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Applied and Environmental Microbiology, Apr. 1992, p. 1082–1088
0099-2240/92/041082-07$02.00/0
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Vol. 58, No. 4

# Purification and Characterization of an α-L-Arabinofuranosidase from *Butyrivibrio fibrisolvens* GS113

ROBERT B. HESPELL* and PATRICIA J. O'BRYAN

*Fermentation Biochemistry Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 N. University Street, Peoria, Illinois 61604*

Received 12 November 1991/Accepted 16 January 1992

**An α-L-arabinofuranosidase (EC 3.2.1.55) was purified from the cytoplasm of *Butyrivibrio fibrisolvens* GS113. The native enzyme had an apparent molecular mass of 240 kDa and was composed of eight polypeptide subunits of 31 kDa. The enzyme displayed an isoelectric point of 6.0, a pH optimum of 6.0 to 6.5, a pH stability of 4.0 to 8.0, and a temperature optimum of 45°C and was stable to 55°C. The $K_m$ and $V_{max}$ for *p*-nitrophenyl-α-L-arabinofuranoside were 0.7 mM and 109 μmol/min/mg of protein, respectively. The enzyme was specific for the furanoside configuration and also readily cleaved methylumbelliferyl-α-L-arabinofuranoside but had no activity on a variety of other nitrophenyl- or methylumbelliferyl glycosides. When the enzyme was incubated with cellulose, carboxymethyl cellulose, or arabinogalactan, no release of sugars was found. Arabinose was found as the hydrolysis product of oatspelt xylan, corn endosperm xylan, or beet arabinan. No activity was detected when either coumaric or ferulic acid ester linked to arabinoxylobiose was used as substrates, but arabinoxylobiose was degraded to arabinose and xylobiose. Since *B. fibrisolvens* GS113 possesses essentially no extracellular arabinofuranosidase activity, the major role of the purified enzyme is apparently in the assimilation of arabinose-containing xylooligosaccharides generated from xylosidase, phenolic esterase, xylanase, and other enzymatic activities on xylans.**

Hemicelluloses compose a large fraction of plant cell walls and are a heterogeneous mixture of polysaccharides that include xylans, glucans, mannans, galactans, and arabinans. Xylans constitute the major portion of the hemicellulose fraction. Generally, xylans contain a β-1,4-linked xylose backbone with side chains of 3-O-linked L-arabinose and 2-O-linked 4-O-methylglucuronate, glucuronate, or acetate. Phenolic acids and/or ligninlike polymers can be ester linked to the arabinoses. All of these side chain substituents can be unevenly distributed in xylan polymers. Microbial degradation and metabolism of xylans require a variety of enzymes (11, 33). The major degradative enzymes include xylanase, xylosidase (XS), glucuronidase, arabinofuranosidase (AF), acetylxylan esterase, and phenolic acid esterase.

Enzymes involved in xylan degradation have been studied mostly with aerobic fungi and bacteria such as *Bacillus* sp. or *Pseudomonas* sp. (33). Even though diets of ruminants contain large amounts of xylans and ruminal microbial populations actively ferment xylans, much less is known about these enzymes with species of ruminal bacteria. Strains of *Butyrivibrio fibrisolvens*, *Bacteroides ruminicola*, *Fibrobacter* (*Bacteroides*) *succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are capable of xylan degradation (8, 11, 22). The most common xylanolytic bacterium isolated from ruminal contents is *B. fibrisolvens* (6, 22). Unlike many strains of ruminal cellulolytic species, all known strains of *B. fibrisolvens* effectively utilize xylan breakdown products for growth (7, 8, 14). We have been studying *B. fibrisolvens* as a model system for exploring the biochemistry and genetics of xylan degradation by strictly anaerobic bacteria in order to improve feedstuff conversions in ruminants (10, 27) and/or utilize this species for biomass fermentations.

With most *B. fibrisolvens* strains, xylanase activity is extracellular, XS is cell associated, and AF activity can be found in both locations (13, 14). Various patterns of regulation of these enzymes occurs with different *B. fibrisolvens* strains (13, 14). With *B. fibrisolvens* GS113, the major xylan-degrading enzymes are induced by growth on xylans (25, 26). The current study reports on the isolation and characterization of a cell-associated α-L-AF from *B. fibrisolvens* GS113. This AF is the first xylan-degrading enzyme to be characterized from a *B. fibrisolvens* strain and differs in many respects from the AF isolated from *R. albus* (9).

## MATERIALS AND METHODS

**Growth conditions.** *B. fibrisolvens* GS113 was routinely grown anaerobically at 37°C on RGM medium, a complex carbohydrate-yeast extract-Trypticase medium (14) with soluble oatspelt xylan or sugars as the energy source. The soluble oatspelt xylan was prepared by heating a 10% solution of xylan in distilled water for 60 min at 50°C. After cooling to room temperature, the solution was centrifuged at 650 × *g* at 22°C for 5 min to remove the insoluble fraction. A two-stage culture procedure was used for maximal yields of induced cells. Culture bottles containing 3 liters of 0.2% xylose-RGM medium were inoculated with 100 ml of a late-logarithmic-stage culture in the same medium. After 24 to 30 h of incubation, soluble oatspelt xylan was added to a final concentration of 0.2% and the culture bottles were incubated for another 24 to 30 h before harvesting. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The cell pellets were washed twice by resuspension and centrifugation with a 5% culture volume of sodium phosphate (50 mM, pH 6.5)–0.1 mM dithiothreitol (DTT)–1.0 mM EDTA buffer (PDE buffer). The final PDE wash buffer also contained 0.1 mM phenylmethylsulfonyl fluoride. The washed cell pellets were used immediately or were frozen and stored at −20°C until needed (no longer than 3 weeks).

