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Purification, Characterization, and Mode of Action of Endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85

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Two different endoxylanases (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8), designated 1 and 2, have been purified by column chromatography to apparent homogeneity from the nonsedimentable extracellular culture fluid of the strictly anaerobic, ruminal bacterium *Fibrobacter succinogenes* S85 grown on crystalline cellulose. Endoxylanases 1 and 2 were shown to be basic proteins of 53.7 and 66.0 kDa, respectively, with different pH and temperature optima, as well as different substrate hydrolysis characteristics. The K_m and V_{max} values with water-soluble oat spelts xylan as substrate were 2.6 mg ml^{-1} and 33.6 μmol min^{-1} mg^{-1} for endoxylanase 1 and 1.3 mg ml^{-1} and 118 μmol min^{-1} mg^{-1} for endoxylanase 2. Endoxylanase 1, but not endoxylanase 2, released arabinose from water-soluble oat spelts xylan and rye flour arabinoxylan, but not from arabinan, arabinogalactan, or aryl-α-L-arabinofuranosides. With an extended hydrolysis time, endoxylanase 1 released 62.5 and 50% of the available arabinose from water-soluble oat spelts xylan and rye flour arabinoxylan, respectively. Endoxylanase 1 released arabinose directly from the xylan backbone, and this preceded hydrolysis of the xylan to xylooligosaccharides. Endoxylanase 2 showed significant activity against carboxymethyl cellulose but was unable to substantially hydrolyze acid-swollen cellulose. Both enzymes were endo-acting, as revealed by their hydrolysis product profiles on water-soluble xylan and xylooligosaccharides. Because of their unique hydrolytic properties, endoxylanases 1 and 2 appear to have strategic roles in plant cell wall digestion by *F. succinogenes* in vivo.

Fibrobacter succinogenes is an important fibrolytic ruminal bacterium and plays a significant role in hemicellulose degradation within the rumen (13, 35, 47). In addition to its multiple endoglucanase (38, 39) and cellobiosidase (25, 26) enzymes, *F. succinogenes* has been shown to possess extracellular endoxylanase activity (16), and a gene coding for endoxylanase activity has been cloned from *F. succinogenes* and expressed in *Escherichia coli* (45). Other hemicellulases, including α-L-arabinofuranosidase and ferulic acid esterase have also been detected (37), and an acetyl xylan esterase has been recently purified from the extracellular culture fluid (36).

To increase our understanding of the *F. succinogenes* enzymes involved in heteroxylan hydrolysis, we undertook to purify and characterize endoxylanases from this bacterium. Here we report the purification, characterization, and mode of action of two endoxylanases purified from *F. succinogenes* S85. Endoxylanase 1 was found to exhibit arabinose-debranching activity, releasing arabinose from arabinoxylans prior to hydrolysis of the xylan backbone to xylooligosaccharides. Endoxylanase 2, in addition to its xylan-hydrolyzing activity, showed significant activity on carboxymethyl cellulose (CMC), although it was unable to hydrolyze amorphous (acid-swollen) cellulose.

MATERIALS AND METHODS

Bacterium and growth conditions. The bacterium used in this study was *Fibrobacter succinogenes* subsp. *succinogenes* S85 (40), formerly *Bacteroides succinogenes* (ATCC 19169). It was grown in a medium identical to that of Scott and Dehority (44), except that ammonium sulfate was used as the sole nitrogen source with 0.4% (wt/vol) Avicel microcrystalline cellulose (PH-105; FMC Corp., Philadelphia, Pa.) or 0.5% (wt/vol) glucose as the carbon source. The culture conditions and anaerobic technique were as previously described by McDermid et al. (37). Large-scale cultures of *F. succinogenes* consisting of 20- or 40-liter volumes of cells were prepared as described by McDermid et al. (36). Solid medium (plates) contained in addition to those components in the liquid medium, 0.125 mg of hemin per 100 ml, 0.2 mg of vitamin K per 100 ml, 0.2% (wt/vol) tryptone, 0.05% (wt/vol) yeast extract, 0.5% (wt/vol) glucose, 0.1% (wt/vol) cellobiose, and 0.2% (wt/vol) maltose and were solidified with 1.5% (wt/vol) agar (Difco).

Concentration and fractionation of extracellular culture fluid. Concentrated culture fluid was prepared from 20- or 40-liter batches of culture and dialyzed in situ as described previously by McGavin and Forsberg (38). In the purification of endoxylanase 1, the concentrated culture fluid from a 20-liter fermentation was centrifuged (100,000 × *g*, 2 h, 4°C), and the supernatant was carefully removed and then recentrifuged under the same conditions, resulting in the sedimentation of additional membranous material. The supernatant from this second centrifugation was carefully removed, while the pelleted material from the first two centrifugations was combined and washed twice by centrifugation (conditions as described above) with 20 mM phosphate buffer, pH 6.5. For purification of endoxylanase 2, the concentrated culture fluid from 40 liters of culture was ultracentrifuged once as described above to sediment the majority of membranous materials. The nonsedimentable fraction and washes (as appropriate) were pooled and concentrated about fourfold by ultrafiltration and used as a source of enzymes for purification experiments.

Enzyme assays. Endoxylanase, endoglucanase, and other endoglycanase activities were determined in triplicate as described previously (16), and reducing sugar produced was

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estimated by the Nelson-Somogyi assay (3). All substrates were included at a final concentration of 1% (wt/vol), except arabinan, which was used at 0.5% (wt/vol). α-L-Arabinofuranosidase and β-D-xylosidase assays were performed as previously described (36) and contained substrates at 1 and 5 mM final concentration, respectively, when column fractions were assayed. For analysis of the substrate specificity of endoxylanases 1 and 2, all *p*-nitrophenyl substrates were included at a final concentration of 5 mM. Aryl esterase activity was determined by the method of Hespell and O'Bryan-Shah (21) with α-naphthyl acetate at a final concentration of 1 mM. Assays with 4-*O*-methylumbelliferyl substrates were performed at a final concentration of 1.0 mM. The reactions were stopped by addition of sodium carbonate to a final concentration of 0.5 M. The A_{365} values were measured and related to a standard curve of 4-*O*-methylumbelliferone analyzed concurrently. Enzyme extracts containing endoxylanase, α-L-arabinofuranosidase, or β-D-xylosidase activities were assayed in 50 mM sodium or potassium phosphate buffer, pH 6.5. Endoxylanase 1 was assayed under optimal conditions in 50 mM MOPS (morpholinopropanesulfonic acid)-KOH buffer (pH 7.0)–1 mM $CaCl_2$. Endoxylanase 2 was assayed in 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-KOH buffer (pH 6.3)–1 mM $MgCl_2$. In all cases, enzyme activity was determined (in triplicate) by relating absorbance values, after correction for background, to standard curves of product. One unit of enzyme activity is defined as the quantity of enzyme required to liberate 1 μmol of product in 1 min at 39°C. Specific activity is defined as units per milligram of protein.

Column chromatography. Polybuffer exchanger 94, Polybuffer 74, Q-Sepharose CL-6B, and CM-Sepharose CL-6B were obtained from Pharmacia, Uppsala, Sweden. Hydroxylapatite HTP was from Bio-Rad Laboratories Ltd., Richmond, Calif. The experiments involving column chromatography were performed at 4°C. All buffers were either adjusted for pH changes if diluted from stock solutions or were prepared at the concentration to be used and contained 0.01% (wt/vol) sodium azide to prevent microbial contamination.

