

## A Novel Family 8 Xylanase, Functional and Physicochemical Characterization\*

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Xylanases are generally classified into glycosyl hydrolase families 10 and 11 and are found to frequently have an inverse relationship between their pI and molecular mass values. However, we have isolated a psychrophilic xylanase that belongs to family 8 and which has both a high pI and high molecular mass. This novel xylanase, isolated from the Antarctic bacterium *Pseudoalteromonas haloplanktis*, is not homologous to family 10 or 11 enzymes but has 20–30% identity with family 8 members. NMR analysis shows that this enzyme hydrolyzes with inversion of anomeric configuration, in contrast to other known xylanases which are retaining. No cellulase, chitosanase or lichenase activity was detected. It appears to be functionally similar to family 11 xylanases. It hydrolyzes xylan to principally xylotriose and xylotetraose and is most active on long chain xylo-oligo-saccharides. Kinetic studies indicate that it has a large substrate binding cleft, containing at least six xylose-binding subsites. Typical psychrophilic characteristics of a high catalytic activity at low temperatures and low thermal stability are observed. An evolutionary tree of family 8 enzymes revealed the presence of six distinct clusters. Indeed classification in family 8 would suggest an  $(\alpha/\alpha)_6$  fold, distinct from that of other currently known xylanases.

Xylanases (EC 3.2.1.8, endo-1,4- $\beta$ -xylan xylanohydrolase) are O-glycoside hydrolases that catalyze the random hydrolysis of internal  $\beta$ -1,4-D-xylosidic linkages of xylan, a major component of plant hemicellulose and constituting 15–30% of the cell wall content of hardwoods, 7–10% of softwoods, and up to 30% of annual plants (1). Xylan is a complex, highly branched heteropolymer of variable size (70–130 units in softwoods and 150–200 units in hardwoods). Depending on the source, the xylan backbone may contain a varying degree of glucuronosyl, 4-O-methyl-D-glucuronopyranosyl,  $\alpha$ -L-arabinofuranosyl, acetyl, feruloyl, and/or p-coumaroyl substituents. Unsubstituted linear xylans also exist and have been isolated from esparto grass and seaweed (2).

The wide diversity of xylan structures is paralleled by a large

variety of xylanases with widely different hydrolytic activities, physicochemical properties, and structures. In an attempt to contend with this diversity of enzymes, Henrissat (3) introduced a classification system for glycoside hydrolases based on sequence homologies and designed to integrate both structural and mechanistic features. Currently 87 families have been identified, and members of each family are believed to have evolved from a common ancestral sequence. Divergent evolution to acquire new substrate specificity has resulted in more than one-third of the families being polyspecific (4) and in different families having a related fold. As the structures of proteins are better conserved than their sequences, the grouping of several families in “clans” has thus been introduced (5). In contrast, convergent evolution has resulted in enzymes with identical substrate and reaction specificity being found in totally unrelated families with disparate three-dimensional folds (6).

Endo- $\beta$ -1,4-D-xylanases have been assigned to two distinct families, 10 (formerly F) and 11 (formerly G) (7–9). Indeed, this separation into two families is in agreement with an earlier classification by Wong *et al.* (10) who indicated that xylanases generally either have a high molecular mass (>30 kDa) and low pI or a low molecular mass (<30 kDa) and high pI. It has been found that family 10 generally groups acidic high molecular mass enzymes, whereas family 11 members are generally much smaller basic proteins, although acidic pI values have been observed for some enzymes of fungal origin (11).

A significant difference in the structure and catalytic properties of the two families also occurs. Family 10 members present an  $(\alpha/\beta)_8$  barrel fold (12), belong to clan GH-A, the 4/7 superfamily (13), and have ~40% of the secondary structure in an  $\alpha$ -helical form (14). Members of family 11 are ~3–5%  $\alpha$ -helical in nature, have a  $\beta$ -jelly roll fold conformation (15), and belong to clan GH-C. In relation to their catalytic properties, it has been found that family 10 xylanases typically have smaller substrate-binding sites, lower substrate specificities (frequently having endoglucanase activity), and hydrolyze heteroxylans to a higher degree (16) as compared with family 11 xylanases (true xylanases). The common feature of the two families is in the mode of action. All xylanases characterized to date retain the anomeric configuration of the glycosidic oxygen following hydrolysis in which two conserved glutamates function as the catalytic nucleophile and acid/base catalyst (17).

In the present study we have isolated a novel cold-active xylanase that belongs to glycoside hydrolase family 8 (formerly D), a family that is mainly composed of endoglucanases (EC 3.2.1.4) as well as lichenases (EC 3.2.1.73) and chitosanases (EC 3.2.1.132) and that typically operates with inversion of anomeric configuration. The cloning, overexpression, purification, and characterization of this novel xylanase as well as the

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ / EBI Data Bank with accession number(s) AJ427921.

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evolutionary relationships within family 8 members are described. Catalytic properties including the specificity, action pattern, and mode of action of this enzyme are also assessed.

#### EXPERIMENTAL PROCEDURES

Unless otherwise stated products were obtained from Sigma.

**Bacterial Strain and Culture Conditions**—The xylanase producing bacterial strain was isolated from soil samples collected in the vicinity of the French Antarctic station in Dumont D'Urville, Terre Adelie, Antarctica (66°40' S; 140°01' E). Screening for xylanase activity was carried out on marine agar (5 g/liter tryptone (Difco), 1 g/liter yeast extract (Difco), 33 g/liter marine salts (Wiegandt), 18 g/liter agar (Difco)) supplemented with 0.15% RBB<sup>1</sup>-xylan at 4 °C.

Microscopic identification, Gram staining and fatty acid methyl ester fingerprint analyses were used to identify the isolate. Cellular fatty acids were extracted and quantified by gas-liquid chromatography and identified using the Microbial Identification System data base (18), TSBA version 4.1 (Microbial ID Inc.).

Growth and xylanase production at 4 and 28 °C were examined in marine broth by monitoring the absorbance ( $A_{550\text{ nm}}$ ) and xylanase activity of the culture supernatant at various time points. Growth and enzyme production at 4 °C in various culture media (marine broth, Luria Bertani, minimal media M63/M9 (19)) and with various supplements (xylan and salts) were also examined.

