New α-L-arabinofuranosidase produced by *Streptomyces lividans*: cloning and DNA sequence of the *abfB* gene and characterization of the enzyme

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A fully secreted α-L-arabinofuranosidase was cloned from the homologous expression system of *Streptomyces lividans*. The gene, located upstream adjacent to the previously described xylanase A gene, was sequenced. It is divergently transcribed from the *xlnA* gene and the two genes are separated by an intercistronic region of 391 nt which contains a palindromic AT-rich sequence. The deduced amino acid sequence of the protein shows that the enzyme contains a distinct catalytic domain which is linked to a specific xylan-binding domain by a linker region. The purified enzyme has a specific arabinofuranose-debranching activity on xylan from *Gramineae*, acts synergistically with the *S. lividans* xylanases and binds specifically to xylan. From small arabinoxylo-oligosides, it liberates arabinose and, after prolonged incubation, the purified enzyme exhibits some xylanolytic activity as well.

INTRODUCTION

Most of the hemicelluloses of softwoods and grasses contain L-arabinose [1]. Although the content of L-arabinose in plants is relatively low, its presence as a side-group substituent of the backbone of hemicelluloses restricts hydrolysis by glycanases. Xylan, the major hemicellulose, generally carries side chains of L-arabinofuranose, 4-*O*-methylglucuronic acid, ferulic and/or acetic acid. Complete hydrolysis of heteroxylans thus requires several specific enzymes [2]. α-L-Arabinofuranosidases (Abfs; EC 3.2.1.55) generally catalyse the cleavage of terminal α-L-arabinofuranosyl residues of arabinoxylan, arabinan and arabinogalactan [3]. In some cases, Abfs also possessing β-xylosidase activity or xylanases with arabinofuranosidase activity have been described [4–7]. In some instances, synergism between xylanase and Abf has been observed [8,9]. αAbfs have been purified and characterized from fungi such as *Aspergillus niger* [10], *Trichoderma reesei* [8] and *Aspergillus awamori* [11]. These enzymes have also been isolated from bacteria such as *Bacillus subtilis* [12], *Clostridium acetobutylicum* [13], *Butyrivibrio fibrisolvens* [14], *Bacillus stearothermophilus* [15] and *Streptomyces* sp. [16–18]. *abf* genes coding for an intracellular enzyme [19] have been cloned from *Pseudomonas fluorescens* [20] and *Streptomyces lividans*.

In this paper, we describe the cloning and DNA sequence of a second *abf* gene, abf*B*, as well as the purification and characterization of the enzyme.

MATERIALS AND METHODS

Substrates and chemicals

Xylan, purified from oat spelts, birchwood or larchwood, as well as arabinogalactan were obtained from Sigma (St. Louis, MO, U.S.A.). Arabinoxylan from rye and wheat flour, linear α-1,5-arabinan and sugar beet arabinan as well as oligoxylo-saccharides (degree of polymerization 2–6) were obtained from Megazyme (Sydney, NSW, Australia). The synthetic substrates *p*-nitrophenyl α-L-arabinofuranoside (pNPA) and 4-methylumbelliferyl α-L-arabinofuranoside (MUA) were also purchased from Sigma. Thiostrepton was a gift from Bristol-Myers-Squibb (Montréal, Qué., Canada). *p*-Hydroxybenzoic acid hydrazide (PAHBAH) was purchased from ICN Biochemicals (Montréal, Qué., Canada). The arabinoxylo-oligosaccharides were prepared by Dr. Catherine Manin as described previously [20].

Organisms and vectors

S. lividans IAF10-164 (*msiK*⁻), a xylanase- and cellulase-negative mutant, served as host strain for cloning of the gene [21]. Plasmid pIAF31 had been obtained by screening a homologous gene bank constructed from DNA isolated from *S. lividans* 1326 and partially digested with *Sau*3A1 using the multicopy plasmid pIJ702 as vector [22].

Cloning of the *abfB* gene

The gene coding for AbfB was found on a 7 kb DNA fragment from pIAF31. The structural *abfB* gene was amplified by PCR using the following oligonucleotides: 5′-GGGGATCCAAGCTTAAAGTAGTGGTCACGGA-3′ and 5′-GGGGATCCAAGCTTGTGCACCGACGGTCT-3′. The amplification product was digested by *Bam*HI and inserted into the unique *Bg1*II site of pIJ702. Protoplasts of *S. lividans* IAF10-164 were prepared and transformed as described by Hopwood et al. [23]. The screening of transformants was carried out on solid Stewart minimal agar [22] containing 4-MUA as chromogenic substrate. The AbfB-expressing clones were identified under UV light by the presence of fluorescence around the colonies after incubation at 34 °C for 1 or 2 days.

DNA manipulation and sequencing

All DNA manipulations in *S. lividans* were carried out as described by Hopwood et al. [23]. Single-stranded DNA was

Abbreviations used: Abf, α-L-arabinofuranosidase; *abfB,* gene coding for AbfB; MUA, 4-methylumbelliferyl α-L-arabinofuranoside; ORF, open reading frame; pNPA, *p*-nitrophenyl α-L-arabinofuranoside; pNPX, *p*-nitrophenyl β-D-xyloside; XBD, xylan-binding domain; XlnA, XlnB and XlnC, xylanase A, B and C from *Streptomyces lividans;* PAHBAH, *p*-hydroxybenzoic acid hydrazide.

