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Purification and Characterization of an Extracellular α-L-Arabinofuranosidase from *Fusarium oxysporum*

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Abstract

An α-L-arabinofuranosidase from *Fusarium oxysporum* F3 was purified to homogeneity by a two-step ion exchange intercalated by a gel filtration chromatography. The enzyme had a molecular mass of 66 kDa and was optimally active at pH 6.0 and 60°C. It hydrolyzed aryl α-L-arabinofuranosides and cleaved arabinosyl side chains from arabinoxylan and arabinan. There was a marked synergistic effect between the α-L-arabinofuranosidase and an endo-(1→4)-β-D-xylanase produced by *F. oxysporum* in the extensive hydrolysis of arabinoxylan.

Index Entries: *Fusarium oxysporum*; α-L-arabinofuranosidase; purification; characterization; synergism.

Introduction

Xylans, the most abundant of the hemicelluloses of land plants, are β-1,4-linked polymers of xylopyranose units with a degree of polymerization ranging from 70 to 200 *(1,2)*. These heteropolysaccharides can be substituted with α-L-arabinofuranose, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative, and acetyl groups. The complete degradation of substituted xylans involves the action of several hydrolytic enzymes: endoxylanases, β-xylosidases, glucuronoxylan hydrolases, and enzymes capable of hydrolyzing substituents from the xylan backbone

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such as α-L-arabinofuranosidases (AFs), acetyl and feruloyl esterases, uronidases, and glucosidases *(3)*.

Arabinofuranosidases include AFs (EC 3.2.1.55) which are capable of hydrolyzing terminal nonreducing α-L-arabinofuranosyl residues from α-L-arabinofuranosides, arabinans, arabinoxylans, and arabinogalactans *(4)*. Among these polysaccharides, arabinoxylans are important animal feed components, and some studies have shown a beneficial response to dietary enzyme supplementation, with improvement noted for growth and feed conversion, as well as for digestibility *(5)*. The principal enzyme required for the prehydrolysis of animal feed is endoxylanase. However, AFs are also necessary for the complete hydrolysis of heteroxylans. Despite their obvious importance in the degradation of xylans and other arabinose-containing polysaccharides and the fact that AF activity is known to be produced by various fungi and bacteria, a relatively small number (compared to other hemicellulases) of these enzymes have been isolated and characterized *(2,6–11)*.

In this article, we report on the purification and characterization of an AF produced by *Fusarium oxysporum* F3.

Materials and Methods

Microorganism and Growth Conditions

F. oxysporum strain F3, used in the present study, was isolated from cumin *(12)*. The fungus was grown in 500-mL Erlenmeyer flasks for 4 d at 30°C and pH 7.0 in a growth medium described previously *(13)*. Four percent (w/v) sugar beet pulp and 5% (w/v) corn steep liquor were added as carbon and nitrogen sources, respectively.

Enzyme Assay

p-Nitrophenyl-α-L-arabinofuranoside (1 m*M*) (Sigma, St. Louis, MO) in 50 m*M* citrate-phosphate buffer, pH 6.0, was used as substrate to measure AF activity. The A_{410} of the liberated *p*-nitrophenol was measured using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Reactions, after temperature equilibration at 40°C, were initiated by the addition of 10 μL of enzyme sample in a total volume of 210 μL. All activities were expressed as international units, in which 1 U was defined as the amount of the enzyme required to release 1 μmol of nitrophenol per minute. Endo-β-1,4-xylanase activity was assayed as described elsewhere *(13)*.

Crude Enzyme Production

Biomass was removed from the culture broth by filtration (Whatman no. 4 filter paper). The filtrate was centrifuged (9000*g*, 20 min, 4°C), and the clear supernatant was concentrated approx 100-fold by ultrafiltration (PM-10, Amicon, Beverly, MA). The resulting retentate was dialyzed against distilled water and stored at –20°C.

Enzyme Purification

One hundred milligrams of crude enzyme in 50 m*M* sodium acetate buffer, pH 4.0, was loaded on an SP-Sepharose (Pharmacia, Upsala, Sweden) column (1.5 × 30 cm), equilibrated with the same buffer. The column was initially washed with 45 mL of the equilibration buffer followed by the application of a linear gradient of 0–0.5 *M* NaCl in 330 mL of the same buffer, at a flow rate of 90 mL/h. Fractions of 7.5 mL were collected and assayed for AF activity. Two activity peaks (fractions 6–10 and 26–38) appeared. The second-peak fractions were combined (total volume 97.5 mL) and concentrated 10-fold by ultrafiltration (PM-10, Amicon).

