Purification and characterization of an α-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (*abf*A)

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The gene encoding an α-L-arabinofuranosidase (*abf*A) was homologously cloned in *Streptomyces lividans* and its DNA sequence was determined. The enzyme was purified from the cytoplasm of the hyperproducing clone *S. lividans* IAF116. Its M_r was estimated by gel filtration and found to be approx. 380000. Since SDS/PAGE indicated a native protein of M_r 69000, it can be concluded that the native protein consists of several subunits of that size. The pI value was 4.6. The kinetic constants determined with *p*-nitrophenyl α-L-arabinofuranoside as substrate were a $V_{max.}$ of 180 units/mg of protein and a K_m of 0.6 mM. The specific activity of the purified enzyme on this substrate was 153 units/mg of protein. Optimal enzyme activity was obtained at 60 °C and pH 6.0. The enzyme cleaved *p*-nitrophenyl α-L-arabinofuranoside, but had no activity on a variety of other *p*-nitrophenyl glycosides, except on *p*-nitrophenyl β-D-xylopyranoside. The enzyme showed no activity on oat-spelts (*Avena sativa*) xylan or arabinogalactan, but acted on beet (*Beta*) arabinan or arabinoxylan. Hydrolysis occurred on arabino-oligoxylosides obtained from oat-spelts xylan after digestion with xylanases. Since *S. lividans* normally does not secrete arabinofuranosidase, this enzyme may play a role in the assimilation of arabinose moieties from arabinose-containing xylo-oligosaccharides generated by β-xylosidases or xylanases.

INTRODUCTION

L-Arabinose residues are found widely distributed among many heteropolysaccharides of different plant tissues. They constitute monomeric and/or oligomeric side chains on β-1,4-linked xylose or galactose backbones in xylans, arabinoxylans and arabinogalactans and are the core in arabinans forming α-1,5-linkages (Ward and Moo-Young, 1989). These side chains may restrict the enzymic hydrolysis of hemicelluloses in different applications of hemicellulases, such as in the improvement of digestibility of animal feed by ruminants (Brice and Morrison, 1982) or biobleaching of wood pulps (Wong et al., 1988). Enzymes which cleave α-L-arabinofuranosidic linkages are arabinosidases which include α-L-arabinofuranosidases (AFs; EC 3.2.1.55), that hydrolyse terminal non-reducing α-L-1,2- or α-L-1,3-arabinofuranosyl residues from xylans, arabinoxylans and arabinogalactans, and endo-1,5-α-L-arabinanases (EC 3.2.1.99), which hydrolyse α-L-1,5-arabinofuranosidic linkages from arabinans (Dekker and Richards, 1976; Kaji, 1984). α-L-AFs are also involved in the hydrolysis of grape monoterpenyl glycosides during wine fermentation (Gunata et al., 1989).

L-AFs have been purified and characterized from fungi such as *Aspergillus niger* (Kaji et al., 1969; Rombouts et al., 1988), *Corticium rolfsii* (Kaji and Yoshihara, 1970), *Trichoderma reesei* (Poutanen, 1988) and *Dichomitus squalens* (Brillouet et al., 1985). These enzymes have also been isolated from bacteria such as *Bacillus subtilis* (Weinstein and Albersheim, 1979), *Clostridium acetobutylicum* (Lee and Forsberg, 1987), *Ruminococcus albus* (Greve et al., 1984), *Butyrivibrio fibrisolvens* (Hespell and O'Bryan, 1992) and *Streptomyces* sp. (Kaji et al., 1981; Komae et al., 1982; Tajana et al., 1992).

In our studies on hemicellulases from *Streptomyces lividans*, we have homologously cloned several xylanase, β-mannanase and cellulase genes by a shot-gun cloning from a gene bank prepared by partial digestion of the chromosome (Mondou et al., 1986; Shareck et al., 1987, 1991; Théberge et al., 1992; Arcand et al., 1993). The same gene bank was used to screen for genes coding for various debranching enzymes related to the xylanolytic system. In the present paper we describe the cloning and DNA sequence of an α-L-AF gene (*abf*A) as well as the purification and characterization of the enzyme produced by clone *S. lividans* IAF116.

MATERIALS AND METHODS

Organisms

Streptomyces lividans 66 strain 1326 was obtained from D. A. Hopwood (John Innes Institute, Norwich, U.K.). The host strain used for cloning was a xylanase- and cellulase-negative mutant, *S. lividans* IAF10-164, obtained by mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Mondou et al., 1986). The multicopy plasmid pIJ702, which served as cloning vector, was kindly supplied by Dr. E. Katz (Katz et al., 1983).

Cloning of the α-L-AF gene

The α-L-AF gene was isolated from a homologous gene bank previously constructed in a xylanase- and cellulase-negative mutant *S. lividans* IAF10-164 and containing approx. 25000 clones (Mondou et al., 1986). The screening was carried out on solid agar TSB medium (Difco Laboratories, Detroit, MI, U.S.A.) by spraying with 1 mM 4-methylumbelliferyl α-L-

Abbreviations used: *abf*A, gene coding for α-L-arabinofuranosidase (AF); DTT, dithiothreitol; *p*-NP, *p*-nitrophenol; TSB, tryptic soy broth; ORF, open reading frame.

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The nucleotide sequence data reported in this paper will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U04630.

arabinofuranoside (Sigma Chemical Co., St. Louis, MO, U.S.A.). The AF-expressing clones were identified under u.v. light by their fluorescence after 10 min of incubation at 37 °C in the dark.