**Preparation of cell extracts.** The cell pellets were sus-

* Corresponding author.



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pended or thawed in an equal volume of PD buffer containing 0.1 mM phenylmethylsulfonyl fluoride, and the cells were disrupted by three successive passages of the cell suspension through a cold French pressure cell operated at 16,000 lb/in$^2$. The resultant suspension was centrifuged at 24,000 × *g* at 4°C for 30 min to remove large cell debris. The supernatant fluid (crude cell extract) was retained and centrifuged at 150,000 × *g* at 4°C for 60 min to remove membrane materials. This second supernatant fluid and the pellet constituted the cytoplasmic cell extract and membrane fractions, respectively.

**Ammonium sulfate fractionation and ultrafiltration.** Solid ammonium sulfate was slowly added with stirring to the cytoplasmic cell extract to a 40% saturation level at 0°C. The mixture was stirred for another 60 min, and the resulting precipitate was removed by centrifugation at 10,000 × *g* at 4°C for 10 min. The supernatant fluid was further treated with ammonium sulfate to a 65% saturation, stirred, and centrifuged as before. The pellet was dissolved in 10 ml of PD buffer and concentrated to 2 ml by filtration with an Amicon ultrafiltration cell fitted with an 100-kDa nominal cutoff filter (OM-1000-43; Omega 100, Pharmacia, Piscataway, N.J.).

**Column chromatography.** The ultrafiltration retentate was adjusted to 1.5 M ammonium sulfate by slow addition of granular ammonium sulfate and loaded onto a column (1.5 by 15 cm) packed with phenyl-agarose (Sigma Chemical Company, St. Louis, Mo.). The column was previously equilibrated with 1.5 M ammonium sulfate in PD buffer and then eluted (1.0 ml/min) at room temperature with a decreasing ammonium sulfate gradient in PD buffer. For this column and subsequent column chromatography, protein elution profiles were determined by monitoring at 280 nm by using a flow-through cell (HR10; Pharmacia). Fractions (2.0 ml) were collected, and AF-containing fractions were pooled and then concentrated-desalted by ultrafiltration procedures described above.

The retained proteins were washed off the filter with 20 mM Tris-Cl buffer (pH 7.5) containing 0.1 mM DTT and adjusted to a volume of 2.0 ml by further filtration. These proteins were loaded onto an ion-exchange column (0.5 by 5.5 cm; Mono Q, Pharmacia) which was eluted (1.0 ml/min) at room temperature by using an increasing sodium chloride gradient in 20 mM Tris-Cl buffer (pH 7.5)–0.1 mM DTT. AF-containing fractions (1.0 ml each) were pooled and then concentrated-desalted by ultrafiltration as before.

The AF was further concentrated by ultrafiltration with microfilter units (100-kDa nominal cutoff; Millipore). The retained proteins were washed off the filter with 0.1 ml of PD buffer and loaded onto a gel filtration column (1.5 by 66 cm; TSK-Gel Toyopearl HW55s, Supelco). The column was previously equilibrated and then eluted (0.2 ml/min) at room temperature with PD buffer. AF-containing fractions (0.2 ml each) were pooled and concentrated by ultrafiltration. The purified AF was stored at ice temperature in PD buffer or at −20°C after addition of glycerol to a final concentration of 20%.

**Enzyme assays.** For assays with *p*-nitrophenol (*p*-NP) glycosides as substrates, enzyme activities were determined by measuring the amount of *p*-NP released from the substrates. The routine assay contained 1 mM substrate in 0.05 mM DTT–25 mM sodium phosphate (pH 6.5) buffer (DP buffer) in a final reaction volume of 0.25 ml. The incubation temperature was 37°C, unless indicated otherwise. The reaction was terminated by the addition of 0.25 ml of 2.0% sodium carbonate. The released *p*-NP was determined by the $A_{405}$ increase, and *p*-NP was used as the standard. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP per h. Assays involving methylumbelliferyl (MU) glycosides were performed in a similar manner, but the $A_{365}$ was measured, and methylumbelliferone (ε = 22,500) was used as the standard. For assays with polysaccharides as substrates, activities were determined by measuring the amount of sugars released with the phenol sulfuric acid method (1) for total sugars or the orcinol assay (24) for pentoses, with glucose or xylose, respectively, as the standards.

**Thin-layer chromatography (TLC).** Sugar and other xylan degradation products were concentrated from assay contents or other sources by lyophilization. The dried residue was suspended in a small amount (10 to 100 μl) of distilled water and spotted onto Whatman silica gel plates (K-5, 250-μm particles). The plates were developed once with solvent system one (nitroethane-ethanol-water, 1:3:1 [vol/vol/vol]) or developed four times with system two (acetonitrile-water, 9:1 [vol/vol]). The separated products were visualized by spraying the plates with *N*-(1-naphthyl)ethylenediamine and heating (3).

For samples containing *O*-[5-*O*-((E)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX), *O*-[5-*O*-((E)-feruloyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (FAXX), or the breakdown products of PAXX or FAXX, the TLC plates were developed four successive times by using solvent system two. To detect free phenolic acids or other UV-absorbing products, the air-dried plates were briefly exposed to ammonia vapors and examined with a hand-held 365-nm light prior to spraying for detection of carbohydrate products. The obtained PAXX and FAXX were purified as previously described (2).

