of enzyme activity (approx. 67%) in the ultrafiltration step. The purified Man3 from *Streptomyces ipomoea* showed a final purification yield of 13% (Montiel, Hernández, Rodríguez, & Arias, 2002). Furthermore, taking into account that β-mannanase activity was not detected in the ultrafiltrate, during purification of Man I at least three β-mannanase activities were present. Since these enzymes act synergistically for the complete hydrolysis of mannans, the yield and fold values were probably underestimated.

The purification step procedures provided an preparation of Man I containing one protein band, as determined by SDS-PAGE (Fig. 5). Man I exhibited a monomeric structure. This protein migrated as 32.5 kDa band on SDS-PAGE stained with silver nitrate. This is in good agreement with the value of 36.5 kDa determined by gel filtration on Sephacryl S-300 column (result not shown). In support to SDS-PAGE result, native PAGE of Man I also revealed one protein band coincident with that staining for mannanase activity (Fig. 5). A clear hydrolysis activity zone was formed against a dark background (Fig. 5). The treatment of Man I with glycosidases did not interfere in its electrophoretic moblitiy under denaturing and non-denaturing conditions (results not shown), suggesting that it is not a carbohydrate-associated enzyme. Further evidence is required to suggest that it is not a glycoprotein.

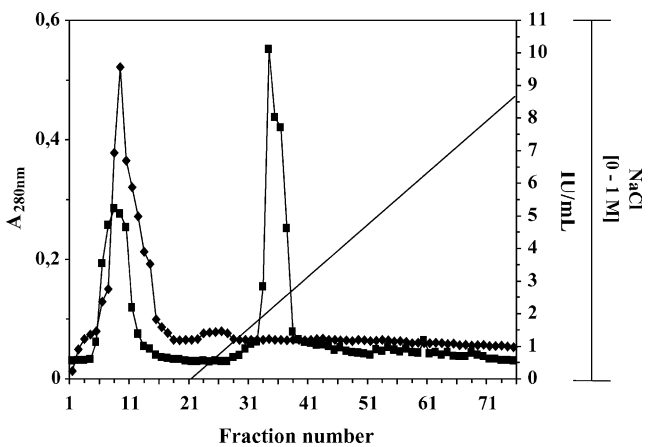

Fig. 4. Elution profile of β-mannanase on DEAE-Sepharose chromatography. (◆) Fractions containing β-mannanase activity; (■) Protein profile at 280 nm.

3.3. Characterization of Man I

Man I exhibited no detectable activity towards carboxymethyl celulose, filter paper, oat spelt xylan, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-β-D-mannopyranoside. The specificity of Man I for mannan as substrate is an important parameter for its use in pulp

Table 1
Summary of the purification of Man I

Steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (-fold)	Yield (%)
Crude extract	81.69	108.96	1.33	1.00	100
Ultrafiltration	13.90	36.22	2.61	1.96	33.24
Sephacryl S-100	8.24	28.70	3.48	2.62	26.34
Sephadex G-50	0.17	3.95	23.24	17.47	3.63
DEAE-Sepharose	0.08	3.47	43.38	32.62	3.18

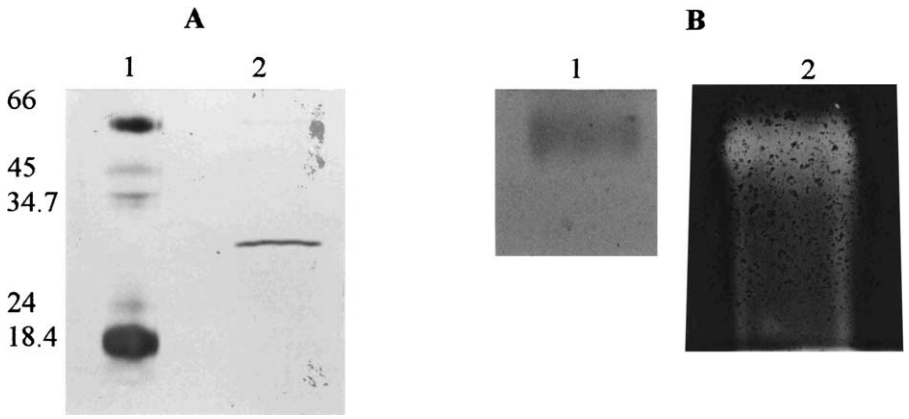

Fig. 5. Homogeneity of the purified β-mannanase from *Trichoderma harzianum* strain T4. (A) SDS-PAGE: Lane 1, molecular weight markers (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa) and lactoglobulin (18.4 kDa); lane 2, Man I. (B) Native electrophoresis: Lane 1, β-mannanase I.; lane 2, Man I activity.

bleaching, whereas in this process the enzyme has to be cellulase free (Silveira et al., 1999). The K_m, V_{max}, and K_{cat} values for locust bean gum as the substrate were 1.3 mg/ml, 3.6 IU/ml and 4.4 s^{-1}, respectively. Man I exhibited a lower apparent K_m value than β-mannanases from *Bacillus stearothermophilus*, *Rhodotermus marinus* and *Mytilus edulis* (Ethier, Tlabot, & Sygusch, 1998; Politz, Krah, Thomesen, & Borriss, 2000; Xu et al., 2002a).