**Production and Purification of Wild-type Xylanase**—The Antarctic isolate was cultivated in 1 liter of modified marine broth (5 g/liter tryptone (Difco), 1 g/liter yeast extract (Difco), 20 g/liter marine salts) supplemented with 1.5% birchwood xylan for 72 h at 4 °C. After centrifugation for 1 h at 18,000  $\times g$  and 4 °C the supernatant was concentrated to ~100 ml by ultrafiltration on a Millipore PBGC 10000 NMWL membrane and dialyzed against buffer A (50 mM BICINE, 10 mM NaCl, pH 8.5). The dialysate was loaded on a Q-Sepharose Fast Flow (Amersham Biosciences) column (9  $\times$  2.5 cm) equilibrated in the same buffer, and the void was collected and immediately loaded on a S-Sepharose Fast Flow (Amersham Biosciences) column (7  $\times$  2.5 cm), also equilibrated in the above-mentioned buffer, and eluted with a linear NaCl gradient (0–100 mM in 350 ml).

**Cloning of the Cold-adapted Xylanase Gene**—The psychrophilic xylanase gene was isolated and sequenced using a procedure similar to that described previously (20, 21). Degenerate primers based on the amino-terminal sequence (5'-GCITTYAAYAAAYCC-3') and an internal peptide sequence determined after CNBr cleavage (see "Analytical Procedures") (5'-RTTTRTTTAICCRACAT-3') allowed amplification of a 101-bp fragment from the genomic DNA. The complete xylanase gene sequence was identified from an enriched *PstI* (3–6-kb fragments) genomic library in pUC19 by PCR screening using specific primers derived from the 101-bp isolated sequence (5'-CGTTTAATA-ATAACCCATCGAGTAGGTAGGC-3' and 5'-GTTGTAGCCGAACATATT-GTCAAAGTACTAT-3'). Double-stranded sequencing was carried out with an ALF DNA sequencer (Amersham Biosciences).

**Overexpression of the Cold-adapted Xylanase**—The xylanase gene, including its signal sequence, was PCR-amplified using *Pwo* polymerase (Europogene), with the sense primer (5'-GGGCATATGAAAGTAT-TTTTTAAATAACAACTT-3') containing an *NdeI* site (underlined) and the antisense primer (5'-GCTACTTAGGTAGTGTTATTTTAATTAACGTGTTTATAA-3') containing the stop codon (underlined). The PCR product was cloned into the PCR-Script Amp SK(+) cloning vector (Stratagene), excised with *NdeI* and *XhoI* and ligated into the pET22b(+) cloning vector (Novagen). The resulting recombinant plasmid was transformed to *Escherichia coli* BL21 (DE3) cells (Stratagene).

**Production and Purification of the Recombinant Xylanase**—Five ml of an overnight preculture (18 °C) of the *E. coli* BL21 (DE3) cells carrying the xylanase gene was centrifuged at 10,000  $\times g$  for 1 min, and the pellet was resuspended in 300 ml of Terrific broth (12 g/liter Bacto-tryptone (Difco), 24 g/liter yeast extract (Difco), 4 ml/liter glycerol, 12.54

<sup>1</sup> The abbreviations used are: RBB, Remazol Brilliant Blue; NMWL, nominal molecular weight limit; ALF, automated laser fluorescence; DSC, differential scanning calorimetry; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; DNS, dinitrosalicylic acid; *pNp*, *para*-nitrophenol; X<sub>1</sub>, xylose; X<sub>2</sub>, xylobiose; X<sub>3</sub>, xylotriose; X<sub>4</sub>, xylotetraose; X<sub>5</sub>, xylopentaose; X<sub>6</sub>, xylohexose; IsoX<sub>3</sub>, isomeric xylotriose; IsoX<sub>4</sub>, isomeric xylotetraose; IsoX<sub>5</sub>, isomeric xylopentaose; IsoX<sub>6</sub>, isomeric xylohexose; MU, 4-methylumbelliferyl.

g/liter K<sub>2</sub>HPO<sub>4</sub>, 2.31 g/liter KH<sub>2</sub>PO<sub>4</sub>) containing 200  $\mu$ g/ml ampicillin in a 3-liter shake flask. The culture was incubated at 18 °C and 250 rpm until an absorbance at 550 nm of between 3 and 4 was reached, whereupon the expression of the enzyme was induced with 1 mM isopropyl-1-thio- $\beta$ -galactopyranoside. Following a further 20-h incubation at 18 °C, the cells were harvested by centrifugation at 18,000  $\times g$  for 30 min at 4 °C, resuspended in modified buffer A (no NaCl), disrupted in a prechilled cell disrupter (Constant Systems Ltd.) at 28 K pounds/square inch, centrifuged at 40,000  $\times g$ , and dialyzed against modified buffer A (no NaCl). The dialysate was then subjected to Q-Sepharose Fast Flow (50  $\times$  2.5 cm) and S-Sepharose Fast Flow purification as described for the wild-type enzyme but using modified buffer A (no NaCl). Active fractions were pooled, concentrated by ultrafiltration (Millipore PBGC 10,000 NMWL), and further purified on a Sephadryl S-100 (Amersham Biosciences) column (90  $\times$  2.5 cm) equilibrated in 20 mM MOPS, 100 mM NaCl, pH 7.5, at 1 ml min<sup>-1</sup>.

**Physicochemical Characterization**—The 20,585-Da mesophilic family 11 xylanase (Xyl1) from *Streptomyces* sp. S38 was obtained from J. Georis (see Ref. 22). The temperature optimum was determined by monitoring activity over 5 min at pH 6.5 between 5–65 °C and 20–80 °C for the psychrophilic and mesophilic enzymes, respectively. Thermostability of the enzymes was evaluated by measuring the residual activity as a function of time at 55 °C in 20 mM MOPS, 100 mM NaCl, 500 mM 3-(1-pyridinio)-1-propanesulfonate, pH 7.5. Kinetic parameters of the psychrophilic xylanase were determined at 25 °C by non-linear regression using the Michaelis-Menten equation of initial rates determined between 0 and 30 mg/ml soluble birchwood xylan. The inability to measure activity at higher substrate concentrations due to substrate interference and high viscosity meant substrate saturation was not achieved and thus extrapolation was used in determination of the kinetic values, giving rise to apparent values. The pH dependence of the psychrophilic xylanase was determined at 25 °C between pH 4.5 and 12 using the following buffer mix: 40 mM sodium acetate, 20 mM MES, 20 mM MOPS, 20 mM TAPS, 20 mM CHES, and 20 mM CAPS. The effect of various heavy metals, chelators, and ions was examined by determining the activity in the presence of 1 and 10 mM of the various compounds.