(i) Purification of endoxylanase 1. For purification of endoxylanase 1, the nonsedimentable extracellular culture fluid, equilibrated in starting buffer (0.025 M imidazole-HCl, pH 7.4) and containing 462 mg of protein was applied to a Polybuffer Exchanger 94 chromatofocusing column (1.5 by 50 cm) previously equilibrated in starting buffer. Following sample application, the pH gradient was developed with 1,320 ml of dilute Polybuffer 74 (Polybuffer-H_2O [1:8, vol/vol]-HCl, pH 4.0), and then a 400-ml gradient of 0 to 0.4 M NaCl in 20 mM potassium phosphate buffer, pH 6.5, was applied. Remaining proteins were eluted from the column with 200 ml of 1.0 M NaCl in 20 mM phosphate buffer, pH 6.5. The initial flow rate was 32 ml h^{-1}, and 7.5-ml fractions were collected. Fractions containing endoxylanase activity as determined by enzyme assays were pooled, desalted by ultrafiltration if necessary, and concentrated by ultrafiltration through a PM-10 membrane (Amicon Canada Ltd., Oakville, Ontario, Canada). The concentrated fraction containing the void volume from chromatofocusing was applied to a column (2.5 by 26 cm) of CM-Sepharose CL-6B equilibrated with 20 mM potassium phosphate buffer, pH 6.5. After sample application, the column was washed with 1 volume of starting buffer, and proteins were eluted with a 500-ml linear gradient of 0 to 0.5 M NaCl in starting buffer. Finally, the column was washed with 1.6 volumes of 1.0 M NaCl in starting buffer to remove any remaining bound proteins. The initial flow rate was 45.6 ml h^{-1}, and 5-ml fractions were collected. Fractions from this column containing endoxylanase activity were pooled and concentrated by ultrafiltration, and that portion of the applied endoxylanase activity eluting in the gradient was applied to a hydroxylapatite column (0.9 by 15 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.5. Following sample application, the column was washed with 1 volume of starting buffer, followed by a 100-ml linear gradient of 0 to 0.4 M KCl in starting buffer. The column was then washed with 2 volumes of 1.0 M KCl in starting buffer. The initial flow rate was 39 ml h^{-1}, and 1-ml fractions were collected. Before pooling fractions, 25 μl of each fraction showing endoxylanase activity was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to assess enzyme purity. Fractions containing purified enzyme were pooled, desalted, and concentrated by ultrafiltration using a PM-10 membrane, and stored frozen at −20°C. Endoxylanase 1 was also purified by sequential chromatography on Q-Sepharose CL-6B and CM-Sepharose CL-6B, although in this case the enzyme was contaminated by a single protein that was detectable by SDS-PAGE but which lacked glycanase activity.

(ii) Purification of endoxylanase 2. Endoxylanase 2 was purified by using a combination of Q-Sepharose CL-6B and CM-Sepharose CL-6B chromatography. A total of 1,520 mg of nonsedimentable extracellular culture fluid protein (6,690 U of endoxylanase activity) in 20 mM potassium phosphate buffer, pH 6.5, was applied to a Q-Sepharose CL-6B Fast Flow column (2.5 by 80 cm) preequilibrated with the same buffer. Following sample application, the column was washed with 500 ml of starting buffer at an initial flow rate of 39.2 ml h^{-1}, and bound proteins were eluted in a 500-ml gradient of 0 to 0.5 M NaCl in 20 mM potassium phosphate buffer, pH 6.5. Fractions of 5 ml were collected and assayed, and the void volume fractions were pooled, concentrated by ultrafiltration, and stored frozen at −20°C.

The void volume sample (94.5 ml, 1,190 U of endoxylanase activity) from the Q-Sepharose column was thawed for CM-Sepharose chromatography. The buffer was exchanged by concentrating the solution twofold by ultrafiltration with a PM-10 membrane, dilution with 10 volumes of MES-KOH buffer (pH 5.5), and concentration again to a volume of 50 ml. This concentrated fraction was then centrifuged (100,000 × *g*, 2 h, 4°C) to sediment denatured proteins, and the supernatant was carefully removed. The supernatant proteins (42 mg, 367 U of endoxylanase activity) were applied to a CM-Sepharose CL-6B column (2.5 by 33 cm) equilibrated with 20 mM MES-KOH buffer, pH 5.5. The column was then washed with 175 ml of starting buffer, and the proteins were eluted with a linear 500-ml gradient from 0 to 0.2 M KCl in starting buffer. The column was then washed with 250 ml of 1.0 M KCl in starting buffer. Fractions (5 ml) from the 1.0 M KCl wash containing endoxylanase 2 were pooled, desalted by ultrafiltration, and stored frozen at −70°C.

Gel electrophoresis and analytical IEF. Gel electrophoresis was performed in the presence of SDS as described by Laemmli (29) with 4% (wt/vol) stacking and 12% (wt/vol) separating gels. Isoelectric focusing (IEF) was performed essentially as described by Huang and Forsberg (24), with the following modifications. In some experiments, *N*,*N'*-methylene bisacrylamide was replaced with 0.17% (wt/vol) piperazine diacrylamide (Bio-Rad) as the cross-linking agent. When gels were prepared through the pH range of 6 to 10.5, broad-range ampholytes were substituted with 1.1% (wt/vol) pH 6 to 8 Ampholine (LKB) and 1.1% (wt/vol) pH 8

to 10.5 Pharmalyte (Pharmacia). For the later gels, 1 M NaOH and 2% (wt/vol) pH 5 to 7 Ampholyte (LKB) were the cathode and anode solutions, respectively. Gels were stained and destained as described in the Bio-Phoresis horizontal electrophoresis cell instruction manual (Bio-Rad). Zymogram analysis of endoxylanase activity in IEF gels was determined at 37°C for 1.5 h with Remazol brilliant blue (RBB)-dyed larchwood xylan as the substrate (7).

Enzymatic hydrolysis of xylan, arabinogalactan, amorphous cellulose, and xylooligosaccharides. To determine the extent of hydrolysis and product distribution from the action of endoxylanases 1 and 2 0.022 U of enzyme ml^{-1} was incubated in the appropriate reaction mixture described under enzyme assays for 20 h, and samples were removed at intervals. Percent hydrolysis was calculated as (micromoles of reducing sugars released/micromoles of xylan as xylose) × 100. The remaining volume of each sample was analyzed for xylooligosaccharides by high-pressure liquid chromatography (HPLC) on a HPX-42A column (Bio-Rad). Arabinose release was quantified by HPLC with the same column with L-arabinose as an external standard.

To determine the products of hydrolysis of xylooligosaccharides in the range xylobiose through xylohexaose, 0.056 U ml^{-1} endoxylanase 1 or 2 was incubated with 1.0 mg of xylobiose, xylopentaose, or xylohexaose ml^{-1} or 0.5 mg of xylotriose or xylotetraose ml^{-1} in reaction mixtures identical to that for xylan hydrolysis. For endoxylanase 1, samples were removed at 0 and 24 h, and immediately heated at 100°C for 15 min to inactivate the enzyme. Endoxylanase 2 appeared to retain residual activity under the same conditions; therefore, heat-treated enzyme controls were prepared by autoclaving an aliquot of enzyme at 121°C for 60 min and then incubating an amount identical to that in the active preparations for 24 h. Products were analyzed by HPLC.

Tritiation of arabinoxylan hydrolysis products. To determine the sequence of appearance of mono- and xylooligosaccharide products from the hydrolysis of rye flour arabinoxylan by endoxylanase 1, the products were labelled with ^3H by using tritiated sodium borohydride (NaB^3H_4) (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada; specific activity, approximately 263 mCi $mmol^{-1}$). Tritiated sodium borohydride was prepared by dissolving the solid in 2.5 ml of filter-sterilized 0.05 M NaOH and immediately aliquoting the material in 50- or 100-μl volumes and freezing in liquid nitrogen. Products were obtained at each time point exactly as described above for arabinoxylan hydrolysis and were frozen immediately at −20°C. Reducing sugars in each sample were converted to their corresponding ^3H-alditols by adding 666 μl of a cold (4°C) solution of 10 μCi of NaB^3H_4 in 0.05 M $NaBH_4$ dissolved in 0.1 M NaOH and incubating for 4 h at 25°C. The total amount of $NaBH_4$ added was equal to or greater than a fivefold molar excess, based on a monosaccharide concentration of 1.0 mg ml^{-1}. Excess $NaBH_4$ was decomposed by dropwise addition of 75 μl of 50% (vol/vol) acetic acid in a fumehood because of the release of $^3H_2$ gas. Residual borate was removed from each sample by rotoevaporation in the presence of methanol (2 by 5 ml, 40°C) to dryness and then with water (2 by 5 ml, 40°C), again to dryness. The residue was quantitatively resuspended in 500 μl of water, and two 20-μl aliquots were removed for scintillation counting. Each sample was then treated with 25 mg of mixed-bed ion exchanger [Amberlite IR-120 (H^+), Rexyn 201 (CO_3^{2-}), 1:1 (vol/vol)], and again, two 20-μl aliquots were removed for counting. Samples were then filtered through a 0.45-μm-pore-size filter, and two 20-μl aliquots of the filtrate were removed for counting.

