Effect of Glycosylation on the Biochemical Properties of β-Xylosidases from *Aspergillus versicolor*

Alexandre Favarin Somera[1], Marita Gimenez Pereira[1], Luis Henrique Souza Guimarães[1],
Maria de Lourdes Teixeira de Moraes Polizeli[1], Héctor Francisco Terenzi[1], Rosa Prazeres Melo Furriel[2],
and João Atílio Jorge[1]*

*[1]Departamentos de Biologia e Química, [2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo,
Avenida Bandeirantes, 3900, CEP 14040-901 Ribeirão Preto, SP, Brasil*

Aspergillus versicolor grown on xylan or xylose produces two β-xylosidases with differences in biochemical properties and degree of glycosylation. We investigated the alterations in the biochemical properties of these β-xylosidases after deglycosylation with Endo-H or PNGase F. After deglycosylation, both enzymes migrated faster in PAGE or SDS-PAGE exhibiting the same R_f. Temperature optimum of xylan-induced and xylose-induced β-xylosidases was 45°C and 40°C, respectively, and 35°C after deglycosylation. The xylan-induced enzyme was more active at acidic pH. After deglycosylation, both enzymes had the same pH optimum of 6.0. Thermal resistance at 55°C showed half-life of 15 min and 9 min for xylose- and xylan-induced enzymes, respectively. After deglycosylation, both enzymes exhibited half-lives of 7.5 min. Native enzymes exhibited different responses to ions, while deglycosylated enzymes exhibited identical responses. Limited proteolysis yielded similar polypeptide profiles for the deglycosylated enzymes, suggesting a common polypeptide core with differential glycosylation apparently responsible for their biochemical and biophysical differences.

Keywords: β-xylosidase, xylobiase, glycosylation, deglycosylation, glycoprotein, *Aspergillus versicolor*

β-Xylosidases (β-D-xyloside xylohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyze xylobiose and short xylooligosaccharides releasing xylose (Wong *et al.*, 1998), although most purified forms prefer xylobiose as substrate (Kulkarni *et al.*, 1999). These enzymes have been detected in several bacteria and fungi, and are the best known members of the xylanolytic system, along with xylanases (Kulkarni *et al.*, 1999; Polizeli *et al.*, 2005). β-Xylosidases are essential components of the xylanolytic system, since their action contributes to decrease the end product inhibition of xylanases (Kulkarni *et al.*, 1999). However, most β-xylosidases described so far are partially or totally inhibited by low xylose concentrations (Poutanen *et al.*, 1987; Dobberstein and Emeis, 1991; Bütner and Bode, 1992; Kulkarni *et al.*, 1999). β-Xylosidases have been studied in several microorganisms, but little is known about the regulation of its biosynthesis (Kristufek *et al.*, 1995). Moreover, information on posttranslational events related to β-xylosidase production is scarce.

Aspergillus versicolor is a good producer of cellulase-free xylanolytic enzymes (Carmona *et al.*, 1997; Carmona *et al.*, 1998). The synthesis of *A. versicolor* β-xylosidase requires an inducer, which may be either xylooligosaccharides, resulting from xylan hydrolysis, xylose, or even some sugars originated by transglycosylation reactions (Andrade *et al.*, 2004). The two best carbon sources for *A. versicolor* β-xylosidase production are xylose and xylan (Andrade *et al.*, 2004). However,

β-xylosidases produced in media containing xylose or xylan as carbon source exhibit significant differences in some biochemical properties, such as pH and temperature optimum, response to ions, PAGE migration, and glycosylation degree (Andrade *et al.*, 2004). Particularly, the xylan-induced β-xylosidase exhibits 47% of carbohydrates, while the xylose-induced enzyme contains 21% only (Andrade *et al.*, 2004). Multiple forms of xylanolytic enzymes are usually encountered in bacteria and fungi (Wong *et al.*, 1998). It is not known whether this multiplicity arises from gene diversity, posttranscriptional processing, or a combination of both.

