Appl Microbiol Biotechnol (1995) 44:112–117

ORIGINAL PAPER

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Purification and Characterization of an arabinofuranosidase from *Bacillus polymyxa* expressed in *Bacillus subtilis*

Received: 19 December 1994/Revised revision: 14 March 1995/Accepted: 3 April 1995

Abstract Two polypeptides showing α-L-arabinofuranosidase activity have been purified to homogeneity from culture supernatants of a *Bacillus subtilis* clone harbouring the *xynD* gene [Gosalbes et al. (1991) J Bacteriol 173: 7705–7710] from *Bacillus polymyxa*. Both polypeptides, with determined molecular masses of 64 kDa and 53 kDa, share the same sequence at their N termini, which also coincides with the sequence deduced for the mature protein from the previously determined sequence of nucleotides (Gosalbes et al. 1991). The two polypeptides have been biochemically characterized. Arabinose is the unique product released from arabinose-containing xylans which are substrates for both enzyme forms. Other natural arabinose-containing polysaccharides, such as arabinogalactans, are not attacked by them but some artificial arabinose derivatives are good substrates for both polypeptides. Their arabinose-releasing activity on arabinoxylans facilitates the hydrolysis of the xylan backbone by some endoxylanases from *Bacillus polymyxa*.

Introduction

Hemicellulose is present together with cellulose and pectin in plant cell walls. Xylan is the major component of hemicellulose but arabinans and mannans are also present. Xylan is a complex polymer with a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, glucuronyl and arabinofuranosyl residues and the natural degradation of xylan involves different enzymatic activities (Biely 1985). Xylanases (1,4-β-D-xylan xylano- hydrolase, EC 3.2.1.8) and β-xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) hydrolyse the xylosidic linkages occurring within the xylan backbone and xylooligosaccharides respectively. Acetyl esterases, glucuronidases and arabinofuranosidases (α-L-arabinohydrolase, EC 3.2.1.55) remove the xylan side-chain substituents. Arabinofuranosidases may also be involved in arabinan degradation. Some arabinose-releasing activities are very specific for one particular type of polymer (e.g. linear arabinan, branched arabinan, arabinoxylan or arabinogalactan) and they do not attack the others. In addition, *Aspergillus awamori* has an enzyme, named arabinoxylan arabinohydrolase (Komerlink et al. 1991), which is only active on arabinoxylans but is unable to degrade the artificial arabinose derivative compound *p*-nitrophenyl arabinofuranoside (NpAra*f*), which acts as a substrate for most of the arabinofuranosidases described to date. The substrate specificities of these enzymes differ from one to another and classification into a few groups is rather difficult. Some of these enzymes are able to release arabinose from many substrates such as NpAra*f*, branched or linear arabinan, arabinofuranose oligomers and arabinoxylan, but in other cases the activity of the enzyme depends on the type of linkage (α-1,2, α-1,3, or α-1,5) of arabinose substituents or whether they are terminal or stub arabinosyl linkages (for a review see Coughlan and Hazlewood 1993; Kaneko et al. 1994).

Few arabinofuranosidases have been characterized from the genus *Bacillus* (Weinstein and Albersheim 1979; Bezalel et al. 1993; Kaneko et al. 1994) *Bacillus polymyxa* shows a rich xylanolytic complex (Piñaga et al. 1993). Purification and characterization of three alkaline endoxylanases from this strain have been reported previously (Morales et al. 1993) as has the purification and characterization of two *B. polymyxa* acidic endoxylanase and arabinofuranosidase activities (Morales et al. 1995). A gene encoding another acidic endoxylanase from this bacterium has also been cloned (Yang et al. 1988). *xynD* from *B. polymyxa*, which

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encodes an alkaline arabinofuranosidase, has been cloned and expressed in *Escherichia coli* and *Bacillus subtilis* (Gosalbes et al. 1991) showing in both hosts two active peptide forms named AF64 and AF53. In this report we present the purification of these two peptides from a *B. subtilis* clone and their characterization. The amino acid sequences of both polypeptides at their N termini have also been determined and coincide with the sequence deduced for the *xynD* gene encoding mature protein (Gosalbes et al. 1991).