The concentrate was applied on a Sephacryl S-200 column (Pharmacia); preequilibrated with 50 m*M* sodium acetate buffer, pH 4.0; and eluted with the same buffer (flow rate of 60 mL/min). The column yielded one major AF active fraction (fraction 51–57), which was collected and concentrated by ultrafiltration (PM-10, Amicon, Beverly, CA). From this fraction 19.2 mg was applied onto a 1 × 15 cm MonoSP-5PW column (Waters, Milford, MA), equilibrated with the same buffer (pH 4.0). The column was first washed with 10 mL of the equilibrating buffer, and then a linear gradient of 0–0.25 *M* NaCl in 50 mL of the same buffer was applied, at a flow rate of 60 mL/h. Fractions (2 mL) containing AF activity were pooled and concentrated with ultrafiltration (PM-10, Amicon). This purification step yielded one AF active fraction (fraction 15–16), and its homogeneity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein Estimation

Protein concentration was determined by the method of Bradford *(14)* using bovine serum albumin as standard. Protein concentration in column effluents was monitored spectrophotometrically at 280 nm.

Synergism Studies

Synergism of AF in combination with endo-(1,4)-β-xylanase II was studied by incubating 1% (w/v) wheat arabinoxylan solution with 10 μg/mL of purified AF and 5 μg/mL (1 U) of endo-(1,4)-β-xylanase II at 40°C in 50 m*M* sodium acetate buffer, pH 5.0, for 24 h, separately and in combination. The reaction mixtures were analyzed for hydrolysis products, by thin-layer chromatography (TLC) on microcrystalline cellulose (DC-Alufolien Cellulose, Merck, Darmstadt, Germany) using the solvent system ethyl acetate:acetic acid:water (3:2:1). Reducing sugars were visualized using the aniline-hydrogen phthalate reagent.

Determination of Molecular Mass

Molecular mass was determined by SDS-PAGE using the PhastSystem electrophoresis unit (Pharmacia) and a 10–15% polyacrylamide gel.

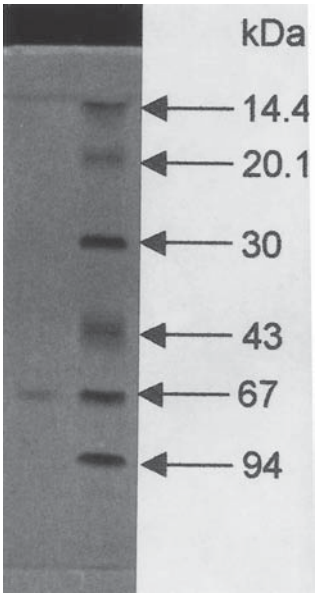

Fig. 1. SDS-PAGE of the purified arabinofuranosidase from *F. oxysporum* under denaturing conditions. (**Right lane**), standard protein markers; (**left lane**), purified arabinofuranosidase.

Table 1
Purification Summary of *F. oxysporum* Arabinofuranosidase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Culture filtrate	5.5	97.0	0.057	—	100.0
SP-Sepharose	3.0	7.5	0.4	17.5	54.5
Sephacryl S-200	1.2	0.25	4.8	84.2	21.8
Mono SP	0.9	0.14	6.42	112.6	16.4

Results and Discussion

When grown on sugar beet pulp, *F. oxysporum* exhibited extracellular AF activity. The enzyme was purified to apparent electrophoretic homogeneity by simple procedures, employing gel-filtration and ion-exchange chromatography (Fig. 1; *see also* Materials and Methods). The results of the purification are summarized in Table 1 and show that the AF was purified 113-fold and had a specific activity of 6.4 U/mg of protein on nPh-Araf as substrate. The molecular mass of the enzyme was found to be about 66 kDa (SDS-PAGE) (Fig. 1). This value was similar to those reported for purified AFs from *Dichromatus squalens* (60 kDa; *[6]*), *Aspergillus niger* (60 kDa; *[7]*), *Aspergillus awamori* (65 kDa; *[8]*), *Trichoderma reesei* (53 kDa; *[9]*), *Bacillus subtilis* (65 kDa; *[10]*), and *Ruminococcus albus* (75 kDa; *[11]*).