Products in each sample (three 50-μl injections) were separated by HPLC on the HPX-42A column. Fractions were collected for 0.33-min intervals into 1.5-ml microcentrifuge tubes, and L-[^3H]arabinitol and [^3H]xylooligosaccharide alditols were pooled separately. The exact retention times for D-[^3H]xylitol, L-[^3H]arabinitol, and D-[^3H]xylobitol were determined by preparing each of these compounds as described above for the hydrolysis mixture by using 200 μl (5 mg ml^{-1} solutions in water) of D-xylose, L-arabinose, or D-xylobiose, respectively. The only deviation in this last procedure was that the sugar solutions were resuspended with 1.0 ml of water after the last rotoevaporation step. The pooled fractions from each sample were concentrated by rotoevaporation at 40°C to dryness and quantitatively resuspended in 1.0 ml of water. Samples of 100 μl were counted from each fraction in triplicate. The resulting incorporation data were corrected for both background labelling of the reducing ends in the arabinoxylan, as well as for binding of labelled products to the ion exchanger.

Analytical methods. Scintillation counting was performed with samples in duplicate or triplicate by using a Tri-Carb 2000 liquid scintillation analyzer (Packard Instrument Company, Downers Grove, Ill.). Aliquots of samples were made to 100 μl with water, and the solution was added to 10 ml of ACS aqueous counting scintillant (Amersham Canada Ltd., Oakville, Ontario, Canada), thoroughly mixed, and counted in an energy range of 0 to 167 kV for 10 min. Counting efficiency for ^3H was determined by using *n*-[^3H]hexadecane (Amersham; specific activity, approximately 1.88×10^6 dpm ml^{-1}) as an external standard. Binding of enzyme to amorphous cellulose was assayed as described previously (38). Monosaccharide compositions of xylan from oat spelts, larchwood, and rye flour were determined by trifluoroacetic acid hydrolysis, and HPLC analysis as described by Lee et al. (31). Quantitation of monosaccharides was accomplished by using D-mannitol as an internal standard. Protein was determined by the method of Bradford (10) by using bovine serum albumin (BSA) as the standard. Protein contents of column fractions were determined as the A_{280}.

Enzyme substrates and chemicals. Water-soluble and -insoluble xylan fractions were prepared from oat spelts xylan (Sigma; lot 14F-0421) by suspending 4% (wt/vol) of the whole xylan in water, by stirring for 4 h at 20°C, and then by centrifugation at 16,000 × *g* for 10 min, and the supernatant fluid and sediment were freeze dried. Acid-swollen (amorphous) cellulose was prepared from Avicel microcrystalline cellulose (PH-105) essentially as described by Wood (52), except that the cellulose was neutralized with 1 M Na_2CO_3 and washed twice with distilled water by centrifugation (8,000 × *g*, 10 min, 4°C) rather than by dialysis. Birchwood xylan (lot 69F0410), larchwood arabinogalactan (lot 63F-7704), carboxymethyl cellulose CMC (low viscosity, sodium salt), lichenan, laminarin, barley β-glucan, and all *p*-nitrophenyl- and 4-methylumbelliferyl substrates were from Sigma. Larchwood xylan (lot 4428kk) was from Aldrich. Xylobiose (lot 16591) was obtained from Pfanstiehl Laboratories, Waukegan, Ill. Purified xylooligosaccharides (xylotriose through xylohexaose) and a mixture of soluble xylooligomers from birchwood were the generous gifts of Mark Bray, Department of Microbiology, University of Guelph. Sugar beet arabinan (neutral fraction [30]) was kindly provided by Song F. Lee. Rye flour arabinoxylan was prepared as described by Antoniou et al. (2). RBB-dyed larchwood

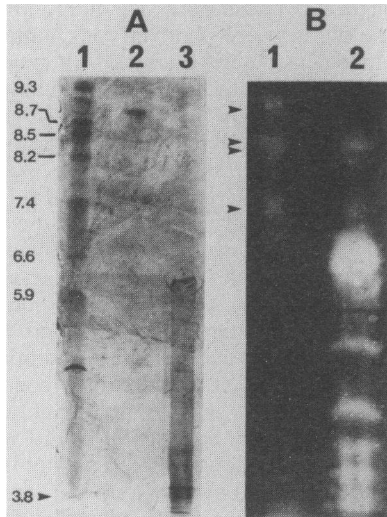

FIG. 1. Analytical isoelectric focusing and zymogram analysis of endoxylanases from the nonsedimentable extracellular culture fluid and purified endoxylanase 1 from hydroxylapatite chromatography. (A) pH 3 to 10 Coomassie brilliant blue R-250-stained IEF gel. Lane 1, broad-range pI markers (Pharmacia); lane 2, 2.1 μg of purified endoxylanase 1 from hydroxylapatite; lane 3, 50 μg of nonsedimentable extracellular culture proteins. (B) RBB-larchwood xylan zymogram analysis. Lane 1, 2.1 μg (0.14 U) of purified endoxylanase 1: lane 2, 50 μg (0.26 U) of nonsedimentable extracellular culture fluid proteins.

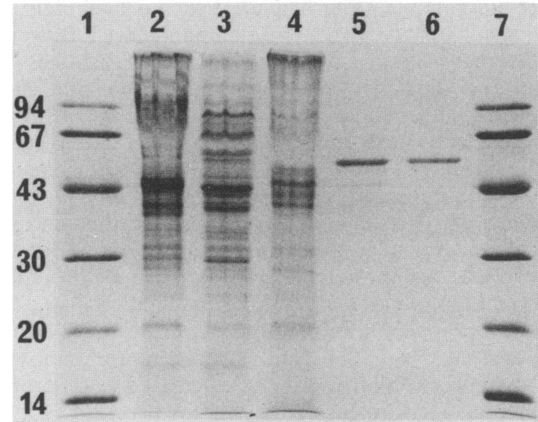

FIG. 2. SDS-PAGE analysis of fractions from the purification of endoxylanase 1. Lanes 1 and 7, low-molecular-mass standards (Pharmacia; in kilodaltons); lane 2, 10 μg of protein from the concentrated extracellular culture fluid; lane 3, 10 μg of protein from the nonsedimentable culture fluid; lane 4, 10 μg of protein from void volume of chromatofocusing; lane 5, 1.8 μg of protein from gradient elution of endoxylanase from CM-Sepharose; lane 6, 0.79 μg of protein from hydroxylapatite.

xylan (dye content, approximately 16% [wt/wt]) was prepared as previously described (8).

RESULTS

Multiplicity of endoxylanases from *F. succinogenes*. Previously, Forsberg et al. (16) showed that endoxylanase activity from *F. succinogenes* is primarily extracellular and that the enzyme is distributed approximately equally between sedimentable and nonsedimentable fractions when extracellular culture fluid is centrifuged at 100,000 × *g* for 2 h. Endoxylanase, β-D-xylosidase, and α-L-arabinofuranosidase enzymes were found in this study to be primarily extracellular and nonsedimentable when *F. succinogenes* was grown on crystalline cellulose (results not shown). Analysis of the nonsedimentable extracellular culture fluid by analytical IEF-RBB xylan zymogram analysis indicated the presence of both basic and acidic forms of endoxylanases having isoelectric points of 8.9, 8.4, 8.3, 7.3, 5.2, 4.9, 3.8, and 3.4 (Fig. 1). An additional activity band corresponding to a pI of 5.7, visible in some gels, was not detectable in Fig. 1 because of the position of sample application. These experiments showed the major endoxylanase activity bands visible in the acidic region of the gel. Coomassie staining of broad-range (pH 3 to 10) IEF gels indicated that most of the proteins in the nonsedimentable extracellular culture fluid were acidic in nature (Fig. 1). An initial attempt at fractionating endoxylanase, β-D-xylosidase, and α-L-arabinofuranosidase through successive anion-exchange, hydrophobic interaction, hydroxylapatite, and chromatofocusing chromatographic treatments proved unsuccessful, with essentially no purification of the enzymes. Since purification of acidic endoxylanases proved to be extremely difficult, an effort was made to purify endoxylanases with a basic charge.