Differential scanning calorimetry (DSC) was carried out in a Microcal MCS-DSC apparatus using the MCS Observer software package for data acquisition, analysis, and deconvolution. Analysis was carried out with 2 mg/ml recombinant protein in 20 mM MOPS, 100 mM NaCl, 500 mM 3-(1-pyridinio)-1-propanesulfonate, pH 7.5, at a scan rate of 1 °C/min and under 1 atmosphere nitrogen pressure. Due to irreversibility of the denaturation, only the apparent *T<sub>m</sub>* (melting temperature) and apparent  $\Delta H_{\text{cal}}$  (calorimetric enthalpy of unfolding) could be calculated.

**Functional Characterization**—Xylanase activity was measured at 25 °C (psychrophile) and 50 °C (mesophile) by a modification of the DNS method as described (23); 3% soluble birchwood xylan (24) in 100 mM citrate-phosphate buffer, pH 6.5, was used as substrate.

The stereoselectivity of hydrolysis was analyzed essentially as described (25); 0.8% *Nothogenia erinacea* xylan, isolated as described previously (26), and 43.5  $\mu$ M enzyme, lyophilized and redissolved twice in D<sub>2</sub>O, were used. Proton NMR spectra were recorded with a Bruker Digital NMR Avance 500 spectrometer at 25 °C using a 5-mm sample tube. Spectra were recorded immediately after mixing and at 15-min intervals for 1 h.

Activity on birchwood xylan, beechwood xylan, oat spelt xylan, CM-cellulose (Fluka), microcrystalline cellulose (Aldrich), cellobiose, arabino-galactan (from larchwood), lichenane (from *Cetraria islandica*), laminarin (from *Laminaria digitata*), and starch (from potato) was determined by the DNS method at 25 °C with a final substrate concentration of 3%. Due to interaction of the chitosan with the citrate-phosphate buffer and insolubility at pH 6.5, the assay for chitosanase activity was carried out in potassium hydrogen phthalate buffer at pH 6.0. Activity was measured at 25 °C by monitoring the decrease in viscosity over 30 h with a Brookfield LVDV viscometer. Chitoclear™ low viscosity (3% acetylation) and FC222B (17% acetylation) chitosan (Primex Ingredients) were examined, and appropriate controls were included in all cases. Activity on *pNp*- $\beta$ -D-glucopyranoside, *pNp*- $\alpha$ -D-glucopyranoside, *pNp*- $\beta$ -D-cellobioside, *pNp*- $\beta$ -D-galactopyranoside, *pNp*- $\beta$ -D-glucuronide, *pNp*- $\alpha$ -D-maltoside, *pNp*- $\beta$ -D-xylopyranoside, and *pNp*- $\alpha$ -D-xylopyranoside was examined at 25 °C at a final concentration of 5 mM.

To determine the extent of hydrolysis of birchwood, oat spelt and *Palmaria palmata* xylan, 25 nM enzyme was incubated at 25 °C, pH 6.5, with a 3% substrate solution containing 0.01% sodium azide (Merck), samples were removed at various time points, boiled for 4 min, filtered through a 0.45- $\mu$ m Millex membrane (Millipore) and analyzed for reducing sugars by the DNS method.

Products of enzymatic hydrolysis were analyzed using a high performance anion exchange chromatography system (Dionex Corp.) equipped with an ED40 pulsed amperometric detector (HPAE-PAD). The Dionex CarboPac PA100 column ( $4 \times 250$  mm) was equilibrated at  $1 \text{ ml min}^{-1}$  in  $100 \text{ mM NaOH}$ ,  $20 \text{ mM}$  sodium acetate, and product separation was obtained with a four-step linear gradient:  $20\text{--}40 \text{ mM}$  sodium acetate in  $20 \text{ ml}$ ,  $40\text{--}100 \text{ mM}$  sodium acetate in  $10 \text{ ml}$ ,  $100\text{--}160 \text{ mM}$  sodium acetate in  $10 \text{ ml}$ , and  $160\text{--}200 \text{ mM}$  sodium acetate in  $1 \text{ ml}$ . Xylo-oligosaccharides,  $X_1\text{--}X_6$  (Megazyme), aldopentaouronic and aldo-hexaouronic acid (Megazyme), and mixed linkage ( $\beta 1,4; \beta 1,3$ ) standards, Iso $X_3$  and Iso $X_4$ , were used for peak identification. The kinetics of hydrolysis of xylo-oligosaccharides was determined as described by Bray and Clarke (27) using the Dionex HPAE-PAD system. Xylo-oligosaccharide separation was achieved with a linear gradient of  $20\text{--}40 \text{ mM}$  sodium acetate in  $20 \text{ ml}$ . Reactions were carried out at  $25^\circ\text{C}$  using  $0\text{--}10 \text{ mM } X_1, X_2, X_3, X_4, 0\text{--}6.3 \text{ mM } X_5$ , and  $0\text{--}3.8 \text{ mM } X_6$ , and kinetic parameters were determined by non-linear regression of initial rates of product formation using the Michaelis-Menten equation.

The action of  $0.2 \mu\text{M}$  enzyme on  $1 \text{ mM MU-xylobioside}$ ,  $1 \text{ mM MU-xylotrioside}$ ,  $100 \mu\text{M MU-cellotrioside}$ , and  $100 \mu\text{M MU-cellopentaoside}$  (28) was examined by fluorescence and analysis on the Dionex HPAE-PAD system.

**Analytical Procedures**—Protein concentration was determined by the Bradford method (29) and/or by absorbance at  $280 \text{ nm}$  using an extinction coefficient of  $95,420$  and  $62,930 \text{ M}^{-1} \text{ cm}^{-1}$  for the psychrophilic and mesophilic xylanase, respectively. Analytical SDS-PAGE was run essentially as described by the instrument supplier (Hoefer). The isoelectric point was determined essentially as described (30). Amino-terminal sequencing was carried out using a Procise 492 pulsed liquid phase protein sequencer (Applied Biosystems) with  $\sim 20\text{--}30 \text{ pmol}$  of protein. CNBr cleavage of the protein was carried out overnight under atmospheric nitrogen in  $70\%$  formic acid with  $2\% (\text{v/v})$   $5 \text{ M}$  CNBr (Fluka). Resulting fragments were separated on  $17.5\%$  SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and subjected to amino-terminal sequencing. Carboxyl-terminal sequencing was performed on a Procise 494C Sequencer (PE Biosystems) with  $1 \text{ nmol}$  of enzyme as described (31). The molecular mass was determined by nano-electrospray ionization spectrometry on a Q-TOF mass spectrometer (Micromass) with  $20 \text{ pmol}$  of enzyme.