DNA manipulations and sequencing

All DNA manipulations in *S. lividans* were carried out as described by Hopwood et al. (1985). Single-stranded DNA was prepared using M13mp18 and M13mp19 phages and *E. coli* DH11S as described by Sambrook et al. (1989). The nucleotide sequence of both strands were determined by the dideoxy chain-termination method of Sanger et al. (1977) using Sequenase (USB Biochemicals) and analysed on a Pharmacia automatic sequencer using the ALF Manager program. To fill the remaining gaps, custom oligonucleotide primers were synthesized with a Gene Assembler Plus (Pharmacia-LKB). DNA sequences were assembled and analysed using the Pustell Sequence Analysis Programs of International Biotechnologies Inc. (New Haven, CT, U.S.A.). A homology search with sequences in the Genbank/EMBL databases was carried out with the software package of the Genetics Computer Group programs installed on a VAX computer at the Université de Montréal.

Culture conditions

The cultivation of the strains was carried out as previously described by Kluepfel et al. (1990). For large-scale enzyme production, 2-litre Erlenmeyer flasks containing 400 ml of modified M13 medium were used. The medium contained oat-spelts xylan (Sigma; 4 g), $(NH_4)_2SO_4$ (1.4 g), K_2HPO_4 (5.5 g), KH_2PO_4 (1.0 g), $MgSO_4,7H_2O$ (0.3 g), $CaCl_2,2H_2O$ (0.3 g) and Tween 80 (2 ml) in 1 litre of distilled water; 1 ml of a trace-metal solution, containing $CoCl_2,6H_2O$ (200 mg), $FeSO_4,7H_2O$ (500 mg), $MnSO_4,H_2O$ (160 mg) and $ZnSO_4,7H_2O$ (140 mg), in 100 ml of distilled water, was added. $CaCl_2$ and $MgSO_4$ were added aseptically after sterilization to prevent the formation of precipitates. The cultures were incubated at 34 °C on a rotary shaker at 240 rev./min for 48 h. The inoculum size was 6 % (v/v) and was obtained from a vegetative tryptic-soy-broth (TSB) culture.

Enzyme recovery

The fermentation broth was centrifuged for 30 min at 4 °C and 10000 ***g***. The mycelium was washed, then suspended in an equal volume of 50 mM sodium phosphate (pH 6.8)/0.5 mM dithiothreitol (DTT)/1.0 mM EDTA/0.5 mM phenylmethanesulphonyl fluoride. The cells were disrupted by two successive passages through a French pressure cell operated at 96.6 MPa (14000 lbf/in²). The resulting suspension was centrifuged at 4 °C and 24000 ***g*** for 30 min. The supernatant represented the cytoplasmic fraction containing the enzyme activity.

Enzyme purification

$(NH_4)_2SO_4$ was slowly added, with stirring, to the cytoplasmic fraction to 35 % saturation level at 4 °C. The mixture was stirred for another 60 min, and the resulting precipitate was removed by centrifugation at 4 °C and 10000 ***g*** for 30 min. The supernatant was adjusted to 65 % satn., stirred, then centrifuged as described above. The pellet was dissolved in 20 mM piperazine (pH 6.0)/0.5 mM DTT/1.0 M $(NH_4)_2SO_4$ and loaded on to a column (5 cm × 15 cm) packed with phenyl-Sepharose CL-4B (Pharmacia). The column was previously equilibrated with the same buffer and then eluted at a rate of 2.0 ml/min with a decreasing gradient of 1.0–0 M $(NH_4)_2SO_4$. Active fractions were collected, pooled, passed through a low-protein-binding Acro Disc filter (0.45 μm pore size; Gelman Science, Ann Arbor, MI, U.S.A.) and absorbed directly on to a semi-preparative anion-exchange h.p.l.c. column (Protein-Pak DEAE 5 PW; Waters-Millipore, St. Laurent, Qué., Canada) which had been equilibrated with 20 mM piperazine (pH 6.0)/0.5 mM DTT. The enzyme was eluted in the same buffer with a linear gradient of 0.1–1.0 M NaCl. The active fractions were pooled, concentrated and desalted in 0.1 M sodium phosphate (pH 7.0)/0.5 mM DTT by ultrafiltration with in a Centriprep concentrator 100 (100000-M_r nominal cut-off; Amicon Division, Danvers, MA, U.S.A.). Final purification was carried out by gel filtration on two h.p.l.c. columns in series (Protein Pak 300 SW; Waters–Millipore). The columns were previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.0)/0.5 mM DTT and then eluted with the same buffer at an elution rate of 0.5 ml/min. AF-containing fractions were pooled and concentrated by ultrafiltration. All the enzyme purification steps were carried out at room temperature. The purified α-L-AF was stored at −70 °C after addition of glycerol to a final concentration of 5 %.

Enzyme assays

The assay solution contained 2 mM *p*-nitrophenyl α-L-arabinofuranoside as substrate in 0.4 ml of 0.1 M McIlvaine buffer, pH 6.0. The reaction was initiated by addition of 0.02 ml of appropriately diluted enzyme, incubated for 10 min at 60 °C and terminated by addition of 0.6 ml of 1.0 M Na_2CO_3. The absorbance of this mixture was determined at 400 nm and converted into μmol of *p*-nitrophenol (*p*-NP) released, calculated from a standard curve with known concentrations of this compound. The enzyme activity was expressed in units, where 1 unit is the amount of enzyme that releases 1 μmol of *p*-NP/min. AF assays on polysaccharide were carried out at substrate concentration of 1 % (suspended in 0.1 M McIlvaine buffer, pH 6.0, to which 1 μg of enzyme was added and incubated at 37 °C for 16 h, followed by h.p.l.c. analysis. Substrates used in these assays were oat-spelts xylan (Sigma), rye- and wheat-flour arabinoxylan (Megazyme Ltd., Sydney, Australia), arabinogalactan (Sigma), and linear and debranched arabinan (Megazyme). AF assays were carried out with arabino-oligoxylosides at concentrations of 50 mM, to which 1 μg of enzyme was added, and the mixture incubated at 37 °C for 2 h, followed by t.l.c.

