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Purification and partial characterization of a new β-xylosidase from *Humicola grisea* var. *thermoidea*

T. Iembo[1], M.O. Azevedo[1], C. Bloch Jr.[2] and E.X.F. Filho[3,*]
[1]*Laboratório de Biologia Molecular, Departamento de Biologia Celular, Universidade de Brasília, Brasília-DF, Brazil*
[2]*Laboratório de Espectrometria de Massa, Cenargen-EMBRAPA, Brasília-DF, Brazil*
[3]*Laboratório de Enzimologia, Departamento de Biologia Celular, Universidade de Brasília, Brasília-DF, Brazil*
**Author for correspondence: Tel.: +55-61-3307-2152, Fax: +55-61-3273-4608, E-mail: eximenes@unb.br*

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Summary

The thermophilic fungus *Humicola grisea* var. *thermoidea* produces a mycelium-associated β-xylosidase activity when grown in liquid-state cultures on media containing oat spelt xylan as the carbon source. The β-xylosidase was purified to apparent homogeneity by gel filtration and anion exchange chromatography. Its molecular weight was 37 and 50 kDa, as determined by MALDI/TOF mass spectrometry and SDS-PAGE, respectively. The purified enzyme exhibited maximum activity at 55 °C and pH 6.5. It was also active at pH 8.8, retaining 60% of its activity after 6 h of incubation at 50 °C. β-xylosidase was strongly inactivated by NBS and slightly activated by DTT and β-mercaptoethanol. The enzyme was highly specific for PNPX as the substrate. The purified β-xylosidase showed K_m and V_{max} values of 1.37 mM and 12.98 IU ml^{-1}, respectively.

Introduction

The hydrolysis of the xylan backbone is brought about by the synergistic action of endoxylanase and β-xylosidase activities (Ximenes *et al.* 1996). Endoxylanase (EC 3.2.1.8) cleaves off the β-1,4 bonds in the xylan main-chain polysaccharide liberating a mixture of xylo-oligosaccharides, while β-xylosidase (EC 3.2.1.37) hydrolyses the xylo-oligosaccharides from the non-reducing end to produce xylose. Acessory enzymes, such as α-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase and *p*-coumaric esterase are essential for complete saccharification of the xylan molecule (Zanoelo *et al.* 2004). Xylanolytic enzymes have been reported to be produced by a variety of microorganisms, such as fungi, actinomycetes and bacteria (Collins *et al.* 2005) These enzymes have been used in a wide range of industrial processes, including the pulp and paper industry (Collins *et al.* 2005).

The thermophilic fungus *Humicola grisea* var. *thermoidea* is an efficient producer of carbohydrate-degrading enzymes, including β-xylanase, β-glucosidase and glucoamylase (Tosi *et al.* 1993; Almeida *et al.* 1995; Filho 1996). It shows a high expression of β-xylanase activity when cultivated on medium containing complex natural substrates, especially agricultural waste (Ximenes *et al.* 1997; Lucena-Neto & Filho 2004).

In a previous article, Almeida *et al.* (1995) reported the properties of a purified mycelial-bound β-xylosidase activity from *H. grisea* var. *thermoidea*. In this present work, we describe the production, purification and partial characterization of a new mycelium-associated β-xylosidase from *H. grisea* var. *thermoidea*.

Materials and methods

Chemicals

Oat spelt xylan, *p*-nitrophenyl-β-D-xylopyranoside (PNPX), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), CM-cellulose, L-cysteine, β-mercaptoethanol, tryptophan, dithiothreitol (DTT), 2,2′-dithiodipyridine (DTP), *N*-bromosuccinimide (NBS), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), diethyl pyrocarbonate (DEPC) and *N*-ethylmaleimide (NEM) and molecular weight markers were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Sephacryl S-300 and DEAE–Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Organism and enzyme production