**Construction of Evolutionary Tree**—Family 8 members were identified from the carbohydrate-active enzymes server (afmb.cnrs-mrs.fr/CAZY) and by a protein database (SWALL) fasta33-t search (32) with the isolated psychrophilic xylanase sequence. Surplus or additional regions and domains were removed, leaving essentially the catalytic core. The tree was then constructed with the Drawtree program (33) by application of the neighbor-joining method (34) to multiple alignments from the ClustalW program (35).

## RESULTS

**Identification and Growth Characteristics of Isolated Strain**—The xylanase-producing strain was identified as the Gram-negative bacterium *Pseudoalteromonas haloplanktis* with a Microbial Identification Score of  $0.391$  and no alternative was proposed. Fig. 1 shows that this bacterium has typical cold-adapted characteristics as xylanase production and stability are much higher at  $4^\circ\text{C}$  than at  $28^\circ\text{C}$ . Furthermore, the optical density at  $28^\circ\text{C}$  drops rapidly to zero, indicating cell lysis at this temperature.

Optimal growth occurs in marine broth containing high concentrations of marine salts ( $20 \text{ g/liter}$ ) and optimal xylanase production was obtained with high concentrations ( $1.5\%$ ) of xylan, birchwood being more suitable than oat spelt due to foaming induced by the less soluble oat spelt xylan at high concentrations. Low quantities of xylanase activity were detected in the absence of xylan in the production medium indicating that it is produced constitutively by the isolate. This organism is, however, unable to utilize xylan as a carbon source, as witnessed from its inability to grow on xylan-supplemented minimal media.

**Purification of Wild-type Xylanase**—A simple two-step purification protocol taking advantage of the high pI (pH 9.5) of the extracellular enzyme resulted in an 11-fold purification with a final yield of  $55\%$ , equivalent to  $1.9 \text{ mg}$  of pure xylanase per liter of culture. Only one xylanase was detected for this organ-

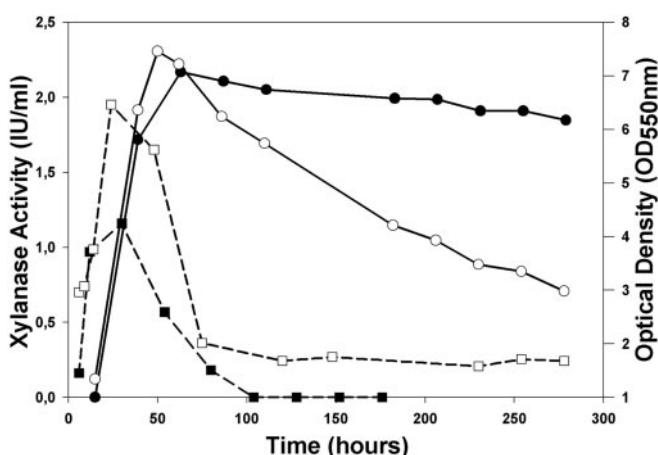


FIG. 1. Growth curves as monitored by optical density (open symbols) and xylanase activity in the culture supernatant (closed symbols) of the *P. haloplanktis* strain at  $4^\circ\text{C}$  (circles, solid lines) and  $28^\circ\text{C}$  (squares, dashed lines).

nism. Low concentrations of salts ( $10 \text{ mM NaCl}$ ) were used throughout the purification procedure so as to prevent interaction between the enzyme and the xylan used for production. SDS-PAGE and mass spectrometry indicated that the enzyme was  $>98\%$  pure with a molecular mass of  $45,982 \text{ Da}$ .

**Cloning and Sequence Analysis of Recombinant Xylanase**—PCR screening allowed isolation of one clone containing a fragment of  $\sim 11 \text{ kb}$ . An open reading frame of  $1278 \text{ bp}$  encoding a protein of  $426$  amino acids was identified (Table I). Amino-terminal and carboxyl-terminal sequencing as well as analysis using the SignalP version 1.1 signal prediction program (36) indicated that the mature xylanase gene has a signal sequence of  $21$  amino acids and a predicted molecular mass of  $45,981.81 \text{ Da}$ , in good agreement with that determined by mass spectrometry.

The deduced amino acid sequence shows highest identity to the *Bacillus halodurans* xylanase Y ( $32.6\%$ ) with  $24.7\%$  identity to the family 8 chitosanase/lichenase of *Bacillus circulans*,  $23.6\%$  identity to the family 8 endoglucanase precursor of *Clostridium thermocellum*, and low identity to the other family 8 enzymes. Furthermore, a FingerPRINTScan against PRINTS using the InterPro Scan search program (37) indicated that the isolated sequence contained the glycosyl hydrolase family 8 fingerprint. Sequence alignment (not shown) indicates that the proposed family 8 catalytic residues, glutamate and aspartate (38), are conserved in the isolated xylanase. Moreover,  $8$  residues are strictly conserved in the  $20$  family 8 enzymes (Glu-78, Trp-124, Ala-142, Asp-144, Ala-150, Ala-156, Trp-225, and Arg-284, cold-adapted xylanase numbering). In addition, two residues are strictly conserved in all enzymes except for the xylanases; one is replaced by a residue conserved within the xylanases (Glu-146 in xylanases-Asp), whereas the other is substituted by an unconserved residue (Val-121 or Tyr in xylanases-Leu).

An unrooted evolutionary tree of family 8 members (see Fig. 2) indicates that this family consists of six major subclasses. Subclasses A-C group endoglucanases from Gram-negative proteobacteria. Subclass A groups enzymes from bacteria belonging to the  $\alpha$ -proteobacterial subdivision, whereas subclasses B and C group, respectively, enzymes from aerobes (*E. coli*, *Salmonella*, and *Pseudomonas*) and facultative anaerobes (*Erwinia* and *Pectobacterium*) belonging to the  $\gamma$ -proteobacterial subdivision. Subclass D contains xylanases from Gram-negative or  $\beta$ -positive bacteria, whereas subclasses E and F contain enzymes from Gram-positive bacteria. Subclass E groups endo-1,4-glucanases from Clostridia (strict anaerobes)

TABLE I  
Physicochemical characteristics of the cold adapted xylanase

DNA sequence	1278 bp
Mature protein sequence	405 amino acids
Signal sequence	21 amino acids
Molecular weight (sequence)	45,981.81 Da
Molecular weight (mass spectrometry)	45,982.35 Da
Isoelectric point	Approximately pH 9.5
pH optimum (25 °C)	pH 5.3–8
pH stability range (25 °C)	pH 6–9
Temperature optimum (5 min assay)	35 °C
Apparent $T_m$ (scan rate, 1 °C/min) <sup>a</sup>	53.5 °C
$\Delta H_{cal}^b$ molar (scan rate, 1 °C/min)	252.8 kcal/mol
$\Delta H_{cal}$ specific (scan rate, 1 °C/min)	0.627 kcal/residue
Apparent $K_m$ (25 °C, soluble BWX <sup>c</sup> )	28 ± 4.5 mg/ml
Apparent $k_{cat}$ (25 °C, soluble BWX)	1247 ± 120 s <sup>-1</sup>
Inhibitors (% inhibition at 1 mM)	Hg <sup>2+</sup> (31%), Cu <sup>2+</sup> (16%), Zn <sup>2+</sup> (14%), Ni <sup>2+</sup> (6%)
Stereoselectivity	Inverting

<sup>a</sup>  $T_m$ , melting temperature.