**Other.** Protein contents of cell extracts or column fraction were determined by the dye-binding method of Bradford (4) with the commercial reagent (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard. For solutions containing very low concentrations, protein levels were estimated by $A_{260}$ and $A_{280}$ measurements (4a). Protein-containing fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (19). The stacking and resolving gels contained 4 and 15% acrylamide, respectively. The gels were fixed and stained with 0.25% Coomassie brilliant blue R-250 in 7% acetic acid, 25% methanol, and 68% distilled water for 30 min. The gels were destained by three successive washes with 7% acetic acid–25% methanol–68% water (vol/vol/vol) followed by 10% (vol/vol) acetic acid washes as needed to reduce background color. Corn endosperm xylan was obtained from lyophilized culture fluids from tissue cultures (21) of corn endosperm cells. The dried residues were dialyzed extensively against distilled water and then relyophilized. Neutral sugar analysis (28) of the corn xylan indicated the major sugar components were arabinose, xylose, galactose, and glucose in a 1.8:2.2:0.3:0.4 ratio. All other chemicals and reagents were purchased from Sigma.

## RESULTS

**Growth conditions and enzyme activities.** When glucose or xylose was used as the growth substrate, *B. fibrisolvens* GS113 cultures displayed no detectable xylanase, XS, or AF activities. With L-arabinose as the substrate, essentially the same results were obtained, but trace AF activity was found. Cultures grown with oatspelt, larchwood, or any of a number

of other xylans displayed high levels of all three enzyme activities. More than 95% of the XS and xylanase activities were always cell associated and extracellular, respectively. Generally, the AF activity was cell associated, but about 10% of the total culture activity could be detected extracellularly, depending upon the xylan substrate used and stage of culture growth (late logarithmic or early stationary). Both AF and XS activities could be readily measured with intact whole cells. Treatment of whole cells with Triton X-100 or other mild detergents did not affect these activities or cause release of them from the cells.

**Purification of AF activity.** Crude cell extracts prepared by French pressure cell disruption displayed levels of AF and XS activities equivalent to those found with intact cells. More than 95% of these activities remained with the cytoplasmic fraction obtained after low-speed centrifugation and ultracentrifugation of the crude extract. Treatment of the cytoplasmic extract with ammonium sulfate resulted in a protein precipitate obtained from 40 to 65% saturation that contained more than 85% of the AF activity and 48% of the XS activity. Ultrafiltration of this protein mixture with a filter having a nominal 100-kDa retention porosity resulted in almost complete retention of the AF activity, but only about 56% of the XS activity was retained.

The retentate obtained from ultrafiltration was subjected to phenyl-agarose column chromatography. Proteins were eluted from the column by using a decreasing salt gradient of ammonium sulfate. The bulk of the protein eluted before the AF activity which eluted between 0.55 and 0.35 M salt (Fig. 1A). The AF activity was free of XS activity, which eluted at about 1.1 to 0.85 M salt. The AF-containing fractions were pooled, concentrated by ultrafiltration, and loaded onto a Mono Q ion-exchange column. The column was eluted by using a variable sodium chloride gradient. The AF activity eluted as a sharp, symmetrical peak at about 0.35 M salt (Fig. 1B). After pooling and desalting the AF-containing fractions by ultrafiltration, the material was subjected to gel filtration chromatography with a TSK Toyapearl column. The AF activity eluted as a single peak that corresponded to a molecular mass of about 230 to 240 kDa (Fig. 1C). However, when this gel filtration step was performed with the carbohydrate-based Superose 6 resin, the AF eluted as a single peak with an apparent molecular mass of 150 to 160 kDa (data not shown). On some occasions, the AF obtained from the Toyapearl column had a small amount of other protein contamination that yielded a band of about 56 kDa on SDS-PAGE analysis. This contaminating protein could be removed when the AF was passed through a further gel filtration column (ca. 1.5 by 100 cm) employing Sephadex G-75 resin.

A summary of the purification steps is shown in Table 1. The enzyme obtained was purified about 300-fold from crude cell extracts. SDS-PAGE analysis was performed on the pooled fractions obtained at the various purification steps. The results of this analysis are shown in Fig. 2 and indicate the purified protein consisted of a polypeptide that migrated with an apparent subunit molecular mass of about 31 kDa. These data suggest the native enzyme consists of a octamer of this polypeptide.

**General properties of the enzyme.** The purified AF lost less than 20% of its activity in 30 days when stored at −20°C. The enzyme was relatively stable up to 45°C and displayed maximal activity at 55°C (Fig. 3). Optimal activity occurred at pH 6.0 to 6.5, and the purified enzyme was fairly stable from pH 4.0 to 8.0 (Fig. 4). The $K_m$ and $V_{max}$ metabolism were 0.66 mM *p*-NP-α-L-AF and 109 μmol of *p*-NP released

