Cloning, sequencing and expression of the cDNA of endoxylanase B from *Penicillium purpurogenum*

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Abstract

The cDNA for xylanase B from *Penicillium purpurogenum* was cloned and sequenced. This DNA encodes a protein of 208 amino acids which is expected to yield a protein of 183 residues upon processing of the N terminus. The sequence of the predicted protein is very similar to that of 40 other xylanase domains which belong to family G of cellulases/xylanases (73–21% identity).

Keywords: Endoxylanase; *P. purpurogenum*; Nucleotide sequence; Glycosyl hydrolase; PCR; Sequence alignment; Homology comparison; Codon usage

1. Introduction

The soft-rot fungus *Penicillium purpurogenum* is an active producer of cellulases and xylanases (Steiner et al., 1994). The fungus secretes to the medium several endoxylanases, two of which (called endoxylanases A and B) have been purified and characterized (Belancic et al., 1995). These two enzymes show differences in molecular weight, p*I*, amino terminal sequence and lack antiserum cross-reactivity. These findings suggest that both enzymes are the product of different genes (Belancic et al., 1995). In this work, a cDNA library was prepared, a clone carrying cDNA coding for endoxylanase B was isolated and sequenced, and the cDNA was expressed in *E. coli*.

(a) S I N Y V Q N Y N G N L G A F S Y N E G A G T F S

(b) N Y Q V M A V E

(c) Left primer:

5' YTA-YGT-NCA-RAA-YTA-YAA-YGG 3' (256 degenerations)

Right primer:

5' CVA-CNG-CCA-TVA-CCT-GGT-AGT 3' (36 degenerations)

Fig. 1. Design of PCR primers. (a) N terminal amino acid sequence of XynB (Belancic et al., 1995). (b) Sequence from xylanase C from *A. kawachii* (Ito et al., 1992) used to design the right primer. This sequence is highly conserved, as found by alignment of several xylanases using the program CLUSTAL (Higgins and Sharp, 1988). (c) Sequences of the degenerate primers (N = A, C, G or T; R = A or G; Y = C or T; V = A, C or G). **Methods**: Oligonucleotide primers for PCR were synthesized by Bios Chile. PCR reactions (100 µl) were performed in an MJ Research thermocycler. The amplification conditions were: denaturation for 1 min at 94°C followed by 35 amplification cycles of 1 min at 94°C, 1 min at 48°C and 1 min at 72°C, and a final elongation of 10 min at 72°C. Products were separated by agarose gel electrophoresis; a product of about 480 bp was purified from the gel with Glassmax (GIBCO-BRL). This DNA was labeled with biotin using the BioNick (GIBCO-BRL) system and used as a probe to screen the cDNA library.

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Abbreviations: *A.*, *Aspergillus*; aa, amino acids; *B.*, *Bacillus*; bp, base pair(s); *C.*, *Cellvibrio*; cDNA, complementary DNA; *E.*, *Escherichia*; *H.*, *Humicola*; kb, kilobase(s) or 1000 bp; nt, nucleotide (s); ORF, open reading frame; *P.*, *Penicillium*; PCR, polymerase chain reaction; *S.*, *Schyzophyllum*; *T.*, *Trichoderma*; *xynB*, gene encoding endoxylanase B from *P. purpurogenum*; XynB, endoxylanase B.

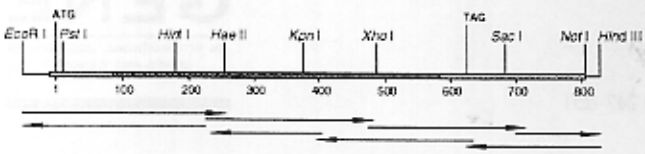

Fig. 2. Sequencing strategy for the full length cDNA of *P. purpurogenum xynB*. The shaded area represents the cDNA. The thin line represents vector sequence. The dark shaded area shows the region covered by the PCR fragment used as probe to screen the cDNA library. The extension and orientation of sequence obtained with various primers are indicated by arrows. Some unique restriction sites are also indicated.

2. Experimental and discussion

2.1. Isolation of cDNA clones encoding endoxylanase B

Degenerate PCR primers were designed as follows: the left primer was based on the amino terminal sequence of XynB (Belancic et al., 1995) and the right primer was derived from a highly conserved region from four family G endoxylanase sequences (Fig. 1). A PCR product of about 480 bp was purified and used to screen a cDNA library of *P. purpurogenum* for clones containing the *xynB* gene.