<sup>b</sup>  $\Delta H_{cal}$ , calorimetric enthalpy of unfolding.

<sup>c</sup> BWX, birchwood xylan.

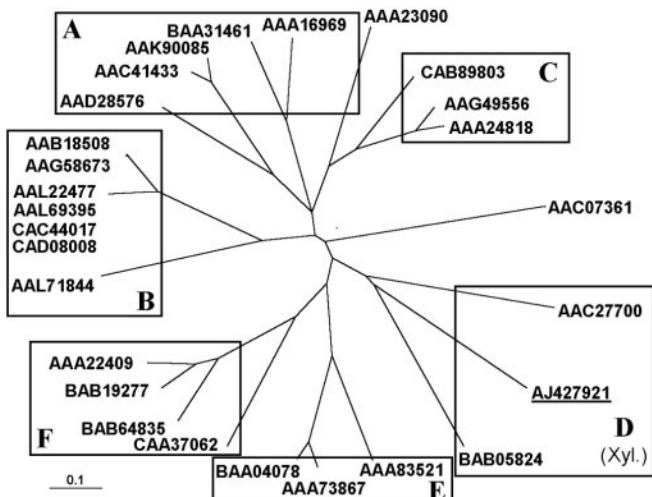


FIG. 2. Unrooted evolutionary tree of glycosyl hydrolase family 8 members. GenBank™/GenPept accession codes are given. The cold-adapted xylanase of this study is underlined. The microbial origin and EC numbers (if known) are as follows: AAD28576 (*Rhizobium leguminosarum*, not determined (ND)), AAC41433 (*Agrobacterium tumefaciens*, N.D.), AAK90085 (*A. tumefaciens* C58, ND), BAA31461 (*Aacetobacter xylinus* BPR2001, EC 3.2.1.4), AAA16969 (*A. xylinus* ATCC 23769, EC 3.2.1.4), AAA23090 (*Cellulomonas uda*, EC 3.2.1.4), CAB89803 (*Erwinia rhamontica* NCPPB2989, EC 3.2.1.4), AAG49556 (*Pectobacterium chrysanthemi* PY35, EC 3.2.1.4), AAA24818 (*Erwinia chrysanthemi* 3937, EC 3.2.1.4), AAC07361 (*Aquifex aeolicus*, ND), AAC27700 (*Bacillus* sp. KK-1, EC 3.2.1.8), AJ427921 (*P. haloplanktis* TAH 3a, EC 3.2.1.8), BAB05824 (*B. halodurans* C-125, EC 3.2.1.8), AAA83521 (*C. thermocellum* NCIB 10682/JW20, EC 3.2.1.4), AAA73867 (*C. cellulolyticum*, EC 3.2.1.4), BAA04078 (*Clostridium josui*, EC 3.2.1.4), CAA37062 (*B. circulans* WL-12, EC 3.2.1.132 and 3.2.1.73), BAB64835 (*Bacillus* sp. d-2, EC 3.2.1.132), BAB19277 (*Bacillus* sp. No.7-M, EC 3.2.1.132), AAA22409 (*Bacillus* sp. KSM-330, EC 3.2.1.4), AAL71844 (*Pseudomonas fluorescens* SBW25, ND), CAD08008 (*Salmonella enterica* subsp. *Enterica* serovar *Typhi* CT18, ND), CAC44017 (*Salmonella typhimurium* ATCC 14028, ND), AAL69395 (*S. typhimurium* UR1, ND), AAL22477 (*S. typhimurium* LT2, ND), AAG58673 (*E. coli* O157:H7 EDL933, ND), AAB18508 (*E. coli* K12/MG1655, EC 3.2.1.4); Xyl., xylanase subclass.

whereas subclass F groups endo-1,4-glucanases, lichenases, and chitosanases from Bacilli (aerobes and facultative anaerobes).

**Overexpression and Purification of Recombinant Xylanase—** Cloning of the xylanase gene, including its proper signal sequence, in a pET22b(+) vector in *E. coli* and cultivation in Terrific broth resulted in production of ~85 mg of the enzyme per liter. The protein was purified with a procedure similar to

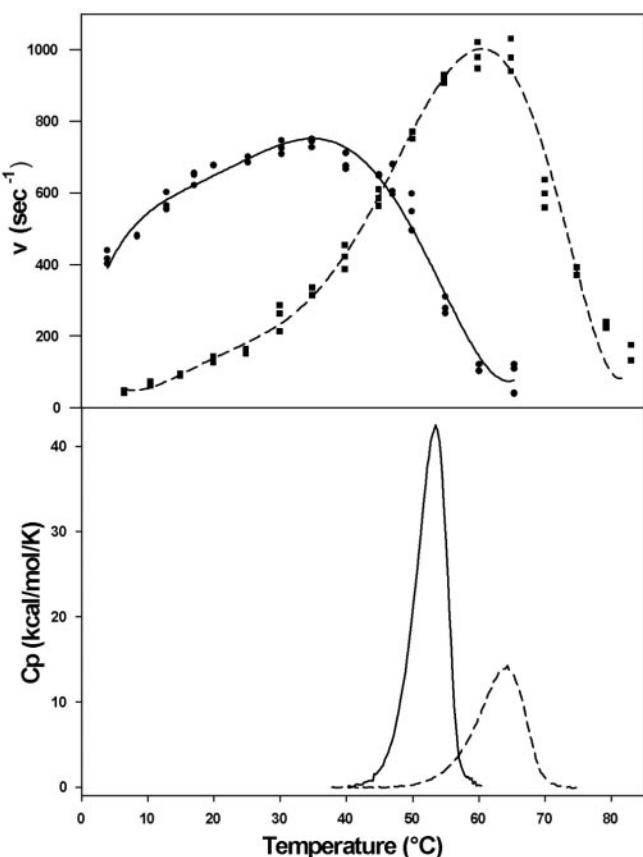


FIG. 3. Thermodependence of activity on 30 mg/ml soluble birchwood xylan (top) and unfolding as monitored by DSC (bottom) of the cold-adapted *P. haloplanktis* xylanase (circles, solid lines) and the mesophilic *Streptomyces* sp. S38 xylanase (squares, dashed lines). Base-line subtracted DSC data have been normalized for protein concentration.

that used for the wild-type enzyme; however, due to the absence of added xylan, no salt was required. An additional gel filtration step was also included, resulting in a final yield of 58 mg/liter and equivalent to a recovery of 68%.