Materials and methods

Strains and culture conditions

B. subtilis DB104 (*his*, *nprR2*, *nprE18*, *aprA3*) transformed with plasmid pBSX1.101 (Fig. 1) was used as the source of enzyme. Plasmid pBSX1.101 contains the *xynD* gene from *B. polymyxa* CECT153, which encodes two polypeptides with arabinofuranosidase activty, subcloned into the pGDV1 plasmid vector, which contains a choramphenicol resistance marker (Gosalbes et al. 1991).

B. subtilis DB104 (pBSX1.101) was grown at 37°C in Luria-Bertani broth. The medium (10 l) was sterilized in 1-l aliquots in 2-l conical flasks, supplemented with 5 $\mu g\,ml^{-1}$ chloramphenicol and inoculated with 0.5% fresh culture. Growth was carried out in an orbital incubator with agitation (200 rpm) for 26 h.

Purification of polypeptides AF64 and AF53

Cells were removed from culture fluid by centrifugation at 9000 rpm for 20 min in 250-ml bottles. The supernatant was then concentrated (final volume, 80 ml) and dialysed against 10 mM MES [2-(*N*-morpholino)ethanesulphonic acid] buffer pH 6.5 through membrane with a 10-kDa molecular-mass cut-off in an Amicon dialysis/concentration cartridge.

The concentrated, dialysed culture fluid was applied to a CM-Biogel A column (1.6 × 50 cm) previously equilibrated with 20 mM MOPS [3-(*N*-morpholino)propanesulphonic acid] buffer pH 7.2. Elution was carried out at a flow rate of 40 $ml\,h^{-1}$ with equilibration buffer. Once a stable basal A_{280} value was established, a linear gradient from 0 to 1 M NaCl in the same buffer was applied for 30 min. Fractions corresponding to adsorbed and NaCl-eluted proteins were collected manually, dialysed against 10 mM MES-NaOH pH 6.5 and concentrated on a CM-Biogel A column (1.6 × 10 cm) previously equilibrated with the buffer 20 mM MES/NaOH pH 6.5. Protein was eluted with 1 M NaCl in the same buffer and desalted by dialysis against 20 mM MES/NaOH pH 6.5 buffer.

Fast protein liquid chromatography (FPLC) was carried out on a pre-packed Mono S HR 5/5 column (Pharmacia). Eluents used were buffer A (20 mM HEPES/NaOH, pH 7.2) and buffer B (1 M NaCl in buffer A). A flow rate of 0.5 $ml\,min^{-1}$ was used. The elution was carried out as follows: 0% buffer B for 2 min, 0–5% buffer B for the next 8 min and 5%–100% buffer B for the last 5 min. The column was washed for 10 min with 100% buffer B and then re-equilibrated. Protein elution was monitored at 280 nm and fractions were collected manually. Polypeptide AF64 eluted free of contaminants. Fractions containing AF53 were re-chromatographed following the same protocol.

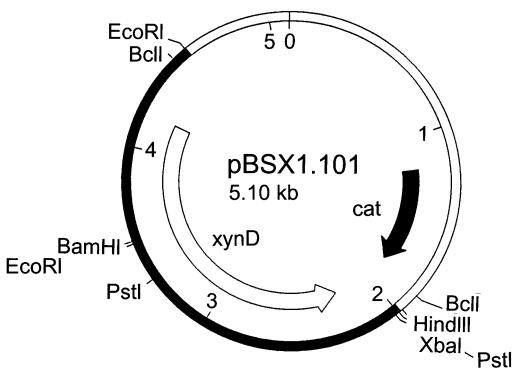

Fig. 1 Map of plasmid pBSX1.101 containing the *xynD* gene from *B. polymyxa*. The *arrows* indicate the position of the genes *xynD* (AF64- and AF53-encoding gene, *open arrow*) and *cat* (chloramphenicol-acetyltransferase-encoding gene, *solid arrow*) and direction of transcription

Gel electrophoresis and analytical isoelectric focusing

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (Laemmli, 1970). Isoelectric focusing was carried out on pre-cast gels with a pH gradient from 3.5 to 9.3 (Pharmacia-LKB) following the instructions of the manufacturer. Titration curves were developed in a Phast-System (Pharmacia) on gels with a pH gradient from 3.5 to 9.3.