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The nucleotide sequence in Figure 2 has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number M64551.

prepared using M13mp18 and M13mp19 phages and *Escherichia coli* DH11S (Gibco–BRL, Mississauga, Ont., Canada) [24]. The nucleotide sequence of both strands was determined by the chain-terminating dideoxy method [25] using the Auto-Read Sequencing Kit from Pharmacia and analysed on a Pharmacia automatic sequencer using the ALF Manager program. DNA sequences were assembled and analysed using the Pustell Sequence Analysis Programs of International Biotechnologies Inc. (New Haven, CT, U.S.A.). Homology search with sequences in GenBank/EMBL was carried out using the BLAST program of NCBI [26] available through the Internet.

Culture conditions

The clones were cultivated in M_{14} minimal medium as previously described [20]. The main carbon source for large-scale enzyme production was 1 % (v/v) xylose. The cultures were incubated at 34 °C on a rotary shaker at 240 rev./min for 72 h.

Enzyme purification

The fermentation broth was recovered by centrifugation at 11000 ***g*** for 30 min. The supernatant was filtered on to glass wool to retain any floating particles and concentrated by ultrafiltration with a Pellicon system (Waters Millipore) using a 10 kDa cut-off membrane. Crude enzyme preparations were obtained by $(NH_4)_2SO_4$ precipitation at 50 % saturation. The precipitate settled overnight at 4 °C, and was recovered by centrifugation at 18000 ***g*** for 30 min. For further purification, 45 mg of the precipitate was dissolved in 50 ml of 20 mM malonate buffer, pH 6.15, and dialysed three times against distilled water. The dialysate was adsorbed on a cation-exchange chromatography column (10 × 1.5 cm), packed with CM-Sepharose CL-6B (Pharmacia Biotech) and equilibrated with a 20 mM malonate buffer, pH 6.15. Enzyme was eluted with this buffer and a linear gradient of 0–1.0 M NaCl at a flow rate of 2.0 ml/min. The proteins were monitored by a UV detector at 280 nm. The active fractions were collected, pooled and dialysed against distilled water. Finally, enzyme was concentrated on an Omega Cell (Filtron, Clinton, MA, U.S.A.) equipped with a 10 kDa cut-off membrane and lyophilized. The purified protein was conserved at −70 °C.

Preparation of enzyme substrate

Soluble fraction from oat spelt xylan was used for characterization of enzyme. Substrate was prepared by boiling 35 g of xylan oat spelt in 2 litres of distilled water for 1 h. The soluble fraction was recovered by centrifugation at 13325 ***g*** for 35 min and lyophilization of the supernatant.

Enzyme assays

The AbfB activity was determined by a modification of the PAHBAH method [27]. Enzyme assay was carried out by mixing 900 μl of a 1 % solution of xylan with 100 μl of appropriately diluted enzyme. The mixture was incubated under agitation in a water bath at 55 °C for 10 min. The reaction was stopped by transferring 100 μl of the sample to 300 μl of 0.25 % PAHBAH solution and heating for 5 min at 95 °C. The reducing sugars released were determined by spectrophotometry at 405 nm using L-arabinose as standard.

Enzyme activity is expressed as units, where 1 unit corresponds to the amount of enzyme that released 1 μmol of arabinose/min.

Protein determination

The protein content of the enzyme preparations was determined by the Bio-Rad (Mississauga, Ont., Canada) Protein Assay reagent using γ-globulin as standard.

Antibody production and Western immunoblotting

Polyclonal antibodies were raised against AbfB in New Zealand White female rabbits by injecting 900 μg of purified enzyme combined with RIBI adjuvant (Ribi ImmunoChem Research, Hamilton, MT, U.S.A.) followed by one additional injection of 900 μg of AbfB at an interval of 3 weeks. Detection of AbfB after Western blotting was carried out using anti-AbfB antibodies coupled to ^{125}I-protein A (ICN Canada) [22].

Molecular mass and glycosylation

The purity and molecular mass of the AbfB was verified by SDS/PAGE using the Pharmacia PhastSystem on a polyacrylamide gel followed by silver staining.

Glycosylation of the protein was investigated by using the DIG Glycan Detection kit (Boehringer-Mannheim, Laval, Qué., Canada).

Determination of pI and confirmation of purity

Analytical isoelectrofocusing was carried out on PhastGel containing ampholites in the pH range 3.0–9.0 using the PhastSystem. The gel was silver stained after the run.

The electrophoretic titration method of Pharmacia served to verify the purity of the enzyme.

Analysis of products of enzyme hydrolysis

The products of enzyme hydrolysis of rye/wheat flour arabinoxylans or arabino-oligoxylosides were analysed on an Aminex HPX 42-A column (Bio-Rad). To 400 μl of the reaction mixtures containing 10 mg/ml substrate, dissolved in 50 mM sodium phosphate buffer, pH 6.0, was added 5 μg of enzyme to start the reaction. After an incubation time lasting from 10 min to 1 h at 55 °C, the enzyme was inactivated in a boiling water bath for 5 min. Studies on synergism were carried out by combining the enzymes under the same conditions but with incubation lasting for 24 h. The sample was centrifuged and filtered through a 0.45 μm pore-size Acrodisc™ filter before injection on to the Aminex HPLC column heated to 80 °C with water as eluent at a flow rate of 0.5 ml/min.

Substrate-binding studies

Insoluble xylan was used as substrate. It was obtained by boiling oat spelt xylan (Sigma) for 30 min in distilled water and recovering the residues by filtration. The binding assays were carried out in Eppendorf tubes with 50 mg of the substrate suspended in 100 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and mixed with 3 units of AbfB at 4 °C (final volume 500 μl). At intervals of 0, 1 and 60 min, samples were withdrawn and centrifuged. The supernatant was recovered and assayed for activity as described above. To exclude non-specific protein adsorption, in some experiments 50 mg of the insoluble substrates were pretreated with 100 μg of bovine IgG with a pI of 7.2, similar to that of AbfB, incubated for 30 min at room temperature, washed three times with the sodium phosphate buffer and used immediately for the binding assay.