Purification of endoxylanases 1 and 2. Endoxylanase 1 was purified to apparent electrophoretic homogeneity by consecutive chromatography on chromatofocusing pH 7 to 4, CM-Sepharose, and hydroxylapatite columns. The elution profiles on chromatofocusing of endoxylanase, β-D-xylosidase, and α-L-arabinofuranosidase enzymes were found to be similar to those from Q-Sepharose chromatography, with the major activity peaks for each enzyme often overlapping (results not shown). Of the applied enzyme activity, 29, 26, and 27% of endoxylanase, α-L-arabinofuranosidase, and β-D-xylosidase, respectively, eluted in the void volume from chromatofocusing chromatography. The bulk of the enzymes which bound to the column could only be eluted upon application of the NaCl gradient. Cation-exchange chromatography of the void volume from chromatofocusing chromatography on a CM-Sepharose column equilibrated at pH 6.5 resulted in 6.8% of the applied endoxylanase activity eluting in the sodium chloride gradient, while 56% of the activity eluted in the column void volume. This step resulted in a 23-fold purification of the enzyme. To remove contaminating proteins, the fraction eluting in the gradient was further purified by hydroxylapatite chromatography. The enzyme eluted as a single symmetric peak coincident with the protein profile and appeared homogeneous by SDS-PAGE analysis as a single band of 53.7 kDa (Fig. 2, lane 6). A summary of the purification of endoxylanase 1 is presented in Table 1.

Endoxylanase 2 was purified by sequential anion- and cation-exchange chromatography on Q-Sepharose CL-6B and CM-Sepharose CL-6B, respectively. In an effort to enhance binding of endoxylanases to the CM-Sepharose CL-6B column, chromatography was performed at pH 5.5 rather than pH 6.5. Endoxylanase activity eluting in the void volume from a Q-Sepharose column was chromatographed on CM-Sepharose at pH 5.5, resulting in two endoxylanase activity peaks eluting with the application of 1.0 M potassium chloride. Analysis of the pooled fractions by SDS-PAGE indicated the presence of a protein with a molecular weight corresponding to that of endoxylanase 1 eluting at the

TABLE 1. Summary of the purification of endoxylanases 1 and 2 from *F. succinogenes* S85

Enzyme and purification method	Total protein (mg)	Total U	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
Endoxylanase 1					
Total concentrated ECF[a]	1,340	4,530	3.4	100	1.0
NSECF[b]	462	1,450	3.1	32	0.9
Chromatofocusing	124	387	3.1	8.5	0.9
CM-Sepharose	0.4	32.1	80.3	0.7	24
Hydroxylapatite	0.03	2.0	66.7	0.04	20
Endoxylanase 2					
Total concentrated ECF[a]	2,400	8,450	3.5	100	1.0
NSECF[b]	1,530	6,690	4.4	79	1.3
Q-Sepharose	275	1,250	4.6	15	1.3
Q-Sepharose, nonsedimentable[c]	43.3	379	8.8	4.5	2.5
CM-Sepharose	0.2	33.6	168	0.4	48

[a] Extracellular culture fluid.
[b] Nonsedimentable extracellular culture fluid.
[c] 100,000 × *g* supernatant from the thawed void volume fractions after Q-Sepharose column chromatography (see Materials and Methods).

lower ionic strength, while that eluting in the 1.0 M potassium chloride was essentially a single protein with a molecular mass of 66 kDa which stained poorly with Coomassie (Fig. 3, lane 5). This second protein, designated endoxylanase 2, was desalted, concentrated, and stored at −70°C. A summary of the purification of endoxylanase 2 is presented in Table 1.

Characterization of endoxylanases 1 and 2. Endoxylanase 1 and endoxylanase 2 showed single Coomassie-stained protein bands by SDS-PAGE corresponding to molecular masses of 53.7 and 66 kDa, respectively. As the native molecular mass for neither protein was determined, it is not possible to conclusively state that the proteins are monomeric, although such a result would be consistent with the known characteristics of endoxylanases (51).

With the purified endoxylanase 1 isolated from hydroxylapatite chromatography, only a single Coomassie-stained protein band with an isoelectric point of 8.9 was detectable by analytical IEF through the pH range of 3 to 10, although

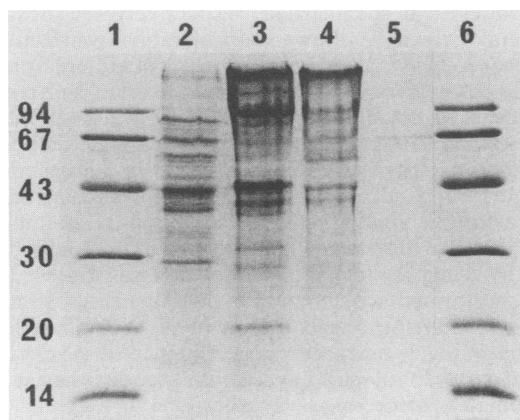

FIG. 3. SDS-PAGE analysis of fractions from the purification of endoxylanase 2. Lanes 1 and 6, low-molecular-mass standards (Pharmacia; in kilodaltons); lane 2, 15 μg of nonsedimentable extracellular culture fluid proteins; lane 3, 20 μg of protein from Q-Sepharose void volume; lane 4, 20 μg of protein from 100,000 × *g* supernatant of Q-Sepharose void volume; lane 5, 1.4 μg of protein from peak 2 of CM-Sepharose column.

a multiplicity of activity bands were found in the zymogram, corresponding to isoelectric points of 8.9, 8.3, 8.1, and 7.3 (Fig. 1). A similar analysis of endoxylanase 2 through the same pH range revealed a major protein band with an isoelectric point of 8.0, and a less intense doublet with a pI of about 7.7, and a third, weakly staining band with a pI of 7.4. Endoxylanase zymogram analysis of this preparation showed three distinct activity bands which aligned with the three protein bands (results not shown).

For endoxylanase 1, the K_m and V_{max} for hydrolysis of water-soluble oat spelts xylan at pH 7.0 were 2.6 mg ml^{-1} and 33.6 U of xylanase activity per mg of protein, respectively, as calculated from an Eadie-Hofstee plot (22). Analysis of initial reaction rates at various concentrations of water-soluble oat spelts xylan for endoxylanase 2 at pH 6.3 revealed an unexpected decrease in the rates at substrate concentrations greater than or equal to 2.5 mg ml^{-1}. Plotting the data by the method of Lineweaver and Burk (33) yielded a curve which suggested that classical substrate inhibition kinetics were occurring at the higher substrate concentrations. By extrapolating the linear region of the Lineweaver-Burk plot, an apparent K_m of 1.3 mg ml^{-1} and V_{max} of 118 U of xylanase activity per mg of protein were determined for endoxylanase 2. Values for K_{cat}, the turnover number, were 30 s^{-1} for endoxylanase 1 and 129 s^{-1} for endoxylanase 2, respectively. Endoxylanase 2, acting on low-viscosity CMC as substrate at pH 6.3, showed a K_m of 30.6 mg ml^{-1} and V_{max} of 66.1 U of activity per mg of protein as estimated from a Lineweaver-Burk plot. Because of the high K_m the endoglucanase activity of endoxylanase 2 could not be saturated with substrate, and thus it is not known if substrate inhibition kinetics also occur with this substrate. For endoxylanase 2 hydrolysis of CMC, the value of K_{cat} was 72.7 s^{-1}.