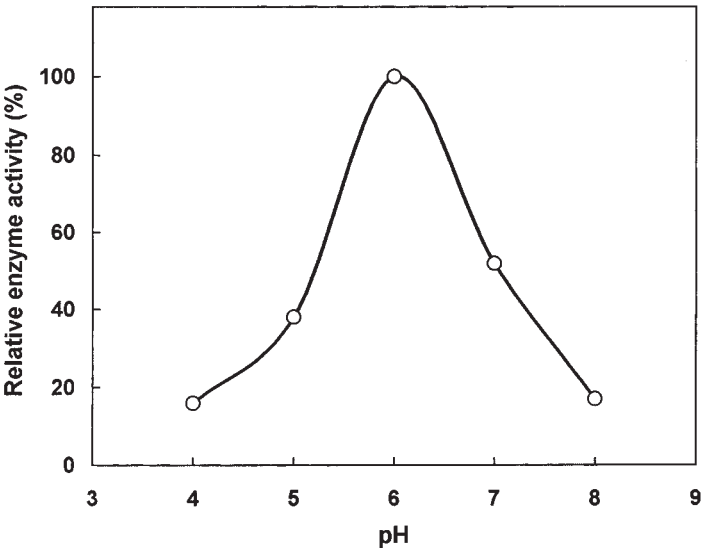

Fig. 2. Effect of pH on arabinofuranosidase activity.

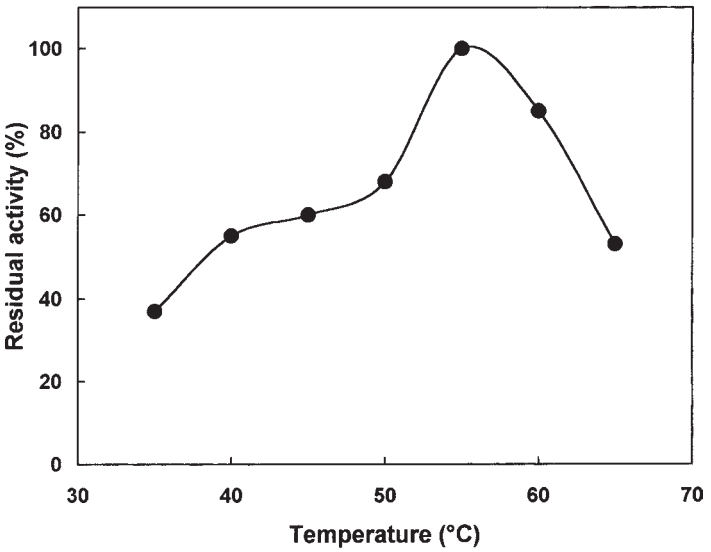

Fig. 3. Effect of temperature on arabinofuranosidase activity.

Measured on nPh-Araf as substrate, the enzyme's characteristics, such as pH *(6)* (Fig. 2) and temperature (55–60°C) optima (Fig. 3), were similar to those reported for AFs from other mesophilic fungi *(2)*.

The specificity of the enzyme was restricted to the furanose form of arabinose. It was active on *p*-nitrophenyl-α-L-arabinofuranoside but did not hydrolyze *p*-nitrophenyl-α-L-arabinopyranoside. However, polysaccharides containing arabinose (arabinan, arabinoxylan) showed an increase in reducing power on incubation with the enzyme (Table 2).

Table 2
Substrate Specificity
of Purified AF from *F. oxysporum*[a]

Substrate	Activity (%)
4nPh-α-L-arabinofuranoside	100
4nPh-α-L-arabinopyranoside	Nil
Arabinoxylan (wheat straw)	85
Arabinan (sugar beet pulp)	76

[a]Enzyme activity was determined spectrophotometrically by liberating *p*-nitrophenol from *p*-nitrophenyl glycosides in 0.1 *M* citrate phosphate buffer (pH 6.0) at 40°C or by assaying reducing sugars with the dinitrosalicylic acid reagent.

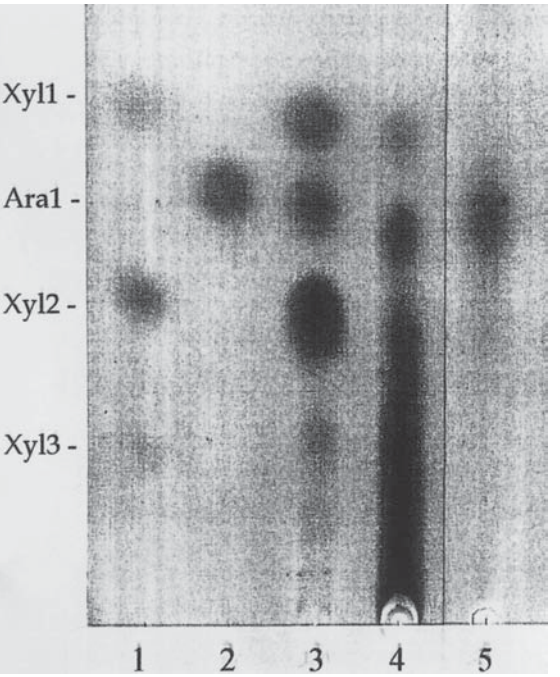

Fig. 4. TLC analysis of degradation products of arabinoxylan. Wheat straw xylan was incubated with the corresponding enzymes as indicated. Lane 1, xylooligosaccharide standards; lane 2, arabinose; lane 3, synergistic action of AF and xylanase; lane 4, xylanase alone; lane 5, AF alone. Xyl3, xylotriose; Xyl2, xylobiose; Xyl1, xylose; Ara1, arabinose.

