A Novel Family 8 Xylanase: Functional and Physico-chemical Characterization

Tony Collins¹, Marie-Alice Meuwis¹, Ingeborg Stals², Marc Claeyssens², Georges Feller¹ and Charles Gerday¹*

¹ Laboratory of Biochemistry, Institute of Chemistry B6, University of Liège, B-4000 Liège, Belgium
² Department of Biochemistry, Physiology and Microbiology, K.L. Ledeganckstraat 35, University of Gent, B-9000 Gent, Belgium

*Corresponding author: Charles Gerday, Laboratory of Biochemistry, Institute of Chemistry B6, University of Liège, B-4000 Liège, Belgium. Tel.: +32 4 366 33 40; Fax: +32 4 366 33 64; Email: ch.gerday@ulg.ac.be

Running Title: A Novel Family 8 Xylanase
Summary

Xylanases are generally classified into glycosyl hydrolase families 10 and 11 and are found to frequently have an inverse relationship between their pl and molecular mass values. We however have isolated a psychrophilic xylanase which belongs to family 8 and which has both a high pl 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-oligosaccharides. 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 (α/α)₆ fold, distinct from that of other currently known xylanases.
Introduction

Xylanases (EC 3.2.1.8, endo-1,4-β-xylan xylanohydrolase) are O-glycoside hydrolases which catalyze the random hydrolysis of internal β-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, 150–200 units in hardwoods). Depending on the source, the xylan backbone may contain a varying degree of glucuronosyl, 4-O-methyl-D-glucuronopyranosyl, α-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-β-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 (> 30Kda) and low pl or a low molecular mass (<30Kda) and high pl. It has been found that family 10 generally groups acidic high molecular mass enzymes while family 11 members are generally much smaller basic proteins, although acidic pl’s 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 (α/β)₈ barrel fold (12), belong to clan GH-A, the 4/7 superfamily, (13) and have approximately 40% of the secondary structure in an α-helical form (14). Members of family 11 are approximately 3-5% α helical in nature, have a β-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 to 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 anomic 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 which belongs to glycoside hydrolase family 8 (formerly D), a family which is mainly comprised of endoglucanases (EC 3.2.1.4), but also lichenases (EC 3.2.1.73) and chitosanases (EC 3.2.1.132) and which typically operate with inversion of anomic configuration. The cloning, overexpression, purification and characterization of this novel xylanase as well as the 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 (5g/l tryptone (Difco), 1g/l yeast extract (Difco), 33g/l marine salts (Wiegandt), 18g/l agar (Difco)) supplemented with 0.15% RBB-xylan at 4°C.

Microscopic identification, Gram staining and fatty acid methyl ester fingerprint analysis were used to identify the isolate. Cellular fatty acids were extracted and quantified by gas-liquid chromatography and identified using the Microbial Identification System (MIS) 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 optical density (O.D.550nm) 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, salts) were also examined.

Production and Purification of Wild-Type Xylanase.

The Antarctic isolate was cultivated in 1 liter of modified marine broth (5g/l tryptone (Difco), 1g/l yeast extract (Difco), 20g/l marine salts) supplemented with 1.5% birchwood xylan for 72 hours at 4°C. After centrifugation for 1 hour at 18000 g and 4°C the supernatant was concentrated to approximately 100ml by ultrafiltration on a Millipore PBGC 10000 NMWL membrane and dialysed against buffer A (50mM BICINE, 10mM NaCl, pH 8.5). The dialysate was loaded on a Q-Sepharose Fast Flow (Pharmacia) column (9 x 2.5cm) equilibrated in the same buffer and the
void was collected and immediately loaded on a S-Sepharose Fast Flow (Pharmacia) column (7 x 2.5cm), also equilibrated in the above mentioned buffer, and eluted with a linear NaCl gradient (0-100mM in 350ml).