Humicola grisea var. *thermoidea* was isolated from Brazilian soil. The fungus was maintained on MYG medium [0.5% (w/v) malt extract, 0.25% (w/v) yeast extract, 1%(w/v) glucose, 2% (w/v) agar]. For enzyme production, a spore suspension of 1×10^7 spores ml^{-1} from routine subcultures was used to inoculate Erlenmeyer flasks containing 0.5% (w/v) oat spelt xylan and 100 ml of minimal liquid medium (adjusted to pH 7.0) with the following composition (w/v): 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4{\cdot}7H_2O$, 0.1% $(NH_4)_2SO_4$ supplemented with 0.06% yeast extract. Cultures were grown for 12 h at 40 °C in a rotatory shaking (150 - rev min^{-1}). The content of each flask was filtered through whatman No.1 filter paper and β-xylosidase activity was extracted from mycelia (5 g) by liquid nitrogen. The suspension obtained with 10 ml of 50 mM sodium phosphate buffer, pH 6.5 was centrifuged at $10{,}000\times g$ for 5 min, and the supernatant, hereafter called crude extract, used as source of β-xylosidase.

Enzyme assays

β-Xylosidase activity was routinely determined by measuring the amount of *p*-nitrophenol released from PNPX. The assay was carried out at 50 °C in 50 mM sodium phosphate buffer (pH 6.5), with 50 μl of 10 mM PNPX plus an appropriately diluted enzyme solution, in a total volume of 0.5 ml. The *p*-nitrophenol released was measured by monitoring the increase in the absorbance at 410 nm following 10 min of incubation. The reaction was stopped by the addition of 1.0 ml of 1.0 M sodium carbonate. One unit of β-xylosidase activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol min^{-1} ml^{-1} (IU ml^{-1}). Xylanase, mannanase and cellulase (CMCase, FPAse and β-glucosidase) activities were determined as reported elsewhere (Silveira *et al*. 1997; Salles *et al*. 2000; Ferreira & Filho 2004) All activity values reported in this paper are mean of triplicate experiments.

Protein determination

Protein concentration was determined by the Bradford method, with BSA as the standard.

Enzyme purification

The crude extract was applied to a Sephacryl S-300 column (3×66 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.5. Fractions showing β-xylosidase activity were pooled and applied to a DEAE–Sepharose ion-exchange column (1.1×5 cm), pre-equilibrated with 50 mM sodium phosphate buffer pH 6.5, followed by 50 ml of a linear gradient (0–1 M) of NaCl. Fractions showing β-xylosidase activity were pooled and dialysed at 4 °C overnight against distilled water. For both columns, proteins were eluted at a flow rate of 0.4 ml min^{-1} and fractions of 4 ml were collected.

Electrophoresis

SDS-PAGE (12%) was performed as described by Laemmli (1970). Protein bands in the gel were visualized by the silver staining method (Blum *et al*. 1987).

The effect of pH and temperature

The influence of temperature on β-xylosidase activity was measured performing the standard activity assay at temperatures ranging from 35 to 65 °C. The temperature stability of β-xylosidase was determined by pre-incubating the enzyme at 50 °C and removing aliquots at intervals to measure the activity as described before. The optimal pH for β-xylosidase activity was carried out by incubating the enzyme at pH values from 3.0 to 10.0 and 50 °C. The following buffers were used: 50 mM sodium acetate (pH 3.0–6.0), sodium phosphate (pH 5.5–7.0) and glycine–NaOH (pH 8.0–10.0). All buffers, regardless of pH, were adjusted to the same ionic strength with NaCl.

Determination of kinetic parameters

For determination of kinetic parameters, the enzyme assay was performed at PNPX concentration varying from 0.1 to 2.5 mM at 50 °C in 50 mM sodium phosphate buffer, pH 6.5. K_m and V_{max} values were obtained by Michaelis–Menten equation with a non-linear regression data analysis program (Leatherbarrow 1987).

Effect of some reagents

The activity of β-xylosidase was also performed in the presence of DTT, β-mercaptoethanol, NBS, EDC, cysteine, glycerol, glycine, DEPC, NEM, iodoacetamide, DTP and xylose. The reaction mixtures contained individual reagents, previously solubilized in distilled water, in a final concentration range of 0.5–20 mM.