Table 1
Codon usage of *Penicillium purpurogenum xynB*

Ala	GCT	6	Gly	GGT	8	Pro	CCT	3
	GCC	6		GGC	11		CCC	1
	GCA	4		GGA	5		CCA	1
	GCG	2		GGG	0		CCG	1
Arg	CGT	0	His	CAT	1	Ser	TCT	7
	CGC	0		CAC	1		TCC	5
	CGA	3					TCA	1
	CGG	1	Ile	ATT	1		TCG	6
	AGA	0		ATC	2		AGT	3
	AGG	1		ATA	1		AGC	8
Asn	AAT	2	Leu	TTA	1	Thr	ACT	5
	AAC	16		TTG	1		ACC	6
				CTT	2		ACA	3
Asp	GAT	2		CTC	2		ACG	7
	GAC	2		CTA	1			
				CTG	1	Trp	TGG	4
Cys	TGT	0						
	TGC	2	Lys	AAA	0	Tyr	TAT	3
				AAG	1		TAC	11
Gln	CAA	3						
	CAG	6	Met	ATG	2	Val	GTT	5
							GTC	9
Glu	GAA	1	Phe	TTT	1		GTA	2
	GAG	4		TTC	10		GTG	4

The numbers indicate the frequency of use of each codon for a total of 208 amino acids.

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-10   ATCAATCATCATGAAGGTCACTGCAGCTTTCGCAGGCCTCTTGGCACGACACTCGCCG
  1              M  K  V  T  A  A  F  A  G  L  L  A  R  H  S  P

 49  CCCCTGTCCACCGAGCTAGTGACGCGAAGTATCAACTACGTCCAGAACTACAACGGCAAC
 17  P  L  S  T  E  L  V  T  R  S  I  N  Y  V  Q  N  Y  N  G  N

109  CTTGGTGCCTTTAGCTACAACGAGGGTGCCGGAACATTCTCTATGTACTGGCAGCAAGGA
 37  L  G  A  F  S  Y  N  E  G  A  G  T  F  S  M  Y  W  Q  Q  G

169  GTCAGCAACGATTTCGTTGTTGGTTTAGGCAGGAGCACTGGTTCCTCTAACCCTATCACC
 57  V  S  N  D  F  V  V  G  L  G  R  S  T  G  S  S  N  P  I  T

229  TACTCTGCCTCCTATAGCGCCTCTGGTGGCTCGTACCTCGCTGTGTACGGCTGGGTCAAC
 77  Y  S  A  S  Y  S  A  S  G  G  S  Y  L  A  V  Y  G  W  V  N

289  TCTCCTCAGGCTGAATACTACGTTGTCGAGGCCTATGGTAATTACAACCCTTGCAGCTCG
 97  S  P  Q  A  E  Y  Y  V  V  E  A  Y  G  N  Y  N  P  C  S  S

349  GGCTCGGCTACAAACCTTGGTACCGTGTCCTCTGATGGAGGCACCTACCAAGTCTGCACC
117  G  S  A  T  N  L  G  T  V  S  S  D  G  G  T  Y  Q  V  C  T

409  GACACTCGGGTTAACCAGCCATCGATAACGGGAACGAGCACGTTCACGCAATTCTTCTCC
137  D  T  R  V  N  Q  P  S  I  T  G  T  S  T  F  T  Q  F  F  S

469  GTTCGACAGGGCTCGCGCACATCTGGAACGGTGACTATTGCCAACCATTTCAACTTCTGG
157  V  R  Q  G  S  R  T  S  G  T  V  T  I  A  N  H  F  N  F  W

529  GCGAACGACGGCTTCGGCAACAGCAACTTCAATTATCAGGTCGTAGCGGTAGAGGCATGG
177  A  N  D  G  F  G  N  S  N  F  N  Y  Q  V  V  A  V  E  A  W