**Physicochemical Characterization—**The enzyme has both a high molecular weight and a high pI (Table I). It has a wide pH activity range with maximum activity occurring between pH 5.3 and 8. While acknowledging the limitations of the test utilized for determination of the kinetic parameters, it can be seen that the apparent  $K_m$  and  $k_{cat}$  values of the cold xylanase at 25 °C are relatively high (2). Heavy metals such as Hg<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Ni<sup>2+</sup> were found to be inhibitory to activity, whereas Mg<sup>2+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, PO<sub>4</sub><sup>3-</sup> and Cl<sup>-</sup> as well as chelating agents (EDTA and EGTA) had no effect.

The thermostability and thermodependence of activity of the isolated xylanase are compared with that of a mesophilic xylanase in Figs. 3 and 4. The cold enzyme shows a shift in apparent optimal activity of ~25 °C toward low temperatures. Activity at 5 °C is 60% of the maximum, compared with the mesophilic xylanase where activity at this temperature is less than 5% of the maximum. It can also be seen that the isolated xylanase is much less stable than the mesophilic xylanase with a 10 °C lower melting temperature (52.6 versus 63.1 °C) and a 12 times shorter half-life at 55 °C (1.9 versus 23 min).

**Functional Characterization—**Fig. 5 shows that the cold enzyme is a true xylanase: no cellulase (endo-1,4-β-glucanase), cellobiose, lichenase (endo-β-1,3-1,4-glucanase), chitosanase (chitosan N-acetylglucosaminohydrolase), laminarinase (endo-1,3-β-glucanase), or amylase (1,4-α-D-glucan-glucohydrolase) activity was detected. In addition, no activity was detected on

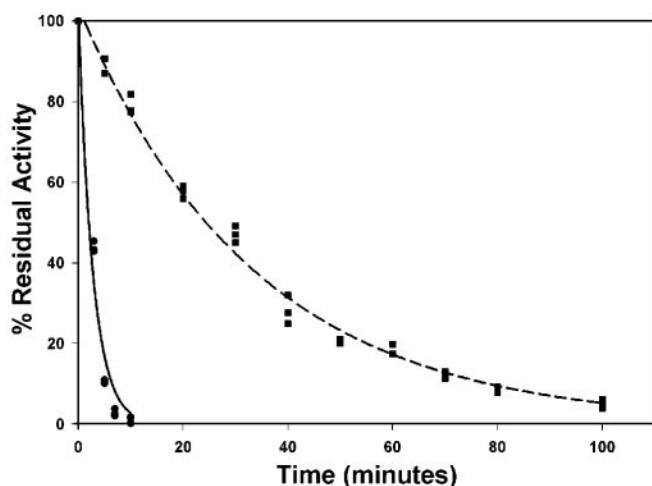


FIG. 4. Thermal stability of activity at 55 °C of the cold-adapted *P. haloplanktis* xylanase (circles, solid lines) and the mesophilic *Streptomyces* sp. S38 xylanase (squares, dashed lines).

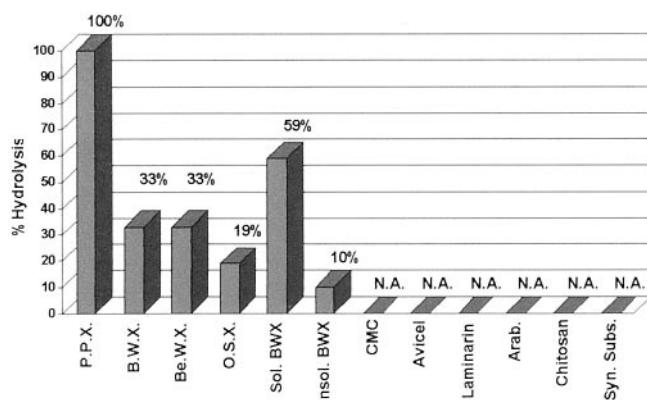


FIG. 5. Activity of the cold-adapted xylanase on various substrates. Expressed as a percentage of the activity on *P. palmata* xylan. P.P.X., *P. palmata* xylan; B.W.X., birchwood xylan; Be.W.X., beechwood xylan; O.S.X., oat spelt xylan; Sol. B.W.X., soluble fraction of birchwood xylan; Insol. B.W.X., insoluble fraction of birchwood xylan; CMC, carboxymethylcellulose; Arab., arabinogalactan; Syn. Subs., synthetic substrates (*p*Np-labeled, MUX-labeled). N.A., no activity, i.e. <0.5%.

any of the synthetic (*p*Np- or MU-labeled) substrates tested. Lack of activity on aryl- $\beta$ -glycosides of cellobiose, cellotriose, or cellopentaose confirms the inability of this enzyme to act on  $\beta$ 1,4-glucose chains. Of note is the inability of this enzyme to cleave aryl- $\beta$ -glycosides of X<sub>1</sub>, X<sub>2</sub> or X<sub>3</sub>, under the conditions used, in agreement with the inability of the enzyme to hydrolyze X<sub>2</sub> or X<sub>3</sub> (Table II). Indeed activity on X<sub>4</sub> is also negligible, with minute quantities of X<sub>3</sub> + X<sub>1</sub> being produced only on prolonged incubation (24 h) with high enzyme and substrate concentrations (0.5  $\mu$ M and 10 mM, respectively). The activity of the enzyme on X<sub>5</sub> is extremely low, giving principally X<sub>3</sub> + X<sub>2</sub>; minute quantities of X<sub>4</sub> + X<sub>1</sub> are also formed, but formation is too low to allow accurate determination of its kinetic parameters and can be taken as negligible. The catalytic efficiency on X<sub>6</sub> is ~46-fold higher than on X<sub>5</sub>, with X<sub>3</sub> being preferentially formed as well as low amounts of X<sub>4</sub> + X<sub>2</sub>. At no point were xylo-oligomer products larger than the original substrates detected, demonstrating the absence of transglycosylation reactions and in good agreement with the NMR analysis which showed that this enzyme hydrolyzes with inversion of the anomeric configuration (results not shown). In this study, on addition of enzyme to substrate a rapid increase and subsequent decrease of a doublet at 5.18 ppm, assigned to the  $\alpha$  configura-

tion of the anomeric proton, was observed. The resonance of the  $\beta$  configuration at 4.56 ppm slowly increases due to mutarotation of the initially formed  $\alpha$ -anomeric proton until equilibration of the  $\alpha$  and  $\beta$  forms is eventually reached.