Mass spectrometry

The purified β-xylosidase was analysed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA) in positive detection reflector mode. The purified enzyme sample was applied on the MALDI target plates after dilution into a mixture containing 1% (w/v) α-cyano-4-hydrocinnamic acid, 0.3% (v/v) trifluoroacetic acid (TFA) and 50% (v/v) acetonitrile. Sequazyme standard kit (Applied Biosystems) was used for external mass calibration of the equipment.

Results and discussion

Enzyme purification

A β-xylosidase has been purified from *H. grisea* var. *thermoidea* mycelium grown on oat spelt xylan as sole carbon source. A summary of the purification procedure is shown in Table 1. It was purified 27-fold with a 9.2% yield. The enzyme was purified by a simple two step procedure involving gel filtration (Sephacryl S-300) and ion-exchange (DEAE–Sepharose) chromatography. Most of the purification schemes for xylanolytic enzymes adopt a three-step strategy (Sá-Pereira *et al*. 2003). β-xylosidase from *Bacillus thermantarcticus* was purified to homogeneity by Sephacryl S-200, Q-Sepharose FF and Phenyl–Sepharose columns (Lama *et al*. 2004). Besides, various purification procedures use in the early stages ammonium sulfate precipitation and/or ultrafiltration (Ximenes *et al*. 1996; Sá-Pereira *et al*. 2003). The mycelial β-xylosidase from *Scytalidium thermophilum* was purified by ammonium sulfate fractionation and chromatography on Sephadex G-100 and DEAE–Sephadex A-50 (Zanoelo *et al*. 2004). In this present article, it was not necessary to use any concentration procedure, because the level of enzyme activity continued to be high even after its purification. The elution in Sephacryl S-300 chromatography resulted in the separation of three peaks of proteins. β-Xylosidase, present in the third peak, was further purified. DEAE–Sepharose chromatography of the above material showed that one peak of β-xylosidase activity eluted as a single peak with a linear NaCl gradient. SDS-PAGE of the β-xylosidase showed one protein band with a molecular mass of 50 kDa, which was far apart from that estimated by mass spectrometry (Figure 1). Some proteins migrate anomalously under the conditions of SDS-PAGE. The molecular mass of β-xylosidase was more accurately determined by using MALDI/TOF mass spectrometry (Figure 2). A monomer of 37 kDa was obtained for the native enzyme. The molecular mass value of β-xylosidase from *H. grisea* var. *thermoidea* was within the range detected for β-xylosidases from other sources (Ramsom & Walton 1997; Lama *et al*. 2004; Zanoelo *et al*. 2004). However, the molecular mass of β-xylosidase differed from the one purified from crude extract samples of the same fungus strain, which was 43 kDa (Almeida *et al*. 1995), and those of *Streptomyces* sp. CH7, *Trichoderma koningii* G-39, *Fusarium verticillioides* and *Fusarium proliferatum* (Li *et al*. 2000; Saha 2001, 2003; Pinphanichakarn *et al*. 2004).

Enzyme characterization

The purified enzyme was most active at 55 °C and pH 6.5. This result was similar to a β-xylosidase from *Streptomyces* sp. CH7 (Pinphanichakarn *et al*. 2004). In comparison, β-xylosidases from *Trichoderma harzianum* and *Aspergillus nidulans* exhibited optimum activities at pH 4.0–4.5 and 5.0, respectively (Ximenes *et al*. 1996; Kumar & Ramon 1996). The properties cited above are different from the other mycelial-bound β-xylosidase isolated from the same fungus (Almeida *et al*. 1995). The latter had optimal pH and temperature of 6.0 and 50 °C, respectively.

After 4 h of incubation at 55 °C, β-xylosidase retained 50% of its original activity. The degree of thermostability of the purified enzyme was higher than the values found for β-xylosidases from *Trichoderma reesei* and *Streptomyces* sp. CH7 (Herrmann *et al*. 1997). β-xylosidase was quite stable at pH 6.5 and 8.8 with 80 and 60% activity remaining, respectively after 6 h of incubation at 50 °C, while the other enzyme from *H. grisea* var. *themoidea* was stable up to 60 min in a pH range of 4.0–9.0 (Almeida *et al*. 1995). The high thermo- and alkaline pH tolerance qualifies the present enzyme for applications in pulp bleaching where the alkaline pH of the pulp requires thermo-alkaliphilic xylan-degrading enzymes (Collins *et al*. 2005). The β-xylosidase from *F. proliferatum* was stable at pH 7.0 and 40 °C with 94% remaining activity and presented only 13% activity at pH 8.0 (Saha 2003).