Protein

The protein content of enzyme preparations was determined as described by Lowry et al. (1951), using BSA fraction V (Sigma) as standard.

Determination of isoelectric point

Analytical isoelectric focusing was carried out on PhastGel containing Pharmalyte carrier ampholytes in the pH range 3–10 using the PhastSystem. The gels were silver-stained after the run. (Pharmacia-LKB Separation and Development Technique Files nos. 100 and 210).

Determination of M_r

The M_r of the purified AF was estimated by SDS/PAGE using the PhastSystem. The protein bands were detected by silver staining (Pharmacia-LKB Separation and Development Tech-

nique Files nos. 111 and 210). The M_r of the native protein was determined by using two Pharmacia Superose 6 HR 10/30 columns in series, calibrated with standard proteins (Sigma Kit no. MW-1000), using 0.1 M sodium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min.

Purification of arabino-oligoxylosides

A digest of oat-spelts xylan by xylanases B and C (Kluepfel et al., 1990, 1992) was passed through a column of Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories, Mississauga, Ont., Canada) using water as eluent at a flow rate of 1 ml/min and at 50 °C (John et al., 1969). The different fractions were collected and freeze-dried. Linear oligoxylosides and branched arabino-oligoxylosides were separated by preparative paper chromatography on Whatman 3MM paper using the solvent system butanol/pyridine/water (6:4:3, by vol.), by the descending method. The oligosaccharides were detected with aniline/hydrogen phthalate reagent. The corresponding bands were cut out, the sugars were eluted in water, and then freeze-dried. Their M_r values were determined by fast-atom-bombardment m.s. by Dr. O. A. Mamer (Biomedical Mass Spectrometry Unit, McGill University, Montréal, Qué., Canada).

Analysis of oligosaccharides

Mixture of oligosaccharides were analysed either by h.p.l.c. on an Aminex HPX-42A column (Bio-Rad) at 80 °C with water as eluent at a flow rate of 0.5 ml/min, or by t.l.c. on microcrystalline cellulose plates (Merck, Darmstadt, Germany) in the solvent system butanol/pyridine/water (6:4:3, by vol). Reducing sugars were revealed with aniline/hydrogen phthalate reagent.

Antibodies and Western immunoblotting

Antibodies were raised against AF in New Zealand White rabbits by injecting 100 μg of pure enzyme combined with complete Freund's adjuvant, followed by three additional injections of 200 μg of AF in incomplete Freund's adjuvant at intervals of 1, 2 and 7 weeks respectively. Western blotting was carried out using anti-AF antibodies coupled to ^{125}I-Protein A (Amersham Canada Ltd., Oakville, Ont., Canada) as previously described (Mondou et al., 1986)

RESULTS

Cloning of the α-L-AF gene

Screening of a gene bank of *Streptomyces lividans*, constructed in *S. lividans* IAF10-164 as described by Mondou et al. (1986), resulted in the isolation of two clones showing α-L-AF activity. These clones harboured plasmids pIAF112 and pIAF116, which had chromosomal DNA insertions of 7.8 kb and 11.4 kb respectively. The insert's size was reduced by *Kpn*I and *Pst*I digestions respectively, and led to plasmids pIAF113 and pIAF117 (Figure 1). Transformation of *S. lividans* IAF10-164 with these plasmids gave AF-positive clones. Southern hybridization with a 3.2 kb *Kpn*I–*Pst*I fragment from pIAF116 revealed identical sequences in all the clones (results not shown). Restriction fragments from plasmid pIAF116 served for the determination of the DNA sequence of the *abf*A gene. AF activity was determined for each clone and compared with that of the wild-type *S. lividans* 1326 and the mutant *S. lividans* IAF10-164 serving as host strain. All clones showed improved enzyme production (Table 1). *S. lividans* IAF116 produced AF at a level 48 times that of the wild type, and therefore was chosen for further enzyme purification.

Nucleotide-sequence analysis

The *abf*A gene was located on the 3.2 kb *Pst*I--*Kpn*I fragment in plasmid pIAF116 (Figure 1). This fragment and the internal *Sal*I fragments were subcloned into M13mp18 and M13mp19 and the nucleotide sequence was determined for both strands. The last