**Cloning of the Cold-Adapted Xylanase Gene.**

The psychrophilic xylanase gene was isolated and sequenced using a procedure similar to that described (20), (21). Degenerate primers based on the amino-terminal sequence (5’-GCIT TYAAAYAYAYCC-3’) and an internal peptide sequence determined after CNBr cleavage (see Analytical Procedures) (5’-RTTRTTAICCRAACAT-3’) allowed amplification of a 101bp fragment from the genomic DNA. The complete xylanase gene sequence was identified from an enriched PstI (3 – 6Kb fragments) genomic library in pUC19 by PCR screening using specific primers derived from the 101bp isolated sequence, (5’-CGTTTAATAAATACCATCGAGTGTAG GC-3’ and 5’-GTTTAGCCGAACATATTGTCAAAAGTACTAT-3’). Double stranded sequencing was carried out with an ALF DNA sequencer (Amersham, Pharmacia).

**Overexpression of the Cold-Adapted Xylanase.**

The xylanase gene, including its signal sequence, was PCR amplified using Pwo polymerase (Eurogentec), with the sense primer ( 5’-GGGCATATGAAAGTATTTTTAAACTT-3’) containing an Nde I site (underlined) and the antisense primer (5’-GCTACTTAGGTAGTTTAATTAAACGTGTTGTTATAA-3’) containing the stop codon (underlined). The PCR product was cloned into the PCR-Script Amp SK(+) cloning vector (Stratagene), excised with Ndel and XhoI and ligated into the pET22b(+) cloning vector (Novagen). The resulting recombinant plasmid was transformed to *E.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 10000 g for 1 minute and the pellet was resuspended in 300ml Terrific broth (12g/l Bacto tryptone (Difco), 24g/l yeast extract (Difco), 4ml/l glycerol, 12.54g/l K$_2$HPO$_4$, 2.31g/l KH$_2$PO$_4$) containing 200µg/ml ampicillin in a 3 liter shake flask. The culture was incubated at 18°C and 250 rpm until an absorbance at 550nm of between 3 – 4 was reached whereupon the expression of the enzyme was induced with 1mM isopropyl-1-thio-β-galactopyranoside. Following 20 hours further incubation at 18°C the cells were harvested by centrifugation at 18000 g for 30 minutes at 4°C, resuspended in modified buffer A (no NaCl), disrupted in a prechilled cell disrupter (Constant Systems Ltd.) at 28Kpsi, centrifuged at 40000 g and dialyzed against modified buffer A (no NaCl). The dialysate was then subjected to Q-Sepharose Fast Flow (50 x 2.5cm) 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 10000 NMWL) and further purified on a Sephacryl S-100 (Pharmacia) column (90 x 2.5cm) equilibrated in 20mM MOPS, 100mM NaCl, pH 7.5 at 1mlmin$^{-1}$.

**Physico-chemical Characterization.**

The 20,585Da mesophilic Family 11 xylanase (Xyl1) from *Streptomyces* sp. S38 was obtained from J. Georis (22). The temperature optimum was determined by monitoring activity over 5 minutes 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 20mM MOPS, 100mM NaCl, 500mM 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 – 30mg/ml soluble birchwood xylan. 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 – 12 using the following buffer mix: 40mM sodium acetate, 20mM MES, 20mM MOPS, 20mM TAPS, 20mM CHES and 20mM CAPS. The effect of various heavy metals, chelators and ions was examined by determining the activity in the presence of 1mM and 10mM of the various compounds.

Differential scanning calorimetry (DSC) was carried out in a MicroCal MCS-DSC apparatus (Microcal) using the MCS Observer software package for data acquisition, analysis and deconvolution. Analysis was carried out with 2mg/ml of recombinant protein in 20mM MOPS, 100mM NaCl, 500mM 3-(1-Pyridinio)-1-propanesulfonate, pH 7.5 at a scan rate of 1°C/min and under 1atm. nitrogen pressure. Due to irreversibility of the denaturation only the apparent Tm (melting temperature) and apparent Hcal (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 dinitrosalicylic acid method as described (23), 3% soluble birchwood xylan (24) in 100mM citrate-acetate 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 in (26) and 43.5µM of the enzyme, lyophilized and redissolved twice in D2O were used. Proton NMR spectra were recorded with a Bruker Digital NMR Avance 500 spectrometer at 25°C using a 5mm sample tube. Spectra were recorded immediately after mixing and at 15 minute intervals for 1 hour.