In this context, our focus is the effect of glycosylation on the biochemical properties of the two distinct β-xylosidases produced by *A. versicolor* in media containing xylose or xylan. We show that the deglycosylation of both forms alters the biochemical properties of the native enzymes and that, after such treatment, both enzymes share quite similar properties. Apparently, both β-xylosidases share a common polypeptide core with different degrees of glycosylation.

Materials and Methods

Microorganism and culture conditions
An *A. versicolor* strain, isolated from Brazilian soil, was maintained at 25°C on slants of Vogel's solid medium (Vogel, 1956) containing 2% (w/v) sucrose as carbon source. Conidia from 10-day-old cultures were inoculated into liquid Vogel's medium containing 1% (w/v) xylose or 1% (w/v) xylan (oat spelt - Sigma Chemical Co., USA) and incubated at 30°C for 96 h, in a rotary shaker at 140 rpm.

* To whom correspondence should be addressed.
(Tel) 55-16-3602-4679; (Fax) 55-16-3602-4886
(E-mail) joajorge@usp.br

Cellular distribution of *β*-xylosidases

Cellular distribution of xylan- or xylose-induced *β*-xylosidases was analyzed as recommended by Mandels (Mandels, 1953) for conidia, with the modifications suggested by Zanoelo *et al.* (2004) for the study of intact mycelium.

Preparation of crude enzyme

The mycelium was harvested by filtration on Whatman No. 1 paper, rinsed with chilled distilled water, blotted on filter paper, and frozen for at least 12 h. The mycelial pads were ground in a mortar with 2 volumes of glass beads at 0°C and extracted with distilled water. The slurry was centrifuged at 12,000×g for 20 min at 4°C. The supernatant was the source of crude enzyme.

Purification of *β*-xylosidases and enzymatic assay

β-Xylosidases were purified to apparent electrophoretic (PAGE) homogeneity according to the procedure described by Andrade *et al.* (2004).

   β-Xylosidase activity was routinely assayed using the synthetic substrate *p*-nitrophenyl *β*-D-xylopyranoside (PNP-xyl) at a final concentration of 7.4 mM, McIlvaine buffer, pH 6.0, at 40°C for xylose-induced form, or pH 5.5, at 45°C for xylan-induced form. At different time intervals, the reaction was interrupted by the addition of two volumes of saturated sodium tetraborate solution to the reaction medium, and *p*-nitrophenolate concentration was estimated spectrophotometrically at 405 nm. One enzyme unit (U) was defined as the amount of enzyme producing 1 μmol product per minute. Specific activity was expressed as units per mg protein (U/mg).

   Kinetic parameters K_m and V_{max} for PNP-xyl hydrolysis were determined in the presence of 0.25 mM calcium chloride. The values were calculated from Lineweaver and Burk plots.
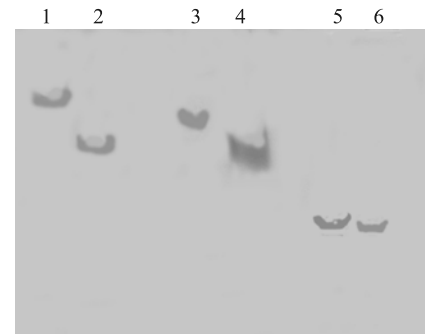
Polyacrylamide gel electrophoresis and protein estimation

Electrophoresis under nondenaturing conditions was carried out by the method of Davis (1964) using 7% acrylamide. SDS-PAGE was carried out according to Laemmli (1970). Molecular mass standards were from Invitrogen Co. Protein was quantified according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Deglycosylation and proteolytic digestion of *A. versicolor* *β*-xylosidases

Purified *β*-xylosidases were deglycosylated using endo-*β*-acetylglucosaminidase H (Endo-H, Sigma Chemical Co.) or N-glycosidase F (PNGase F) (Sigma Chemical Co.). Treatment with Endo-H was carried out in a 10-mM sodium citrate buffer, pH 5.0, for 8 h at 37°C, at an Endo-H : *β*-xylosidase ratio of 5 U:100 mg protein. Treatment with PNGase F followed the supplier recommendations. After treatment with Endo-H or PNGase F, samples were diluted in large volumes of distilled water (or a convenient buffer) and concentrated in microcon tubes (Millipore Corp., USA) with a 10 kDa cut-off. This procedure was repeated at least three times in order to eliminate the buffers used in the reactions.

