Purification and characterization of an \(\alpha\)-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (\(abfA\))

Catherine MANIN, François SHAREEK, RolF MOROSOLI and Dieter KLUEPPEL*
Centre de recherche en microbiologie appliquée, Institut Armand-Frappier, Université du Québec, 531 boulevard des Prairies, Laval-des-Rapides, Québec, Canada H7N 4Z3

The gene encoding an \(\alpha\)-L-arabinofuranosidase (\(abfA\)) 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 Mr was estimated by gel filtration and found to be approx. 380000. Since SDS/PAGE indicated a native protein of Mr, 69000, it can be concluded that the native protein consists of several subunits of that size. The pl value was 4.6. The kinetic constants determined with p-nitrophenyl \(\alpha\)-L-arabinofuranosidase as substrate were a \(V_{\text{max}}\) 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 \(\alpha\)-L-arabinofuranoside, but had no activity on a variety of other p-nitrophenyl glycosides, except on p-nitrophenyl \(\beta\)-D-xylopyranoside. The enzyme showed no activity on oat-sprouts (*Arenia sativa*) xylan or arabinoxylan, but acted on beet (\(\Beta\)) arabinan or arabinoxylan. Hydrolysis occurred on arabin-oligooligosides obtained from oat-sprouts 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 \(\beta\)-xylosidases or xylanases.

**INTRODUCTION**

\(\alpha\)-Arabinose residues are found widely distributed among many heteropolysaccharides of different plant tissues. They constitute monomeric and/or oligomeric side chains on \(\beta\)-1,4-linked xylose or galactose backbones in xylans, arabinoxylans and arabinogalactans and are the core in arabinitols forming \(\alpha\)-1,5-linkages (Ward and Moo-Young, 1989). These side chains may restrict the enzymic hydrolysis of hemicelluloses in different applications of hemicelluloses, 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 \(\alpha\)-L-arabinofuranosidic linkages are arabinosidases which include \(\alpha\)-L-arabinofuranosidases (AFs; EC 3.2.1.55), that hydrolyze terminal non-reducing \(\alpha\)-1,2- or \(\alpha\)-1,3-arabinofuranosyl resi-

utes from xylans, arabinoxylans and arabinogalactans, and endo-1,5-\(\alpha\)-L-arabinanases (EC 3.2.1.99), which hydrolyse \(\alpha\)-1,5-arabinofuranosidic linkages from arabinitols (Dekker and Richards, 1976; Kaji, 1984). \(\alpha\)-AFs are also involved in the hydrolysis of grape monoterpenyl glycosides during wine fermentation (Gunata et al., 1989).

\(\alpha\)-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’Byran, 1992) and *Streptomyces* sp. (Kaji et al., 1981; Komae et al., 1982; Tajana et al., 1992).

In our studies on hemicelluloses from *Streptomyces lividans*, we have homologously cloned several xylanase, \(\beta\)-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; Theberge 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 \(\alpha\)-L-AF gene (\(abfA\)) 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 multi-copy plasmid pIJ702, which served as cloning vector, was kindly supplied by Dr. E. Katz (Katz et al., 1983).

**Cloning of the \(\alpha\)-L-AF gene**

The \(\alpha\)-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 \(\alpha\)-L-
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 was 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 Pestell 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-splets xylan (Sigma; 4 g), (NH₄)₂SO₄ (1.4 g), K₂HPO₄ (5.5 g), KH₂PO₄ (1.0 g), MgSO₄·7H₂O (0.3 g), CaCl₂·2H₂O (0.3 g) and Tween 80 (2 ml) in 1 litre of distilled water; 1 ml of a trace-metal solution, containing CoCl₂·6H₂O (200 mg), FeSO₄·7H₂O (500 mg), MnSO₄·H₂O (160 mg) and ZnSO₄·7H₂O (140 mg), in 100 ml of distilled water, was added. CaCl₂ and MgSO₄ 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