In order to detect xylan-degrading activities, SDS-PAGE of the samples (denatured by 2 h incubation at 37°C in loading buffer) was carried out on gels incorporating 0.3% oat spelt xylan. Immediately after electrophoresis, SDS was removed by soaking the gel in 50 mM phosphate buffer (pH 6.5) containing 2.5% Triton X-100 for 30 min. The gel was then incubated 24 h at 37°C and stained with Congo red by the method of Béguin (1983). In the case of isoelectric focusing analyses the gels were placed on 1.25% agarose detection gels containing 0.3% oat spelt xylan. The sandwich was incubated for 24 h at 37°C. The agarose gel was then stained with Congo red.

Enzyme assays

Arabinofuranosidase activity was determined using *p*-nitrophenyl-α-L-arabinofuranoside (NpAra*f*) as substrate. An appropriate dilution of enzyme was mixed with the substrate to a final concentration of 0.8 $mg\,ml^{-1}$ using 80 mM MES/NaOH pH 6.5 buffer for AF64 and 80 mM MOPS/NaOH pH 6.5 for AF53. $MgCl_2$ at final concentrations of 5 mM for AF64 and 2 mM for AF53 was included in mixtures when indicated. Samples were incubated for 15 min at 55°C. After incubation, one volume of 1 M Na_2CO_3 was added and the A_{420} measured. The absorbance values were transformed to micromoles of *p*-nitrophenol. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol min^{-1}.

Activity on xylan was measured by mixing an appropriate dilution of the enzyme with a suspension of substrate in the buffers indicated above and incubation of the samples at 55°C for 15 min. Blanks were prepared by incubating enzyme and substrate solutions separately. The reducing sugars formed were measured by the Somogyi (1952) method. One unit of activity was defined as the amount of enzyme releasing 1 μmol reducing sugar equivalent min^{-1}.

β-Xylosidase activity on *p*-nitrophenyl β-D-xyloside (NpXyl) or 4-methylumbelliferyl β-D-xyloside (MeUmb-Xyl) and α-L-arabinofuranosidase activity on 4-methylumbelliferyl α-L-arabinofuranoside (MeUmb-Ara*f*) or 4-methylumbelliferyl α-L-arabinopyranoside (MeUmb-Ara*p*) determined as described before for NpAra*f*. For methylumbelliferyl substrates the A_{365} was determined instead of A_{420} and transformed to micromoles of methylumbelliferone.

Measurement of enzyme properties

Thermostability was monitored by incubating the enzyme at a fixed temperature and removing aliquots at intervals to test arabinofuranosidase activty. The efect of temperature on reaction rates was assessed by incubating reaction mixtures at different temperatures in the range 25–65°C. For the corresponding study of pH effects, several buffers were used at a final concentration of 50 mM: MES/NaOH for the pH range 5.5–6.5, MOPS/NaOH for 6.5–7.5 and TRIS/HCl for 7.5–8.5. The influence of the metal ions Na^+, K^+, Cu^{2+}, Ca^{2+} and Mg^{2+} on activity was also tested. The effect on enzyme thermostability was also determined for those ions that showed an influence on activity. Activity measurements were carried out by incubating 300-µl mixtures containing 0.8 mg ml^{-1} NpAra*f* and 7 µg ml^{-1} AF64 or 4.5 µg ml^{-1} of AF53 in the appropriate buffer for 15 min at 55°C. Samples to test thermostability were assayed at a lower temperature: 45°C for 15 min.