The enzyme was most active at pH 3.0. It showed a slow decrease on alkaline side. About the same pH result was found for β-mannanases from *Sclerotium rolfsii* and *Aspergillus aculeatis* (Gúbitz et al., 1996; Setati, Ademark, van Zyl, Hahn-Hägerdal, & Stalbrand, 2001). The purified Man3 from *Strepotmyces ipomoea* had optimum pH of 7.5 and it was stable at pH 7.5–8.0 (Montiel et al., 2002). However, the enzymes from *Sclerotium rolfsii* were not stable under alkalyne conditions. β-Mannanases from *Mytilus edulis* exhibited optimal activity at pH 5.2 with a rapid decrease on both acid and alkalyne sides (Xu et al., 2002a). The acidic and basic tolerant properties of Man I demonstrate potential to be used in industrial processing, especially in the fruit and paper industries. Man I showed maximum activity at 55 °C. Like β-mannanases from *Sclerotium rolfsii* (Gúbitz et al., 1996), temperature above 65 °C resulted in a more rapid inactivation of Man I. The optimum temperature of β-mannanases F4 from *Bacillus* sp was below 55 °C, while β-mannanase from *Thermotoga neapolitana* 5068 displayed a higher activity at 90–92 °C (Duffaud et al., 1997; Hossain et al., 1996). Man I exhibited half-lives of approx. 5 and 4 h at 55 and 60 °C, respectively. And at 70 °C, Man I was less stable with a half-life of 1 h. It was more stable than β-mannanases from *Trichoderma reesei*, *Aspergillus aculeatis* and *Bacillus* sp. KK01 which only retained stability at 50 °C and below (Ademark et al., 1998; Setati et al., 2001).

The involvement of some modifying agents on Man I activity was investigated (Table 2). Man I was activated by L-cysteine, DTT, DTNB and β-mercaptoethanol and inhibited by NEM and iodoacetamide. The assay of Man I at 70 °C showed 138, 147, 156, and 166% of activation in the presence of DTNB, β-mercaptoethanol L-cysteine and DTT, respectively. These effects may indicate the presence of cysteine residues at the active site. L-Cysteine is thought to be involved in hydrogen-bonding with the substrate and may be involved with enzyme folding and the formation of the covalent glycosyl-galactomannan intermediate (Coughlan, 1992). Incubation of Man I with 2,2-dithiodipyridine resulted in 100% retention of enzyme activity. Man I was strongly inhibited by NBS, a strong oxidizng agent of L-tryptophan. Small inactivation of Man I activity by DEPC and EDC suggests the involvement of histidine and carboxyl groups.

It must be said from above, however, that those modifying agents may not be specific; essential but inaccessible groups may not be modified by the agent used and modification of groups at a distance from the active site may effect conformational changes and consequent loss of enzyme activity (Coughlan, 1992). NBS is capable of eliciting a variety of effects on proteins (Clarke, 1987).

Comparison of some physicochemical properties of Man I and mannanases from other microorganisms are listed in Table 3. Such enzymes had optimal activity varying from

Table 2
Effect of reagents on the activity of Man I

Reagent	Concentration (mM)	Relative activity (%)
–	–	100
NBS	10	10.9
Iodoacetamide	10	67.3
EDC	10	74.8
DEPC	10	75.5
NEM	10	76.9
2,2-Dithiodipyridine	10	100
DTNB	10	200.7
β-Mercaptoethanol	20	223.8
L-Cysteine	20	273.5
DTT	20	323.1