Activity on birchwood xylan, beechwood xylan, oat spelt xylan, CM-cellulose (Fluka), microcrystalline cellulose (Aldrich), cellobiose, arabinogalactan (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-acetate 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 hours with a Brookfield LVDV viscometer. Chitoclear™ low viscosity (3% acetylation) and FC222B (17% acetylation) chitosan (Primex Ingredients) were examined, 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 5mM.

To determine the extent of hydrolysis of birchwood, oat spelt and Palmaria palmata xylan, 25nM 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 minutes, filtered through a 0.45µm 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 x 250mm) was equilibrated at 1ml min$^{-1}$ in 100mM NaOH, 20mM sodium acetate and product separation was obtained with a four step linear gradient: 20 – 40mM sodium acetate in 20ml, 40 – 100mM sodium acetate in 10 ml, 100 – 160mM sodium acetate in 10 ml and 160 – 200mM sodium acetate in 1ml. Xylo-oligosaccharides, $X_1$ – $X_6$ (Megazyme), aldopentaouronic and aldohexaouronic acid...
(Megazyme) and mixed linkage ($\beta1,4;\beta1,3$) standards, IsoX$_3$ and IsoX$_4$, 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 - 40mM sodium acetate in 20 ml. Reactions were
carried out at 25°C using 0-10mM X$_1$, X$_2$, X$_3$, X$_4$, 0 – 6.3mM X$_5$ and 0 – 3.8mM X$_6$, kinetic
parameters were determined by non-linear regression of initial rates of product formation using
the Michaelis-Menten equation.

The action of 0.2\muM enzyme on 1mM MU-xylobioside, 1mM MU-xylotrioside, 100\muM MU-
cellotrioside and 100\muM 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
280nm using an extinction coefficient of 95,420 M$^{-1}$cm$^{-1}$ and 62,930 M$^{-1}$cm$^{-1}$ for the
psychrophilic and mesophilic xylanase, respectively. Analytical SDS-PAGE was run essentially
as described by the instrument supplier (Hoefer Scientific). 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 approximately 20 – 30pmol of
protein. CNBr cleavage of the protein was carried out overnight under atmospheric nitrogen in
70% formic acid with 2% (v/v) 5M CNBr (Fluka). Resulting fragments were separated on 17.5%
SDS-PAGE, electroblotted on a PVDF membrane and subjected to amino-terminal sequencing.
Carboxy-terminal sequencing was performed on a Procise 494C Sequencer (PE Biosystems)
with 1nmol enzyme as described (31). The molecular mass was determined by nano-
electrospray ionization spectrometry on a Q-TOF mass spectrometer (Micromass) with 20pmol
of enzyme.
**Construction of Evolutionary Tree.**

Family 8 members were identified from the carbohydrate-active enzymes server (http://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, and the tree was constructed with the Drawtree program (33) by application of the neighbor-joining method (34) to multiple alignments from the CLUSTAL W 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 being proposed. Figure 1 shows that this bacterium has typical cold-adapted characteristics as xylanase production and stability are much higher at 4°C than at 28°C. Furthermore, the optical density at 28°C drops rapidly to zero, indicating cell lysis at this temperature.

Optimal growth occurs in marine broth containing high concentrations of marine salts (20g/l) while 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 pl (pH 9.5) of the extracellular enzyme resulted in an 11 fold purification with a final yield of 55%, equivalent to 1.9
mg of pure xylanase per litre of culture. Only one xylanase was detected for this organism. Low concentrations of salts (10mM 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 Da.

