Modification of the Carbohydrate Composition of Sulfite Pulp by Purified and Characterized β-Xylanase and β-Xylosidase of *Aureobasidium pullulans*

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Both β-xylanase and β-xylosidase were purified to homogeneity from a xylose-grown culture of *Aureobasidium pullulans*. Cellular distribution studies of enzyme activities revealed that β-xylanase was an extracellular enzyme, during both the exponential and stationary phases, whereas β-xylosidase was mostly periplasmic associated. The β-xylanase exhibited very high specificity for xylan extracted from *Eucalyptus grandis* dissolving pulp, whereas the β-xylosidase was only active on *p*-nitrophenyl xyloside and xylobiose. Comparison of k_{cat}/K_m ratios showed that the β-xylanase hydrolyzed xylan from dissolving pulp 1.3, 2.1, and 2.3 times more efficiently than *Eucalyptus* hemicellulose B, *Eucalyptus* hemicellulose A, and larchwood xylan, respectively. The β-xylosidase exhibited a transxylosylation reaction during the hydrolysis of xylobiose. When applied on acid sulfite pulp, both enzymes released xylose and hydrolyzed xylan to a different extent. Although β-xylosidase (0.4 U/g pulp) liberated more xylose from pulp than β-xylanase (4.7 U/g pulp), it was responsible for only 3% of xylan solubilization. Treatment of pulp with β-xylanase liberated 51.7 μg of xylose/g and hydrolyzed 10% of xylan. The two enzymes acted additively on pulp and removed 12% of pulp xylan. A synergistic effect in terms of release of xylose from pulp was observed when the enzyme mixture of β-xylanase and β-xylosidase was supplemented with β-mannanase. However, this did not result in further enzymatic degradation of pulp xylan. Both β-xylanase and β-xylosidase altered the carbohydrate composition of sulfite pulp by increasing the relative cellulose content at the expense of reduced hemicellulose content of pulp.

Introduction

The major component of hardwood hemicellulose is xylan, a polymer of β-1,4-linked xylose monomers with acetyl and 4-*O*-methylglucuronosyl side chains (Fengel and Wegener, 1994). Due to its complex structure, complete breakdown of naturally occurring branched xylan requires the action of several hydrolases. Important are *endo*-1,4-β-D-xylanase (EC 3.2.1.8) that degrades the xylan backbone randomly (acting in an endo-manner), liberating xylooligomers, and 1,4-β-D-xylosidase (EC 3.2.1.37) that converts xylooligomers to xylose. Furthermore, β-xylanases are produced by many microorganisms and have been extensively investigated (Wong et al., 1988). These enzymes have potential in industrial processes, including bioconversion of lignocellulose-derived sugars into fuels, bread making, and clarification of beer and juices (Royer and Nakas, 1989). In recent years there has been an increasing interest in the application of xylanases to the pulp and paper industry to facilitate bleaching and to improve fiber properties (Bajpai and Bajpai, 1997).

The high levels of cellulase-free β-xylanase and β-xylosidase produced by *Aureobasidium pullulans* have made this yeast-like fungus especially suitable for biotechnological applications (Deshpande et al., 1992). Two isoenzymes have been so far purified from *A. pullulans* strain Y-2311-1: a 20 kDa xylanase (Leathers, 1989) and a 25 kDa xylanase (Li et al., 1993). The latter was subsequently cloned in *Escherichia coli* (Li and Ljungdahl, 1994) and later expressed in *Saccharomyces cerevisiae* (Li and Ljungdahl, 1996). Another strain of *A. pullulans* CBS 58475 was also used to produce and purify β-xylanase (Dobberstein and Emeis, 1989) and β-xylosidase (Dobberstein and Emeis, 1991). Previously we reported on the use of the enzyme complex of *A. pullulans* in biobleaching of sulfite pulps (Christov and Prior, 1997). In this work we studied some physical and kinetic properties of *A. pullulans* purified β-xylanase and β-xylosidase, which are relevant to the application of these enzymes on sulfite pulp, with the aim to examine their cooperative action and importance in the carbohydrate modification of sulfite dissolving pulp. This information would be especially useful in designing the optimum enzyme combination for the selective enzymatic hydrolysis of xylan in dissolving pulp, which is the raw material for the manufacture of high-quality viscose rayon and other cellulose derivatives (Christov and Prior, 1996).