Kinetic parameters were determined on oat spelt xylan and wheat flour arabinoxylan. The concentration of AF64 in mixtures with oat spelt xylan was 850 ng ml^{-1} and 250 ng ml^{-1} in mixtures with wheat flour arabinoxylan. Samples were incubated in 50 mM MES/NaOH buffer pH 6.5 with 5 mM $MgCl_2$ for 10 min at 55°C. The concentration of AF53 was 356 ng ml^{-1} in oat spelt xylan and 182 ng ml^{-1} in wheat flour arabinoxylan. Samples incubated in 50 mM MOPS/NaOH buffer pH 6.5 with 2 mM $MgCl_2$ for 15 min at 55°C. Oat spelt xylan was assayed in the concentration range 0–60 mg ml^{-1} with AF64 and 0–84 mg ml^{-1} with AF53; wheat flour arabinoxylan was assayed in the concentration range 0–30 mg ml^{-1} with both polypeptides. The reducing sugars liberated were determined by the Somogyi (1952) method. Specific activity on different substrates was determined using the same method.

Enzymatic hydrolysis of xylans, xylooligosaccharides and arabinooligosaccharides

The analysis of products from the hydrolysis of xylans from different sources was performed by HPLC on a SugarPack 1 column (Waters) placed in an oven. The temperature of the column was adjusted to 85°C and that of the refractive-index detector (Waters) to 40°C. The eluent used was water at a flow rate of 0.5 ml min^{-1}. Samples of 20 µl from reaction mixtures were injected. Xylose, arabinose (both from Sigma), (1 → 3)-β-D-xylooligosaccharides having two to four units of xylose and (1 → 5)-α-L-arabinooligosaccharides haing two to eight units of arabinose (all from Megazyme, North Rocks, Australia) were used as standards. Enzymes were incubated at a concentration of 8.5 µg ml^{-1} for AF64 and 4 µg ml^{-1} for AF53 with 60 mg ml^{-1} oat spelt xylan or 20 mg ml^{-1} wheat flour arabinoxylan. Incubation was carried out at 40°C for 20 h. Samples corresponding to AF64 were incubated in 50 mM MES/NaOH buffer containing 5 mM $MgCl_2$ and samples corresponding to AF53 were incubated in 50 mM MOPS/NaOH containing 2 mM $MgCl_2$.

Similar mixtures and hydrolysis conditions were used to determine the products of hydrolysis of xylobiose, xylotriose, xylotetraose, arabinobiose, arabinotriose, arabinopentaose and arabinooctaose but in these cases the substrate concentration was 2 mg ml^{-1} for each oligosaccharide.

Studies of cooperation between different enzymes from *B. polymyxa*

Cooperation between different enzymes was studied by analysis of products of hydrolysis in long-term incubations. Xylanases X22 or X34E from *B. polymyxa* (Morales et al. 1993) together with AF64 or AF53 were used in these assays.

For X34E/AF64 combinations, 60 mg ml^{-1} oat spelt xylan or 20 mg ml^{-1} wheat flour arabinoxylan in 50 mM MES buffer pH 6.5 containing 5 mM $MgCl_2$ was incubated with 8 µg ml^{-1} X34E and/or 8.5 µg ml^{-1} AF64 at 40°C.

For X22/AF64 combinations, 20 mg ml^{-1} wheat flour arabinoxylan was incubated in 50 mM MES buffer pH 6.5 containing 5 mM $MgCl_2$ at 40°C with 8.5 µg ml^{-1} AF64 and/or 1.2 µg ml^{-1} X22.

For X34E/AF53 combinations, 60 mg ml^{-1} oat spelt xylan or 30 mg ml^{-1} wheat flour arabinoxylan was incubated in 50 mM MOPS buffer pH 6.5 containing 2 mM $MgCl_2$ at 40°C with 4 µg ml^{-1} AF53 and/or 8 µg ml^{-1} X34E.

For X22/AF53 combinations, 20 mg ml^{-1} wheat flour arabinoxylan was incubated with 1.2 µg ml^{-1} X22 and/or 2.5 µg ml^{-1} AF53 in 50 mM MES buffer pH 6.5 containing 2 mM $MgCl_2$ at 40°C.