Endoxylanases 1 and 2 showed similar pH optima of 7.0 and 6.3, respectively, although their overall pH-activity profiles appeared quite different (Fig. 4). Through the pH interval of 5.5 to 8.5, endoxylanase 1 showed a bell-shaped profile, while endoxylanase 2 appeared to have a more broad pH-activity relationship. At pH 5.5, endoxylanase 1 showed 23% of the maximum activity at pH 7, while endoxylanase 2 showed about 79% of its maximum activity at the same pH. Adjusting the ionic strength of the buffers to 43 mM with potassium chloride was found to be necessary to obtain similar relative activities at overlapping pH values for en-

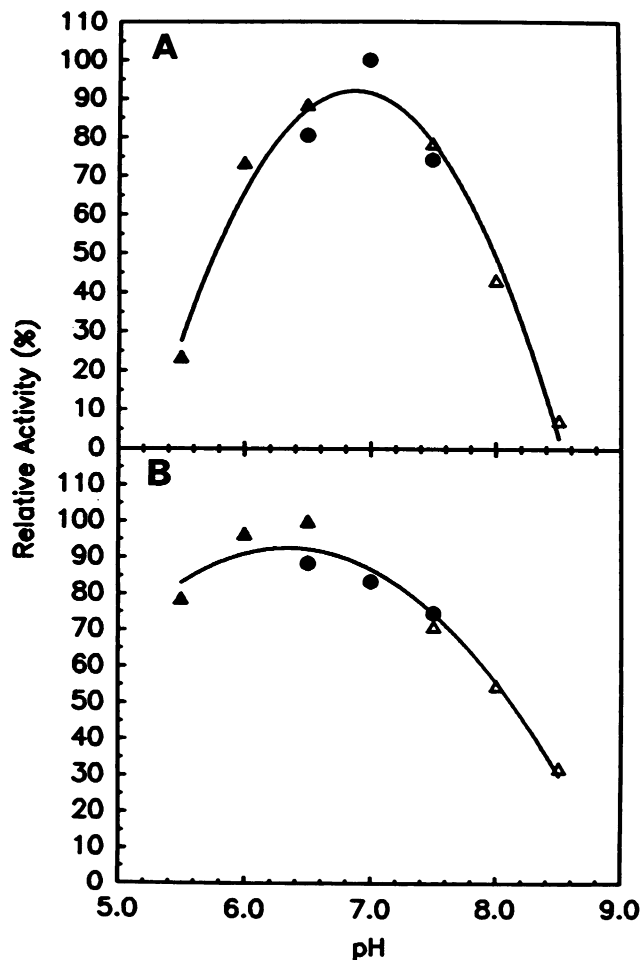

FIG. 4. Effects of pH on the activity of endoxylanase 1 (A) and endoxylanase 2 (B). The buffers used included 50 mM MES-KOH at pH 5.5, 6.0, and 6.5 (▲); 50 mM MOPS-KOH at pH 6.5, 7.0, and 7.5 (●); and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid)-KOH at pH 7.5, 8.0, and 8.5 (△). The quantity of enzyme used for each assay was 2.76 mU for endoxylanase 1 and 7.49 mU for endoxylanase 2, as determined at 39°C in 50 mM potassium phosphate buffer, pH 6.5, with 1% (wt/vol) water-soluble oat spelts xylan as substrate. All buffers were adjusted to a final ionic strength of 43 mM, with solid KCl in the case of endoxylanase 2 only.

doxylanase 2, although this was not a problem with endoxylanase 1. Endoxylanase 1 showed essentially identical pH-activity profiles in the presence or absence of 1 mM $CaCl_2$.

Temperature-activity profiles were determined for endoxylanases 1 and 2 through the temperature intervals of 25 to 60°C and 15 to 70°C, respectively. Endoxylanase 1 exhibited a temperature optimum of 39°C, while endoxylanase 2 had a temperature optimum of 55°C. At 39°C, the physiological temperature of the rumen, the relative activity of endoxylanase 2 was only 41% of that of endoxylanase 1. The activation energy, E_A, calculated for endoxylanase 2 gave a value of 51.1 kJ mol^{-1} with water-soluble oat spelts xylan as substrate. It was also found that endoxylanase 2 was more thermostable than endoxylanase 1, on the basis of the observation that endoxylanase 2 retained residual activity on xylooligosaccharides following heat treatment of the enzyme at 100°C for 30 min, while endoxylanase 1 was totally inactivated under the same conditions. Heating endoxylanase 2 at 121°C for 60 min totally inactivated the enzyme.

TABLE 2. Effects of metal ions and chelating agents on the enzymatic activity of endoxylanases 1 and 2

Ion or chelator[a] (mM)	% Relative activity[b]	
	Endoxylanase 1	Endoxylanase 2
None	100	100
$CaCl_2$ (1.0)	114	103
$CaCl_2$ (10.0)	90	114
$MgCl_2$ (1.0)	106	147
$MgCl_2$ (10.0)	94	143
$CuCl_2$ (1.0)	5.6	39
$HgCl_2$ (1.0)	0	0
EDTA (1.0)	39	156
EGTA (1.0)	41	142

[a] EDTA and EGTA were adjusted to pH 7.0 with NaOH.
[b] Enzymes were assayed in 50 mM MOPS-KOH buffer, pH 7.0 (endoxylanase 1), or 50 mM MES-KOH buffer, pH 6.3 (endoxylanase 2), at 39°C with additives at the indicated concentrations. The metal ions at the concentrations used showed no interfering effect on the endoxylanase assay.

The effects of several metals and chelating agents on the activity of endoxylanases 1 and 2 are presented in Table 2. Endoxylanase 2 showed enhanced activity in the presence of the divalent metal cation Mg^{2+}, with 1 mM $MgCl_2$ being most stimulatory. In each case, the enzymes were partially inactivated by Cu^{2+} and totally inactivated by Hg^{2+}. While endoxylanase 1 was inhibited by 1 mM EDTA or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), endoxylanase 2 was stimulated by both chelating agents at the same concentration. Addition of excess $MgCl_2$ to endoxylanase 1 following treatment with 1 mM EDTA resulted in only partial reactivation (65%) of the enzyme, indicating that reactivation was only partially reversible. Addition of excess $MgCl_2$ following treatment of endoxylanase 2 with 1 mM EDTA did not result in any significant difference in activity compared with that in the controls.

A summary of the substrate specificities of endoxylanases 1 and 2 is presented in Table 3. Endoxylanases 1 and 2 showed maximum activity with rye flour arabinoxylan and water-soluble oat spelts xylan, respectively, as substrates. While endoxylanase 1 showed considerably lower activity in the presence of the same concentrations of either larchwood or birchwood xylans, the activity of endoxylanase 2 on these substrates was comparable to that on water-soluble oat spelts xylan. Endoxylanase 2 showed higher activity on CMC (24% of activity on water-soluble xylan) than endoxylanase 1 (10%). Endoxylanases 1 and 2 showed generally low activity on other polysaccharide substrates. Essentially no acetyl esterase or exoglycosidase activities could be detected by using a variety of model aryl substrates. In the assays performed with 4-methylumbelliferyl substrates, it was found that the concentration of *N*,*N*,-dimethylformamide used (32.3 mM) did not influence the activity of endoxylanase 1 but partially inhibited endoxylanase 2 by approximately 45%.

Hydrolysis of polysaccharides and xylooligosaccharides by endoxylanases 1 and 2. Analysis of reducing sugars released by endoxylanases 1 and 2 acting on rye flour arabinoxylan showed that endoxylanase 1 hydrolyzed the substrate to a greater extent (15.6%) after 20 h than did endoxylanase 2 (6.5%), with identical quantities of both enzymes (Fig. 5). In contrast, endoxylanase 2 showed the ability to substantially

TABLE 3. Substrate specificity of endoxylanases 1 and 2[a]

Substrate (%[b])	Endoxylanase 1		Endoxylanase 2	
	Sp act[c]	%[d]	Sp act[c]	%[d]
H_2O-soluble xylan[e] (1.0)	26.8	100	91.7	100
Arabinoxylan[f] (1.0)	28.2	105	54.1	59.0
Oat spelts xylan (1.0)	19.8	73.8	75.4	82.2
Larchwood xylan (1.0)	4.9	18.1	76.3	82.9
Birchwood xylan (1.0)	4.4	16.4	71.5	78.0
H_2O-insoluble xylan[g] (1.0)	2.7	10.1	22.2	24.3
CMC[h] (1.0)	2.7	10.0	22.1	24.1
Lichenan (1.0)	0.0	0.0	2.0	2.2
Barley β-glucan (1.0)	0.8	3.0	0.0	0.0
Mannan (1.0)	0.3	1.0	0.0	0.0
Laminarin (1.0)	0.5	1.9	0.4	0.4
Arabinan (0.5)	0.6	2.2	0.5	0.6