The purified enzyme was not active against xylan, β-mannan, CMC, filter paper and PNPG (results not shown). The substrate specificity of β-xylosidase was restricted to PNPX. The kinetic parameters of β-xylosidase were investigated using PNPX as substrate. The enzyme had K_m and V_{max} values of 1.37 mM and 12.98 IU ml^{-1}, respectively. The K_m value was close to reported to β-xylosidases from *A. nidulans* and *S. thermophilum* (Kumar & Ramon 1996; Zanoelo *et al*. 2004). Compared with the other β-xylosidase isolated from *H. grisea* var. *thermoidea* (Almeida *et al*. 1995), it showed lower affinity for the substrate. The K_{cat} (0.00073 min^{-1}) and K_{cat} K_m^{-1} (0.00053 min^{-1} mM^{-1}) values were measured taking into account the molecular mass obtained by SDS-PAGE. The low K_{cat} and K_{cat} K_m^{-1} values indicate that it is primarily a β-xylosidase.

The influence of various reagents on β-xylosidase activity from *H. grisea* var. *thermoidea* was investigated. The enzyme was slightly inhibited by xylose (6.2% of inhibition), suggesting a tolerance to xylose inhibition.

Table 1. Summary of the purification of β-xylosidase from *H. grisea* var. *thermoidea*

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	1	0.726	0.726	100	1
Sephacryl S-300	0.0048	0.078	16.5	10.7	22.7
DEAE–Sepharose	0.0034	0.067	19.6	9.2	27

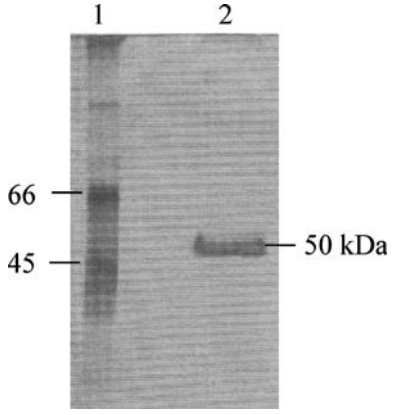

Figure 1. SDS-PAGE of purified β-xylosidase from *H. grisea* var. *thermoidea*. Lane 1: molecular weight standards (from the top): myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa); lane 2: purified β-xylosidase.

The presence of xylose at the same concentration did not affect the activity of the other β-xylosidase from *H. grisea* var. *thermoidea* (Almeida *et al.* 1995). β-xylosidase from *S. thermophilum* was xylose-tolerant at concentrations up to 200 mM (Zanoelo *et al.* 2004). On contrary, a significant effect on β-xylosidase activity from *T. harzianum* was found with xylose at 2 mM concentration (Ximenes *et al.* 1996). Evidence for involvement of L-tryptophan residue at the catalytic site is given by the high inhibition of β-xylosidase activity by NBS, which is a strong inhibitor for xylanolytic enzymes (Medeiros *et al.* 2003). NBS at 0.5 mM concentration inhibited the enzyme activity by 98.16%. On the other hand, the enzyme was slightly stimulated by DTT and β-mercaptoethanol with activation of 106.19 and 109.18%, respectively. Treatment with other thiol inhibitors (NEM and DTP) had negative effects on β-xylosidase activity with inhibition of 52.6 and 38.2%, respectively. However, iodoacetamide effected a little inactivation (8.12% of inhibition). Surprisingly, L-cysteine inhibited by 41.24% the activity of the purified enzyme. L-Cysteine is described as an activator of xylan-degrading enzyme activities from fungi (Salles *et al.* 2000; Medeiros *et al.* 2003). L-Glycine inhibited the enzyme activity by 29.71%. L-Glycerol had no influence in the enzyme catalysis. The failure of DEPC to activate β-xylosidase suggests no involvement of histidine residues in binding or catalysis. The enzyme inhibition by EDC (25.3%) indicates the existence of essential glutamic acid residues in the active site.