   Limited proteolysis of *β*-xylosidases was carried out by in-

Fig. 1. Electrophoretic analysis (PAGE) of native, Endo-H- or PNGase F-deglycosylated *β*-xylosidases. Electrophoresis was performed in a 7% polyacrylamide gel using 20 μg protein per lane: Lanes 1, 3, and 5, xylan-induced enzyme; 2, 4, and 6, xylose-induced enzyme. Lanes 1 and 2, native enzymes (controls); 3 and 4, Endo-H-treated enzymes; and 5 and 6, PNGase F-treated enzymes.

cubation with TPCK-treated trypsin from bovine pancreas (Sigma Chemical Co.) in 100 mM sodium bicarbonate, pH 8.0, containing 1 mM DTT and 1 mM $CaCl_2$ for 30 h at 37°C. Final reaction volume was 50 μl, and a protease:*β*-xylosidase ratio of 0.2 μg:1 μg was employed. Proteolysed samples were immediately submitted to SDS-PAGE (15%), and the gels were stained, as suggested by Wray *et al.* (1981).

Results

Effect of deglycosylation on *A. versicolor* *β*-xylosidases electrophoretic mobility

PAGE analysis of native *β*-xylosidases revealed that the xylose-induced enzyme migrated faster than the xylan-induced
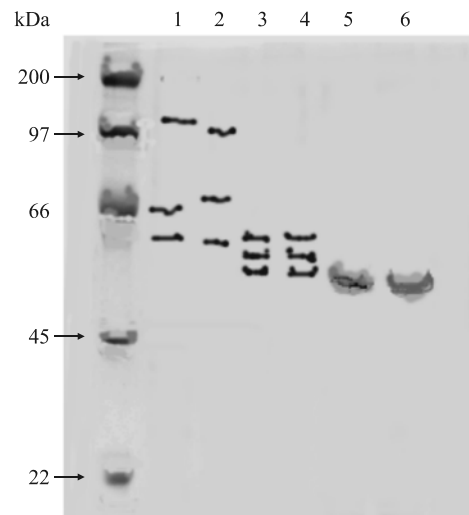
Fig. 2. Electrophoretic analysis (SDS-PAGE) of native enzyme forms or deglycosylated *β*-xylosidases. Electrophoresis was performed in a gradient polyacrylamide gel (5~20%) using 30 μg protein per lane. Lanes 1, 3, 5, xylan-induced enzyme; 2, 4, 6, xylose-induced enzyme; 1 and 2, native enzymes; 3 and 4, endo -H-treated enzymes; 5 and 6, PNGase F-treated enzymes.

one (Fig. 1). A similar pattern was observed after treatment with Endo-H, but both forms showed increased migration rates as compared to their glycosylated counterparts. On the other hand, treatment with PNGase F resulted in quite similar migration rates for both deglycosylated forms, faster than those observed for nontreated or Endo-H-treated enzymes. These results suggested a higher efficiency of the PNPGase F in removing the carbohydrate moiety of both *β*-xylosidases.