RESULTS

Detection and sequence analysis of *abfB*

An open reading frame (ORF) was detected 391 nt upstream of the *xlnA* gene present in pIAF31, a plasmid coding for xylanase A (XlnA) of *S. lividans* (Figure 1) [22]. The 1434 nt ORF, shown in Figure 2, starts at nt 400 and stops at a TGA codon at nt 1834. The base composition of this ORF (68.3 % G+C) is in agreement with the G+C content of *Streptomyces* DNA with more than 92 % G or C at the third position [28]. Consequently, 14 codons are not used and 17 are used four times or less. This ORF is divergently transcribed from the previously identified *xlnA* and encodes a 478-amino acid polypeptide of molecular mass 50826 Da. The first 37 amino acids conform to the classical biochemical characteristics of a signal peptide of 3775 Da with a hydrophilic (R) arginine-rich N-terminus, followed by a hydrophobic core (A, L and V), then a proline (P) residue which introduces a break in the helix and exposes the AQA sequence to the signal peptidase. Thus the mature secreted protein has a molecular mass of 47051 Da, which matches closely that estimated for the purified protein by SDS/PAGE (see Figure 5b).

The AbfB protein is composed of two distinct domains (Figure 3). First, the N-terminus comprising 124 amino acids exhibited 63 % identity with the C-terminus of the XlnA located in the vicinity (Figure 2 nt 1052–762) and presumably arose from gene duplication. This homology was also detectable using specific anti-XlnA antibodies, which showed a cross-reaction with this domain but none with the catalytic domain (Figure 4).

Figure 1 Restriction map of plasmid pIAF31

The line and open box indicate respectively the vector pIJ702 and the DNA insert. The solid arrow is the *xlnA* gene and the cross-hatched arrow the *abfB* gene. The two small open arrows represent the DNA duplication that encodes the xylan-binding domain (XBD) present in both proteins.