589  AGTGGTACTGGCACCGCTAGTGTCACGGTCTCAGCTTAGGAGATTAGGAGATGAGGGGCT
197  S  G  T  G  T  A  S  V  T  V  S  A  *

649  TAGTGGTCAAAAAACTTGAAAGCCGAGCTCTCTTGCAAGTGGTGTGATGATCGACTAGGG

709  GTTTAGCAATTTCAAACGTCGAGACCGTATATAGTGTGGCGGGGCGGACGTGTCGCCGGT

769  CGCGTTTTCAACTCACACAGAAAAAAAAAAAAAAAAAA
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Fig. 3. Nucleotide and deduced aa sequences of the cDNA of *P. purpurogenum xynB*. The N-terminal aa sequence of the mature enzyme obtained by protein sequencing is underlined. The presumed signal peptide is indicated with a dotted underline. Putative catalytic residues E101 and E194 are shown in bold. The nucleotide sequence data reported here have been deposited in the EMBL/Genbank/DDBJ databases under accession No. Z50050. **Methods**: DNA manipulations were carried out using standard methods (Sambrook et al., 1989). The fungus was grown in liquid medium (Belancic et al., 1995) on oat spelts xylan (1% w/v) at 28°C and 200 rpm for 5 days. Total RNA was prepared and mRNA was obtained by oligo(dT) cellulose chromatography. cDNA was synthesized with the Superscript Preamplification System (GIBCO-BRL) using an oligo(dT)-adaptor which provides a *Not*I site. Double-stranded cDNA was generated and *Sal*I adaptor arms were ligated; digestion with *Not*I afforded asymmetric ends. This DNA was ligated to λZiplox arms, subjected to in vitro packaging using the Lambda Packaging system (GIBCO-BRL) and used to infect *E. coli* Y1090(ZL). Approximately 20 000 recombinant clones were obtained. Phage plaques were transferred to Photogene membrane. The cDNA phage library was screened by plaque hybridization with the probe described in Fig. 1 using the Photogene detection system (GIBCO-BRL). Positive plaques were used to infect *E. coli* DH10B(ZIP) to generate colonies. Plasmids, derived from the phage vector by self excision, were purified and sequenced. The cDNA was entirely sequenced on both strands by the chain termination method (Sanger et al., 1977) using the Sequenase v.2.0 (US Biochemical) sequencing kit.

2.2. Sequencing of the xynB gene

The DNA for *P. purpurogenum* XynB contained in one of the clones was entirely sequenced on both strands. The sequencing strategy employing M13 primers and specific primers is shown in Fig. 2. The cDNA insert was 816 bp long. An open reading frame encoding a product of 208 amino acids was identified. The nucleo-