Both purified native *β*-xylosidases were resolved into three protein bands with very similar migrations in SDS-PAGE (Fig. 2). The native xylan-induced form was resolved in 123, 67, and 60 kDa polypeptide bands, while the xylose-induced one was resolved in 98, 77, and 59 kDa polypeptide bands

(Fig. 2, lanes 1~2). This is in agreement with data from Andrade *et al.* (2004), showing that both native enzymes were resolved into three polypeptide bands in SDS-PAGE, corresponding to apparent molecular masses of about 110, 66, and 50 kDa. Endo-H treatment leads to different migration of the polypeptide bands, which exhibited apparent molecular masses around 62, 60, and 57 kDa for both *β*-xylosidase forms (Fig. 2, lanes 3~4). In contrast, both enzymes exhibited a single polypeptide band in SDS-PAGE analysis after PNGase F treatment, corresponding to molecular masses of about 49 kDa (Fig. 2, lanes 5~6).

The deglycosylation treatments affected the activity of both xylan- and xylose-induced *β*-xylosidases. PNGase F led to complete inactivation of the enzymes, thus precluding
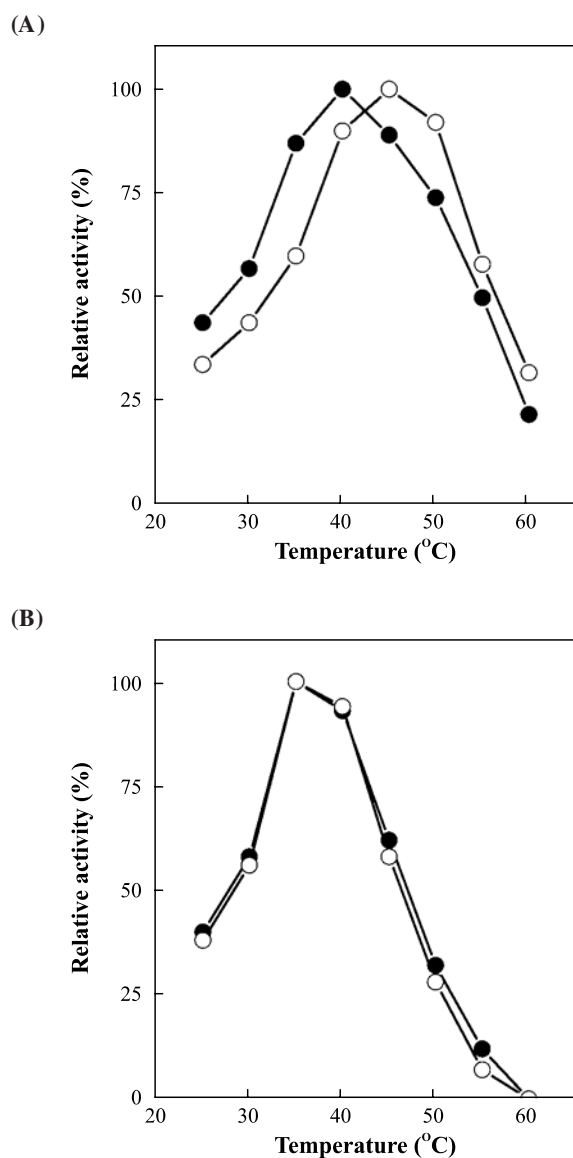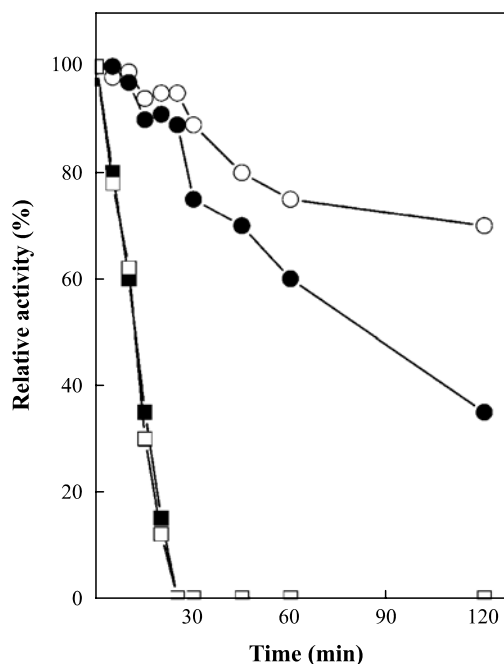