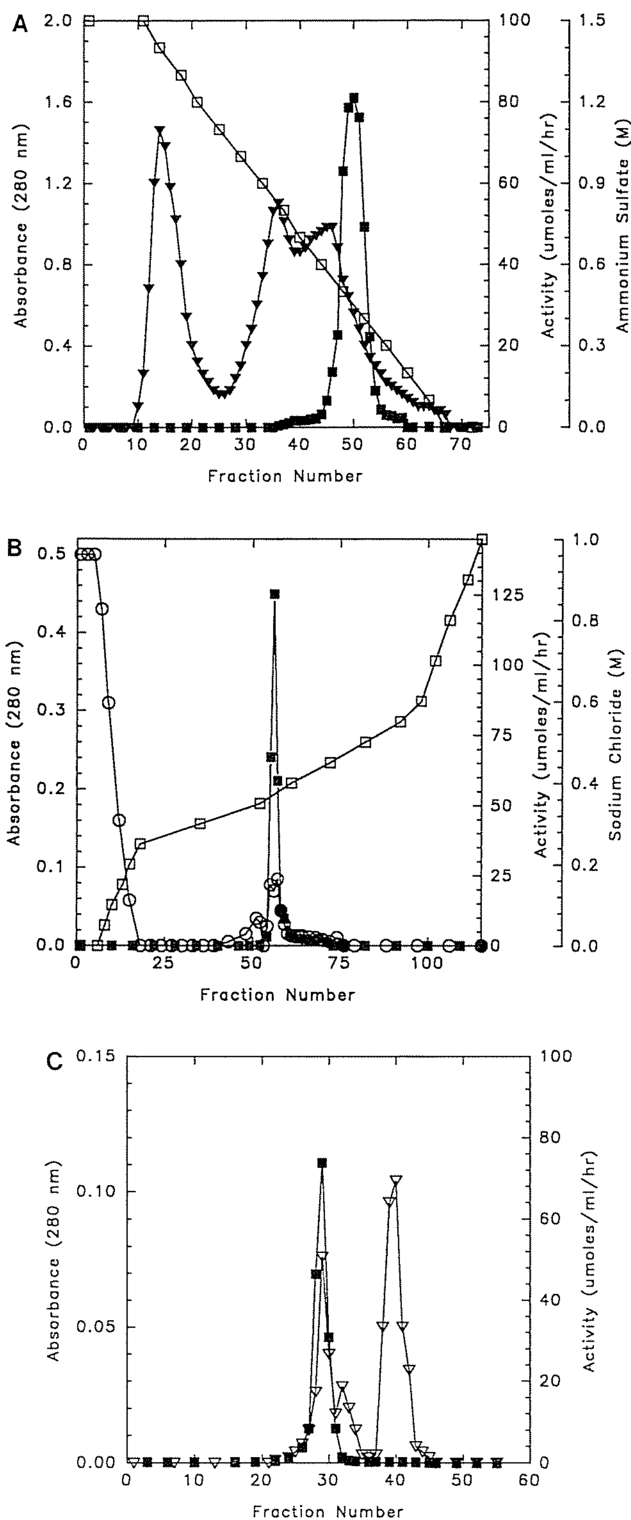


FIG. 1. Column chromatography profiles of AF. (A) Phenyl agarose: absorbance (▼), ammonium sulfate concentration (□), and activity (■). (B) Mono Q ion exchange: absorbance (○), sodium chloride concentration (□), and activity (■). (C) TSK gel filtration: absorbance (▽) and activity (■).

TABLE 1. Purification of AF from *B. fibrisolvens* GS113

| Step | Total activity (U) | Total protein (mg) | Sp act (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cytoplasmic extract | 7,779 | 309 | 25.1 | 1.0 | 100 |
| Ammonium sulfate | 6,000 | 160 | 37.5 | 1.5 | 77 |
| Ultrafiltration | 6,885 | 156 | 44.0 | 1.8 | 88 |
| Phenyl agarose | 5,450 | 47 | 115.8 | 4.6 | 70 |
| Mono Q | 2,438 | 7 | 338.1 | 13.5 | 31 |
| TSK gel filtration | 681 | 0.09 | 7,093.8 | 282.2 | 9 |

per min/mg of protein. The isoelectric point was 6.05 as determined by analytical isoelectrofocusing-PAGE.

**Effects of metals and other compounds.** Prior to assaying, the purified AF was incubated for 30 min at room temperature in 50 mM phosphate buffer containing any one of a number of potential inhibitors at 1 mM. The chloride salts of barium, calcium, magnesium, or manganese as well as EDTA had no effect on the AF. Mercurous chloride or cuprus sulfate caused a loss of more than 90% of AF activity. Similar additions of phenylmethylsulfonyl fluoride, SDS, or ethanol at 1% (vol/vol) also had no effect on AF activity.

**Substrate specificities.** When any of a variety of *p*-NP-glycosides were used as a substrate, the purified AF displayed high activity only with *p*-NP-L-AF or the MU derivative of this substrate (Table 2). No end product inhibition was evident when the purified AF was assayed in the presence of up to 50 mM L-arabinose. No detectable release of sugars could be measured when the enzyme was incubated with cellulose, carboxymethyl cellulose, or arabinogalactan as the substrate.

Sugar release was detected when the AF was incubated

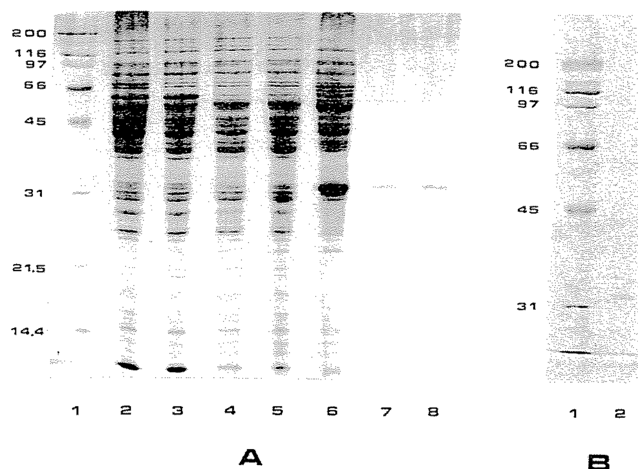


FIG. 2. SDS-PAGE of AF fractions at various purification stages. (A) 15% gel. Lane 1, standard proteins (from top): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 2, crude extract; lane 3, cytoplasmic fraction; lane 4, 40 to 65% ammonium sulfate; lane 5, 100-kDa ultrafiltrate; lane 6, phenyl agarose; lane 7, Mono Q ion exchange; lane 8, TSK gel filtration. (B) 10% gel. Lane 1, standard proteins (same as 15% gel, lane 1); lane 2, 1.4 μg of ultrapure enzyme from Sephadex G-75 gel filtration.