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Cellular Localization and Distribution of β-Xylanase and β-Xylosidase Activities

The yeast *A. pullulans* NRRL Y-2311-1 was obtained from C. P. Kurtzman, Northern Regional Research Centre, Peoria, IL. The culture was routinely maintained on YM slants containing (g/L) D-glucose (Merck, Darmstadt, Germany) (10), yeast extract (3), malt extract (3), peptone (5), and agar (20). The enzyme production of β-xylanase and β-xylosidase by *A. pullulans* using oats spelts xylan (Sigma, St. Louis, MO) was previously described (Myburgh et al., 1991).

The intracellular, periplasmic, and extracellular activities of β-xylanase and β-xylosidase were determined according to the method of the supplier of the Lyticase (Sigma). Cells (5 mL) from exponential- and stationary-phase xylose-grown culture were harvested by centrifugation (18000*g*, 5 min). The supernatant represented the extracellular fraction. The formation of spheroplasts by using Lyticase was microscopically monitored. The fragile spheroplasts were removed from the supernatant containing the periplasmic enzymes by low-speed centrifugation (1000*g* for 20 min at 4 °C). Thereafter the spheroplast pellet was resuspended in 5 mL of 50 mM sodium acetate (Merck) buffer, resulting in lysis of the spheroplasts. The cell debris was removed by centrifugation (18000*g*, 5 min), and the supernatant was held for analyses.

β-Xylanase, β-xylosidase, and cellulase activities were then assayed in the various fractions. β-Xylanase and cellulase activities were routinely assayed using oat spelts xylan (Sigma) or microcrystalline cellulose (Merck) as reported previously (Christov and Prior, 1996). The concentration of reducing sugars released was determined according to the Somogyi (1952)−Nelson (1944) method using either D-xylose (Merck) or D-glucose (Merck) as a standard. One unit of β-xylanase or cellulase activity (U) was defined as the amount of enzyme that released 1 μmol of reducing equivalents per minute at pH 5.0 and 30 °C. β-Xylosidase activity was routinely measured using *p*-nitrophenyl xyloside (pNPX; Christov and Prior, 1994) or xylobiose as substrate. In the latter case, the reaction products of xylobiose (prepared as described by Christov et al. (1997)) were measured by high-performance liquid chromatography (HPLC) on an Aminex Carbohydrate HPX-42C column (Bio-Rad, Richmond, CA) at 85 °C with double-distilled water as the eluant. A refractive index detector was used, and the column was developed at 0.6 mL/min. D-Ribose (1 mg/mL, Merck) was used as internal standard. One unit of β-xylosidase activity (U) was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol or xylose equivalents per minute at pH 5.0 and 45 °C.

β-Xylanase and β-xylosidase activities were studied for their intracellular, periplasmic, and extracellular distribution in a xylose-grown culture of *A. pullulans* during its exponential and stationary growth phases. It was found that β-xylanase either was located in the extracellular fluid (51%) or was cell-bound (49%) in the exponential phase; the amounts are 63% and 37% in the stationary phase, respectively. On the other hand, β-xylosidase was primarily periplasmic in both growth stages: 85% and 74% in the exponential and stationary phases, respectively. However, some intracellular β-xylosidase activity was also found: 8% for the exponantial phase and 9% for the stationary phase. Thus, β-xylanase was mainly located in the extracellular fluid, whereas β-xylosidase was primarily periplasmic. Higher levels of these enzymes were detected in the culture fluid when the culture attained the stationary phase than during exponential growth phase cultivation, a finding in agreement with results reported for *Cryptococcus albidus* culture (Biely et al., 1980). No cellulase activity could be found in the cytoplasmic, periplasmic, or extracellular phase of *A. pullulans*, which confirmed the inability of the organism to produce cellulase when grown on xylose (Leathers, 1986; Dobberstein and Emeis, 1989).