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1    5'GGAGCCCATGACTGTGCCTCCAAAGTAGTGGTCACGGAAGGACTGGAGCGCAGGGAAGTCAGACAGGAATGATTGAGGTGTTGA
85     CGATGCTCGGTCAATACTTGGCGCCGGAAAAGTTTCAGTTATTGTGCGAAAGTTTCGAAATCGAGTGAGTCGGGCCTGATCTGC
169    GAGGTCGTGAGCCGTCACGGGGCGGCCTGCCGTGTGACGTGCGGCATCGGGTAGATCGCCTCTCATGAATGTTTCGCGGTGGCG
253    GCGATACATGCGAGAGGTATTGACGGCGTTCGCCGGTTCTCTATGATGCGGAGCGCTCGACTCTTCGACCGCTGTTCGATATTG
337    CGAACATGTCGTGTCGCTCTCCATCCCGGCAAGGAACCACCCACCCCAGTCGAGGGAGTTCCCATGCACAGAGGAAGTCTCAGC
                                                                     M  H  R  G  S  L  S   7
421    CGCGGGCAACACGTCCGCGGTACTCGCCGCCGTGGTGCGGCCCTGGCCGCGCTGGCGGCGCTGCTCGTCGCAACCGCCCCCGCT
        R  G  Q  H  V  R  G  T  R  R  R  G  A  A  L  A  A  L  A  A  L  L  V  A  T  A  P  A   35
505    CAGGCGGCCGGCAGCGGCGCCCTGCGCGGTGCCGGTTCGAACCGGTGTCTCGACGTGCTGGGCGGCAGCCAGGACGACGGCGCG
        Q  A  a  g  s  g  a  l  r  g  a  g  s  n  r  c  l  d  v  l  g  g  s  q  d  d  g  a   63
589    CTCCTGCAGCTGTACGACTGCTGGGGCGGCACCAACCAGCAGTGGACGTCGACGGACACCGGCCGGCTCACCGTGTACGGCGAC
        l  l  q  l  y  d  c  w  g  g  t  n  q  q  w  t  s  t  d  t  g  r  l  t  v  y  g  d   91
673    AAGTGCCTGGATGTTCCGGGGCACGCCACCGCGCCCGGTACCCGGGTGCAGATCTGGTCCTGCTCCGGCGGACGGAACCAGCAG
        k  c  l  d  v  p  g  h  a  t  a  p  g  t  r  v  q  i  w  s  c  s  g  g  r  n  q  q   119
757    TGGCGGGTGAACTCCGACGGCACGGTCGTCGGTGTGGAGTCCGGGCTGTGCCTGGAGGCCGCGGGCGCCGGCACGCCAAACGGC
        w  r  v  n  s  d  g  t  v  v  g  v  e  s  g  l  c  l  e  a  a  g  a  g  t  p  n  g   147
841    ACGGCGGTGCAGCTCTGGACGTGCAACGGCGGCGGCAACCAGAAGTGGACCGGTCTGACCGGTACGCCGCCGACGGACGGCACG
        t  a  v  q  l  w  t  c  n  g  g  g  n  q  k  w  t  G  L  T  G  T  P  P  T  D  G  T   175
925    TGTGCGCTTCCGTCGACGTACCGGTGGTCCTCGACGGGGGTTCTGGCACAGCCGAAGAGCGGGTGGGTCGCGCTGAAGGACTTC
        C  A  L  P  S  T  Y  R  W  S  S  T  G  V  L  A  Q  P  K  S  G  W  V  A  L  K  D  F   203
1009   ACCACGGTGACGCACAACGGCAGGCACCTGGTCTACGGGTCGACCTCTTCGGGGTCCTCGTACGGCTCGATGGTGTTCAGCCCC
        T  T  V  T  H  N  G  R  H  L  V  Y  G  S  T  S  S  G  S  S  Y  G  S  M  V  F  S  P   231
1093   TTCACCAACTGGCGGACATGGTCGGACATGGCGTCGGCCGGACAGAACGCGATGAACCAGGCCGCGGTGGCGCCCACGCTGTTC
        F  T  N  W  R  T  W  S  D  M  A  S  A  G  Q  N  A  M  N  Q  A  A  V  A  P  T  L  F   259
1177   TACTTCGCGCCCAAGAACATCTGGGTGCTGGGGTACCAGTGGGGCTCGTGGCCCTTCATCTACCGCACGTCGAGCGACCCCACC
        Y  F  A  P  K  N  I  W  V  L  G  Y  Q  W  G  S  W  P  F  I  Y  R  T  S  S  D  P  T   287
1261   GACCCCAACGGCTGGTCCGCTCAGCAGCCGCTGTTCACCGGGAGCATCTCCGGCTCCGACACCGGCCCGATCGATCAGACCCTG
        D  P  N  G  W  S  A  Q  Q  P  L  F  T  G  S  I  S  G  S  D  T  G  P  I  D  Q  T  L   315
1345   ATCGCCGACGGCCAGAACATGTACCTGTTCTTCGCCGGTGACAACGGCAAGATCTACCGGGCGAGCATGCCGATCGGGAACTTC
        I  A  D  G  Q  N  M  Y  L  F  F  A  G  D  N  G  K  I  Y  R  A  S  M  P  I  G  N  F   343
1429   CCGGGCAACTTCGGTTCCTCGTACACGACGATCATGAGCGACACGAAGGCCAACCTGTTCGAGGGCGTACAGGTCTACAAGGTC
        P  G  N  F  G  S  S  Y  T  T  I  M  S  D  T  K  A  N  L  F  E  G  V  Q  V  Y  K  V   371
1513   CAGGGCCAGAACCAGTACCTCATGATCGTCGAGGCGATGGGCGCGAACGGGCGCTACTTCCGCTCCTTCACGGCCTCCAGTCTG
        Q  G  Q  N  Q  Y  L  M  I  V  E  A  M  G  A  N  G  R  Y  F  R  S  F  T  A  S  S  L   399
1597   AGCGGCTCGTGGACCCCGCAGGCCGCCAGTGAGGGCAACCCCTTCGCGGGCAAGGCCAACAGCGGGGCCACCTGGACCAACGAC
        S  G  S  W  T  P  Q  A  A  S  E  G  N  P  F  A  G  K  A  N  S  G  A  T  W  T  N  D   427
1681   ATCAGCCACGGCGACCTGGTCCGCGACAACCCCGACCAGACCATGACCGTCGACCCCTGCAACCTGCAGTTCCTCTACCAGGGC
        I  S  H  G  D  L  V  R  D  N  P  D  Q  T  M  T  V  D  P  C  N  L  Q  F  L  Y  Q  G   455
1765   AAGGCCCCGAACGCGGGCGGGCACTACAACTCGCTGCCGTGGCGGCCGGGAGTCCTCACCCTGCGGCACTGACCTCTCCGACGG
        K  A  P  N  A  G  G  H  Y  N  S  L  P  W  R  P  G  V  L  T  L  R  H  -               478
1849   ACGGTGACCGCATGCGGTGCGGCGAGCGGCGTACGGTGAGTACCGCCAAGTCGTCCTGCTGCGGTCCCTCCGTGAAGGCGTGGA
1933   CGTCGTCGACGAGTGCGTGGGTGAGCCGGCCCGCGGTGATGTCGACGTGCCAGGCGAGGCGGGTCTCCAGCGGGTAGAAGGCGC
2017   CGGCGGGGGAGCGGGCCTCGGTGAGACCGTCGGTGCACAGCAGCAGCGTCGCCCCGGCGGGGAAGGGGAACCAGGACACCGTGC
2101   GCGGCTCGTCGACGAGGTCTGCCAGGCCGAGCGGCGCATGCTCCGTCCCGGCGCCCACGGGGTGACGGTCCCGTCGTGCAGCAG
2185   GTAGGGCGGGATGTGGCCGCACGTGACCAGCTGGGTCTCGGCGCCGGTGTCCACCCCCAGGACGACGGCGTGACGAACCGTTCC
2269   GGTCCACGGCTGTTCCCCGTGACGTTTGTTGCCGGACAACGCGGTTGGGGCGGTTGCCC 3'
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Figure 2 Nucleotide sequence of *abfB*

The putative ribosome-binding site is in **bold**. The first 37 amino acids constituting the signal peptide are underlined. The next 127 amino acids, indicated by lower-case letters, represent the XBD of the AbfB, which is separated from the catalytic site by a Gly-Pro-Thr-rich linker indicated in **bold** (GenBank Accession No. M64551).

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                                             1        10        20
AbfB mhrgslsrgqhvrgtrrrgaalaalaallvatapaqaagsGalrGaGSnRCLDVlggsqdD
XlnA                                         GqikGvGSgRCLDVPdastsD
Glc                                        gtGalr.iGStlCLDVPwadptD
Rpi                                        ttsyvqGyqnn.CiDVPnsdftD

          30        40        50        60        70        80
AbfB GalLQLyDCwGGTNQQWts.TDtGrLtVYGDKCLDvpGhaTapGTrVQIwSCsGgrNQqWRv
XlnA GtQLQLwDChsGTNQQWaa.TDaGeLRVYGDKCLDaaG..TsnGskVQIySCwGgdNQKWRl
Glc  tnQvQLatCsGnaaQQWtrgTDgt.vRaYG.KCLDvarsgTadGTaVwIytCnGtgaQKWty
Rpi  GkQLQvwnCnGtnaQkvsfhpDgt.LRinG.KCLDarwawThnGTeVQImnCnGhiaQKftl