[a] The following substrates were hydrolyzed at less than 1% of the rate of water soluble xylan: p-nitrophenyl-β-D-xyloside, p-nitrophenyl-α-L-arabinofuranoside, and p-nitrophenyl-β-D-cellobioside at 5 mM and α-naphthylacetate, 4-methylumbelliferyl-β-D-xyloside, 4-methylumbelliferyl-α-L-arabinofuranoside, and 4-methylumbelliferyl-α-L-arabinoside, each at 1 mM.
[b] Final concentration in assay in percent (wt/vol).
[c] Units of U mg^{-1} protein.
[d] Percent activity relative to water-soluble fraction of oat spelts xylan.
[e] Water-soluble fraction of oat spelts xylan.
[f] Rye flour arabinoxylan.
[g] Water-insoluble fraction of oat spelts xylan.
[h] Low-viscosity CMC.

hydrolyze water-soluble oat spelts xylan (15.9%) compared with that of rye flour arabinoxylan (6.5%) under the same conditions (data not shown). It is noted that the profiles had not reached their maximum values following 20 h of incubation, and so the true hydrolytic potentials of the enzymes may be underestimated. During hydrolysis of rye flour arabinoxylan by endoxylanase 1, a noticeable decrease in the viscosity of the solution was evident, as was the formation of a white precipitate which appeared to accumulate over time. Endoxylanase 1 did not release any detectable reducing sugars from larchwood arabinogalactan after 20 h of incubation. Endoxylanase 2 showed only a very limited ability (<1% hydrolysis) to release reducing sugars from phosphoric acid-swollen cellulose following 24 h of incubation. In an experiment to examine the ability of endoxylanase 2 to bind to amorphous cellulose, only 15.6% of the endoglucanase activity compared with that of the control without cellulose was no longer associated with the supernatant fraction following 15 min incubation at 39°C. The control containing a 200-fold (by weight) excess of BSA showed 230% greater activity on low viscosity CMC than the control without BSA, perhaps suggesting that BSA may have stabilized the enzyme.

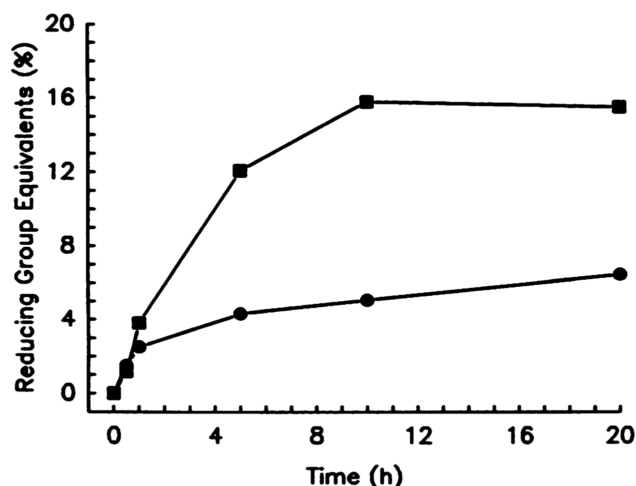

FIG. 5. Release of reducing sugars during hydrolysis of rye flour arabinoxylan by endoxylanases 1 (■) and 2 (●). Enzymes (0.022 U) were incubated with 1% (wt/vol) rye flour arabinoxylan and 0.005% (wt/vol) sodium azide at 39°C under optimal conditions.

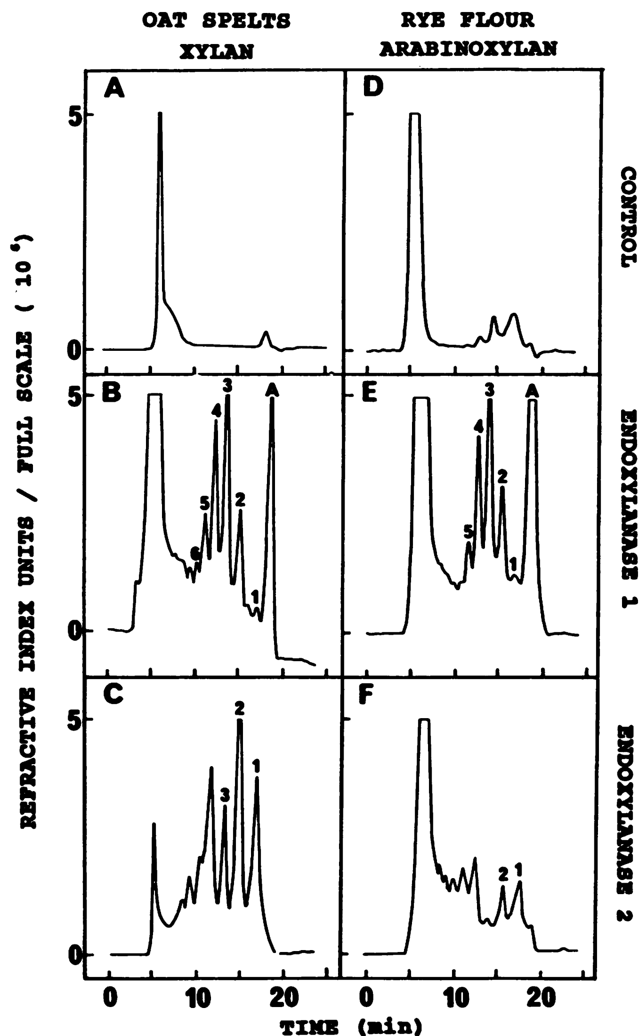

FIG. 6. HPLC analysis of xylan hydrolysis products following incubation for 20 h with endoxylanases 1 or 2. The reaction mixture composition was exactly as described in the legend to Fig. 5. (A to C) Hydrolysis of water-soluble oat spelts xylan: time = 0 control (A), endoxylanase 1 (B), endoxylanase 2 (C). (D to F) Hydrolysis of rye flour arabinoxylan: time = 0 control (D), endoxylanase 1 (E), endoxylanase 2 (F). Symbols: 1, xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose; 5, xylopentaose; 6, xylohexaose; A, arabinose.

Products from the hydrolysis of water-soluble xylan and rye flour arabinoxylan by both endoxylanases were analyzed by HPLC (Fig. 6). Endoxylanase 1 was able to release arabinose from both water-soluble oat spelts xylan and rye flour arabinoxylan but not from arabinogalactan, on the basis of the presence of a peak in the chromatogram eluting with a

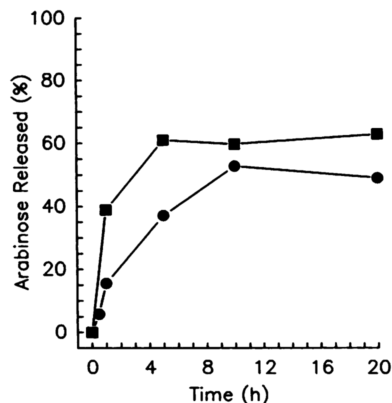

FIG. 7. Time course of arabinose release from water-soluble oat spelts xylan (■) and rye flour arabinoxylan (●) by endoxylanase 1. Percentage of arabinose released expressed as the fraction of available arabinose in each xylan determined from the monosaccharide composition.