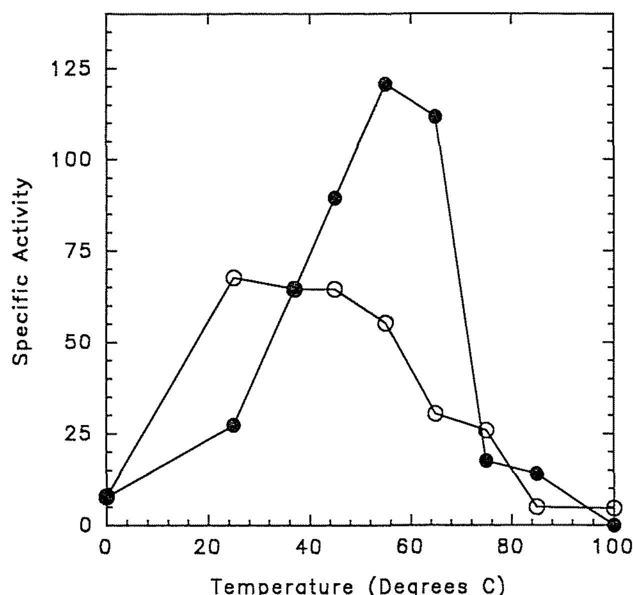


FIG. 3. Effects of temperature on activity (●) and stability (○) of the purified AF. For activity, the enzyme was assayed at the indicated temperatures. For stability, the enzyme was exposed to the indicated temperature for 60 min, and subsequently residual activity was measured at an assay temperature of 37°C.

with either oatspelt or corn endosperm xylans. TLC analysis of the reactions products showed arabinose to be the only released product (Fig. 5). However, the release of arabinose from corn xylan was slow and not extensive, even though the arabinose-to-xylose ratio of this xylan was about 1. Xylose and xylooligosaccharides could also be detected as additional products, if a semipurified XS from *B. fibrisolvens* GS113 and a crude xylanase cloned from *Bacteroides ruminicola* (31) were included in the incubation mixture (data not shown). Even with all these enzymes present, the degradation of the corn xylan was minimal compared with that of the oatspelt xylan. In contrast, when about 65% of the arabinose was removed from the corn xylan by oxalic acid treatment (20 mM, 80°C, 5 h), arabinose release could be detected after 6 h of incubation (Fig. 5B) compared with 18 to 22 h for untreated corn xylan.

With many native xylans, the arabinose side chains often have phenolic acids ester linked at the 5-*O* position (2). To assess whether the purified AF could degrade these substituted arabinose residue, the enzyme was incubated with FAXX or PAXX as a substrate. With incubation times up to 18 h, no degradation of either substrate could be detected. When FAXX or PAXX are incubated in weak alkaline solutions, the ester-linked phenolic acids are readily cleaved off from the arabinose-xylobiose trisaccharide. When these hydrolysate solutions were used as substrates for the AF, arabinose and xylobiose were detected by TLC as reaction products (data not shown).

## DISCUSSION

Microbial AFs display a considerable diversity in a number of properties (Table 3). The native enzymes range from 40 to about 500 kDa in size. While many AFs are single polypeptides, the *B. fibrisolvens* GS113 AF appears to be an octamer of a 30- to 31-kDa polypeptide. The AF of *R. albus*,

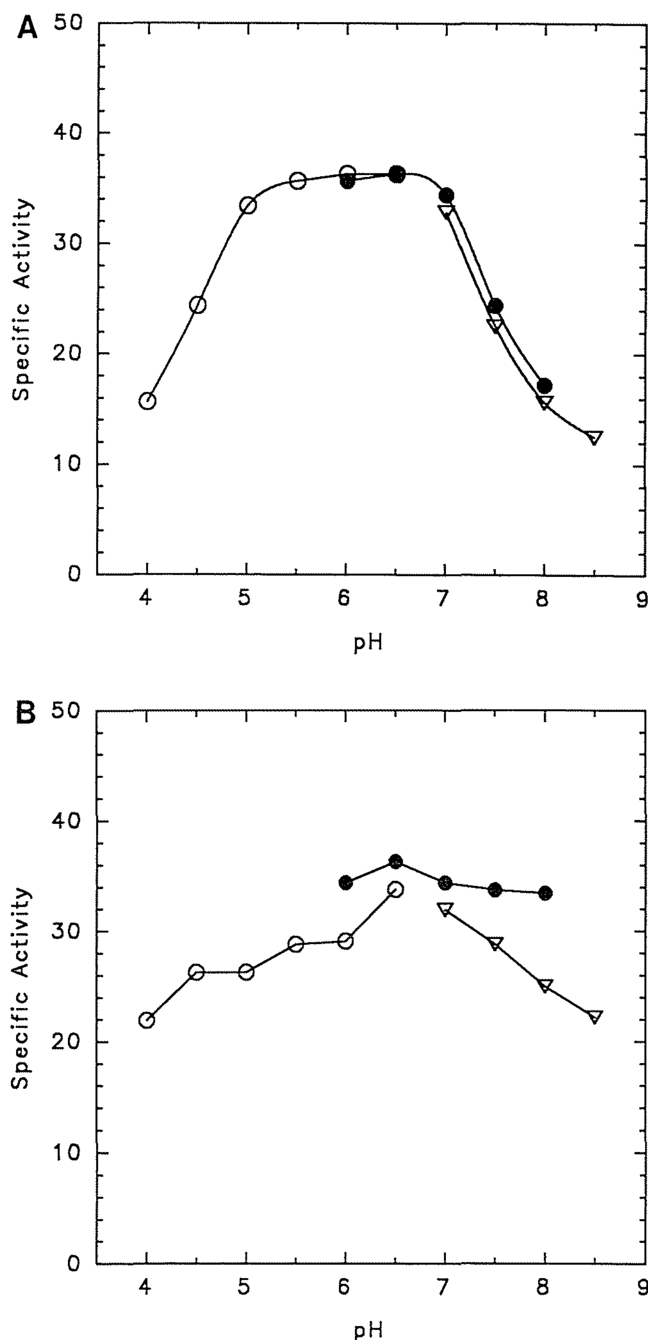


FIG. 4. Effects of pH on activity (A) and stability (B) of the purified AF. Buffers: citrate-phosphate (○), phosphate (●), and Tris (▽). For activity, the enzyme was assayed at the indicated pH. For stability, the enzyme was exposed to the indicated pH for 60 min, and residual activity was measured at an assay pH of 6.5.