         90       100       110       120       130       140
AbfB NSDGtVVGV.eSGlCLeAaGaGTp.NGTavQLWTCngGgNQkWTgltGtpptdgTCaLpsty
XlnA NSDGsVVGV.qSGlCLDAvGnGTa.NGTliQLyTCsnGsNQrWTrt
Glc  dSatkalrnpqSGkCLDAqGgaplrdGqkvQLWTCnqteaQrWTl
Rpi  NgaGdlVnV.hankCvDvkdwGg..qGgklQLWeCsgGaNQkWwrr
XYLC                                               ...320 TCeLkapl

        150       160       170       180       190       200
AbfB RWsSTGvLaqPKs.GWvalKDfttVthNg.rHlvYgsTssgssYgSMvfspFTnWrTwsdma
XYLC RWtSTGpLisPKnpGWisiKDpsiVkyNdtyHv.Y.aTyydtaYrSM.ytsFTnWnTaqqap

        210       220       230       240       250       260
AbfB sagqNamnqaa.VAPtlFYFaPkNiWvLgyQW.GswpfiyrTssDptdPNgWSAqQpLftG
XYLC hismNgsrvgntVAPqvFYFrPhNkWyLitQWaGaya....TtdDirnPN.WSAkQkLlqG

         270       280       290       300       310       320
AbfB sisGsdtgpiDqtlIa.DgqnmYLfFagDnGkiYrasmpigNFPgNFgSsYttiMsDtkaN
XYLC epnGa....lDfwvIcnDthc.YLyFsrDdGvlYvskttlaNFP.NF.SgYsivMeDhrgN

             330       340       350       360       370       380
AbfB ....LFEgvqVYKvqGQNqYLmiVEAmganGRyFrsftasslsgswtpqAasEgNPFAGka
XYLC gnsyLFEaanVYKldGQNrYLlmVEAyis.GRaFsapgqrpawmahgplAdtEaNPFAGmm