Analytical methods

Protein concentration was measured either by the Bradford (1976) method or by A_{280} using albumin as standard.

Results

Purification of AF64 and AF53 polypeptides

Preliminary isoelectric focusing and zymogram analyses performed with culture supernatants from *B. subtilis* DB104 (pBSX1.01) and *B. subtilis* DB104 (pGDV1) showed that the AF64 and AF53 polypeptides have alkaline p*I* (Fig. 2A). Cation-exchange chromatography on a CM-Biogel A column was performed as the first purification step on concentrated culture broth. As expected, both AF64 and AF53 polypeptides adsorbed

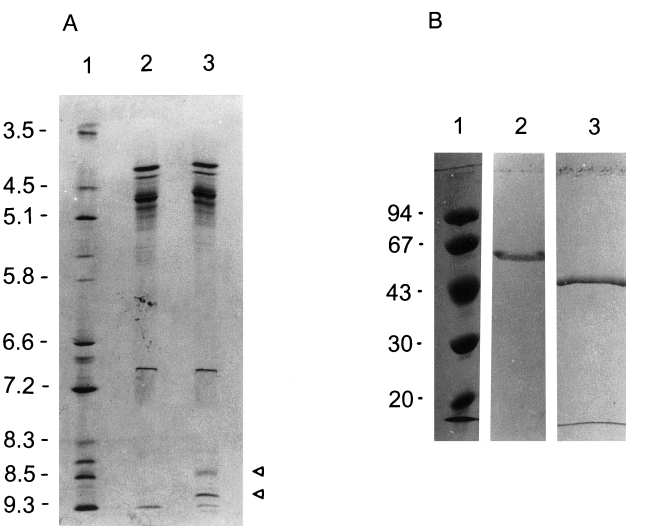

Fig. 2 **A** Isoelectric focusing analysis of cell culture broths. *1* Protein isoelectric point standards, *2 B. subtilis* DB104 (pGDV1) culture broth, *3 B. subtilis* DB104 (pBSX1.101) culture broth. The *arrows* indicate the putative AF64 and AF53 polypeptides. The numbers to the *left* indicate the p*I* of the standards. **B** SDS-PAGE analysis of AF64 and AF53 polypeptides. *1* Protein molecular mass standards, *2* pure AF64 polypeptyde (5 µg), *3* pure AF53 polypeotide (5 µg). The numbers to the *left* indicate the molecular mass (kDa) of the standards

to the column and were detected in the NaCl-eluted fractions, where they constituted the major proteins. A titration curve of this sample indicated that the electrical properties of both polypeptides are similar in the pH range used. The second purification step comprised a cation-exchange FPLC. The working pH selected was close to the p*I* to both polypeptides to enable more effective use to their different electric charges. Three peaks with arabinofuranosidase activity were detected. The first contained pure AF64 polypeptide (see Fig. 2B) and the third showed enrichment of the AF53 polypeptide, as revealed by SDS-PAGE analysis. The latter fraction was re-chromatographed under the same conditions and pure AF53 was obtained (see Fig. 2B). The specific activity of fractions containing the purified enzymes was increased 9- and 12-fold for AF64 and AF53 respectively, in comparison with that of culture supernatant. The activity recoveries in purified fractions were 7.2% and 0.75% for AF64 and AF53 respectively, from a total activity of 108 U in 10 l culture supernatant. The recoveries of protein were 10-fold lower in both cases from a total protein of 1226 mg in the same culture supernatant volume.

Characterization of AF64 and AF53 polypeptides

The molecular masses estimated by SDS-PAGE (see Fig. 2B) were 64 kDa for AF64 and 53 kDa for AF53. From isoelectric focusing analysis the p*I* values determined for AF64 and AF53 were 8.7 and 9 respectively. The N-terminal amino acid sequences obtained (X–N–R–P–L–A–K–I–P–G–N–S–N–P–L–M–D–H–X–L, where X is a non-determined amino acid) for both polypeptides were identical. This sequence coincides with that predicted for the mature protein (i.e. after processing of a putative signal perptide) from nucleotide sequence data (Gosalbes et al. 1991). The molecular mass predicted for the mature enzyme is 64 kDa which is in agreement with the molecular mass determined for AF64 (see above). The AF53 polypeptide could be a product of processing of AF64 is about 100 amino acids were removed from its C terminus.