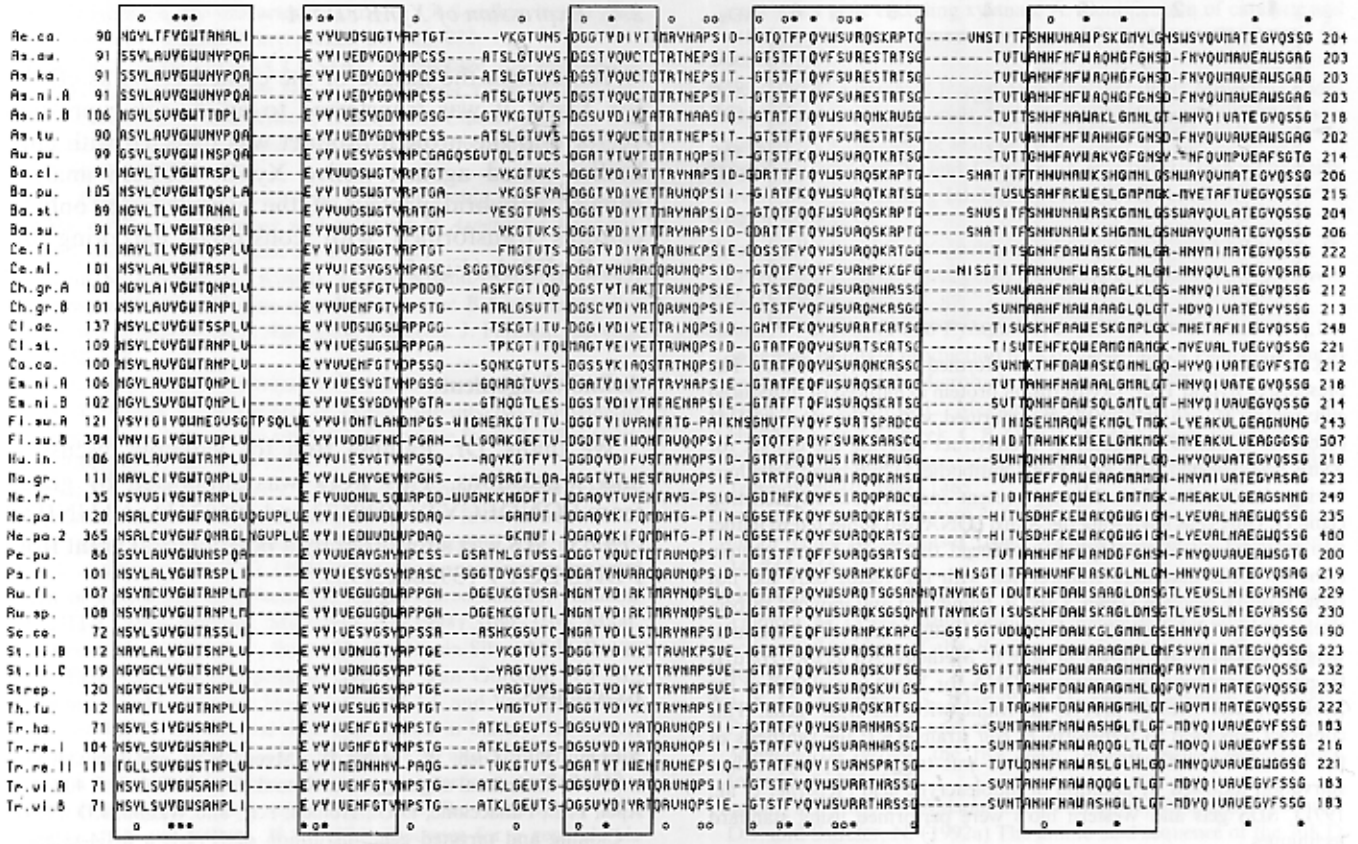

Fig. 4. Alignment of residues 86–200 of XynB with other members of the G family of endoxylanases. The regions of highest homology are boxed. Residues which are entirely conserved are shown in bold and marked by a black circle; residues which are conserved in at least 90% of the 41 sequences are indicated by an open circle. *Aeromonas caviae* (Ae.ca.) xylanase I (D32065); *A. awamori* (As.aw.) EXLA (Hessing et al., 1994); *A. kawachii* (As.ka.) XYNC (Ito et al., 1992); *A. niger* (As.ni.) XYLA (Maat et al., 1992) and XYLB (Kinoshita et al., 1995); *A. tubigensis* (As.tu.) XLNA (De Graaff et al., 1994); *Aureobasidium pullulans* (Au.pu.) (Li and Ljungdahl, 1994); *B. circulans* (Ba.ci.) XLNA (Yang et al., 1988); *B. pumilus* (Ba.pu.) XYNA (Fukusaki et al., 1984); *B. stearothermophilus* (Ba.st.) XYNA (U15985); *B. subtilis* (Ba.su.) XYNA (Paice et al., 1986); *Cellulomonas fimi* (Ce.fi.) xylanase D (Millward-Sadler et al., 1994); *C. mixtus* (Ce.mi.) XynA (Millward-Sadler et al., 1995); *Chaetomium gracile* (Ch.gr.) CgXA and CgXB (Yoshino et al., 1995); *Clostridium acetobutylicum* (Cl.ac) XYNB (Zappe et al., 1990); *Clostridium stercorarium* (Cl.st.) XYNA (Sakka et al., 1993); *Cochliobolus carbonum* (Co.ca.) XYL1 (Apel et al., 1993); *Emericella nidulans* (Em.ni.) XYLA (Z49892) and XYLB (Z49893); *Fibrobacter succinogenes* (Fi.su.) XYNC (domains A and B) (Paradis et al., 1993); *H. insolens* (Hu.in.) XYL1 (Dalboge and Heldt-Hansen, 1994); *Magnaporthe grisea* (Ma.gr.) (Wu et al., 1995); *Neocallimastix frontalis* (Ne.fr.) XYN2 (X82439); *Neocallimastix patriciarum* (Ne.pa.) XYNA (domains 1 and 2) (Gilbert et al., 1992); *Pseudomonas fluorescens* (Ps.fl.) XYLE (Millward-Sadler et al., 1995); *Ruminococcus flavefaciens* (Ru.fl.) XYNA (Zhang and Flint, 1992); *Ruminococcus* sp. (Ru.sp.) xylanase 1 (Z49970); *S. commune* (Sc.co.) XYNA (Oku et al., 1993); *Streptomyces lividans* (St.li) XLNB and XLNC (Shareck et al., 1991); *Streptomyces* sp. (Strp.) EC3 (X81045); *Thermomonospora fusca* (Th.fu.) TfxA (Irwin et al., 1994); *T.harzianum* (Tr.ha.) (Yaguchi et al., 1992a); *T. reesei* (Tr.re.) XYNI and XYNII (Törrönen et al., 1992); *T. viride* (Tr.vi.) IIA (Yaguchi et al., 1992b) and IIB (A44595). Accession numbers are given for unpublished entries. **Methods**: The similarity search was performed using the BLASTP algorithm (Altschul et al., 1990). The boxes of greatest similarity were identified with the program MATCH-BOX (Depiereux and Feytmans, 1991, 1992).

tide sequence and the predicted amino acid sequence are shown in Fig. 3. The location of the sequence of the N-terminal peptide, which was entirely confirmed, predicts a signal peptide of 25 residues and a mature protein of 183 amino acids. No potential N-glycosylation sites are detected. The calculated molecular mass for the mature enzyme is 19 277 Da; the value estimated from SDS-PAGE analysis is ~23 000 Da (Belancic et al., 1995). The difference in molecular weight obtained by these two methods may be due, at least in part, to O-glycosylation.