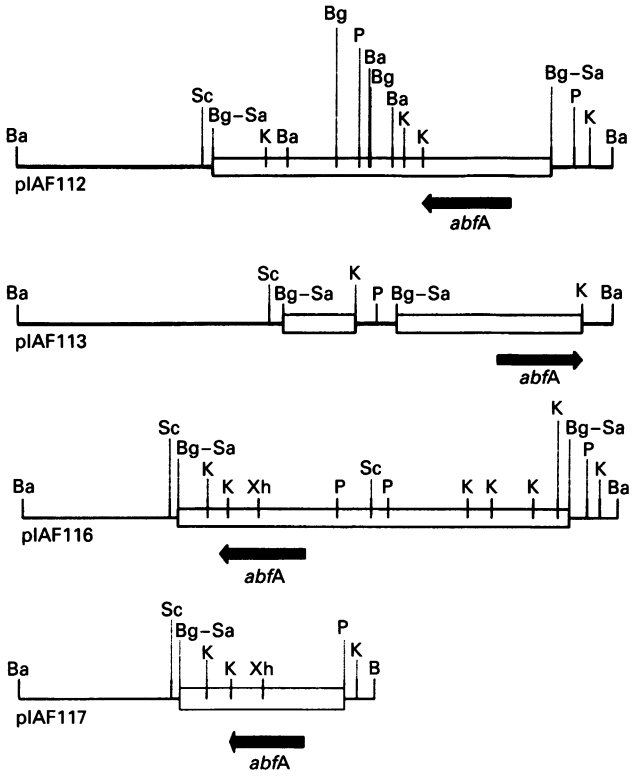

Figure 1 Restriction maps of plasmids pIAF112, pIAF113, pIAF116 and pIAF117

The open boxes represent the inserts cloned into plasmid pIJ702 (thin line). The arrow marked *abf*A indicates the orientation of the *abf*A structural gene. Restriction endonucleases: Ba, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; Sa, *Sau*3A; Xh, *Xho*I.

Table 1 Comparison of intracellular α-L-arabinofuranosidase activity of wild-type *Streptomyces lividans* 1326 and mutant *S. lividans* IAF10-164 and the clones carrying *abf*A recombinant plasmids pIAF112, pIAF113, pIAF116 and pIAF117 after cultivation in submerged cultures for 48h

Streptomyces lividans strain	Activity (units/mg of protein)
1326 (wild-type)	0.05
3131 (wild-type + pIJ702)	0.07
IAF10-164 (*xln*$^-$, *cel*$^-$)	ND*
IAF112	0.26
IAF113	0.84
IAF116	2.41
IAF117	2.04

* ND, not detectable.

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aa#                                                                         nt#
     GG CCG GGC GCC TGA TGG CCG GCC TGC CGC TGA TCG TCG TCT TCG TCT         47
    TCT TCC AGC GCC AGA TCG TGG CGG CGT CGC CCA CAC GGC CTG GCC GGC         95
    CAG TAG CCC CTC ACC CCG TCC CGA AAG GAA AAG ATG CGC ACC GCC CGC        143
                                                M   R   T   A   R
    TTC ACC CTC GAC CCC GCC TTC ACC GTC GGC GCC GTC AAC CCC CGT CTC        191
  6  F   T   L   D   P   A   F   T   V   G   A   V   N   P   R   L
    TTC GGC TCC TTC GTC GAA CAC CTC GGA CGC TGC GTG TAC ACC GGC GTC        239
 22  F   G   S   F   V   E   H   L   G   R   C   V   Y   T   G   V
    TTC GAG CCC GGC CAC CCC ACC GCG GAC GCC GAG GGC CTG CGC CAG GAC        287
 38  F   E   P   G   H   P   T   A   D   A   E   G   L   R   Q   D
    GTG CTG GAG CTG GTC CGG GAA CTC GGC GTC ACC GCC GTC CGC TAC CCC        335
 54  V   L   E   L   V   R   E   L   G   V   T   A   V   R   Y   P
    GGC GGC AAC TTC GTC TCC GGC TAC AAG TGG GAG GAC TCG GTC GGC CCG        383
 70  G   G   N   F   V   S   G   Y   K   W   E   D   S   V   G   P
    GTG GAG GAC CGT CCG CGC CGC CTC GAC CTG GCC TGG CGC TCC ACC GAG        431
 86  V   E   D   R   P   R   R   L   D   L   A   W   R   S   T   E
    ACC AAC CGC TTC GGC CTC TCC GAG TAC ATC GCC TTC CTG AAG AAG ATC        479
102  T   N   R   F   G   L   S   E   Y   I   A   F   L   K   K   I
    GGC CCG CAG GCC GAG CCC ATG ATG GCG GTC AAC CTC GGC ACC CGC GGC        527
118  G   P   Q   A   E   P   M   M   A   V   N   L   G   T   R   G
    GTC GCC GAG GCG CTC GAA CTC CAG GAG TAC GCC AAC CAC CCC TCC GGC        575
134  V   A   E   A   L   E   L   Q   E   Y   A   N   H   P   S   G
    ACG GCC CTG TCC GAC CTG CGC GCC GAG CAC GGC GAC AAG GAC CCC TTC        623
150  T   A   L   S   D   L   R   A   E   H   G   D   K   D   P   F
    GGC ATC AGG CTC TGG TGC CTG GGC AAC GAG ATG GAC GGC CCC TGG CAG        671
166  G   I   R   L   W   C   L   G   N   E   M   D   G   P   W   Q
    ACC GGC CAC AAG ACC GCC GAG GAG TAC GGC CGC GTC GCC GCC GAG ACC        719
182  T   G   H   K   T   A   E   E   Y   G   R   V   A   A   E   T
    GCC CGC GCC ATG CGC CAG ATC GAC CCG GAC GTC GAA CTG GTC GCG TGC        767
198  A   R   A   M   R   Q   I   D   P   D   V   E   L   V   A   C
    GGG TCG TCC GGG CAG TCC ATG GAG ACC TTC GCC GAG TGG GAG GCG ACG        815
214  G   S   S   G   Q   S   M   E   T   F   A   E   W   E   A   T
    GTC CTG AAG GAG ACC TAC GAC CTG GTC GAC CAC ATC TCG CTG CAC GCC        863
230  V   L   K   E   T   Y   D   L   V   D   H   I   S   L   H   A
    TAC TAC GAG CCG CAC GAC GGG GAC GTC GAC TCC TTC CTC GCC TCC GCC        911
246  Y   Y   E   P   H   D   G   D   V   D   S   F   L   A   S   A
    GTG GAC ATG GAG TCG TTC ATC GAG AAC GTC GTC GCC ACC TGC GAC CAC        959
262  V   D   M   E   S   F   I   E   N   V   V   A   T   C   D   H
    GTC GGC GCC CGC CTC AAG TCG AAG AAG AAG ATC AAC CTC TCC TTC GAC       1007
278  V   G   A   R   L   K   S   K   K   K   I   N   L   S   F   D
    GAG TGG AAC GTC TGG TAC ATG ACC AAG ACG CAG GCC GAG GTC AGC GCC       1055
294  E   W   N   V   W   Y   M   T   K   T   Q   A   E   V   S   A
    CTG GAC TGG CCC GAG GCG CCC CGC CTG CTC GAG GAC AAC TAC AGC GTC       1103
310  L   D   W   P   E   A   P   R   L   L   E   D   N   Y   S   V
    ATG GAC GCG GTC GTC TTC GGC TCG CTC CTG ATC GCC CTG CTG CGG CAC       1151
326  M   D   A   V   V   F   G   S   L   L   I   A   L   L   R   H
    GCC GAC CGC GTC ACC GTG GCC TGC CTC GCG CAG CTC GTC AAC GTC ATC       1199
342  A   D   R   V   T   V   A   C   L   A   Q   L   V   N   V   I
    GCG CCG ATC ATG ACC GAG CCG GGC GGC CCG GCC TGG CGC CAG ACG ACG       1247
358  A   P   I   M   T   E   P   G   G   P   A   W   R   Q   T   T