Table 1. Relative Activity of Purified β-Xylanase and β-Xylosidase of *A. pullulans* toward Xylose-Containing Substrates[a]

substrate	β-xylanase (%)	β-xylosidase (%)
oat spelts xylan	100[d]	0
larchwood xylan	95	0.5
hemicellulose A[b]	113	0
hemicellulose B[b]	85	0
pulp xylan[c]	115	0.2
xylobiose	0	30
p-nitrophenyl β-D-xyloside	1.3	100[e]

[a] The concentration of the substrates is 10 mg/mL except for xylobiose and *p*-nitrophenyl β-D-xyloside with a concentration of 1 mg/mL. Values are averages of duplicate determinations. [b] Isolated from *E. grandis* wood. [c] Isolated from dissolving pulp produced from *E. grandis* wood. [d] 483 U/mg with oat spelts xylan as substrate. [e] 33 U/mg with *p*-nitrophenyl β-D-xyloside as substrate.

Substrate Specificity of β-Xylanase and β-Xylosidase

β-Xylanase and β-xylosidase were purified from the culture fluid of *A. pullulans* NRRL Y 2311-1 to homogeneity using size exclusion chromatography followed by anion-exchange chromatography as described by Christov et al. (1997). The purification procedure resulted in 5.0- and 9.21-fold purifications of the β-xylanase and β-xylosidase enzymes, respectively, from the supernatant of a 3-day-old xylose-grown culture. Final yields were 50% and 38.1% with specific activities of 482.8 and 33.4 U/mg protein for the β-xylanase and β-xylosidase enzymes, respectively.

The substrate specificities of purified β-xylanase and β-xylosidase were evaluated using the following xylose-containing substrates: oat spelts xylan (Sigma), larchwood xylan (Sigma), pNPX (Sigma), xylobiose (prepared as described by Christov et al. (1997)), and hemicellulose (A and B) extracted from *Eucalyptus grandis* wood and xylan extracted from *E. grandis* dissolving pulp (using the procedure described by Du Toit et al. (1983)). The polymeric substrates were selected on the basis of the similarities in their chemical composition with the fiber-bound pulp xylan. The β-xylanase activity was very specific for β-xylans, with the highest activity determined on xylan derived from dissolving pulp and hemicellulose A extracted from *E. grandis* wood (Table 1). The former was 15% and the latter 13% higher than the reference activity of 33 U/mg protein obtained with oat spelts xylan as substrate. Except for arabinoxylan, specific activities of this enzyme were also found on chitin and starch (Leathers, 1989). Neither β-xylanase nor β-xylosidase possessed cellulase activity (data not shown). On the other hand, very low but detectable activitiy toward pNPX was observed, indicating that β-xylanase possessed negligible β-xylosidase activity. Therefore, this β-xylanase is typical of the β-glycanase family G which generally lacks cellulase activity (Wong et al., 1988). β-Xylosidase was most active on pNPX (100%) and xylobiose (30%), whereas it exhibited no or negligible activity with xylan-like substrates. Incubation of the β-xylosidase of *A. pullulans* CBS 58475 with larchwood and birchwood

Table 2. Kinetic Constants of β-Xylanase from *A. pullulans*

substrate	K_m (±SEM)[a] (mg/mL)	k_{cat} (±SEM) (s^{-1})	k_{cat}/K_m (mL/s·mg)
oat spelts xylan	12.5 ± 0.9	ND[b]	ND
larchwood xylan	28.4 ± 4.1	44.9 ± 3.6	1.58
hemicellulose A[c]	16.4 ± 1.3	28.5 ± 1.3	1.73
hemicellulose B[c]	16.4 ± 1.9	46.3 ± 2.9	2.82
pulp xylan[d]	29.0 ± 2.5	103.8 ± 6.2	3.58

[a] SEM = standard error of the mean of three replicates. [b] ND = not determined due to substrate inhibition. [c] Isolated from *E. grandis* wood. [d] Isolated from dissolving pulp produced from *E. grandis* wood.

xylans also did not produce any activity (Dobberstein and Emeis, 1991). The enzyme was, however, active on *p*-nitrophenyl α-L-arabinoside (data not shown), similar to results reported for the β-xylosidase of *Trichoderma reesei* (Poutanen and Puls, 1988).