            390       400       410       420       430       440
AbfB nsgatwtndishgdlvrdnpdqtmtvdpcnlqflyqgkapnagghynslpwrpgvltlrh
XYLC fcftmasslkvytcy
```

Figure 3 Homology of AbfB with other proteins

The conserved amino acids are indicated in capital letters. AbfB, arabinofuranosidase B from *S. lividans*; Glc, β-1,3-glucanase from *Oerskovia xanthineolytica* [30]; Rpi, protease I from *Rarobacter faecitabidus* [35]; XlnA, xylanase A from *S. lividans*; XYLC, the C-terminus of the arabinofuranosidase from *Ps. fluorescens* [19].

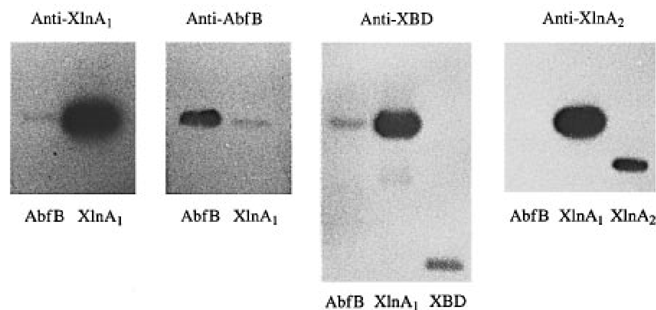

Figure 4 Western-blot analysis comparing purified AbfB, XlnA$_1$, XlnA$_2$ and XBD with anti-AbfB, anti-XlnA$_1$, anti-XlnA$_2$ and anti-XBD antibodies

The amount of purified protein used in each experiment was 5 μg. AbfB, arabinofuranosidase B; Anti-AbfB, anti-AbfB antibodies; XlnA$_1$, xylanase A$_1$; anti-XlnA$_1$, anti-XlnA$_1$ antibodies; XlnA$_2$, xylanase A2; anti-XlnA$_2$, anti-XlnA$_2$ antibodies; XBD, xylan-binding domain; anti-XBD, anti-XBD antibodies.

Subcloning of *abfB*

Since AbfB is a secreted protein with a molecular mass similar to that of XlnA and could hamper its purification, we subcloned by PCR the *abfB* gene into the *Bgl*II site of the multicopy plasmid pIJ702 by using the two oligonucleotides described in the Materials and methods section. Transformants of *S. lividans* IAF10-164 obtained with this preparation were tested for Abf activity. Clone *S. lividans* IAF1, which is Abf-positive and Xln-negative, was selected for characterization of the enzyme.

Production and purification of AbfB

In contrast with the previously described intracellular AbfA [20], AbfB was fully secreted. It was recovered from culture filtrates obtained from submerged cultures using a minimal salt medium with 1 % (w/v) xylose as carbon source. Optimal enzyme levels of 3 units/ml were reached after 72 h of incubation at 34 °C. AbfB production was monitored by SDS/PAGE of the supernatant, which showed one major protein band at 43 kDa. In Western-blot analysis, this protein also reacted with anti-XlnA antibodies which is explained by the two proteins sharing an

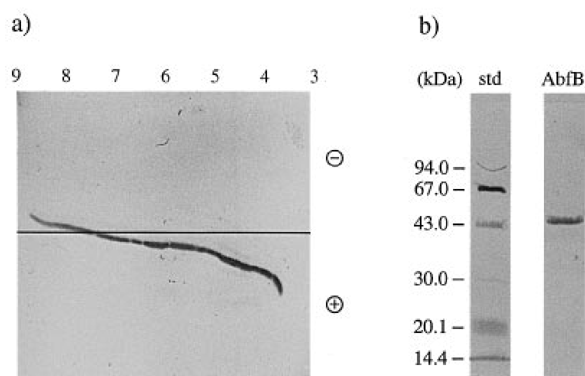

Figure 5 Silver-stained (a) two-dimensional electrophoretic titration curve (PhastGel IEF 3–9) and (b) SDS/PAGE (PhastGel 12.5%) of purified AbfB

The amount of purified AbfB used for both experiments was 4 μg. Marker proteins were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soyabean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa).

homologous substrate-binding domain. AbfB did not cross-react with the antibodies raised against $XlnA_2$, which lacks the substrate-binding domain [31] (Figure 4). The enzyme was purified from the cation-exchange column by elution with a linear NaCl gradient at a concentration of 0.4 M. This one-step purification allowed a 2-fold purification of the enzyme with a yield of 35%. The specific activity of the purified AbfB on oat spelts xylan was 8.75 units/mg, whereas on rye and wheat arabinoxylan this value was 11.3 and 13.4 units/mg respectively. The purity of the enzyme was verified by the electrophoretic titration method and SDS/PAGE (Figures 5a and 5b).

Characterization of AbfB

The apparent molecular mass of the purified enzyme was determined by SDS/PAGE to be 43 kDa (Figure 5a), whereas when calculated from the amino acid sequence it was 47 kDa. The pI determined by analytical isoelectrofocusing was 7.4. The protein was not glycosylated. The optimal activity of AbfB was 55 °C at pH 6.0. In the absence of substrate, the enzyme was stable at 40 °C for 48 h. At 50 °C its half-life was 1 h, whereas at 60 °C all activity was lost within 10 min (results not shown).

The Michaelis–Menten constants of the purified enzyme were determined under optimal assay conditions by the Lineweaver–Burk plot with either wheat arabinoxylan or oat spelts xylan at concentrations varying from 0.1 to 20 mg/ml. The V_{max} and K_m for the former were 18.5 units/mg and 1.17 mg/ml, whereas for the latter they were 17.2 units/mg and 5.12 mg/ml respectively.

Substrate binding

The putative function of the N-terminal region of AbfB as a specific substrate-binding domain for xylan is shown in Table 1. The enzyme adsorbed rapidly to xylan and remained with the associated substrate for up to 1 h. It showed a strong affinity for xylan. Non-specific adsorption of the enzyme was excluded by pretreating the xylan with 100 μg of IgG. These results confirm the existence of a specific XBD similar to that reported recently for XylD of *Cellulomonas fimi* [32].

Table 1 Substrate binding of AbfB to insoluble oat spelt xylan treated and not treated with IgG