another ruminal bacterium, is tetrameric, but the subunit is larger (75 kDa) and is a glycoprotein (9). While most fungal AFs have pH optima of less than 5.0, those for ruminal bacteria are in the range of 6.0 to 7.0, which is the pH range commonly found in the rumen ecosystem. Unlike many other types of glycosidases, AFs usually have a narrow range of substrate specificity. In this regard, the *B. fibrisolvens* GS113 AF is quite normal. This enzyme shows activity only with synthetic substrates having an α-L linkage and a

TABLE 2. Activity of purified *B. fibrisolvens* GS113 AF on synthetic substrates

| Substrate | Sp act (AU) |
|---|---|
| *p*-NP-α-L-arabinofuranoside | 52.9 |
| *p*-NP-α-L-arabinofuranoside | 0.0 |
| *p*-NP-β-D-xylopyranoside | 0.12 |
| *o*-Nitrophenol-β-D-xylopyranoside | 0.07 |
| *p*-NP-α-L-fucopyranoside | 0.33 |
| *p*-NP-α-D-glucopyranoside | 0.12 |
| *p*-NP-β-D-glucopyranoside | 0.06 |
| *p*-NP-β-D-galactopyranoside | 0.17 |
| *p*-NP-β-D-cellobioside | 0.20 |
| *p*-NP-α-D-mannopyranoside | 0.12 |
| MU-α-L-arabinofuranoside | 17.6 |
| MU-β-D-xylopyranoside | 0.0 |

furanoside configuration (Table 2). It also was able to release arabinose from a number of xylans and other substrates with α-linked arabinose side chains. The *B. fibrisolvens* GS113 AF has a rather low $K_m$ and high $V_{max}$ compared with those of most other AFs (Table 3).

The first gene to be cloned from *B. fibrisolvens* was a XS from strain GS113 (26). The recombinant *Escherichia coli* containing this gene produced a new protein of about 60 kDa and the XS hydrolyzed xylooligosaccharides with chain lengths of 2 to 5 U. Recently, this gene has been sequenced and further characterized (29). By using *p*-NP substrates, the protein made in *E. coli* appears to be bifunctional and expresses both XS and AF activities. The properties of this protein and those of the cytoplasmic AF purified from *B. fibrisolvens* GS113 described in this paper indicate that these are apparently different enzymes. A similar multiplicity of AF activities also appears to occur in *Bacteroides ovatus* (32).

Currently, it is not clear what substructure of xylans serves as the optimal substrate for AFs. The *R. albus* AF releases little reducing sugars from intact alfalfa cell walls, but this release is enhanced 50- to 90-fold if xylanase or polygalacturonidase is also present (9). The AF of *Clostridium acetobutylicum* has little activity on oatspelt xylan unless XS and particularly xylanase are present (20). As indicated by TLC analysis of degradation products, we have found similar results with the *B. fibrisolvens* GS113 AF. In addition, the rate and extent of arabinose release was less with corn endosperm xylan than with oatspelt xylan. The corn endosperm xylan contains about equal amounts of arabinose and xylose, whereas oatspelt xylan has one arabinose for every eight xyloses. Treatment of the corn xylan with oxalic acid removed much of the arabinose and considerably improved its susceptibility to AF attack. Thus, these results suggest that the high-arabinose side chain substitution pattern in the corn endosperm xylan in fact sterically hinders the action of the AF. Furthermore, the AF was unable to degrade either FAXX or PAXX. When these substrates were alkali treated to remove the ester-linked phenolics, arabinose and xylobiose were detected as products. These observations indicate that the phenolic residues also may hinder AF activity and the arabinose-xylobiose trisaccharide can be a substrate for AF.

With *B. fibrisolvens* strains, more than 95% of the XS activity is cell associated (13, 14). It is reasonable to assume that xylobiose and possibly other xylooligosaccharides (XOS) can enter the cell and be degraded to monomers by this XS activity. However, unlike many other xylan-degrad-