Fig. 3. Effect of deglycosylation on temperature optima for *β*-xylosidases activity. (A) native enzymes; (B) Endo-H-treated enzymes. Symbols: (○) xylan-induced enzyme; (●) xylose induced-enzyme.
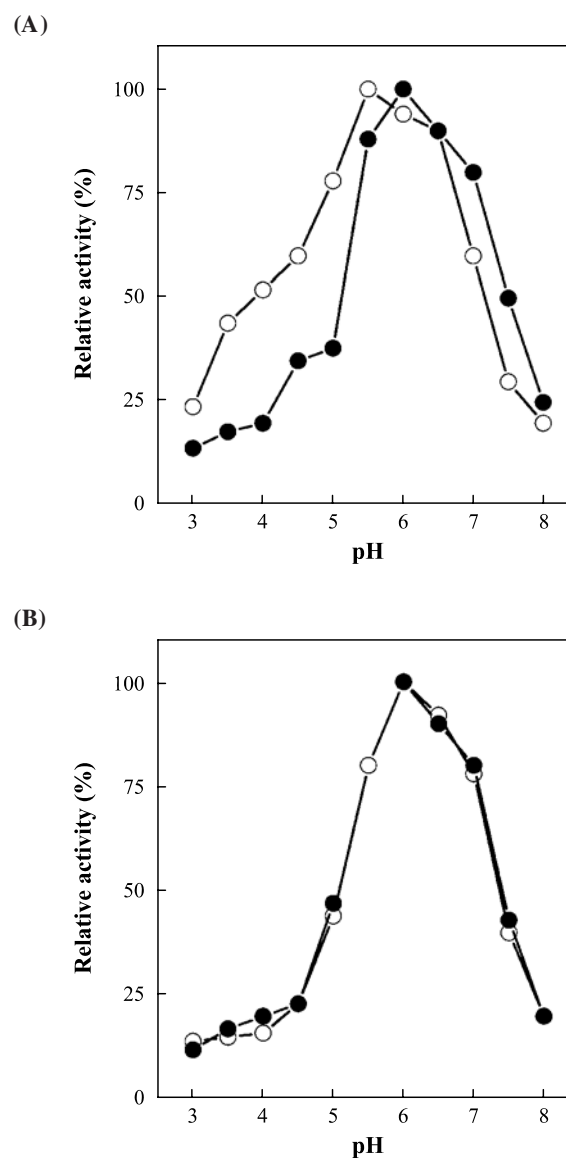
Fig. 4. Effect of deglycosylation on pH optima for *β*-xylosidases activity. (A) native enzymes; (B) Endo-H-treated enzymes. Symbols: (○) xylan-induced enzyme; (●) xylose-induced enzyme.

(A)