The residual activity of AbfB in the supernatant after incubation is shown.

Incubation time (min)	Residual AbfB activity (%)	
	Xylan	IgG-treated xylan
0	100	100
1	0	0
60	1.1	2.9

Substrate-specificity of AbfB

The preferred substrate was the arabinoxylan from cereals such as oats, rye or wheat. No hydrolysis was observed with birchwood or larchwood xylan nor with arabinogalactan or linear α-1,5-arabinan. AbfB did yield arabinose by debranching sugar beet arabinan (results not shown). The enzyme had a very low activity with the synthetic substrates pNPA and MUA, which are often used to determine enzyme activity.

Short-chain arabinoxylo-oligosaccharides, ranging from arabinoxylotriose (A_1X_3) to arabinoxylohexaose (A_1X_6), when incubated with AbfB for 1 h, yielded arabinose as the only hydrolysis product. However, prolonged incubation (for 24 h) led to partial hydrolysis of the oligoxyloside backbone as well (Figures 6a and 6b). The possibility of cross-contamination of the purified AbfB by xylanases was excluded by zymograms with Remazol Brilliant Blue–xylan after isoelectrofocusing. Therefore it must be concluded that the AbfB from *S. lividans* has the ability to hydrolyse slowly short-chain oligoxylosaccharides. This observation was confirmed with unsubstituted xylotetraose and xylopentaose (results not shown).

Figures 7(a) and 7(b) show the HPLC patterns of the synergistic action of AbfB with either XlnA or XlnC on wheat flour xylan. Combining AbfB with X1nA or X1nC dramatically increased the production of oligosaccharides Figure 7. No such synergy was observed with oat spelt xylan.

DISCUSSION

The presence of a second gene encoding an α-L-Abf was shown during the sequencing of the upstream region of a plasmid containing the gene coding for XlnA of *S. lividans*. This plasmid, pIAF31, had been isolated previously from a homologous gene bank by functional complementation of the cellulase- and xylanase-negative mutant *S. lividans* IAF10-164 [22]. DNA sequencing and analysis revealed that the *abfB* gene is divergently transcribed from the *xlnA* gene and that the two genes are separated by an intercistronic region of 391 nt which contains a palindromic AT-rich sequence (CGAAAGTTTCG) which is also found at the 5′ end of other genes from *S. lividans* involved in the biosynthesis of xylanases, namely *xlnB-axeA*, *xlnC* [29,33] and *msiK* [21]. Since all these genes are induced by xylan and repressed by glucose and were mapped in different quadrants of the chromosome [34], it seems reasonable to speculate that specific DNA-binding regulatory proteins might bind to these sequences.

An interesting feature of the primary DNA sequence of this chromosomal locus is a DNA duplication of 354 nt in length located less than 2 kb apart and showing 70% identity. A similar gene duplication, not essential for the catalytic activity, was also encountered in the *xlnB* locus of *S. lividans* [33]. However, no

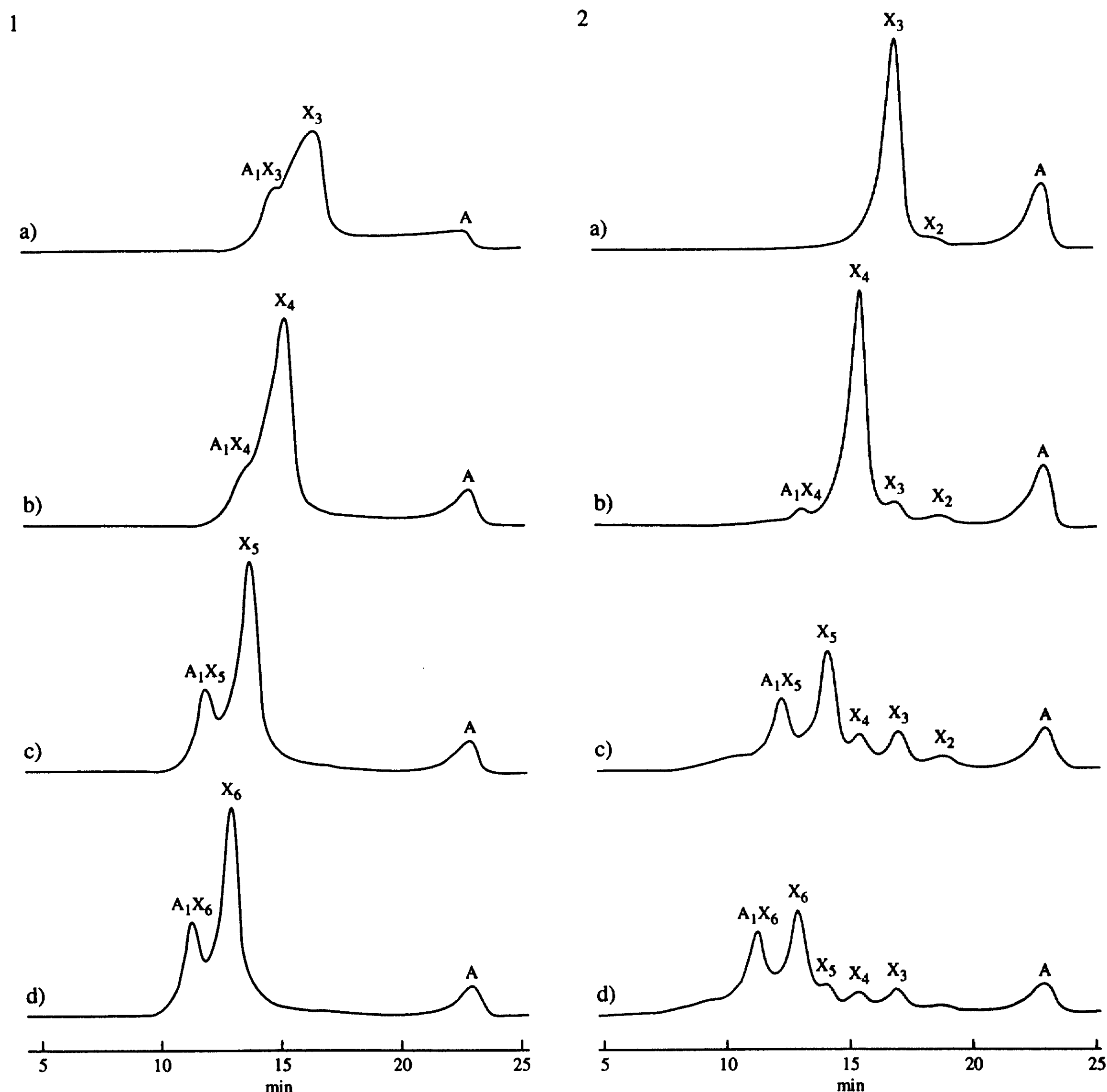

Figure 6 Hydrolysis patterns of arabinoxylo-oligosaccharides with purified AbfB after (1) 1 h incubation and (2) 24 h incubation

(a) Arabinoxylotriose; (b) arabinoxylotetraose; (c) arabinoxylopentaose; (d) arabinoxylohexaose. Soluble arabinoxylo-oligosaccharides at a concentration of 10 mg/ml in 50 mM sodium phosphate, pH 6.0, were hydrolysed with 5 μg of AbfB at 55 °C. A_1X_3, arabinoxylotriose; X_3, xylotriose; X_2, xylobiose; A, arabinose; A_1X_4, arabinoxylotetraose; X_4, xylotetraose; A_1X_5, arabinoxylopentaose; X_5, xylopentaose; A_1X_6, arabinoxylohexaose; X_6, xylohexaose.

obvious nucleotide sequence could be detected to explain the shuffling of an entire functional domain between genes. Thus the deduced amino acid sequence of AbfB revealed that the protein architecture is comprised of two domains. The N-terminus, which is composed of 124 amino acids, exhibited extensive homology (63 % identity) to the C-terminus of xlnA from *S. lividans*, which is the binding domain of the protein to its substrate. This domain also showed considerable homology with mannose-binding domains found in other multidomain proteins such as the yeast-specific, 1,3-glucanase from *Oerskovia xanthineolytica* [30] and the protease I of *Rarobacter faecitabidus* [35].