**Cloning and Sequence Analysis of Recombinant Xylanase.**

PCR screening allowed isolation of one clone containing a fragment of approximately 11 Kb. An open reading frame of 1278 bp encoding a protein of 426 amino acids was identified (Table I). N-terminal and C-terminal sequencing as well as analysis using the SignalP V1.1 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 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 (Glu78, Trp124, Ala142, Asp144, Ala150, Ala156, Trp225, Arg284, 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 (Glu146 in xylanases – Asp) while the other is substituted by an unconserved residue (Val121 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, B and C group endoglucanases from Gram negative proteobacteria. Subclass A groups enzymes from bacteria belonging to the alpha proteobacterial subdivision while subclasses B and C group, respectively, enzymes from aerobes (E. coli, Salmonella, Pseudomonas) or facultative anaerobes (Erwinia, Pectobacteria) belonging to the gamma proteobacterial subdivision. Subclass D contains xylanases from Gram negative or positive bacteria while subclasses E and F contain enzymes from Gram positive bacteria. Subclass E groups endo-1,4-glucanases, from Clostridia (strict anaerobes) whereas sub-class F groups endo-1,4-glucanases, lichenases and chitosanases from Bacilli (aerobes, 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 approximately 85mg of the enzyme per liter. The protein was purified with a procedure similar to 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 58mg/l 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 $k_{cat}$ of the cold xylanase at 25°C are relatively high (2). Heavy metals such as Hg$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ were found to be inhibitory to activity, whereas Mg$^{2+}$, Ca$^{2+}$, Na$^+$, K$^+$, PO$_4^{3-}$ and Cl$^-$ as well as chelating agents (EDTA, EGTA) had no effect.
The thermostability and thermodependence of activity of the isolated xylanase are compared to that of a mesophilic xylanase in figures 3 and 4. The cold enzyme shows a shift in apparent optimal activity of approximately 25°C towards low temperatures. Activity at 5°C is 60% of the maximum, compared to 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°C versus 63.1°C) and a 12 times shorter half life at 55°C (1.9 versus 23 minutes).

**Functional Characterization**

Figure 5 shows that the cold enzyme is a true xylanase, no cellulase (endo-1,4-beta-glucanase), cellobiase, lichenase (endo-beta-1,3-1,4-glucanase), chitosanase (chitosan N-acetylglucosaminohydrolase), laminarinase (endo-1,3-beta-glucanase) or amylase (1,4-alpha-D-glucan-glucohydrolase) activity was detected. In addition, no activity was detected on any of the synthetic (pNp or MU labeled) substrates tested. Lack of activity on aryl-β-glycosides of cellobiose, cellotriose or cellopentaose confirms the inability of this enzyme to act on β1,4 glucose chains. Of note is the inability of this enzyme to cleave aryl-β-glycosides of X1, X2 or X3, under the conditions used, in agreement with the inability of the enzyme to hydrolyze X2 or X3 (Table II). Indeed activity on X4 is also negligible, with minute quantities of X3 + X1 being produced only on prolonged incubation (24 hours) with high enzyme and substrate concentrations (0.5µM and 10mM, respectively). The activity of the enzyme on X5 is extremely low, giving principally X3 + X2, minute quantities of X4 + X1 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 X6 is approximately 46 fold higher than on X5, with X3 being preferentially formed as well as low amounts of X4 + X2. 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 \( \alpha \) configuration of the anomeric proton, is 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 (figure 5) and extensively (figure 6) hydrolyzed while birchwood xylan is more efficiently but less extensively hydrolyzed than oat spelt xylan. Analysis of the products of hydrolysis (figure 7) indicates that for all substrates tested \( X_3 \), \( X_4 \), \( X_5 \), \( X_6 \) and higher xylodextrins are initially formed. As the hydrolysis progresses, \( X_4 \) and in particular \( X_3 \) accumulate while \( X_5 \) and \( X_6 \) are slowly degraded, eventually, after prolonged digestion (up to 21 days in some cases), giving rise to large quantities of \( X_3 \) and \( X_4 \) plus low quantities of \( X_1 \), \( X_2 \) and \( X_5 \). In all cases, varying quantities of mixed linkage or substituted compounds were also detected, these depending on the structure of the substrate used. Due to 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 *Palmaria palmata* xylan, a mixed linkage \( \text{Iso}X_4 \) and most probably \( \text{Iso}X_5 \) and \( \text{Iso}X_6 \) are produced.