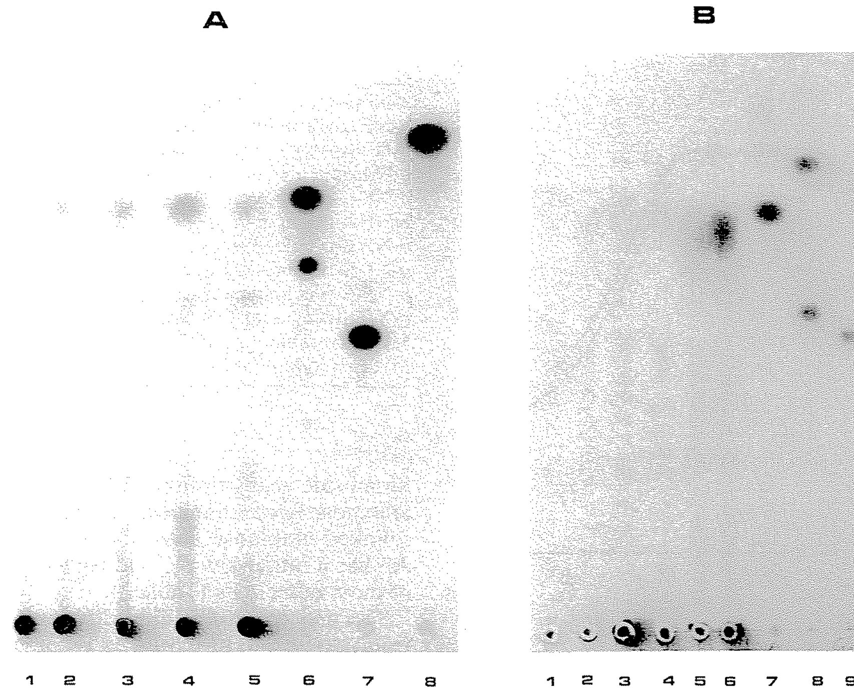


FIG. 5. TLC of soluble products released from xylans by purified AF. (A) Oatspelt xylan. Lanes 1 and 5, xylan incubated for 0 and 18 h, respectively, with no enzyme added; lanes 2, 3, and 4, xylan incubated with enzyme for 1, 3, and 18 h, respectively. Sugar standards: Lane 6, arabinose (top) and xylobiose; lane 7, glucose; and lane 8, xylose. (B) Oxalate-treated corn endosperm xylan. Lanes 1, 2, and 3, xylan incubated for 0, 6, and 22 h, respectively, with no enzyme added; lanes 4, 5, and 6, xylan incubated with enzyme for 0, 6, and 22 h, respectively. Sugar standards: lane 7, arabinose; lane 8, xylose (top) and xylobiose; and lane 9, glucose.

ing microbial species, the majority of AF activity is also cell associated with *B. fibrisolvens* strains. With strain 49, 70 to 80% of the total AF activity is cell associated, but protoplasting of the cells releases about half of this AF activity and none of the XS activity (12). These results suggest that some of the AF activity is located on the cell surface and, in conjunction with extracellular AF activity, would be available to degrade arabinose-containing XOS (AXOS) generated from xylans by the extracellular xylanase. With strain GS113, very little AF activity is extracellular, and attempts to release AF activity from cells by protoplasting were unsuccessful. The cytoplasmic location of AFs in *B. fibrisolvens* strains suggests that AXOS also can enter the cell.

In the rumen ecosystem, polymer-degrading species must compete with many other microbial species. Some species can utilize breakdown products but are incapable of degrading plant cell wall polymers. For example, a number of noncellulolytic species can utilize cellulodextrins (23). A similar situation may exist for usage of XOS or AXOS by various species of ruminal bacteria that are incapable of degrading intact xylans. Evidence for this comes from preliminary experiments indicating that *Selenomonas ruminantium* strains can be cocultured with *B. fibrisolvens* in media that contain oatspelt xylan as the sole added energy source (5). Thus, an efficient uptake of XOS and AXOS by *B. fibrisolvens* may be of ecological importance in allowing this organism to effectively compete in the rumen and minimize losses of these substrates to nonxylanolytic species.

TABLE 3. Comparisons of microbial AFs

| Microorganism | Property | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mol wt (kDa) | Subunit (kDa) | pI | pH optimum | pH stability | $K_m$[a] | $V_{max}$[a] | Sulfhydryl sensitivity | Polymers attacked[b] | Reference |
| *Butyrivibrio fibrisolvens* | 240 | 31 | 6.0 | 6.0–6.5 | 4.0–8.0 | 0.7 | 109 | + | CX, OX, AX, BA | |
| *Ruminococcus albus* | 310 | 75 | 3.8 | 6.9 | ND[c] | 1.6 | ND | + | AH | 9 |
| *Clostridium acetobutylicum* | 94 | 94 | 8.2 | 5.0–5.5 | 5.5–8.0 | 4.0 | 36 | ND | BA | 20 |
| *Bacillus subtilis* | 65 | 65 | 5.3 | 6.5 | ND | ND | ND | − | BA | 30 |
| *Streptomyces purpurascens* | 495 | 62 | 3.9 | 6.5 | ND | 0.08 | 89 | + | A2, A3 | 18 |
| *Streptomyces* sp. strain 17-1 | 92 | 92 | 4.4 | 6.0 | 4.0–9.0 | 3.6 | ND | + | BA, AX, AG | 15 |
| *Aspergillus niger* | 53 | 53 | 3.6 | 3.8–4.0 | ND | 4.9 | ND | ND | BA, AX | 16 |
| *Monilinia fructigena* | 40 | 35 | 6.5 | 4.0 | ND | 0.8 | 1.03 | ND | ND | 17 |

[a] Measured with *p*-NP-AF as the substrate; $K_m$ is given in millimolar. $V_{max}$ (maximum rate of metabolism) is given in micromoles per minute per milligram.
[b] AX, arabinoxylan; CX, corn endosperm xylan; OX, oatspelt xylan; BA, beet arabinan; AH, alfalfa hemicelluloses; AG, arabinogalactan; A2, arabinobiose; A3, arabinotriose.
[c] ND, not determined.

## ACKNOWLEDGMENTS

We thank Scott Borneman for providing the PAXX and FAXX substrates and the chemical characterization of them. The beet arabinan was a gift from C. W. Forsberg. We thank Linda Ericsson for the sugar analyses of the xylans and Jan Miernyk for the corn endosperm tissue culture.

## REFERENCES

1. **Ashwell, G.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3:**73–105.
2. **Borneman, W. S., R. D. Hartley, D. S. Himmelsbach, and L. G. Ljungdahl.** 1990. Assay for *trans-p*-coumaryl esterase using a specific substrate from plant cell walls. Anal. Biochem. **190:** 129–133.
3. **Bounias, M.** 1980. *N*-(1-Naphthyl)ethylenediamine dihydrochloride as a new reagent for nanomole detection of sugars on thin layer plates by a mathematical calibration process. Anal. Biochem. **106:**291–295.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.