The putative catalytic domain, composed of the remaining 300 amino acids revealed significant similarity (66 %) to XylC from *Ps. fluorescens* which has Abf activity [19]. The two domains are separated by a short Pro-Thr-rich linker region that is also found in other hydrolases. The structural organization of *S. lividans* AbfB is a classical example of glycosyl hydrolases, where a catalytic and a substrate-binding domain are found together. Although the catalytic domain of AbfB is highly similar to XylC of *Ps. fluorescens*, their substrate-binding domains are clearly different. This explains why XylC binds specifically to Avicel but not to xylan [19], whereas AbfB, having a binding domain similar to that of XlnA from *S. lividans*, binds specifically to insoluble xylan (P. Vincent, F. Shareck, C. Dupont, R. Morosoli and D. Kluepfel, unpublished work).

From its biochemical characteristics, AbfB resembles the monomeric proteins of lower molecular mass that have been described for *Aspergillus awamori* [11], *Clostridium stercorarium* [9] and *Ps. fluorescens* [19], all of which specifically attack arabinoxylan but not other arabinosaccharides. The enzymes had only low activity with synthetic substrate such as pNPA.

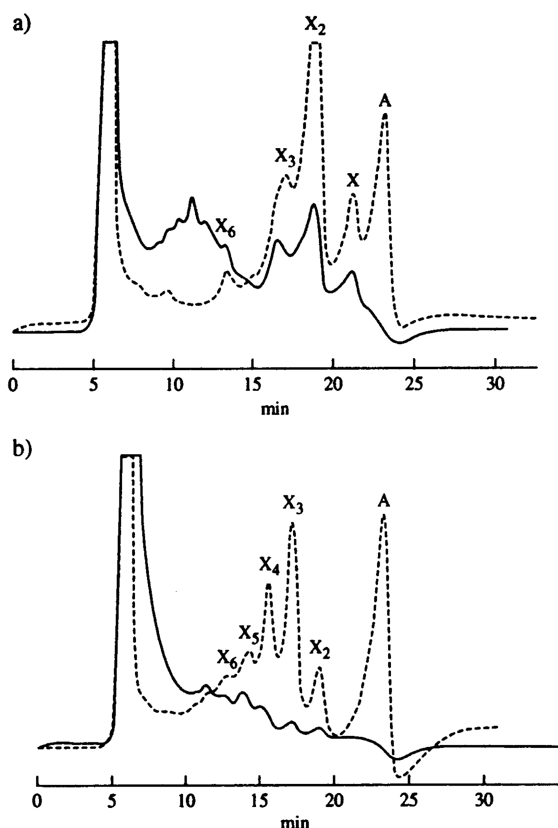

Figure 7 Comparison of hydrolysis patterns of wheat flour arabinoxylan after 24 h incubation with both (a) XlnA and AbfB or (b) XlnC and AbfB

Soluble wheat flour arabinoxylan at a concentration of 10 mg/ml in 50 mM sodium phosphate buffer, pH 6.0, was hydrolysed with both XlnA or XlnC (0.5 μg) and AbfB (5 μg) at 55 °C. ——, XlnA or XlnC; ––––, XlnA + AbfB or XlnC + AbfB.

Acting on arabinoxylans, the AbfB from *S. lividans* liberates only arabinose. From short-chained arabinoxylo-oligosacharides, the enzyme first cleaves the arabinofuranose moiety and then hydrolyses, after prolonged incubation, the oligoxyloside backbone as well. Furthermore a residual β-xylosidase activity was detected on *p*-nitrophenyl β-D-xyloside (PNPX) and 4-methylumbelliferyl β-D-xyloside. This is in contrast with XYLC from *Ps. fluorescens*, which did not show such activity [19]. However, as seen in Figure 6, after 24 h of incubation, no xylose was released from any of the arabinoxylo-oligosaccharides tested with AbfB. This is in contradiction with true β-xylosidase activity as suggested by the results on synthetic substrates. Contamination by any of the *S. lividans* xylanases can be excluded on the basis of the method used for the purification of AbfB. Bifunctional enzymes have been reported, e.g. Abfs from *B. fibrisolvens* [4] and *Cl. stercorarium* [9] and a β-xylosidase from *T. reesei* [8]. The ratio of the activities, determined on pNPA and pNPX respectively, was comparable with that obtained with AbfB of *S. lividans*. However, owing to the low level of activity for each synthetic substrate (16 and 11 m-units/mg for pNPA and pNPX respectively), no further kinetic characterization could be pursued, unfortunately preventing unambiguous assignment of each activity to a unique catalytic site. However, these dual activities might be explained by a rotation about the α-1,3-glycosidic linkage of the arabinofuranose group to the xylose moiety of xylan, which produces a bond conformation resembling that of a β-1,4 bond found in xylosides. Thus a single catalytic site, as has been postulated in *B. fibrisolvens*, could perform the double activity [4]. As was the case in this bacterium, the AbfB of *S. lividans* showed a considerably higher activity on the natural arabinosyl derivatives.

The synergistic mode of action of AbfB is most evident with XlnC which is an endo-acting enzyme, yielding larger oligoxylosaccharides [36]. Without removal of the arabinofuranose side chain, this XlnC is unable to hydrolyse the arabinoxylan to any significant degree (Figure 7b). The combined action with XlnA, which produces mainly xylotriose and xylobiose, is significant but less dramatic (Figure 7a), which shows that, in contrast with XlnC, XlnA can gain some access to the xyloside backbone. No significant synergistic effect was observed on oat spelt xylan. These differences clearly depend on the arabinose content of the substrates, which is high in wheat and rye flour xylan (41–49 % arabinose) but low in oat spelt (9 %).

The advantage that is conferred on *S. lividans* by producing two distinct Abfs is obvious. By acting as a debranching enzyme of long-chain arabinoxylosides synergistically with the endoxylanases, extracellular AbfB creates access for these enzymes to the xylose moieties of xylan. Since this debranching action is unlikely to remove all arabinose molecules from highly arabinosylated xylan, xylanolytic action will yield short-chain arabinoxylo-oligosaccharides (arabinoxylobiose, arabinoxylotriose), some of which can be transported into the cell, where the intracellular AbfA will hydrolyse them further [20]. Such dual mechanisms would definitely give the bacteria a competitive advantage over other lignocellulose-degrading micro-organisms.

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