Regarding codon usage, there is some preference for C in the third position, e.g. TTC vs. TTT for Phe (10/1), TAC vs. TAT for Tyr (11/3) and AAC vs. AAT for Asn (16/2) (Table 1). The coding region is 21.8% A, 23.4% T, 25.3% G and 29.5% C.

2.3. Alignment of XynB and other G-family xylanases

The XynB sequence was compared to similar proteins from the databases (Fig. 4). Significant similarity was found with 38 other fungal and bacterial xylanases representing a total of 40 xylanase domains all of which belong to the G family of cellulases/xylanases (Gilkes

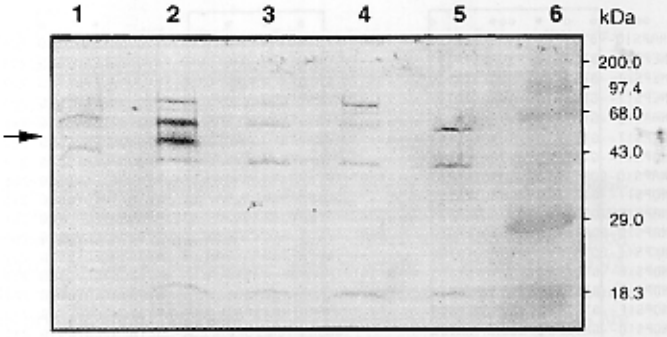

Fig. 5. Western analysis of *E. coli* transformed with XynB cDNA. The presence of hybrid TrpE'-XynB fusion protein in induced or uninduced cultures of *E. coli* RR1 cells transformed with either the pATH3 (Koerner et al., 1991) vector or a construct carrying the cDNA for XynB was detected with anti-XynB antibodies. Uninduced cells harboring the XynB cDNA did not present specific antibody reaction (lane 1). Only cells harboring the XynB cDNA and subjected to induction (withdrawal of tryptophan) produced the XynB fusion protein (lane 2). Cells that were transformed with the vector only did not show positive reaction (lanes 3 and 4, uninduced and induced respectively); neither did the control *E. coli* RR1 culture (lane 5). Molecular weight standards are shown in lane 6. **Methods**: An *Eco*RI/*Hin*dIII fragment containing the complete cDNA for XynB was cloned in the *Eco*RI/*Hin*dIII sites of pATH3 in frame with the *trpE'* gene. This construct was used to transform *E. coli* strain RR1; the synthesis of the hybrid TrpE'-XynB fusion protein was triggered by tryptophan starvation followed by addition of indoleacrylic acid (Koerner et al., 1991). SDS gels and Western blots were performed using standard techniques.

et al., 1991). As might be expected, XynB is more closely related to other fungal endoxylanases than to bacterial enzymes. Among the fungal comparisons the highest similarity found was with xylanases of the genus *Aspergillus*. The shared sequence ranged from 73% identity with xylanase EXLA from *A. awamori* (Hessing et al., 1994) to 30% for XYL1 from *H. insolens* (Dalboge and Heldt-Hansen, 1994). Identity of *P. purpurogenum* XynB with bacterial enzymes ranged from 37% with XYNA from *C. mixtus* (Millward-Sadler et al., 1995) to 21% with XYNA from *B. circulans* (Yang et al., 1988).

The two glutamic acid residues that are conserved in all members of family G and which have been postulated to participate in catalysis in *B. circulans* (Wakarchuk et al., 1994), *B. pumilus* (Ko et al., 1992), and *S. commune* (Bray and Clarke, 1994) correspond to E101 and E194 in XynB. There are other highly conserved residues that have been postulated to be important in substrate binding and in modulation of the enzymatic activity, mainly from crystallographic studies. For example, the residues that surround the active site of *T. reesei* xylanases I and II (Törrönen and Rouvinen, 1995) have their counterparts in XynB residues Y92, W94, Y103, Q153 and Y188. Of the xylanases belonging to family G whose structure has been solved by crystallography, *T. reesei* XYNI is the one most closely related to XynB.

2.4. Expression of XynB cDNA

To confirm the nature of the cloned DNA as cDNA for XynB, it was transferred to an *E. coli* expression vector and the protein product was detected with antibodies raised against native XynB. Western analysis showed a hybrid protein of the expected size only in cultures transformed with constructs harboring the XynB cDNA (Fig. 5).

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