Conclusion

In conclusion, *H. grisea* var. *thermoidea* produced a mycelium-associated β-xylosidase activity when grown in the presence of xylan as the carbon source. In comparison with other β-xylosidase from the same fungus, it has different properties, such as pH and temperature optima, kinetic parameters, thermostability and molec-

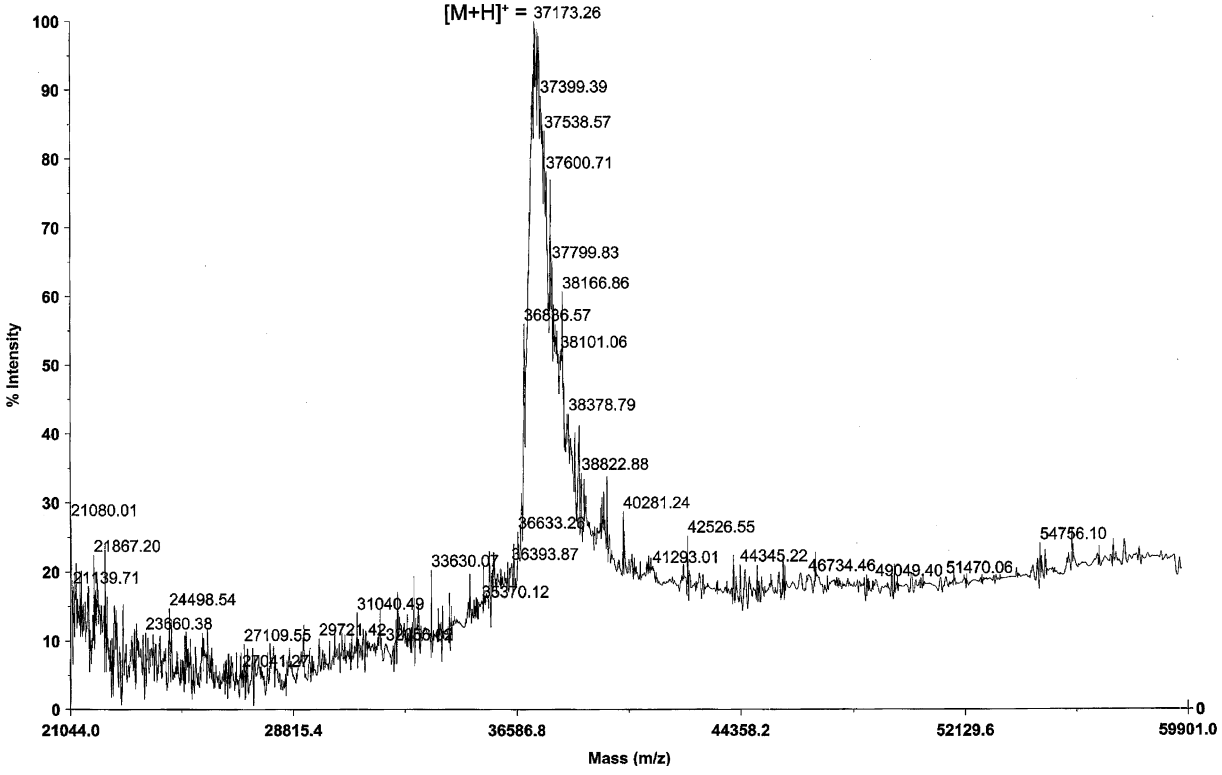

Figure 2. MALDI-TOF/MS spectrum of purified β-xylosidase from *H. grisea* var. *thermoidea*.

ular mass. Further work will be concentrated on the hydrolysis mechanism of xylans in synergism with different xylan-degrading enzymes. In order to determine whether the present enzyme is a true β-xylosidase, it will be incubated with xylobiose and short xylo-oligosaccharides.

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