In relation to the xylan substrates, it can be seen that *Palmaria palmata* xylan is the most efficiently (Fig. 5) and extensively (Fig. 6) hydrolyzed, whereas birchwood xylan is more efficiently but less extensively hydrolyzed than oat spelt xylan. Analysis of the products of hydrolysis (Fig. 7) indicates that for all substrates tested, X<sub>3</sub>, X<sub>4</sub>, X<sub>5</sub>, X<sub>6</sub>, and higher xylo-dextrins are initially formed. As the hydrolysis progresses, X<sub>4</sub> and in particular X<sub>3</sub> accumulate, whereas X<sub>5</sub> and X<sub>6</sub> are slowly degraded and eventually, after prolonged digestion (up to 21 days in some cases), giving rise to large quantities of X<sub>3</sub> and X<sub>4</sub> plus low quantities of X<sub>1</sub>, X<sub>2</sub>, and X<sub>5</sub>. In all cases, varying quantities of mixed linkage or substituted compounds were also detected, these depending on the structure of the substrate used. Because of the lack of suitable standards, all compounds could not be identified. Acidic compounds produced from birchwood and oat spelt xylan are probably aldouronic acids with arabinose-substituted xylo-oligomers of varying lengths also being liberated from oat spelt xylan. In the case of *P. palmata* xylan, a mixed linkage IsoX<sub>4</sub> and most probably IsoX<sub>5</sub> and IsoX<sub>6</sub> are produced.

#### DISCUSSION

The isolated *P. haloplanktis* strain displays characteristics typical for a cold-adapted microorganism. Optimal biomass production occurs at 4 °C, in accord with the description of a psychrophile as any organism capable of growing close to 0 °C (39). At temperatures higher than that of the natural environment, higher growth rates do occur but cell development, as measured by cell biomass, and enzyme production and secretion, as measured by xylanase levels in the culture supernatant, are markedly reduced. This is in agreement with previous studies (40), which suggested that this may be due to alterations in the secretory pathway, membrane fluidity, and/or protein synthesis mechanisms. When compared with the mesophilic xylanase from *Streptomyces* sp. S38, the enzyme displays a lower apparent optimum temperature, a lower thermal stability of activity, as well as a lower conformational stability. Such an influence of temperature points to the adaptation of the enzymes to its cold habitat. Indeed it has been proposed that psychrophilic enzymes have a high flexibility that results in improved activity at low temperatures and concomitantly a decreased stability (41). The enzyme has a drastically improved turnover rate at low temperatures (0–40 °C), which is probably its main adaptation strategy. The apparent  $K_m$  value of the enzyme on soluble birchwood xylan is relatively high, as typically  $K_m$  values of between 0.5 and 5 mg/ml are found (2) and suggests that this parameter is not optimized for the cold-adapted xylanase. It should be noted, however, that high  $K_m$  values for xylanases from *Acrophilophora nainana* (42), *Trichoderma reesei* (43), and *Streptomyces* T7 (44) have been reported. Relatively high  $k_{cat}$  and  $K_m$  values have also been found for other extracellular cold-adapted enzymes where optimization of  $k_{cat}$  was the only relevant parameter and indeed is to be expected for enzymes that normally operate in high concentrations of substrate, e.g. digestive enzymes or enzymes from organisms growing on organic debris (39).

The production of only one xylanase by this organism, its inability to utilize xylan as a source of carbon, as well as the digestion products released by the xylanase would suggest that this enzyme does not partake in production of sugars for cellular metabolism by this microorganism. In the Antarctic environment, sources of xylan and of other polysaccharides are extremely limited, with the main source being the cell walls of

TABLE II  
Kinetic parameters for the hydrolysis of xylo-oligomers by the cold-adapted xylanase

Xylo-oligomer	Products	$K_m$	$k_{cat}$	$k_{cat}/K_m$
$X_2$	No hydrolysis	NA <sup>a</sup>	NA	NA
$X_3$	No hydrolysis	NA	NA	NA
$X_4$	$X_3 + X_1$ (slow)	Not determined	Not determined	Not determined
$X_5$	$X_3 + X_2$	$5.8 \pm 0.4$	$0.7 \pm 0.03$	0.12
$X_6$	$2X_3$	$2 \pm 0.3$	$11 \pm 0.7$	5.5

<sup>a</sup> NA, not applicable.

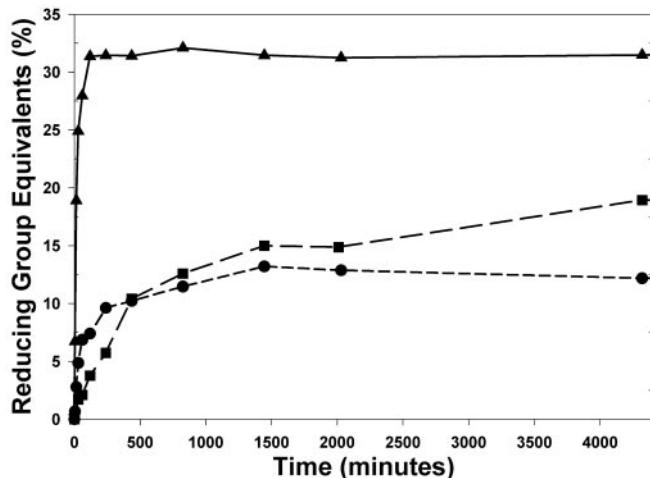


FIG. 6. Release of reducing sugars during hydrolysis of *P. palmata* xylan (triangles), oat spelt xylan (squares) and birchwood xylan (circles) by the cold-adapted xylanase.