4a. **Brewer, J. M., A. J. Pesue, and R. B. Ashworth.** 1974. Experimental techniques in biochemistry, p. 328. Prentice-Hall, Inc., Englewood Cliffs, N.J.

5. **Cotta, M. A.** 1991. Unpublished data.
6. **Dehority, B. A.** 1966. Characterization of several bovine rumen bacteria isolated with a xylan medium. J. Bacteriol. **91:**1724–1729.
7. **Dehority, B. A.** 1967. Rate of hemicelluose degradation and utilization by pure cultures of rumen bacteria. Appl. Microbiol. **15:**987–993.
8. **Dehority, B. A.** 1973. Hemicellulose degradation by rumen bacteria. Fed. Proc. **32:**1819–1825.
9. **Greve, J. C., J. M. Labavitch, and R. E. Hungate.** 1984. α-L-Arabinofuranosidase from *Ruminococcus albus* 8: purification and possible role in hydrolysis of alfalfa cell wall. Appl. Environ. Microbiol. **47:**1135–1140.
10. **Hespell, R. B.** 1987. Biotechnology and modifications of the rumen microbial ecosystem. Proc. Nutr. Soc. **46:**407–413.
11. **Hespell, R. B.** 1988. Microbial digestion of hemicelluloses in the rumen. Microbiol. Sci. **5:**362–365.
12. **Hespell, R. B.** 1991. Unpublished data.
13. **Hespell, R. B., and T. R. Whitehead.** 1990. Physiology and genetics of xylan degradation by gastrointestinal tract bacteria. J. Dairy Sci. **73:**3013–3020.
14. **Hespell, R. B., R. Wolf, and R. J. Bothast.** 1988. Fermentation of xylans by *Butyrivibrio fibrisolvens* and other ruminal bacterial species. Appl. Environ. Microbiol. **53:**2849–2853.
15. **Kaji, A., M. Sato, and Y. Tsutsui.** 1981. An α-L-arabinofuranosidase produced by wild-type *Streptomyces* sp. no. 17-1. Agric. Biol. Chem. **45:**925–931.
16. **Kaji, A., and K. Tagawa.** 1970. Purification, crystallization, and amino acid composition of α-L-arabinofuranosidase from *Aspergillus niger*. Biochim. Biophys. Acta **207:**456–464.
17. **Kelly, M. A., M. L. Sinnott, and M. Herrchen.** 1987. Purification and mechanistic properties of an extracellular α-L-arabinofuranosidase from *Monilinia fructigena*. Biochem. J. **245:**843–849.
18. **Komae, K., A. Kaji, and M. Sato.** 1982. An α-L-arabinofuranosidase from *Streptomyces purpurascens* IFO 3389. Agric. Biol. Chem. **46:**1899–1905.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **Lee, S. F., and C. W. Forsberg.** 1987. Purification and characterization of an α-L-arabinofuranosidase from *Clostridium acetobutylicum* ATCC 824. Can. J. Microbiol. **33:**1011–1016.
21. **Miernyk, J. A.** 1987. Extracellular secretion of acid hydrolases by maize endosperm cells grown in liquid medium. J. Plant Physiol. **129:**19–32.
22. **Morris, E. J., and N. O. van Gylswyk.** 1980. Comparison of the action of rumen bacteria on cell walls from *Eragrostis tef.* J. Agric. Sci. **95:**313–323.
23. **Russell, J. B.** 1985. Fermentation of cellodextrins by cellulolytic and non-cellulolytic rumen bacteria. Appl. Environ. Microbiol. **49:**572–576.
24. **Schneider, W. C.** 1957. Determination of nucleic acids by pentose analysis. Methods Enzymol. **3:**374–381.
25. **Sewell, G. W., H. C. Aldrich, D. Williams, B. M. Mannarelli, A. Wilkie, R. B. Hespell, P. H. Smith, and L. O. Ingram.** 1988. Isolation and characterization of xylan-degrading strains of *Butyrivibrio fibrisolvens* from a Napier grass-fed anaerobic digestor. Appl. Environ. Microbiol. **54:**1085–1090.
26. **Sewell, G. W., E. A. Utt, R. B. Hespell, and L. O. Ingram.** 1989. Xylosidase (*xylB*) from *Butyrivibrio fibrisolvens*: identification of coding region and expression in *Escherichia coli*. Appl. Environ. Microbiol. **55:**306–311.
27. **Smith, C. J., and R. B. Hespell.** 1983. Prospects for development and use of recombinant deoxyribonucleic acid techniques with ruminal bacteria. J. Dairy Sci. **66:**1536–1546.
28. **Stack, R. J.** 1988. Neutral sugar composition of extracellular polysaccharides produced by strains of *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. **54:**878–883.
29. **Utt, E. A., C. K. Reddy, K. F. Keshaw, and L. O. Ingram.** 1991. Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl. Environ. Microbiol. **57:**1227–1234.
30. **Weinstein, L., and P. Albersheim.** 1979. Structure of plant cell walls. IX. Purification and partial characterization of a wall-degrading endo-arabanase and an arabinosidase from *Bacillus subtilis*. Plant Physiol. **63:**425–432.
31. **Whitehead, T. R., and R. B. Hespell.** 1989. Cloning and expression in *Escherichia coli* of a xylanase gene from *Bacteroides ruminicola* 23. Appl. Environ. Microbiol. **55:**893–896.
32. **Whitehead, T. R., and R. B. Hespell.** 1990. The genes for three xylan-degrading activities from *Bacteroides ovatus* are clustered in a 3.8-kilobase region. J. Bacteriol. **172:**2408–2412.
33. **Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler.** 1988. Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol. Rev. **52:**305–317.