The influence of metal ions on the activities of AF64 and AF53 was studied. Whereas Na^+ and K^+ had on effect, Cu^{2+} at 5 mM concentration inhibited 5- and 10-fold the activities of AF64 and AF53, respectively. Ca^{2+} and particularly Mg^{2+} showed positive effects on activity. When the activity was measured on NpAra*f*an an increase of 50% was observed for both polypeptides in the presence of 5 mM $MgCl_2$. When oat spelt xylan was used as substrate the effect of Mg^{2+} ions was stronger. 50-fold increase in activity was observed for AF64 and AF53 at $MgCl_2$ concentrations of 5 mM and 2 mM respectively, in comparison to controls. In the following experiments, carried out in order to characterize the polypeptides, $MgCl_2$ was included in activity assays at the optimal concentration.

The effect of temperature on activity of both polypeptides was very similar, 55°C being their temperature optima. In both cases the activities at 30°C and 65°C were 4-fold lower than those at the optimum. The thermostabilities of AF64 and AF53 were, however, rather different. In the absence of $MgCl_2$ both polypeptides retained 90% activity after 4 h incubation at 45°C, AF64 lost 60% activity within 5 min at 55°C and, in contrast, AF53 retained 60% activity after 4 h incubation at this temperature. The addition of $MgCl_2$ enhanced the thermostability of both polypeptides, the action of Mg^{2+} ions being more effective in the case of AF64, which can then retain 60% of activity after 3 h incubation at 55°C.

The pH optima determined for both polypeptides were the same, namely 6.5. The activities of AF64 and AF53 at pH 5.5 were, respectively, 5- and 3-fold lower than those at pH optimum. At pH 7.5 AF64 and AF53 showed half the activity measured at the optimal pH. AF64 showed higher activity when assayed in MES buffer (11% higher) than in MOPS buffer at the same pH (6.5), whereas at that pH AF53 had lower activity (25% lower) in MES buffer than in MOPS buffer. At alkaline pH values the effect of buffer used was stronger. Both polypeptides showed very poor activity in TRIS buffer. The activities of AF64 and AF53 assayed in TRIS buffer were, respectively, 5- and 3-fold lower than those measured in MOPS buffer at the same pH (7.5).

Analysis of substrate concentration effects on AF64 and AF53 activities when wheat flour arabinoxylan was used as substrate indicated that substrate inhibition takes place. The experimental values obtained fitted the equation $V = (V_{max}S)/(K_m + S + K_iS^2)$ for substrate inhibition kinetics. The kinetic parameters were determined by non-linear parametric regression analysis using the Sigma Plot computer program. The effect of substrate concentration was also analysed using oat spelt xylan as substrate but, in this case, no substrate inhibition was observed. Table 1 summarizes the kinetic parameters of AF64 and AF53 on both substrates.

The specific activities of both polypeptides on polysaccharides of different chemical structure were determined. Some artificial substrates were also tested. Table 2 summarizes the values obtained. No activity was observed on birchwood xylan, arabinan, arabinogalactan, NpXyl, MeUmb-Xyl or MeUmb-Ara*p* with AF64 and AF53.

Hydrolysis of xylans and oligosaccharides by AF64 and AF53

Products of hydrolysis of xylans from different sources by AF64 and AF53 were analysed by HPLC. The unique product released from oat spelt and wheat flour xylans by both polypeptides was arabinose, as can be seen in Fig. 3 for AF64. Neither of the enzymes hydrolysed xylo- or arabinooligosaccharides.