aa#                                                                         nt#
    TTC TTC CCC TTC TCC CAG GCC TCG AAG TAC GGC CGC GGC GAG GTC CTC       1295
374  F   F   P   F   S   Q   A   S   K   Y   G   R   G   E   V   L
    GAC GTC CGC GTG GAC TCC CCG ACG TAC GAC ACC GCG AAG TAC GGC GAG       1343
390  D   V   R   V   D   S   P   T   Y   D   T   A   K   Y   G   E
    GCC GAC CTG CTG CAC GCC ACC GCC GTG GTG CGC GCC AGA CGC TCG GTC       1391
406  A   D   L   L   H   A   T   A   V   V   R   A   R   R   S   V
    ACC GTC TTC GCC GTC AAC CGC TCG CGC ACC GGG GCC CTC CCG CTG GAG       1439
422  T   V   F   A   V   N   R   S   R   T   G   A   L   P   L   E
    GTC GCC CTG AGC GGC CTG GAG CTG ACC GAG GTC GTC GAG CAC AGC GCC       1487
438  V   A   L   S   G   L   E   L   T   E   V   V   E   H   S   A
    CTC GCG GAC GCC GAC CCC GAC GCC CGC AAC ACC CTC GCC GAG CCC GAG       1535
454  L   A   D   A   D   P   D   A   R   N   T   L   A   E   P   E
    CGC GTC GTC CCG CAC CCG GTC GAC GGC ACC TCC CTG AGG GAC GGC CGG       1583
470  R   V   V   P   H   P   V   D   G   T   S   L   R   D   G   R
    CTC ACC GCC GCC CTG GAA CCG CTG TCC TGG AAC TCG ATC CGC TGC GCT       1631
486  L   T   A   A   L   E   P   L   S   W   N   S   I   R   C   A
    GAC CCG GCC CCG GGT CAG CCG CCG CGC CGG CCG GGG GAG GGG ACG GGC       1679
502  D   P   A   P   G   Q   P   P   R   R   P   G   E   G   T   G
    TTC ACC GGC ACT CCT CCG GCC GCG CCG CCC AGC AGC TCC AGC GCA CCG       1727
518  F   T   G   T   P   P   A   A   P   P   S   S   S   S   A   P
    CGC CCG GAC CCG ACG GCG CGC CGT TCG CCG GAC AGG ACC GCG AGG GCC       1775
534  R   P   D   P   T   A   R   R   S   P   D   R   T   A   R   A
    AGG GTG TTG GCG GCC GCC CGG GTG CGC AGG ATG CCG TTC GGC AGG ACG       1823
550  R   V   L   A   A   A   R   V   R   R   M   P   F   G   R   T
    AAG GTG TGC GGG GCA CCC GTG CGG CCG CCC ACG TAC GCG CCC AGG TTC       1871
566  K   V   C   G   A   P   V   R   P   P   T   Y   A   P   R   F
    CAG CCG TTC AGG AAG ACC TGG ACG CGG TGG GCA CCG GCG CCG CGG TCC       1919
582  Q   P   F   R   K   T   W   T   R   W   A   P   A   P   R   S
    GGG TCT CCG TCC AGG CGC AGC CCC ACC GAG GCG TCG ATC CCC GGC GGG       1967
598  G   S   P   S   R   R   S   P   T   E   A   S   I   P   G   G
    ACG TCC AGC CGG AAC GTG GTC CGG TAC CAG GTC ACG CCC TGG CGC CGG       2015
614  T   S   S   R   N   V   V   R   Y   Q   V   T   P   W   R   R
    TCC CCA CCC GGC AGC GCG CCG GGG ACA CCC GCT CCC ACC CGC CGT CGT       2063
630  S   P   P   G   S   A   P   G   T   P   A   P   T   R   R   R
    CGT ACC CGG GCA GGT GCC AGC CGT GGC GCT CCC CGT ACA GCC CGC CGT       2111
646  R   T   R   A   G   A   S   R   G   A   P   R   T   A   R   R
    TGT TGA GCG GCC CGC GCA CCG GGT CGG GGG CGG CCG CGC CCA TGA TCC       2159
662  C   *
    GCC AGC GCA CCT CGG GCG AGG CGC CCT CGA ACG CCG CCG ACG TCA GCC       2207
    CGC GGG CCT CCC GGT ACG CGT CCT GGC CGT GCT GTG TGC GCC GCA CCA       2255
    GGA CGG ACA GGA CGG GGC CGT CGT CCC CTT CGC CGC CCT TGC TCC CCC       2303
    CGC CCG CCC TGC TCC CGG CGG CCC GAC GCA GTG CGG CGC GCA GGT CCT       2351
    CCG GCA GCC CGA ACG CGG CGT GCC GGT CCA CGT GCC CTT CCC GTC GCC       2399
    CGC CCC GTC CGG CTG GTG GCT GCC CAG CGG CAC CCC GTC CAG CCA GGC       2447
    CAT CAG CAG GCC GTC CGC GCC CGT GCT GTA CGC GAG GGA CAC CCG CTC       2495
    CAG CCC CGT GGC GCC GGT GAG GCG GGC GC                                2524
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Figure 2 Nucleotide sequence and deduced amino acid sequence of *abf*A