Kinetic Parameters for β-Xylanase and β-Xylosidase

The Michaelis kinetic parameters, K_m and V_{max}, were determined by fitting data directly to the rate equation using Sigmaplot (Sigma). Substrates were tested at the following approximate concentrations: $K_m/4$, $K_m/3$, $K_m/2$, K_m, and $2K_m$. Due to the viscous nature of xylan, it was not possible to obtain concentrations higher than 40−60 mg/mL. The effect of substrate concentration on β-xylosidase was investigated using pNPX and xylobiose.

β-Xylanase showed (Table 2) the greatest affinity for the substituted oat spelts arabinoxylan (K_m of 12.5 mg/mL) followed by hemicellulose A and B (16.4 mg/mL). The lowest affinity was found for xylan extracted from dissolving pulp followed by larchwood xylan. The last two substrates are known to possess a low degree of substitution (Gamerith and Strutzenberger, 1992; Rozie et al., 1992). Thus, the lower the degree of substitution, the lower the affinity of the enzyme for the substrate. The decrease in affinity with a decrease in the degree of substitution was also previously reported by Tenkanen et al. (1992).

Comparison of k_{cat} values shows that β-xylanase hydrolyzed xylan from dissolving pulp 2.2, 2.3, and 3.6 times faster than hemicellulose B, larchwood xylan, and hemicellulose A, respectively (Table 2). Comparison of k_{cat}/K_m ratios shows that this xylanase hydrolyzed the xylan from pulp 1.3, 2.1, and 2.3 times more efficiently than hemicellulose B, hemicellulose A, and larchwood xylan, respectively, possibly as a result of the unsubstituted nature of xylan in pulp. Due to the large variation in xylan substrates and assay conditions reported in various publications, it proved difficult to compare directly the kinetic constants obtained here with that of other workers. Substrate inhibition of β-xylanase by oat spelts xylan was observed at concentrations greater than 20 mg/mL, while at 40 mg/mL the β-xylanase was almost completely inhibited (data not shown).

The K_m value of β-xylosidase for pNPX (1.43 mM) was in the range of 1−6.8 reported for other filamentous fungi (Matsou and Yasui, 1984). The K_m value for xylobiose (2.55 mM) was, however, lower than the K_m of 17.5 mM reported for β-xylosidase of *A. pullulans* CBS 58475 (Dobberstein and Emeis, 1991). β-Xylosidase had a greater affinity for pNPX than xylobiose and hydrolyzed pNPX 2.2-fold faster and 3.9-fold more efficiently than xylobiose. Xylotriose was detected during HPLC as well as thin-layer chromatography (TLC) analysis of xylobiose hydrolysis (data not shown), thus indicating a transxylosylation reaction. This is in agreement with a similar observation reported by Dobberstein and Emeis (1991) for the β-xylosidase of *A. pullulans* CBS 58475.

Table 3. Xylose and Xylan Removal from Sulfite Pulp by *A. pullulans* β-Xylanase and β-Xylosidase and Their Combination[a]

enzyme	xylose released (μg/g pulp)	xylan hydrolyzed (% of pulp xylan)
control	1.5	0
xln	51.7	10
xls	88.8	3
xln + xls	127.0	12

[a] xln = β-xylanase; xls = β-xylosidase. Enzymes were applied at the following charges (U/g pulp): β-xylanase, 4.7; β-xylosidase, 0.4. Values are the averages of duplicate determinations; the range of determinations was less than 10% of the mean.