retention time of approximately 19.5 min, identical to that of an L-arabinose standard. Subsequent collection of the putative L-arabinose peak, lyophilization, suspension in water, and chromatography on a monosaccharide analysis column (Bio-Rad HPX-87P) revealed that the sugar eluted with the same retention time as an L-arabinose standard (approximately 23.7 min). Arabinose release from both xylans as a function of time, as quantified by HPLC using L-arabinose as an external standard, showed that endoxylanase 1 released a maximum of 62.5% of the arabinose present in the water-soluble xylan and approximately 50% of that available in the rye flour arabinoxylan following 20 h of incubation (Fig. 7). Percentage values were calculated on the basis of monosaccharide compositions determined for both xylans by trifluoroacetic acid hydrolysis and HPLC analysis. Incubation of each of the four electrophoretically distinct endoxylanase 1 variants separated by analytical IEF with water-soluble oat spelts xylan showed the presence of both arabinose and xylooligosaccharides by HPLC analysis, indicating that each form of the enzyme was capable of catalyzing both activities. Endoxylanase 2 did not release arabinose from either water-soluble oat spelts xylan or rye flour arabinoxylan. The xylooligosaccharide products released by the action of endoxylanase 1 on both xylans were similar, xylotriose and xylotetraose being most predominant, with lesser amounts of xylobiose and xylopentaose, and only a trace of xylohexaose and xylose (Fig. 6). Unlike endoxylanase 1, endoxylanase 2 released significant xylose from water-soluble oat spelts xylan, the other products following 20 h of hydrolysis being xylobiose, xylotriose, and xylotetraose. Analysis of the products from rye flour arabinoxylan hydrolysis with endoxylanase 2 showed small amounts of xylose and xylobiose (Fig. 6). Other oligosaccharides present in the chromatogram had retention times somewhat different from xylooligosaccharides in the standard and are most probably various arabinoxylooligosaccharides. Xylooligosaccharides present in the acid hydrolysate of birchwood xylan had the following retention times: xylobiose, 16.1 min; xylotriose, 14.4 min; xylotetraose, 13.1 min; xylopentaose, 12.0 min; and xylohexaose, 11.1 min. Retention times for purified xylooligosaccharides (xylobiose through xylohexaose) chromatographed individually under the same conditions gave values virtually identical for those present in the xylooligomer mixture. Xylose had a retention time of 18.1 min. No cellooligosaccharides could be detected following incubation of an equivalent amount of endoxylanase 2 (endoglucanase units) with acid-swollen cellulose as used to hydrolyze water-soluble oat spelts xylan or rye flour arabinoxylan.

By using purified xylooligosaccharides (xylobiose through xylohexaose), the modes of action of endoxylanases 1 and 2 were examined. Endoxylanase 1 showed no activity on xylobiose or xylotriose, slowly hydrolyzed xylotetraose to xylobiose with a trace of xylotriose and xylose, xylopentaose to mostly xylotriose and xylobiose with a trace of xylotetraose and xylose, and xylohexaose to mostly xylotriose, with less xylotetraose and xylobiose. Unlike endoxylanase 1, endoxylanase 2 hydrolyzed xylotriose and all larger xylooligosaccharides to xylobiose and xylose. Xylobiose was not hydrolyzed further. Neither endoxylanase 1 or endoxylanase 2 exhibited transglycosylase activity at the substrate concentrations used.

Mode of action of endoxylanase 1 as revealed by NaB^3H_4 reduction of arabinoxylan hydrolysis products. Analysis of water-soluble xylan and rye flour arabinoxylan hydrolysis products over time by HPLC suggested that arabinose was released prior to hydrolysis of the xylan backbone, as substantial arabinose could be observed prior to the detection of xylooligosaccharides. Labelling of mono- and xylooligosaccharide products resulting from the action of endoxylanase 1 on rye flour arabinoxylan with NaB^3H_4 was performed to better define the arabinose-debranching and xylan-hydrolyzing activities of this enzyme. In Fig. 8A, the kinetics of ^3H incorporation into arabinose as [^3H]arabinitol and into xylooligosaccharides as their corresponding [^3H]alditols may be seen. No incorporation into xylooligosaccharides was observed until after 1 h of hydrolysis, while by the same time 70% of the total incorporation into the arabinose fraction had taken place (Fig. 8B). Following 20 h of hydrolysis, only 38% of the ^3H incorporated into arabinose had been incorporated into xylooligosaccharides. It should be noted that the incorporation of ^3H into xylooligosaccharides proceeded rapidly only after the initial release of substantial arabinose (Fig. 8B). Quantifying arabinose release as [^3H]arabinitol by using unlabelled L-arabinitol as an external standard showed similar results to those earlier, with 60% of the total arabinose present in the sample being released during the first 20 h of hydrolysis. The ratio of percentage of maximum ^3H incorporated into arabinitol to the percentage of total arabinitol as quantified by HPLC for each time point was nearly equal to unity in each case, indicating that the incorporation data accurately reflected the arabinose release by endoxylanase 1. Under the conditions employed, D-[^3H]xylitol, L-[^3H]arabinitol, and D-[^3H] xylobiitol had retention times of approximately 19.8, 19.2, and 17.1 min, respectively, compared with 19.5, 17.9, and 15.9 min for D-xylose, L-arabinose, and D-xylobiose, respectively. Examining the tritiated xylose, arabinose, and xylobiose standards as well as the time course samples by HPLC indicated that in each case the sugars had been totally reduced to their corresponding alditols.

DISCUSSION

Two endoxylanases, designated 1 and 2, were purified from the nonsedimentable extracellular culture fluid of *F. succinogenes* grown on crystalline cellulose. Endoxylanase 1 was a basic protein of 53.7 kDa, showing a single Coomassie-stained protein band with an isoelectric point of 8.9, but four activity bands by IEF-RBB xylan zymogram

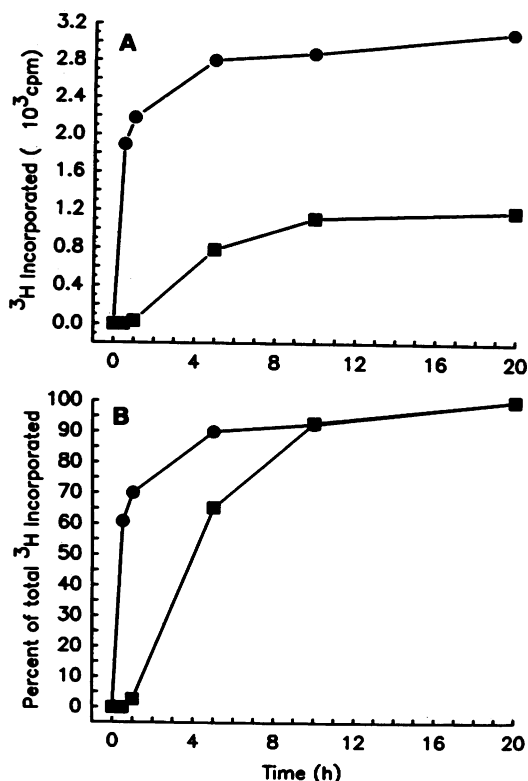

FIG. 8. Incorporation of ^{3}H into arabinitol and xylooligosaccharide alditols from the sodium borohydride reduction of rye flour arabinoxylan hydrolysis products resulting from the action of endoxylanase 1. (A) Time course of ^{3}H incorporation into arabinitol (●) and xylooligosaccharide alditols (■). (B) Percent maximum ^{3}H incorporated into arabinitol (●) and xylooligosaccharide alditols (■).

analysis. The enzyme had a K_m of 2.6 mg ml^{-1} and V_{max} of 33.6 μmol min^{-1} mg^{-1} at pH 7.0 with water-soluble oat spelts xylan as substrate. An interesting property of endoxylanase 1 was its ability to cleave arabinose substituents from water-soluble oat spelts xylan (arabinoglucuronoxylan) and rye flour arabinoxylan, prior to hydrolysis of the xylan backbone to xylooligosaccharides. Under the classification of endoxylanase enzymes proposed by Dekker (14, 15), endoxylanase 1 is a debranching endoxylanase.

In contrast to endoxylanase 1, endoxylanase 2, a protein of 66 kDa, did not hydrolyze arabinose substituents from heteroxylans, and thus belongs to the nondebranching class of endoxylanases (14, 15, 42). This enzyme exhibited substantial (ca. 24%) activity on low-viscosity CMC compared with that of water-soluble oat spelts xylan. The apparent K_m and V_{max} of endoxylanase 2 for water-soluble oat spelts xylan were 1.3 mg ml^{-1} and 118 μmol min^{-1} mg of protein^{-1}, respectively. Despite the endoglucanase activity of endoxylanase 2 on CMC, it had limited hydrolytic activity on acid-swollen cellulose. Like endoxylanase 1, endoxylanase 2 showed multiple activity bands by IEF-RBB xylan zymogram analysis.