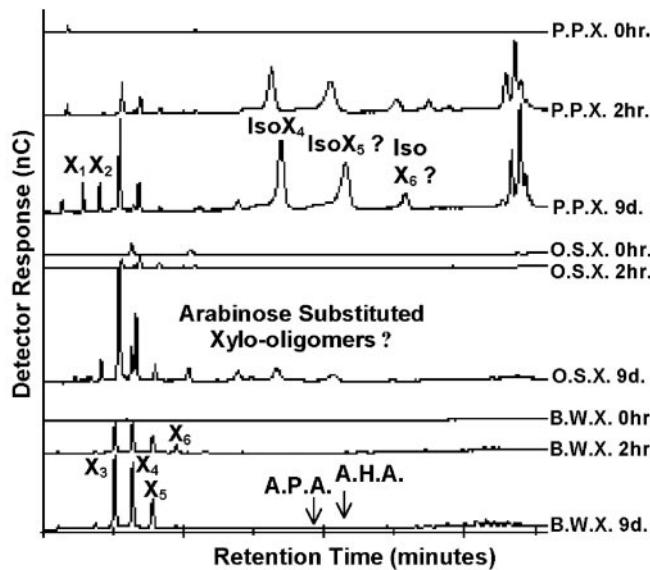


FIG. 7. High performance anion exchange chromatography analysis of the products of hydrolysis of 3% *P. palmata* xylan (P.P.X.), oat spelt xylan (O.S.X.) and birchwood xylan (B.W.X.) by the cold-adapted xylanase after 0 h, 2 h and 9 days of incubation.  $IsoX_4$ , isomeric xylotetraose ( $Xyl\beta 1,3-Xyl\beta 1,4-Xyl\beta 1,4-Xyl$ );  $IsoX_5$ , isomeric xylopentaose;  $IsoX_6$ , isomeric xylohexaose; A.P.A., aldo-pentaouronic acid; A.H.A., aldohexaouronic acid. Xylo-oligomers ( $X_1-X_6$ ), isomeric xylotetraose, aldpentaouronic acid and aldohexaouronic acid determined from retention times of standards.

green and red algae belonging to microphytobenthos (45). It is probable that the xylanase is used to loosen the cell wall structure of algae, thereby allowing better access to the cellulose in the cell walls as well as to the storage polysaccharides.

The xylanase of this study is unique; it has a high molecular weight, in common with most family 10 xylanases, yet it also

has a high pI, typical for family 11 enzymes. The deduced amino acid sequence has no isology with currently identified xylanases of families 10 or 11; however, it does have low similarity with endoglucanases, lichenases, and chitosanases of family 8. As the fold of a particular family is generally conserved among its members, this indicates that the cold-adapted xylanase has an  $(\alpha/\alpha)_6$ -like fold (46), widely different to that of all other known xylanases. In addition, xylanases typically catalyze hydrolysis via a double displacement mechanism in which the anomeric configuration is retained, whereas family 8 enzymes are believed to catalyze hydrolysis with inversion of configuration. Endoglucanase C from *Clostridium cellulolyticum* has been shown to invert the anomeric configuration (47), and in conjunction with the opinion that members of a given family have the same stereoselectivity (25), we have shown that the cold-adapted xylanase also hydrolyzes with inversion of configuration.

Database searches have identified two further xylanases with homology to the investigated xylanase that can also be included as members of family 8. This shows that the cold xylanase is not a unique case and supports the suggestion that the current view, in which xylanases are restricted to families 10 and 11, be revised to include family 8. In addition, database searches also indicate that xylanases have been reported, which can be classified into families 5 and 43; however, apart from xylanase A from *Erwinia chrysanthemi* (8), information, in particular on functional characteristics, is minimal.

Although the enzyme shares isology with endoglucanases, lichenases and chitosanases, it is a true xylanase. The highest activity was found on xylan from *P. palmata*, a linear mixed linkage ( $\beta 1,3$  and  $\beta 1,4$ ) seaweed xylan, and indeed this might be expected as the main source of xylan in the natural environment of this enzyme is probably of algal origin. Our study also shows that the degree of substitution of the substrate influences the activity of the enzyme, with birchwood xylan being the least extensively hydrolyzed due to its high degree of substitution, 15–30% (48, 49), and thus greater steric hindrance. Analysis of the products released from these substrates shows that the cold enzyme generates products similar to those from family 11 enzymes but larger than those from family 10 enzymes. In addition, the cold enzyme liberates  $IsoX_4$  as the shortest mixed linkage fragment from *P. palmata* xylan, in agreement with that shown for family 11 xylanases (16), and on the basis of chromatographic mobility this is suggested to be  $Xyl\beta 1,3-Xyl\beta 1,4-Xyl\beta 1,4-Xyl$ . This indicates that the cold-adapted xylanase can attack the  $\beta 1,4$  linkage that precedes (non-reducing end) a  $\beta 1,3$  linkage but can only cleave the  $\beta 1,4$  linkage two xylosyl residues distal from the reducing end of a  $\beta 1,3$  bond.

The activity on xylo-oligosaccharides was also investigated and it can be seen that here again the enzyme seems to be functionally similar to family 11 xylanases. Based on the catalytic efficiency of  $X_3$  production, it appears that  $X_6$  is the smallest chain length broken rapidly by the enzyme. It is more active on higher xylo-oligosaccharides and appears to have a substrate-binding site of at least six subsites, with the catalytic

site in the middle. In contrast to all tested xylanases (16) but in consonance with the family 8 endoglucanase from *C. thermocellum* (50), the cold-adapted enzyme was not active on aryl- $\beta$ -glycosides of  $X_2$  or  $X_3$ . In conjunction with its inactivity on  $X_2$  and  $X_3$  as well as its slow cleavage of  $X_4$  and  $X_5$ , this suggests that, analogous to the family 8 endoglucanase from *C. thermocellum* (38), the substrate residue at subsite -1 adapts a distorted boat conformation with the energy for this distortion being obtained from that released on substrate binding. It is possible that high subsite occupancy (at least 5 subsites) is required to provide sufficient energy for the substrate to adapt the distorted conformation. However, the fact that principally only  $X_3$  is produced from  $X_6$  indicates that it is also possible that like family 10 and 11 xylanases (51) the subsites adjacent to the catalytic site (*i.e.* -1 and +1) have a negative affinity for monomer units but, unlike other xylanases, the -2 or +2 subsites, or perhaps even both subsites, may also have a negative or weak affinity.

The cold-adapted enzyme is thus unique among xylanases, and in particular at the level of its primary and probably also tertiary structures, it exhibits similarities in its catalytic function to family 11 enzymes while retaining individuality in its stereoselectivity and probably also in its specificity site structure. Further studies, in particular of its three-dimensional structure, should give further information on the structure-function relationship of this novel enzyme.

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