(NH₄)₂SO₄ 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 (NH₄)₂SO₄ and loaded on to a column (5 cm x 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 (NH₄)₂SO₄. 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 FW; 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 Centrprep concentrator 100 (100000-Mₖ) 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 Na₂CO₃. 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%, (suspending 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 arabinoylxylan (Megazyme Ltd., Sydney, Australia), arabinogalactan (Sigma), and linear and debranched arabinan (Megazyme). AF assays were carried out with arabinooligosaccharides 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 M₅₅

The M₅₅ 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 \( M_s \) 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-oligomannosides**

A digest of oat-spelt 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 oligomannosides and branched arabino-oligomannosides 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 \( M_s \) values were determined by fast-atom-bombardment m.s. by Dr. O. A. Mamer (Biomedical Mass Spectrometry Unit, McGill University, Montréal, Que., 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 \( \mu \)g of pure enzyme combined with complete Freund's adjuvant, followed by three additional injections of 200 \( \mu \)g 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 \( \alpha-L \)-arabinofuranosidase 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 \( \alpha-L \)-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 \( \text{PstI} \) and \( \text{XhoI} \) 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 \( \text{PstI} \)–\( \text{XhoI} \) 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 \( \text{abfA} \) 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.

**Table 1** Comparison of intracellular \( \alpha-L \)-arabinofuranosidase activity of wild-type *Streptomyces lividans* IAF10-164 and the clones carrying \( \text{abfA} \) recombinant plasmids pIAF112, pIAF113, pIAF116 and pIAF117 after cultivation in submerged cultures for 48h

<table>
<thead>
<tr>
<th>Streptomyces lividans strain</th>
<th>Activity (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAF10-164 (wild-type)</td>
<td>0.05</td>
</tr>
<tr>
<td>3131 (wild-type + pJU702)</td>
<td>0.07</td>
</tr>
<tr>
<td>IAF10-164 (xIL-; ceIL-)</td>
<td>ND*</td>
</tr>
<tr>
<td>IAF112</td>
<td>0.26</td>
</tr>
<tr>
<td>IAF113</td>
<td>0.84</td>
</tr>
<tr>
<td>IAF116</td>
<td>2.41</td>
</tr>
<tr>
<td>IAF117</td>
<td>2.94</td>
</tr>
</tbody>
</table>

* ND, not detectable.
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.

The amino acids 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 C or G (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, Québec, 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 xylC 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 submerging 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

<table>
<thead>
<tr>
<th>Purification</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>step</td>
<td>(units)</td>
<td>(mg)</td>
<td>(units/mg protein)</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic extract</td>
<td>2826</td>
<td>1970.0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2595</td>
<td>1590.0</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>2116</td>
<td>158.0</td>
<td>13</td>
<td>75</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>1307</td>
<td>17.8</td>
<td>73</td>
<td>46</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>581</td>
<td>3.8</td>
<td>153</td>
<td>21</td>
</tr>
</tbody>
</table>

Figure 4  Western-blot analysis of cytoplasmic extract (50 µg of protein) with anti-α-L-AF antibodies
Lane St., ¹⁴C-labelled proteins standards: myosin (200 000 U), phosphorylase b (97 400 U), BSA (69 000 U), ovalbumin (46 000 U), carbonic anhydrase (30 000 U), trypsin inhibitor (21 500 U) and lysozyme (14 300 U); lane A, S. lividans 1326 (wild-type); lane B, S. lividans IAF10-164 (cot-”, xln-” mutant); lane C, S. lividans IAF116 (abA); lane D, pure AF (1 µg of protein).

Figure 5  T.l.c. analysis of α-L-af activity on arabinogalactan oligosaccharides
Lane St., standard sugars: xylitol, arabinose, xylobiose (X₂), xylooligosaccharide (X₃), xylooligosaccharide (X₄); lane 1, arabinobiose (X₂A); lane 2, X₂A hydrolysed with AF; lane 3, arabinoligosaccharide (X₃A); lane 4, X₃A hydrolysed with AF; lane 5, arabinoligosaccharide (X₄A); lane 6, X₄A hydrolysed with AF; lane 7, arabinoligosaccharide (X₅A); lane 8, X₅A hydrolysed with AF; lane 9, arabinoligosaccharide (X₆A); lane 10, X₆A hydrolysed with AF.