The amino acids that are identical with the N-terminus of the protein are shaded (residues 1–23). The putative ribosome-binding site is shown in **bold** face.

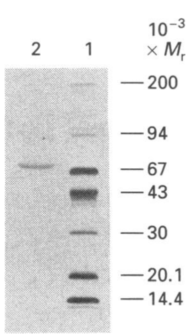

Figure 3 Silver-stained SDS/PAGE (12.5% polyacrylamide gel) of purified α-L-AF

Lane 1, standard protein markers: β-amylase (M_r 200000), phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400); lane 2, purified α-L-AF.

41 amino acids of the C-terminal end of the protein were beyond the *Kpn*I site and were deduced from the nucleotide sequence of the 0.6 kb *Kpn*I fragment isolated from plasmid pIAF116. Figure 2 shows the nucleotide sequence of *abf*A. An open reading frame (ORF) starts at an ATG codon located at nt 128 and stops at the TGA codon at nt 2117. This 1986 nt ORF encodes a protein of 662 amino acids totalling 72492 Da. This value is close to the M_r of the purified protein estimated by SDS/PAGE (Figure 3). As reported for many genes from streptomycetes, the base composition of the coding region is 73.5% rich in G+C and the third base of the codon is highly biased (95%) in favour of G or C (Wright and Bibb, 1992). Accordingly, 17 codons out of 61 are unused, and eight are used only once. The first 23 amino acids of the N-terminus of the purified intracellular protein were determined (shaded amino acid residues in Figure 2) using the Edman degradation method (carried out by F. Dumas, Biotechnology Research Institute, Montréal, Qué., Canada). These results enabled us to validate the nucleotide sequence. The putative ORF is preceded by a potential ribosome-binding site (AGGA) located 8 nt upstream of the starting ATG codon.

A search through the Genbank and EMBL Nucleotide Sequence Databases using FASTA software showed no significant similarity with other AF genes already sequenced, namely the *xyn*C gene from *Pseudomonas fluorescens*, which encodes the XylC protein (Kellett et al., 1990), and the *xyl*B gene of *Butyrivibrio fibrisolvens* (Utt et al., 1991).

Production and isolation of α-L-AF

The α-L-AF was produced in submerged cultures with clone *Streptomyces lividans* IAF116 using the mineral-salt medium M13 containing 1% oat-spelts xylan as carbon source. Optimal enzyme levels of 1 unit/mg of total protein were reached after 48 h of incubation. Crude cell extracts were prepared by disruption of the mycelium in a French press. More than 90% of

Table 2 Purification of an α-L-arabinofuranosidase from *S. lividans* IAF116

Purification step	Total activity (units)	Total protein (mg)	Specific activity) (units/mg of protein)	Yield (%)
Cytoplasmic extract	2826	1970.0	1	100
$(NH_4)_2SO_4$	2595	1590.0	2	92
Phenyl-Sepharose CL-4B	2116	158.0	13	75
DEAE-Sepharose	1307	17.8	73	46
Gel filtration	581	3.8	153	21

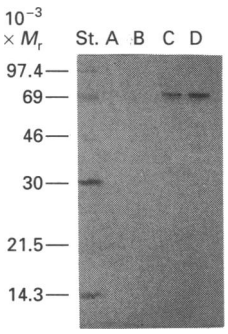

Figure 4 Western-blot analysis of cytoplasmic extract (50 μg of protein) with anti-α-L-AF antibodies

Lane St., ^{14}C-labelled proteins standards: myosin (200000), phosphorylase *b* (97400), BSA (69000), ovalbumin (46000), carbonic anhydrase (30000), trypsin inhibitor (21500) and lysozyme (14300); lane A, *S. lividans* 1326 (wild-type); lane B, *S. lividans* IAF10-164 (*cel*$^-$, *xln*$^-$ mutant); lane C, *S. lividans* IAF116 (*abfA*); lane D, pure AF (1 μg of protein).