Kaji *(15)* divided the AFs into two groups according to their substrate specificity. The first group, that of *Streptomyces purpurascens*, degraded only low molecular mass L-arabinofuranose-containing oligosaccharides and synthetic substrates. The second group, that of *A. niger*, released L-arabino-

syl residues not only from low molecular mass L-arabinofuranose-containing oligosaccharides and synthetic substrates but also from polymeric L-arabinan, arabinoxylan, and arabinogalactan. Based on the substrate specificity results, the AF from *F. oxysporum* belongs to the second group.

The degradation of polymeric arabinoxylan by extended incubation with the AF from *F. oxysporum* showed that the only hydrolysis product, detected by TLC, was arabinose (Fig. 4). The synergistic action of purified AF and the endoxylanase II from *F. oxysporum (16)* was carried out on arabinoxylan. The endoxylanase alone yielded a set of products including xylotriose, xylobiose, xylose, and oligosaccharides, containing presumably arabinose and xylose residues. The incubation of arabinoxylan with AF and endoxylanase II (synergism) yielded arabinose, xylose, xylobiose, and no higher oligosaccharides (Fig. 4). Greve et al. *(11)* observed synergism in the hydrolysis of alfalfa cell walls when an AF from *R. albus* was used in combination with an endo-β-xylanase. Similar observations were made with *T. reesei* enzymes on wheat-straw arabinoxylan *(17)*. The presence of large amounts of substituents may hinder the formation of enzyme-substrate complexes and thus impede enzymatic hydrolysis *(17)*. Enzymes able to release these substituents are therefore essential in the complete degradation of heteroxylans.

References

1. Biely, P. (1985), *Trends Biotechnol.* **3,** 286–290.
2. Filho, E. X. F., Puls, J., and Coughlan, M. (1996), *Appl. Environ. Microbiol.* **62,** 168–173.
3. Coughlan, M. P. and Hazlewood, G. P. (1993), *Biotechnol. Appl. Biochem.* **17,** 259–289.
4. Kormelink, F. J. M., Searle-Van Leeuwen, M. J. F., Wood, T. M., and Voragen, A. G. J. (1991), *Appl. Microbiol. Biotechnol.* **35,** 753–758.
5. Roche, N., Desgranges, C., and Durand, A. (1994), *J. Biotechnol.* **38,** 43–50.
6. Brillouet, J.-M., Moulin, J.-C., and Agosin, E. (1985), *Carbohydr. Res.* **144,** 113–126.
7. Rombouts, F. M., Voragen, A. G. J., Searle-Van Leeuwen, M. F., Geraeds, C. C. J. M., Schols, H. A., and Pilnik, W. (1988), *Carbohydr. Polymers* **9,** 25–47.
8. Wood, T. M. and McCrae, S. I. (1996), *Appl. Microbiol. Biotechnol.* **45,** 538–545.
9. Poutanen, K. (1988), *J. Biotechnol.* **7,** 271–282.
10. Weinstein, L. and Albersheim, P. (1979), *Plant Physiol.* **63,** 425–432.
11. Greve, L. C., Labavitch, J. M., and Hungate, R. (1984), *Appl. Environ. Microbiol.* **47,** 1135–1140.
12. Christakopoulos, P., Macris, B., and Kekos, D. (1989), *Enzyme Microb. Technol.* **11,** 236–239.
13. Christakopoulos, P., Mamma, D., Nerinckx, B., Kekos, D., Macris, B., and Claeyssens, M. (1996), *Bioresour. Technol.* **58,** 115–119.
14. Bradford, M. B. (1976), *Anal. Biochem.* **72,** 248–254.
15. Kaji, A. (1984), *Adv. Carbohydr. Chem. Biochem.* **42,** 383–394.
16. Christakopoulos, P., Mamma, D., Nerinckx, B., Kekos, D., Macris, B., and Claeyssens, M. (1997), *Carbohydr. Res.* **302,** 191–195.
17. Kormelink, F. J. M. and Voragen, A. G. J. (1993), *Appl. Microbiol. Biotechnol.* **38,** 688–695.