(B)
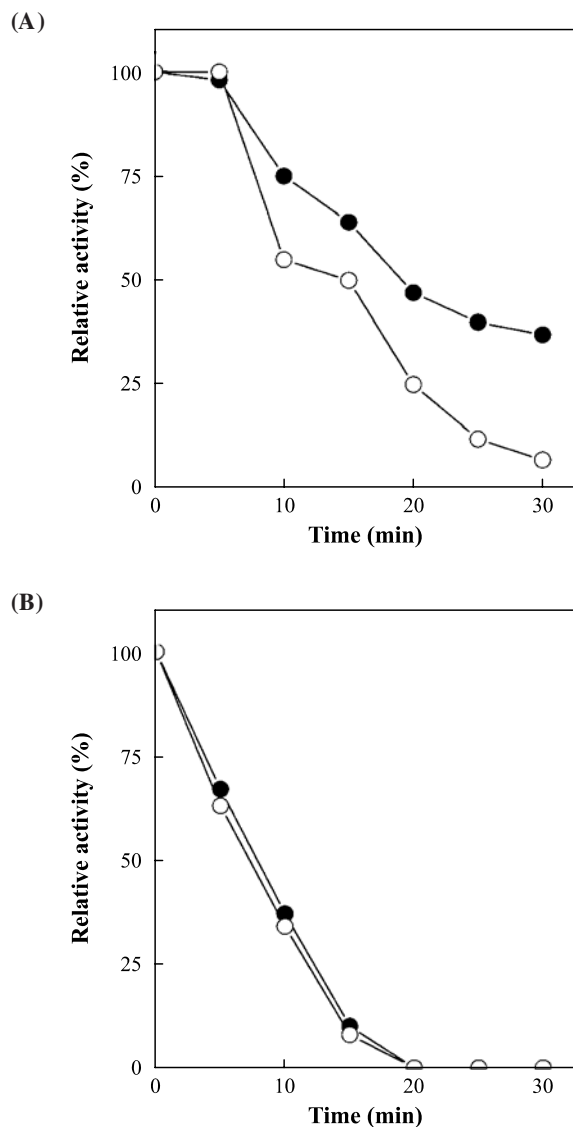
Fig. 5. Effect of deglycosylation on the thermal stability of *β*-xylosidases at 50°C. (A) native enzymes; (B) Endo-H-treated enzymes. Symbols: (○) xylan-induced enzyme; (●) xylose-induced enzyme.

Fig. 6. Effect of deglycosylation on *β*-xylosidases stability at pH 3.5. Symbols: (open) xylan-induced enzyme; (closed) xylose-induced enzyme; circles, native enzymes; squares, endo-H-treated enzymes. Samples were incubated in McIlvaine buffer, pH 3.5, at 37°C.

further functional studies. On the other hand, Endo-H treated *β*-xylosidases retained from 30% to 40% of initial activity and were assayed as described below.

Effect of deglycosylation on temperature optimum and pH optimum of *β*-xylosidases

The temperature optimum for native xylose- and xylan-induced *β*-xylosidases was 40°C and 45°C, respectively (Fig. 3A). Furthermore, the xylose-induced form showed higher activity at low temperatures (25~35°C) than the xylan-induced one (Fig. 3A), while the latter was slightly more active at higher temperatures (50~60°C). After deglycosylation with

Table 1. Effect of ions on the activity of native and endo-H treated β-xylosidases

Effectors	β-Xylosidase activity (%)			
	Non-treated		Endo-H treated	
	Xylan-induced	Xylose-induced	Xylan-induced	Xylose-induced
EDTA (1 mM) (Control)	100	100	100	100
CaCl₂ (0.25 mM)	189	140	88	91
BaCl₂ (0.25 mM)	158	105	98	100
MgSO₄ (0.25 mM)	158	113	102	96
CoCl₂ (1 mM)	147	102	99	100
ZnCl₂ (1 mM)	120	123	94	92
AlCl₃ (1 mM)	26	92	0	0
CuSO₄ (1 mM)	33	26	0	0

Table 2. Effect of deglycosylation on the kinetic parameters for PNP-xyl hydrolysis by *A. versicolor* β-xylosidases

β-Xylosidase	Endo-H treatment	V_{max} (U mg protein^{-1})	K_m (mM)
Xylan-induced	−	1060±206	0.16±0.03
Xylan-induced	+	315±59	0.43±0.04
Xylose-induced	−	815±114	0.31±0.02
Xylose-induced	+	322±82	0.41±0.03

Endo-H, the temperature optimum was the same for both enzymes, 35°C (Fig. 3B). The xylan- and xylose-induced enzymes exhibited pH optimum of 5.5 and 6.0, respectively (Fig. 4). Moreover, the xylan-induced form was somewhat more active at acidic pH (Fig. 4A). Similarly, as observed for the temperature optimum, the pH activity profiles for both *β*-xylosidases overlapped after Endo-H treatment, with a maximum at pH 6.0 (Fig. 4B).