Enzymatic Hydrolysis of Sulfite Pulp by β-Xylanase and β-Xylosidase

Unbleached sulfite pulp, derived from *E. grandis* wood by the acid bisulfite method with calcium as a base (Hinck et al., 1985), was obtained from Sappi Saiccor, Umkomaas, South Africa. Prior to use, pulp was washed with distilled water until a neutral pH of the wash water was obtained and then air-dried. β-Xylanase and β-xylosidase were applied on pulp at dosages of 4.7 and 0.4 U/g oven-dry pulp, respectively. A purified β-mannanase from *Aspergillus niger* (18 U/g pulp) was purchased from Megazyme, Sydney, Australia. The reaction parameters were as follows: 9% pulp consistency; 3 h treatment time; temperature of 55 °C; pH 4.7 (50 mM sodium acetate buffer). Samples were boiled for 10 min to stop the enzymatic reactions. Controls were prepared as above with the exception that neither enzymes nor denatured xylanase (boiled for 2 h at 96 °C) was added. Each experiment was done in duplicate.

The enzyme-treated pulp samples were hydrolyzed with sulfuric acid according to the method of Wright and Wallis (1996). The filtrates (0.22 μm; Micron Separations Inc., Westborough, MA) obtained after the enzyme treatment of pulp as well as the acid hydrolysates were analyzed according to Sullivan and Douek (1994) on a Dionex DX 500 HPLC system (Dionex, Sunnyvale, CA) equipped with a GP40 gradient pump, an ED40 electrochemical detector, a Dionex CarboPac PA guard column, and a Dionex CarboPac PA1 analytical column. The standard error of the determination in all experiments was less than 10%.

Both enzymes released xylose from pulp and hydrolyzed pulp xylan (Table 3). Although β-xylosidase removed more xylose from pulp (88.8 μg of xylose/g), it was responsible for only 3% of xylan solubilization. Treatment of pulp with β-xylanase liberated 51.7 μg of xylose/g and hydrolyzed 10% of xylan. The enzyme combination released the greatest amount of xylose from pulp (127 μg/g) and hydrolyzed 13% of xylan. However, the sum of the amount of xylose released from pulp by treatment with the single enzymes (141 μg/g) was 10% higher than that produced by the enzyme combination.

A synergistic effect in terms of release of xylose from pulp was observed when the enzyme mixture of β-xylanase and β-xylosidase was supplemented with β-mannanase (Table 4). In this case the amount of xylose released by the combined treatment with the three enzymes (211.6 μg/g) exceeded by 49% the sum of the sugar obtained by the separate enzyme treatments (142 μg/g). Since it is believed that xylan microfibrils are partially covered by amorphous mannan (Fengel and

Table 4. Effect of β-Mannanase on Xylose and Xylan Removal from Sulfite Pulp by *A. pullulans* β-Xylanase and β-Xylosidase[a]

enzyme	xylose released (μg/g pulp)	xylan hydrolyzed (% of pulp xylan)
control	1.5	0
man	1.6	0
xln + man	46.6	10
xln + xls + man	211.6	13

[a] xln = β-xylanase; xls = β-xylosidase; man = β-mannanase. Enzymes were applied at the following charges (U/g pulp): β-xylanase, 4.7; β-xylosidase, 0.4; mannanase, 18.0. Values are the averages of duplicate determinations; the range of determinations was less than 10% of the mean.

Table 5. Carbohydrate Composition of Sulfite Pulp Treated with *A. pullulans* β-Xylanase and β-Xylosidase and Their Combination[a]

	monocarbohydrate composition (% of total)				
enzyme	glucose	xylose	mannose	arabinose	galactose
control	89.65	9.22	0.99	0.10	0.04
xln	91.34	7.67	0.91	0.08	0.00
xls	90.98	8.01	0.92	0.09	0.02
xln + xls	91.64	7.46	0.84	0.06	0.00

[a] Enzymes were applied at the following charges (U/g pulp): β-xylanase, 4.7; β-xylosidase, 0.4. Values are the averages of duplicate determinations; the range of determinations was less than 10% of the mean.

Wegener, 1984), the enzymatic breakdown of some mannan by β-mannanase would then expose more of the xylan polymer to the direct attack of β-xylanase and especially β-xylosidase. The last enzyme is a 224 kDa dimer (Dobberstein and Emeis, 1991) with limited penetration capabilities due to its large size, and apparently the loosening of the hemicellulose structure would improve its diffusability. However, this did not result in further enzymatic degradation of pulp xylan. Moreover, β-xylanase and β-mannanase did not act in synergism when applied together (Table 4). This, however, is in accordance with results obtained on softwood kraft pulps using *T. reesei* β-xylanase and β-mannanase and apparently confirms the suggestion that xylan and mannan may not be intimately associated at least in some parts of the fiber cell wall (Buchert et al., 1993).