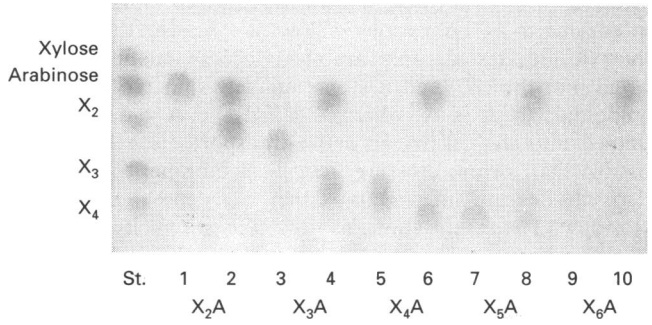

Figure 5 T.l.c. analysis of α-L-AF activity on arabino-oligoxylosides

Lane St., standard sugars: xylose, arabinose, xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4); lane 1, arabino-xylobiose (X_2A); lane 2, X_2A hydrolysed with AF; lane 3, arabinoxylotriose (X_3A); lane 4, X_3A hydrolysed with AF; lane 5, arabinoxylotetraose (X_4A); lane 6, X_4A hydrolysed with AF; lane 7, arabinoxylopentaose (X_5A); lane 8, X_5A hydrolysed with AF; lane 9, arabinoxylohexaose (X_6A); lane 10, X_6A hydrolysed with AF.

the AF activity was intracellular and was recovered in the cytoplasmic fraction obtained after centrifugation. In all, 90% of the activity was recovered in the protein precipitate between 35 and 65% saturation with $(NH_4)_2SO_4$. On phenyl-Sepharose CL-4B column chromatography, the bulk of the protein was eluted before the the AF activity, which was recovered at the end of the $(NH_4)_2SO_4$ gradient. The AF activity loaded on to an h.p.l.c. DEAE anion-exchange column was eluted at an NaCl concentration of 0.26 M. On h.p.l.c. gel-filtration chromatography, the AF activity was eluted as a single peak. The approximate M_r of this protein was 380 kDa, estimated by h.p.l.c. gel filtration on Superose columns. The purity of the α-L-AF was verified by SDS/PAGE followed by silver staining (Figure 3). A summary of the purification steps is shown in Table 2.

The AFs produced by clones IAF 112 and 113 were also purified and showed the same enzyme characteristics as those found for *S. lividans* IAF 116. However, in the case of IAF 113, the purified protein lacked several amino acid residues at the C-terminal end of the molecule.

Characterization of the α-L-AF

The cloned α-L-AF from *S. lividans* IAF116 was compared with that of *S. lividans* 1326 and IAF10-164. SDS/PAGE and Western immunoblotting with anti-AF antibodies showed the identity of the purified enzyme with that of the wild-type (Figure 4). AF was not detectable in the mutant IAF10-164. This confirmed the lack of activity observed for this strain. SDS/PAGE analysis indicated that the purified protein had an apparent M_r of about 69000. This is supported by an M_r of 72492 calculated from the DNA and amino acid sequence analysis. From gel chromatography the M_r of the protein was estimated to be about 380000, which suggests that the native AF has a polymeric nature.

Analytical isoelectric focusing of the enzyme on a pH gradient from 3.0 to 10.0 indicated a pI of 4.6. The AF has an optimum activity at 60 °C and at pH 6.0. The enzyme when incubated without substrate was stable at 4 °C for 12 h at pH 6 .0. At 68 °C and pH 6.0 the half-life was 12 min. Periodate/Schiff staining indicated that the protein was not glycosylated.

The determination of the kinetic constants of the purified α-L-AF was carried out on *p*-nitrophenyl α-L-arabinofuranoside at substrate concentrations between 0.1 and 1.0 mM. Under optimal assay conditions at pH 6.0 and 60 °C and using either the Lineweaver–Burk or the Eadie–Hofstee plots, resulted in a $V_{max.}$ of 180 units/mg of enzyme and a K_m of 0.6 mM.

Towards *p*-nitrophenyl α-L-arabinofuranoside the purified AF displayed high specific activity of 153 units/mg. A faint β-xylosidase activity of 0.18 unit/mg of protein was observed with *p*-nitrophenyl β-D-xylopyranoside. Other *p*-nitrophenyl saccharides with sugar moieties such as α-L-arabinopyranose, β-D-glucose, β-D-galactopyranose, β-D-cellobiose or α-L-mannopyranose were not affected. No end-product inhibition was evident when the purified AF was assayed in the presence of up to 50 mM L-arabinose. No release of sugars could be detected when the enzyme was incubated with either oat-spelts xylan or arabinogalactan. Arabinose release was detected by h.p.l.c. analysis when the AF was incubated with debranched or linear beet arabinan and with rye- or wheat-flour arabinoxylans (results not shown). However, the AF hydrolysed arabino-oligoxylosides to arabinose and the corresponding oligoxyloside (Figure 5).

These saccharides had been obtained from xylanase digest of oat spelts xylan and purified by column and paper chromatography, ranging from arabino-xylobiose to the arabinohexaxyloside.