Thermal inactivation kinetics was studied by incubating purified *β*-xylosidases in water at 55°C (Fig. 5A). Xylose-induced native enzyme (half-life of 20 min) showed higher thermostability as compared to the native xylan-induced one, with a half-life around 15 min. After deglycosylation, both enzymes exhibited identical profiles of thermal inactivation, and their half-lives decreased to about 9 min (Fig. 5B).

Stability studies conducted at pH 3.5 revealed a more pronounced stability for xylan-induced *β*-xylosidase, with a half-life above 2 h. In comparison, the xylose-induced enzyme exhibited a half-life of 83 min (Fig. 6). Again, Endo-H treatment led to the identical profiles of inactivation at pH 3.5 for both forms, with half-lives around 12 min (Fig. 6).

Effect of deglycosylation on the sensitivity of *β*-xylosidases to ions

Xylan-induced native *β*-xylosidase was more sensitive to EDTA inhibition than the native xylose-induced enzyme (Andrade *et al.*, 2004). In addition, while the former enzyme was activated by calcium (89%), barium (58%), magnesium (58%), and cobalt (47%) and inhibited by aluminum (74%) and copper (67%), the latter was insensitive to barium, magnesium, and cobalt but activated by calcium (40%) and inhibited by cooper (74%) (Table 1). After deglycosylation, the enzyme induced by xylan lost its sensitivity to the activators, while the enzyme induced by xylose remained unaffected by barium, magnesium, and cobalt, and it lost its sensitivity to calcium (Table 1). Moreover, both deglycosylated forms were totally inhibited by aluminum and copper. Thus, after Endo-H treatment, *A. versicolor* *β*-xylosidases acquired a very similar sensitivity to ions.

Effect of Endo-H deglycosylation on the kinetic parameters for PNP-xyl hydrolysis by *A. versicolor* *β*-xylosidases

Endo-H-deglycosylation of both *β*-xylosidases resulted in marked decreases in maximal velocities for PNP-xyl hydrolysis (Table 2). Values 3.4-fold and 2.6-fold lower were determined for xylan- and xylose-induced enzymes, respectively. In contrast, Km value remained almost constant for the xylose-induced enzyme, while a 2.7-fold increase was observed for the xylan-induced form. Moreover, a striking similarity in the kinetic parameters for both deglycosylated forms was

observed.

Cellular distribution of *β*-D-xylosidases

Studies on the cellular distribution of *β*-D-xylosidases in cultures grown in 1% xylan or 1% xylose revealed that about 70~74% of the enzymes were surface-bond (e.g., activity detectable in intact cells) and could be partially released by successive washes with distilled water. Sixteen to twenty percent of the enzymes remained strongly attached to the cell walls, while about 10~13% was detected only after cell disruption.

Limited trypsin proteolysis of native and PNGase F-deglycosylated *A. versicolor* *β*-xylosidases

The SDS-PAGE analysis of glycosylated *A. versicolor* *β*-xylosidases submitted to limited proteolysis revealed minor but consistent differences between xylan- and xylose-induced

Fig. 7. Electrophoretic analysis (SDS-PAGE) of tryptic fragments of native (Lanes 2 and 3) and PNGase F-deglycosylated (Lanes 4 and 5) *A. versicolor* *β*-xylosidases. Lanes 2 and 4, xylan-induced enzyme; 3 and 5, xylose-induced enzyme; 1, trypsin; 6, PNGase F, as controls.

forms. Particularly, three major peptide bands showed significant differences in migration rate (Fig.7, lanes 2~3). In contrast, quite coincident profiles were obtained for both enzyme forms after treatment with PNGase F (Fig. 7, lanes 4~5).