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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 (NH₄)₂SO₄. On phenyl-Sepharose CL-4B column chromatography, the bulk of the protein was eluted before the Af activity, which was recovered at the end of the (NH₄)₂SO₄ 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 Mₘ 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 Mₘ of about 69 000. This is supported by an Mₘ of 72 492 calculated from the DNA and amino acid sequence analysis. From gel chromatography the Mₘ of the protein was estimated to be about 380 000, 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 pl 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-arabinofuranosidase 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 Vₘ of 153 units/mg of enzyme and a Kₘ of 0.6 mM.

Towards p-nitrophenyl α-L-arabinofuranosidase 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-arabinopyranosyl, β-D-glucose, β-D-galactopyranosyl, β-D-cellulobiose or α-L-mannopyranosyl 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-sprouts xylan or arabino-galactan. Arabinose release was detected by h.p.l.c. analysis when the AF was incubated with debranched or linear beet arabinoxylan and with rye- or wheat-flour arabinoxylans (results not shown). However, the AF hydrolysed arabinogalactan to arabinose and the corresponding oligosaccharides (Figure 5).
These saccharides had been obtained from xylanase digest of oat spelts xylan and purified by column and paper chromatography, ranging from arabino-xyloligosaccharides to arabinoxylotriose.

**DISCUSSION**

The \( \alpha \)-\( \lambda \)-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 \( \alpha \)-AF activity. The use of the multicycop 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 consistent 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 \( \beta \)-mannanase (Arcand et al., 1993). AFs play an important role in the degradation of the many arabinose-containing hemi-celluloses. In conjunction with acetylxylan esterase, \( \alpha \)-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* (Filippi et al., 1993).

The DNA sequence of the *abfA* gene and its deduced amino acid sequence of the enzyme were determined. No significant similarity was found with the *xynC* gene of *P. fluorescens*, which encodes the XyIC protein or with the *xyfB* gene of *B. fibrisolvens*. Both encode a bifunctional protein which exhibited both \( \beta \)-xylanase and an \( \alpha \)-AF activities. The *S. lividans* enzyme has a low activity on \( \pi \)-nitrophenyl \( \beta \)-D-xylopyranoside, but does not act on xylan. Most \( \alpha \)-AFs exist in nature as monomers, but dimers, tetramers and octamers have also been reported. Their \( M_c \), values range from 53000 to 495000 (Erikkson et al., 1990). From the estimated \( M_c \) 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 copurified and interfered consistently with the isolation.

The \( \alpha \)-\( \lambda \)-AF was produced by submerged cultures in Erlemeyer flasks containing a simple mineral-salts medium with oat-spells 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 *abfA* 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 \( M_c \) (> 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 streptomyces isolated elsewhere.

A comparison of specificities on natural substrates of the characterized AFs shows subtle differences. Thus the enzyme from *Streptomyces* sp. 17-1 hydrolyses arabinoxylan, arabinoxylan or arabinoagalactan (Kaji et al., 1981), whereas the AF from *S. purpurascens* is inactive towards these substrates, but is active on low-\( M_c \) oligoarabinosides (Komae et al., 1982). *S. diastaticus* produces two enzymes that attack oat-spells xylan and arabinoxylan (Tajana et al., 1992). The *S. lividans* AF described here exhibits no activity on oat-spells xylan and arabinoxylan. It acts slowly on arabinoxylans and arabinoxylan from wheat and rye flour by releasing L-arabinofuranose after prolonged incubation (overnight). However, the enzyme hydrolyses rapidly the short-chain arabino-oligoxyllosides prepared by digestion of oat-spells 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 arabinoyxyllosides 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 \( \alpha \)-\( \lambda \)-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 \( M_c \). Among the arabinono-oligoxyllosides tested, only arabinoxyloligosaccharides might be transported into the cell, and it seems unlikely that the larger size compounds (\( X_1 \)-A--\( X_n \)-A, 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 arabinono-oligoxyllosides are affected by either a xylanase or a \( \beta \)-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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**REFERENCES**


OC-L-Arabinofuranosidase from *Streptomyces lividans* 449


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