**Discussion**

The isolated *Pseudoalteromonas haloplanktis* strain displays characteristics typical for a cold adapted micro-organism. 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 to 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 enzymes adaptation to its cold habitat. Indeed it has been proposed that psychrophilic enzymes have a high flexibility which 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 $K_m$ of the enzyme on soluble birchwood xylan is relatively high, as typically $K_m$ values of between 0.5 – 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 Acrophiulophora 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 micro-organism. In the Antarctic environment, sources of xylan and of other polysaccharides are extremely limited, with the main source being the cell walls of 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 pl, 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 while 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 and which 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, are minimal.

Although the enzyme shares isology with endoglucanases, lichenases and chitosanases, it is a true xylanase. Highest activity was found on xylan from *Palmaria palmata*, a linear mixed linkage (β1,3) (β1,4) 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 IsoX₄ as the shortest mixed linkage fragment from *Palmaria 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[β1,3-Xyl]β1,4-Xyl[β1,4-Xyl]. This indicates that the cold-adapted xylanase can attack the β1,4 linkage that precedes (non-reducing end) a β1,3 linkage but can only cleave the β1,4 linkage two xylosyl residues distal from the reducing end of a β1,3 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 X₃ production, it appears that X₆ 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 *Clostridium thermocellum* (50) the cold-adapted enzyme was not active on aryl-β-glycosides of X₂ or X₃. This, in conjunction with its inactivity on X₂ and X₃ as well as its slow cleavage of X₄ and X₅.
suggests that, analogous to the family 8 endoglucanase from *Clostridium 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 X₃ is produced from X₆ 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, 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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Footnotes

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The nucleotide sequence reported in this paper has been submitted to the EMBL Nucleotide Sequence Database with accession number AJ427921.

The abbreviations used are: pNp, para-nitrophenol; $X_1$, xylose; $X_2$, xylobiose; $X_3$, xylotriose; $X_4$, xylotetraose; $X_5$, xylopentaose; $X_6$, xylohexaose; Iso$X_3$, isomeric xylotriose; Iso$X_4$, isomeric xylotetraose; Iso$X_5$, isomeric xylopentaose; Iso$X_6$, isomeric xylohexaose; MU, 4-methylumbelliferyl.
Figure Legends

**Figure 1:** Growth curves as monitored by optical density (open symbols) and xylanase activity in the culture supernatant (closed symbols) of the *Pseudoalteromonas haloplanktis* strain at 4°C (circles, solid lines) and 28°C (squares, dashed lines).

**Figure 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 number (if known) are as follows: AAD28576 (*Rhizobium leguminosarum*, n.d.), AAC41433 (*Agrobacterium tumefaciens*, n.d.), AAK90085 (*Agrobacterium tumefaciens* C58, n.d.), BAA31461 (Acetobacter xylinus BPR2001, 3.2.1.4), AAA16969 (*Acetobacter xylinus* ATCC 23769, 3.2.1.4), AAA23090 (*Cellulomonas uda*; 3.2.1.4), CAB89803 (*Erwinia rhapontica* NCPPB2989, 3.2.1.4), AAG49556 (*Pectobacterium chrysanthemi* PY35, 3.2.1.4), AAA24818 (*Erwinia chrysanthemi* 3937, 3.2.1.4), AAC07361 (*Aquifex aeolicus*, n.d.), AAC27700 (Bacillus sp. KK-1, 3.2.1.8), AJ427921 (*Pseudoalteromonas haloplanktis* TAH 3a, 3.2.1.8), BAB05824 (*Bacillus halodurans* C-125, 3.2.1.8), AAA83521 (*Clostridium thermocellum* NCIB 10682 / JW20, 3.2.1.4), AAA73867 (*Clostridium cellulolyticum*, 3.2.1.4), BAA04078 (*Clostridium josui*, 3.2.1.4), CAA37062 (*Bacillus circulans* WL-12, 3.2.1.132, 3.2.1.73), BAB64835 (*Bacillus* sp. D-2, 3.2.1.132), BAB19277 (*Bacillus* sp. No.7-M, 3.2.1.132), AAA22409 (Bacillus sp. KSM-330, 3.2.1.4), AAL71844 (*Pseudomonas fluorescens* SBW25, n.d.), CAD08008 (*Salmonella enterica* subsp. *Enterica* serpvar *Typhi* CT18, n.d.), CAC44017 (*Salmonella typhimurium* ATCC 14028, n.d.), AAL69395 (*Salmonella typhimurium* UR1, n.d.), AAL22477 (*Salmonella typhimurium* LT2, n.d.), AAG58673 (*Escherichia coli* O157:H7 EDL933, n.d.), AAB18508 (*Escherichia coli* K12 / MG1655, 3.2.1.4); n.d.: not determined; Xyl.: Xylanase.
subclass.