Discussion

This study extends previous observations on the influence of the carbon source on glycosylation and other biochemical properties of β-xylosidases from *A. versicolor* (Andrade *et al.*, 2004). The biochemical differences observed for two forms of β-xylosidases, one produced in xylan and the other in xylose, as carbon source, were tentatively attributed to the different degree of glycosylation exhibited by the enzymes (Andrade *et al.*, 2004). This assumption was strengthened by the present study, in which both enzymes were submitted to deglycosylation and to a comparative analysis of their biochemical and physical properties. Taken together, our data strongly suggest that the two forms of β-xylosidases share a common polypeptide core, which suffers differential glycosylation. Apparently, the mechanisms involved in the glycosylation of these β-xylosidases are affected by the carbon source. It has been reported that the glycosylation of phosphoglucomutase from *Saccharomyces cerevisiae* varies according to the carbon source present in the culture medium (Dey *et al.*, 1994). Also, the glycosylation of cellobiohydrolase I, a cellulase abundantly expressed by most *Trichoderma reesei* strains, is influenced by the carbon source or other nutritional conditions (Klarskov *et al.*, 1997; Maras *et al.*, 1997; Harrison *et al.*, 1998; Hui *et al.*, 2001; Stals *et al.*, 2004a, 2004b). The different deglycosylation patterns exhibited by xylan- and xylose-induced β-xylosidases after treatment with Endo H or PNGase F may be attributed to the fact that Endo-H treated enzymes still retain N-acetyl glucosamine residues.

The temperature optimum estimated for both forms of *A. versicolor* β-xylosidase decreased after deglycosylation with Endo-H. The carbohydrate richer (47%), xylan-induced form revealed to be more active at higher temperatures than the xylose-induced one, which has only 21% carbohydrate. Indeed, several authors have suggested that glycosylation protects enzymes from heat denaturation and proteolytic attack (Kern *et al.*, 1992; Varki, 1993; Meldgaard and Svendesen, 1994; Lige *et al.*, 2001; Venturi *et al.*, 2002). In agreement, glycosylation undoubtedly protected both forms of *A. versicolor* β-xylosidase against thermoinactivation, since deglycosylated enzymes showed significantly lower half-lives at 55°C. Endo-H deglycosylation of *A. versicolor* β-xylosidases also suggested that the glycan moiety might confer some protection to both enzymatic forms against low pH inactivation. The greater resistance of the xylan-induced enzyme to acidic medium may be tentatively attributed to its higher sugar content. Similarly, *Fusarium oxysporium* produces a deglycosylated α-L-fucosidase slightly more unstable at low pH than a glycosylated form (Tsuji *et al.*, 1990).

The xylan-induced β-xylosidase was more susceptible to ion stimulation than the xylose-induced enzyme. However, both enzyme forms gained identical susceptibility to ions after deglycosylation. Moreover, glycosylated *A. versicolor* β-xylosidases showed higher substrate affinity and V_{max} for PNP-xyl hydrolysis and, therefore, higher catalytic efficiencies. On the other hand, glycosylated enzyme forms induced by xylan or xylose showed significantly different K_m and V_{max} values, while for both Endo-H deglycosylated forms close values were determined. According to the literature, enzyme glycosylation may either affect or not the values of K_m and V_{max} (Varki, 1993).

Altogether, our data suggest that differential glycosylation of *A. versicolor* β-xylosidases is responsible for the biochemical and biophysical differences noted for xylan- and xylose-induced forms, particularly considering the coincident migration bands in PAGE and the coincident profiles after limited proteolysis obtained for both enzyme forms after treatment with PNGase F.

When grown in a medium containing equal parts of xylose or xylan *A. versicolor* produces a mixture of xylan- and xylose-induced enzymes (data not shown). The mechanisms employed by *A. versicolor* to produce different forms of β-xylosidase in response to the carbon source remain to be elucidated. However, we can hypothesize that in nature, the production of two forms of xylosidases by an organism simultaneously exposed to xylan and xylose might allow a better performance in the utilization of natural short xylooligosaccharides, since the enzymes are of surface-bound cell localization.

Acknowledgements

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