Table 3
Some physicochemical properties of β-mannanases from microorganisms

Microorganism	Enzyme	kDa	T[a] (°C)	pH[b]	Thermostability (half-life)	K_m (mg/ml)	Reference
Aspergillus aculeatus	Man5A	45[c]	50	3.0	NR[d]	01.5[e]	Setati et al. (2001)
	Man5A[f]	50[c]	50	3.0	NR[d]	0.30[e]	
Aspergillus niger	Mannanase	40[c]	NR[d]	3.5	NR[d]	NR[d]	Ademark et al. (1998)
	F1	NR[d]	60	7.1	NR[d]	Nr[d]	
	F2	NR[d]	55	7.1	NR[d]	NR[d]	
	F3	NR[d]	55	7.1	NR[d]	NR[d]	
Bacillus sp. KK01	F4	NR[d]	50	7.1	NR[d]	NR[d]	Hossain et al. (1996)
	Man A	39.70[g]	50	5.2	20 min (50 °C)	3.95[e]	
Mytilus edulis	Man B	39.26[g]	50	5.2	20 min (50 °C)	NR[d]	Xu et al. (2002a)
	Mannanase[f]	40[c]	50	5.2	NR[d]	NR[d]	Xu et al. (2002b)
	Mannanase	61.2[c]	74	2.9	3.9 h (50 °C)	2.05[h]	
Sclerotium rolfsii	Mannanase	41.9[c]	72	3.3	2.9 h (50 °C)	2.73[h]	Gúbitz et al. (1996)
Thermotoga neapolitana 5068	Manannase	65[c]			24 h (86 °C)	0.55[e]	
		55[i]	92	7.1	1 h (97.5 °C)	0.23[j]	Duffaud et al. (1997)
					5 h (55 °C)		
Trichoderma harzianum		32.5[c]			4 h (60 °C)		
	Man I	36.5[i]	55	3.0	1 h (70 °C)	1.3[e]	This work
Trichoderma reesei	Man5A	53.6[c]	NR[d]	NR[d]	NR[d]	NR[d]	Hägglund et al. (2003)
	Mannanase Pi 4.6	51[c]	70	3.5-4.0	NR[d]	NR[d]	
Trichoderma reesei	Mannanase Pi 5.4	53[c]	70	3.5-4.0	NR[d]	NR[d]	Stalbrand et al. (1993)

[a] Optimum temperature.
[b] Optimum pH.
[c] Determined by SDS-PAGE.
[d] Not reported.
[e] K_m value for locust bean gum as the substrate.
[f] Recombinant enzyme.
[g] Determined by mass spectrometry.
[h] K_m value for ivory nut as the substrate.
[i] Determined by gel filtration.
[j] K_m value for AZO-locust bean gum as the substrate.

50–92 °C and pH values between 2.9 and 7.1. They also differed in their thermostability, K_m and molecular weight values.

In conclusion, we have purified and characterized a β-mannanase (Man I) from *T. harzianum* strain T4. Man I showed acidic tolerance, stability at 50 and 60 °C and no activity against xylan and CMC, encouraging its use in industrial applications. The activity of this enzyme can be enhanced by incubation with β-mercaptoethanol, cysteine, DTNB and DTT.

Acknowledgements

This work was funded by PADCT III/CNPq (Brazil). E.X.F.F. and H.M.F. acknowledge the receipt of research fellowship and posgraduate maintenance scholarship, respectively from CNPq (Brazil).

References

Ademark, P., Varga, A., Medve, J., Harjunpää, V., Drakenbeg, T., Tjerneld, F., & Stalbrand, H. (1998). Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: purification and properties of a β-mannanase. *Journal of Biotechnology*, *63*, 199–210.

Biely, P., Marcovic, O., & Mislovicova, D. (1985). Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. *Analytical Biochemistry*, *144*, 147–151.

Blum, H., Beier, H., & Gross, B. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrilamide gels. *Electrophoresis*, *8*, 93–99.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254.

Brumer, H., III, Sims, P. F. G., & Sinnott, M. L. (1999). Lignocellulose degradation by *Phanerochaete chrysosporium*: purification and characterization of the main α-galactosidase. *Biochemical Journal*, *339*, 43–53.

Clarke, A. J. (1987). Essential tryptophan in cellulase from *Schizophyllum commune*. *Biochimica Biophysica Acta*, *912*, 424–431.

Coughlan, M. P. (1992). Towards an understanding of the mechanism of action of main chain cleaving xylanases. In J Visser, G. Beldman, M. A. K. van Someren, & A. G. J Voragen (Eds.), *Xylans and xylanases* (pp. 111–139). Amsterdam: Elsevier.

Duffaud, G. D., McCutchen, C. M., Leduc, P., Parker, K. N., & Kelly, R. (1997). Purification and characterization of extremely thermostable β-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Applied and Environmental Microbiology*, *63*, 169–177.

Ethier, N., Tlabot, G., & Sygusch, J. (1998). Gene cloning, DNA sequecing, and expression of thermostable β-mannanase from *Bacillus stearothermophilus*. *Applied and Environmental Microbiology*, *64*, 4428–4432.