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

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

**Figure 5:** Activity of the cold-adapted xylanase on various substrates. Expressed as a percentage of the activity on *Palmaria palmata* xylan. P.P.X.: *Palmaria 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 (pNp labeled, MUX labeled). N.A.: No Activity i.e. < 0.5%.

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

**Figure 7:** HPAE-PAD analysis of the products of hydrolysis of 3% *Palmaria palmata* xylan (P.P.X.), oat spelt xylan (O.S.X.) and birchwood xylan (B.W.X.) by the cold-adapted xylanase.
after 0 hours, 2 hours and 9 days incubation. IsoX4 : Isomeric xylotetraose (Xylβ1,3-Xylβ1,4-
Xylβ1,4-Xyl); IsoX5 : Isomeric xylopentaose; IsoX6 : Isomeric xylohexaose; APA. :
alnopentaouronic acid, A.H.A. : aldohexasuronic acid, Xylo-oligomers (X1 – X6), IsoX4, APA
and A.H.A. determined from retention times of standards.

**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) | 45,981.81Da |
| Molecular Weight (Mass Spectrometry) | 45,982.35 Da |
| Isoelectric Point | Approx. pH 9.5 |
| pH Optimum (25°C) | pH 5.3 – 8 |
| pH Stability Range (25°C) | pH 6 - 9 |
| Temperature Optimum (5 minute assay) | 35°C |
| Apparent **Tm** (scan rate : 1°C/min) | 53.5°C |
| **Hcal** Molar (scan rate : 1°C/min) | 252.8 kcal/mol |
| **Hcal** Specific (scan rate : 1°C/min) | 0.627 kcal/residue |
| Apparent **Km** (25°C, Soluble BWX) | 28 ± 4.5 mg/ml |
| Apparent **kcat** (25°C, Soluble BWX) | 1247 ± 120 s⁻¹ |
| Inhibitors (% inhibition at 1mM) | Hg²⁺(31%), Cu²⁺(16%), Zn²⁺(14%), Ni²⁺(6%) |
| Stereoselectivity | Inverting |

**Tm**: melting temperature; **Hcal**: calorimetric enthalpy of unfolding; BWX: birchwood xylan.
Table II: Kinetic parameters for the hydrolysis of xylo-oligomers by the cold-adapted xylanase.

| Xylo-oligomer | Products          | $K_m$ (mM) | $k_{cat}$ (sec$^{-1}$) | $k_{cat}/K_m$ (sec$^{-1}$mM$^{-1}$) |
|---------------|-------------------|------------|------------------------|----------------------------------|
| $X_2$         | No Hydrolysis     | N.A.       | N.A.                   | N.A.                             |
| $X_3$         | No Hydrolysis     | N.A.       | N.A.                   | N.A.                             |
| $X_4$         | $X_3 + X_1$ (slow)| Not Determined | Not Determined | Not Determined                  |
| $X_5$         | $X_3 + X_2$       | 5.8 ± 0.4  | 0.7 ± 0.03             | 0.12                             |
| $X_6$         | 2$X_3$            | 2 ± 0.3    | 11 ± 0.7               | 5.5                              |

N.A.: Not Applicable
Arabinose Substituted Xylo-oligomers?
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