Table 1 Kinetic parameters determined for AF64 and AF53 enzymes

Substrate	AF64			AF53		
	K_m (mg ml^{-1})	V_{max} (U min^{-1} mg^{-1})	K_i (ml mg^{-1})	K_m (mg ml^{-1})	V_{max} (U min^{-1} mg^{-1})	K_i (ml mg^{-1})
Oat spelt xylan	48.63	143.24	0.00	23.24	165.00	0.00
Wheat flour xylan	3.51	275.70	0.01	16.92	501.30	0.03

Table 2 Substrate specificity of AF64 and AF53. *NpAraf* *p*-nitrophenyl α-L-arabinofuranoside, *MeUmb-Araf* methylumbelliferyl α-L-arabinofuranoside

Substrate	Specific activity (U min^{-1} mg^{-1})	
	AF64	AF63
β-glucan	2.9	0.0
Carboxymethylcellulose	4.1	0.0
Oat spelt xylan	45.9	91.3
Methylglucuronoxylan	0.0	16.2
Wheat flour xylan	119.0	135.0
NpAra*f*	0.78	0.98
MeUmb-Ara*f*	0.15	0.20

When a xylanase was included in the arabinoxylan hydrolysis assays together with AF64 or AF53, an increase in the xylanase products such as xylose and xylobiose was detected in comparison to controls comprising only the xylanase. However, the quantity of arabinose was similar in all cases. Thus, when AF64 and the *B. polymyxa* X22 xylanase (Morales et al. 1993) were combined, the release of xylobiose and xylotriose from wheat flour arabinoxylan was increased in comparison to the xylanase control (see Fig. 4). Similarly,

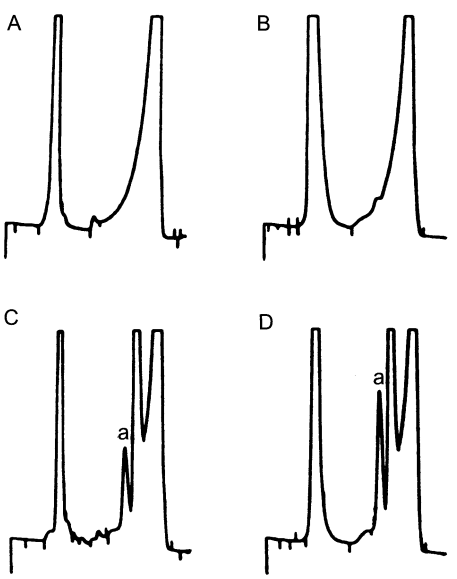

Fig. 3A–D HPLC analysis of xylan hydrolysis products. **A**, **B** Controls of non-hydrolysed substrates. Oat spelt xylan (**A**, **C**) and wheat flour arabinoxylan (**B**, **D**) were the substrates degraded by AF64 polypeptide. Changes in refractive index were detected. *a* Arabinose

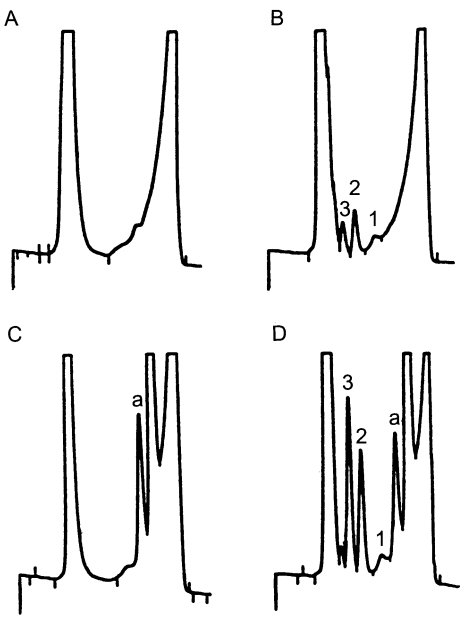

Fig. 4A–D HPLC analysis of wheat flour arabinoxylan hydrolysis products. **A** Control of non-hydrolysed substrate. **B** Products obtained from substrate hydrolysis by xylanase X22. **C** Products obtained from substrate hydrolysis by AF64 polypeptide. **D**. Products obtained from substrate hydrolysis by xylanase X22 and AF64 polypeptide. *1* Xylose, *2* xylobiose, *3* xylotriose, *a* arabinose