It is evident from the data in Table 5 that some alterations in the carbohydrate composition occurred as a result of the pulp hydrolysis by β-xylanase and β-xylosidase. Apparently the relative amount of cellulose (glucose) present in pulp was increased at the expense of reduced hemicellulose content (xylose, mannose, arabinose, and galactose) of pulp. For instance, the glucose content of samples treated with β-xylanase increased by 1.7% over the control (from 89.65% to 91.34%). At the same time the amount of xylose detected in pulp decreased by approximately 1.6% relative to the control (from 9.22% to 7.67%). On the other hand, the mannose and arabinose contents of pulp were affected to a lesser extent in comparison to glucose and xylose. A similar trend of modification of pulp carbohydrates was observed when β-xylosidase and the enzyme combination were used. The latter proved to be again most efficient in removing hemicellulose from pulp. Thus, 12% of xylan in pulp was solubilized when both enzymes were applied on pulp, whereas β-xylanase and β-xylosidase removed 10% and 3%, respectively, when used separately.

Both β-xylanase and β-xylosidase when applied on pulp released xylose and altered the carbohydrate composition of pulp (Tables 3 and 5). The liberation of xylose as a result of β-xylanase action was reported previously for both kraft (Eligir et al., 1995) and sulfite (Christov et al., 1997) pulps. This phenomenon could be explained by the fact that this enzyme may express some β-xylosidase activity, as also demonstrated with the experiments on substrate specificity in the present study. Prolonged incubation of *A. pullulans* β-xylanase with oat spelts xylan and xylooligomers also yielded xylose (Dobberstein and Emeis, 1989; Li et al., 1993). The formation of xylose from pulp xylan by β-xylosidase may be indicative of the following: (1) a xylooligosaccharide fraction of relatively low molecular weight, a suitable substrate for β-xylosidase (Dobberstein and Emeis, 1991), which is attached to the main chain of the xylan polymer and/or physically entrapped in the pulp fibers, could be present in pulp; (2) β-xylosidase could be a multifunctional β-D-xylan xylohydrolase which can act in an exo-manner (Herrmann et al., 1997). However, the last suggestion seems to be unlikely, because it was already shown in this work and elsewhere (Dobberstein and Emeis, 1991) that *A. pullulans* β-xylosidase is inactive on polymeric β-xylans.

As previously observed, the enzymatic hydrolysis of pulp xylan is limited and generally does not exceed 20% (Kantelinen et al., 1993). The major limitation in xylan hydrolysis is the accessibility and composition of the substrate. However, the type of wood pulp (softwood, hardwood) and properties of the enzyme used also play an important role. For instance, when acid sulfite pulp from spruce was treated with purified β-xylanase of *T. reesei*, only 4% of xylan was solubilized (Buchert et al., 1995). Results from the present study indicate 10% hydrolysis of the initial xylan content of eucalyptus acid sulfite pulp by *A. pullulans* β-xylanase. Therefore, it appears that the xylan substrate in hardwood sulfite pulps is more accessible than that in softwood sulfite pulp.

The *A. pullulans* β-xylanase and β-xylosidase acted together on pulp xylan in an additive rather than synergistic way. For an apparent synergism, the combined effect of using both enzymes should exceed the sum of their individual effects (He et al., 1994). However, synergism between β-xylanase and β-xylosidase in hemicellulose degradation has been observed previously (Dekker, 1993; Deshpande et al., 1986; Poutanen and Puls, 1988). The cooperative action between these two enzymes could be improved by optimizing the ratio of β-xylanase to β-xylosidase (Seeta et al., 1989). On the other hand, the degree of xylan hydrolysis could be impaired by the transferase activity exhibited by *A. pullulans* β-xylosidase and end-product inhibition by xylose.

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