DISCUSSION

The α-l-AF gene of *S. lividans* was cloned by functional complementation in the previously described mutant *S. lividans* IAF10-164 (Mondou et al., 1986). This mutant, which does not express either the xylanase or the cellulase genes, was also unable to produce α-AF activity. The use of the multicopy vector pIJ702 permitted the overproduction of the enzyme, reaching 48 times that of the wild-type strain *S. lividans* 1326. This increase in gene expression facilitated significantly the purification of the enzyme. The overexpression is also consistant with the previously reported results for the genes cloned with the same system from *S. lividans* encoding for the xylanases A, B and C (Mondou et al., 1986; Kluepfel et al., 1990, 1992), the cellulases A and B (Théberge et al., 1992; Wittmann et al., 1994), as well as for a β-mannanase (Arcand et al., 1993). AFs play an important role in the degradation of the many arabinose-containing hemicelluloses. In conjunction with acetylxylan esterase, α-methylglucuronidase and xylanases, their action is required as debranching enzyme for the complete biodegradation of xylans which are found in Nature in various heteropolymeric forms (Biely, 1985). Although AFs are found in many plants, fungi and bacteria, comparatively few have been cloned, purified and characterized. The heterologous cloning approach, using *Escherichia coli*, has been used to clone the AF-encoding genes from *Pseudomonas fluorescens* (Kellett et al., 1990), *Butyrivibrio fibrisolvens* (Utt et al., 1991), *Clostridium stercorarium* (Schwarz et al., 1990) and, more recently, for *Aspergillus niger* (Flipphi et al., 1993).

The DNA sequence of the *abf*A gene and its deduced amino acid sequence of the enzyme were determined. No significant similarity was found with the *xyn*C gene of *P. fluorescens*, which encodes the XylC protein or with the *xyl*B gene of *B. fibrisolvens*. Both encode a bifunctional protein which exhibited both β-xylosidase and an α-AF activities. The *S. lividans* enzyme has a low activity on *p*-nitrophenyl β-D-xylopyranoside, but does not act on xylan. Most α-AFs exist in Nature as monomers, but dimers, tetramers and octamers have also been reported. Their M_r values range from 53000 to 495000 (Eriksson et al., 1990). From the estimated M_r of 380000 the *S. lividans* enzyme in its natural form appears to consist also of several subunits. The enzyme is located inside the cells and was purified from the cytoplasmic fraction during the exponential growth of the microorganism (up to 72 h). It is also released, after prolonged fermentation, into the culture filtrate in its natural form, possibly as a result of cell lysis. Attempts to purify the protein from the supernatant were unsuccessful, owing to a pigment that co-purified and interfered consistently with the isolation.

The α-L-AF was produced by submerged cultures in Erlenmeyer flasks containing a simple mineral-salts medium with oat-spelts xylan. Under these conditions the wild-type strain *S. lividans* 1326 produced 0.05 unit/mg of protein. The clone *S. lividans* IAF116 containing the *abf*A gene on the multicopy plasmid produced under the same conditions 2.41 units, corresponding to a 48-fold increase in enzyme production. This production level is amongst the highest reported in the literature (Kaji et al., 1981; Komae et al., 1982; Greve et al., 1984; Lee and Forsberg 1987; Hespell and O'Bryan, 1992; Tajana et al., 1992).

DNA sequence and N-terminal amino acid analysis of the enzyme indicated the absence of a signal peptide, which confirmed the intracellular location of protein. AFs purified from actinomycetes such as *S. diastaticus* (Tajana et al., 1992), *Streptomyces* sp. 17-1 (Kaji et al., 1981) and *S. purpurascens* (Komae et al., 1982) were all recovered from culture supernatants. While the first enzymes isolated after only 24 h of incubation appeared likely to have been secreted, the latter two, of high M_r (> 92000) were isolated in the later stages (more than 4 days), possibly released during cell lysis. The biochemical characteristics of the *S. lividans* AF are similar to those reported for many of the enzymes isolated from both eukaryote and prokaryote origins (Hespell and O'Bryan, 1992). The specific activity of the enzyme from *S. lividans* is 153 units/mg of protein, significantly higher than those of streptomycetes reported elsewhere.

A comparison of specificities on natural substrates of the characterized AFs shows subtle differences. Thus the enzyme from *Streptomyces* sp. 17-1 hydrolyses arabinan, arabinoxylan or arabinogalactan (Kaji et al., 1981), whereas the AF from *S. purpurascens* is inactive towards these substrates, but is active on low-M_r oligoarabinosides (Komae et al., 1982). *S. diastaticus* produces two enzymes that attack oat-spelts xylan and arabinan (Tajana et al., 1992). The *S. lividans* AF described here exhibits no activity on oat-spelts xylan and arabinogalactan. It acts slowly on arabinan and arabinoxylan from wheat and rye flour by releasing L-arabinofuranose after prolonged incubation (overnight). However, the enzyme hydrolyses rapidly the short-chain arabino-oligoxylosides prepared by digestion of oat-spelts xylan with xylanases. Owing to the difficulty of preparing sufficient quantities of these compounds in pure form, testing of these substrates had to be limited to arabinoxylosides containing two to six xylose moieties as backbone substituted with one arabinose molecule. From these results and from the hydrolysis patterns obtained with the different *p*-nitrophenyl derivatives, the *S. lividans* enzyme can be classified among the α-L-AFs (EC 3.2.1.55).

The substrate specificity of the AF of *S. lividans* is likely correlated with its intracellular location, which limits access to the enzyme to carbohydrates of low M_r. Among the arabino-oligoxylosides tested, only arabinoxylobiose might be transported into the cell, and it seems unlikely that the larger size compounds (X_3A–X_6A, where X = Xyl and A = Ara) can be pumped into the cell by an active-transport mechanism. It is possible that, before crossing the cell membrane, the arabino-oligoxylosides are affected by either a xylanase or by a β-xylosidase. The results show also that this specificity is characteristic and remains unchanged, even when AF is released into the culture filtrates during the idiophase of the fermentation. It remains to be established whether the enzyme retains a role in the microbial metabolism at this stage.

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