the production of xylose and xylobiose was also increased by combination of either AF64 or AF53 with the *B. polymyxa* X34E xylanase (Morales et al. 1993) with respect to controls in which no arabinofuranosidase was present (data not shown). In both cases the arabinose released was similar to that from controls in which only the arabinofuranosidase enzyme was present. However, the production of oligosaccharides larger than xylobiose was less than that observed in the xylanase control as a result of the combined action of the arabinose-releasing activity and the activity shown by X34E on xylooligosaccharides.

Discussion

The sequences of the N termini determined for both AF64 and AF53 purified polypeptides are identical to that deduced for the mature protein from the *B. polymyxa xynD* nucleotide sequence (Gosalbes et al. 1991). AF53 seems to be a product of proteolytic processing of AF64, in which about 100 amino acids are

cleaved from the C terminus of the protein. An *E. coli* clone harbouring a plasmid with a deletion in the 3′ region of the *xynD* gene, corresponding to 71 amino acids of the C terminus of the encoded protein, has arabinofuranosidase activity. However, activity was not detected in a clone with a deletion corresponding to 198 amino acids in the C terminus of the protein. The protein may therefore retain activity after deletion of about 100 amino acids from the C terminus, as is the case for the AF53 polypeptide. The processing of proteins by proteolytic cleavage removing amino acids from the C terminus is not unusual among cellulolytic enzymes when the corresponding genes are cloned in a host microorganism (Lo et al. 1988; Fierobe et al. 1991; Ahn et al. 1993). In some cases reiterated sequences posessing cellulose-binding functions are removed, usually resulting in improved enzymatic characteristics.

From substrate specificity analysis it is clear that AF64 and AF53 are not able to degrade bonds other than the $\alpha(1 \rightarrow 3)$bond between arabinose and xylose in arabinose-containing xylans but differ from the so-called arabinoxylan arabinofuranohydrolases (Komerlink et al. 1991) in their ability of hydrolyse NpAra*f* and MeUmb-Ara*f*, substrates which are not hydrolysed by the latter type of enzyme but can be hydrolysed by AF64 and AF53. Both polypeptides are specific for the furanose configuration as they do not act on MeUmb-Ara*p*. The first 465 amino acids of the deduced protein sequence of the *xynD* gene product are 60% homologous to the corresponding sequence of the *Clostridium stercorarium xylA* gene product (Sakka et al. 1993), an enzyme that has β-xylosidase and arabinofuranosidase activities. However no β-xylosidase activity was observed for AF64 and AF53.

The higher arabinose content of wheat flour arabinoxylan makes it a better substrate for the enzymes than arabinoxylan from oat spelts and can also account for the substrate inhibition detected with the former. Substrate inhibition was more marked in the case of AF53 and for this reason the apparent K_m value of this enzyme on wheat flour arabinoxylan was higher than the AF64 apparent K_m value. The V_{max} value is lower for the latter. Other low-arabinose-content xylans or other polysaccharides demonstrate virtually no susceptibility to these enzymes.

The addition of the arabinose-releasing enzymes AF64 or AF53 to xylan-degrading mixtures containing one of the xylanases previously purified from *B. polymyxa* facilitated the action of the endoxylanase as the production of smaller xylooligosaccharides increased. However, xylan-chain degradation by xylanases did not produce an increase in the arabinose released by the arabinofuranosidases. Therefore, the action of the arabinofuranosidases seems to be independent of the length of the arabinoxylan chain.

Acknowledgements This work has been supported by grant ALI91-0336 from the Comisión Interministerial de Ciencia y Tecnología (CICYT) of the Spanish Government. Thanks are due to A. Martinez for the protein N-terminal sequencing and to A.P. MacCabe for critical reading of the manuscript. P. Morales was the recipient of a fellowship from Bancaja.

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