Filho, E. X. F. (1994). The xylan-degrading enzyme system. *Brazilian Journal of Medical and Biological Research*, *27*, 1093–1109.

Filho, E. X. F. (1998). Hemicellulases and biotechnology. *Recent Research Developments in Microbiology*, *2*, 165–176.

Gúbitz, G. M., Hayn, M., Sommerauer, M., & Steiner, W. (1996). Mannan-degrading enzymes from *Sclerotium rolfsii*: characterisation and synergism of two endo β-mannanases and a β-mannosidase. *Bioresource Technology*, *58*, 127–135.

Hägglund, P., Eriksson, T., Collén, A., Nerinckx, W., Claeyssens, M., & Stalbrand, H. (2003). A cellulose-binding module of the *Trichoderma reesei* β-mannanase Man5A increases the mannan-hydrolysis of complex substrates. *Journal of Biotechnology*, *101*, 37–48.

Hossain, M. Z., Abe, J.-I., & Hizukuri, S. (1996). Multiple forms of β-mannanase from *Bacillus* sp. KK01. *Enzyme Microbial and.Technology*, *18*, 95–98.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Leatherbarrow, R. J. (1987). *Enzfitter manual*. London: Biosoft.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Montiel, M. D., Hernández, M., Rodríguez, J., & Arias, M. E. (2002). Evaluation of na endo-β-mannanase produced by *Streptomyces ipomoea* CECT 3341 for the biobleaching of pine kraft pulps. *Applied Microbiology and Biotechnology*, *58*, 67–72.

Politz, O., Krash, M., Thomsen, K. K., & Borriss, R. (2000). A highly thermostable endo-(1,4)-β-mannanase from the marine bacterium *Rhodothermus marinus*. *Applied Microbiology and Biotechnology*, *53*, 715–721.

Stalbrand, H., Siika-aho, M., Tenkanen, M., & Viikari, L. (1993). Purification and characterization of two β-mannanases from *Trichoderma reesei*. *Journal of Biotechnology*, *29*, 229–242.

Setati, M. E., Ademark, P., van Zyl, W. H., Hahn-Hägerdal, B., & Stalbrand, H. (2001). Expression of the *Aspergillus auleatus* endo-β-1, 4-mannanase encoding gene (man1) in *Saccharomyces cerevisae* and characterization of the recombinant enzyme. *Protein Expression and Purification*, *21*, 105–114.

Silveira, F. Q. P., Melo, I. S., & Filho, E. X. F. (1997). Carbohydrate-hydrolysing enzyme activity production by solid-state cultures of *Trichoderma harzianum* strains. *Brazilian Journal of Microbiology*, *28*, 152–156.

Silveira, F. Q. P., Sousa, M. V., Ricart, C. A. O., Milagres, A. M. F., Medeiros, C. L., & Filho, E. X. F. (1999). A new xylanase from a *Trichoderma harzianum* strain. *Journal of Industrial Microbiology and Biotechnology*, *23*, 682–685.

Singh, S., Madlala, A. M., & Prior, B. A. (2003). *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews*, *27*, 3–16.

Wong, K. K. Y., Tan, L. U. L., & Saddler, J. N. (1988). Multiplicity of β-1, 4-xylanase in microorganisms: functions and applications. *Microbiology Reviews*, *52*, 305–317.

Wong, K. K. Y., & Saddler, J. N. (1993). Applications of hemicellulases in the food, feed, and pulp and paper industries. In G. P. Hazlewood, & H. J. Gilbert (Eds.), *Hemicellulose and hemicellulases* (pp. 127–143). London: Portland Press.

Ximenes, F. A., Fonseca, A. S., Ximenes, E. A., Silveira, F. Q. P., Silva, C. H. C., Lucena, S. A., Ribeiro, W. R. C., & Filho, E. X. F. (1997). Xylan-degrading enzyme production by solid-state cultures of aerobic fungi. *Brazilian Journal of Microbiology*, *5*, 22–28.

Xu, B., Hägglund, P., Stalbrand, H., & Janson, J.-C. (2002a). Endo-β-1,4-mannanases from blue mussel, *Mytilus edulis*: purification, characterization, and mode of action. *Journal of Biotechnology*, *92*, 267–277.

Xu, B., Selllos, D., & Janson, J.-C. (2002b). Cloning and expression in *Pichia pastoris* of a blue mussel (*Mytilus edulis*) β-mannanase gene. *European Journal of Biochemistry*, *269*, 1753–1760.