The yield of purified endoxylanases 1 and 2 was low and in the same range as the purified acetyl esterase from *F. succinogenes* (36). This can be attributed to several reasons. First, both column chromatography and IEF-RBB xylan zymogram analysis of the nonsedimentable extracellular culture fluid indicated that *F. succinogenes* produces a multiplicity of endoxylanases, both acidic and basic in nature. Second, aggregation of basic and acidic proteins with acidic cellular components could have resulted in some of the basic proteins adhering to the Q-Sepharose and chromatofocusing columns, despite having the same charge as the ionizable groups of the matrix. Third, it is not clear if or how much of endoxylanases 1 and 2 are associated with the sedimentable protein fraction; if they were enriched within this fraction, only a small proportion would then be available for purification from the soluble protein.

Analysis of endoxylanases 1 and 2 by SDS-PAGE revealed only a single Coomassie-stained protein band and by this criterion appeared to be pure, although IEF-RBB xylan zymogram analysis of both endoxylanases 1 and 2 showed multiple activity bands. In the case of endoxylanase 1 each electrophoretically distinct form of the enzyme which released xylooligosaccharides also released arabinose substituents as well. With endoxylanase 2, all of the protein bands appeared to correspond directly with the three activity bands in the xylan zymogram. Microheterogeneity has been observed in both purified endoxylanase preparations (9, 27), as well as cloned endoxylanase-encoding gene products (34) and may be attributed to a variety of posttranslational modification events.

Endoxylanase 1 was found to cleave arabinose from arabinoglucurono- and arabinoxylans, and thus may contribute to the overall α-L-arabinofuranosidase activity of this bacterium. It is noteworthy that the enzyme showed 65 and 62% lower specific activity on larchwood and birchwood xylans, respectively, than did endoxylanase 2. It must be emphasized that because of the kinetics of arabinose cleavage by endoxylanase 1, most of the reducing sugar released from water-soluble oat spelts xylan in the short-term (30-min) assay is in fact arabinose and thus is a measure of the α-L-arabinofuranosidase activity of the enzyme rather than the endoxylanase activity. The low degree of branching in larchwood (4) and birchwood (43) xylans and the corresponding low activity of endoxylanase 1 on these substrates compared with that of oat spelts xylan and rye flour arabinoxylan would seem to suggest an intrinsically lower specificity of this enzyme for cleaving the β-(1,4) xylan linkage than the α-(1,2) or α-(1,3) arabinofuranose linkages compared with endoxylanase 2. Alternatively, the endoxylanase activity of endoxylanase 1 may be specific for more highly substituted xylans and cleave at or near branch points, as has been shown with two endoxylanases from *Aspergillus niger* (17).

Arabinose release by endoxylanase I was accompanied by a decrease in the viscosity of the reaction mixture and formation of a white precipitate. The arabinose side chains of endospermic arabinoxylans contribute to its high-viscosity, gellike structure (1, 12). At higher ratios of arabinose to xylose, the structure of the arabinoxylan molecule is rodlike, but as the ratio decreases (i.e., arabinose substituents are removed), the xylan becomes more flexible, and finally the individual molecules aggregate and precipitate from solution once extensive removal of arabinose has been achieved (1). Incubation of arabinoxylan or α-(1,5)-L-arabinan with a purified α-L-arabinofuranosidase from *A. niger* resulted in the release of approximately 90% of the α-(1,3)-linked arabinofuranosyl residues after 40 h and the formation of a white precipitate which upon analysis was mostly D-xylose with only a trace of L-arabinose (48). Thus, precipitation of the partially dearabinosylated xylan during action of endoxylanase 1 may have resulted in a decrease in accessibility of the

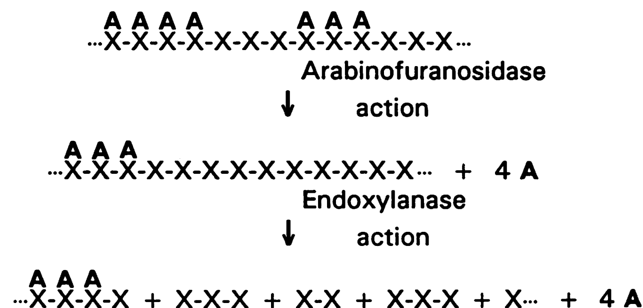

FIG. 9. Model illustrating the mode of action of endoxylanase 1. Xylosyl (X) residues β-(1,4) linked to form the xylan chain are substituted on O-2 and O-3 positions with α-linked L-arabinofuranosyl (A) residues. The arabinofuranosyl residues are initially cleaved by arabinofuranosidase action, and this is followed by hydrolysis of the xylan backbone by endoxylanase action. Whether backbone cleavage occurs at unsubstituted locations on the xylan chain is unknown. The dot triplex indicates an extended polymer structure.

enzyme for substrate, resulting in lower endoxylanase compared to α-L-arabinofuranosidase activity. The arabinose substituents of rye flour arabinoxylan are attached only to the O-3 positions of the xylose residues (6) and are randomly distributed along the xylan chain (5). Further studies will be required to determine whether endoxylanase 1 hydrolyzes arabinoxylans in a manner dependent upon the arabinose substitution pattern or the specific linkage at which the arabinose moiety is attached to the xylan backbone, as has been shown with acetyl group hydrolysis by an acetyl xylan esterase purified from *Trichoderma reesei* (41).

The arabinose-cleaving endoxylanase of *F. succinogenes* appears to be similar to a xylanase purified from *Streptomyces roseiscleroticus* (19) and xylanase 1 from *Trichoderma koningii* (53) in that all three liberate arabinose directly from the xylan backbone and neither the *T. koningii* nor the *F. succinogenes* enzymes were shown to exhibit activity with *p*-nitrophenyl-α-L-arabinofuranoside as a substrate while the *Streptomyces* enzyme was not tested. Wood and McCrae (53) rationalized that since only small amounts of arabinoxylotriose and arabinoxylotetraose accumulated during xylan hydrolysis by the *T. koningii* enzyme, arabinose groups were cleaved from the xylan backbone prior to hydrolysis of the xylan to xylooligosaccharides. Analysis of rye flour arabinoxylan hydrolysis products resulting from the action of *F. succinogenes* endoxylanase 1 by labelling with tritiated sodium borohydride indicated that arabinose release preceded hydrolysis of the xylan backbone, as illustrated in Fig. 9. It would appear that xylan hydrolysis only proceeds following removal of sufficient arabinose residues to permit the enzyme access to the xylan backbone. This in a sense demonstrates directly the synergistic relationship between arabinose release and xylan hydrolysis previously observed by mixing purified α-L-arabinofuranosidase and endoxylanase enzymes of *Clostridium acetobutylicum* (30) and *Ruminococcus albus* 8 (20) in that, for some endoxylanases, the arabinose substituents present a steric barrier to the enzyme. It is interesting to note, however, that synergistic action has not been found to occur between purified arabinose-debranching and nondebranching endoxylanases of *T. koningii* (53). As endoxylanase 2 from *F. succinogenes* showed a limited ability to hydrolyze rye flour arabinoxylan to short-chain oligosaccharides compared with that of the less-substituted water-soluble oat spelts xylan, it would be of interest to see if the purified enzymes from *F. succinogenes* could act synergistically on the arabinoxylan substrate. The fact that the xylan tends to precipitate as it is debranched complicates the issue, however, since endoxylanase 2 showed low activity on insoluble compared with water-soluble oat spelts xylan.

While endoxylanase 2 showed significant activity on low-viscosity CMC, very little reducing sugar and no cellooligosaccharides could be detected when the enzyme was incubated with acid-swollen cellulose. This inability to hydrolyze amorphous cellulose may at least in part be attributed to two reasons. First, the K_m for hydrolysis of low-viscosity CMC by endoxylanase 2 was 23.5-fold greater than the K_m for hydrolysis of water-soluble oat spelts xylan, indicating a much lower substrate affinity for amorphous cellulose compared with xylan. Secondly, endoxylanase 2 showed a very low affinity for binding to acid-swollen cellulose. A number of purified bacterial endoxylanases have been shown to possess endoglucanase (carboxymethyl cellulase) activity, including an enzyme from a thermophilic *Bacillus* sp. 11-1S (49), an endoxylanase from *Streptomyces exfoliatus* (46), and endoxylanase A of *C. acetobutylicum* (32). In addition, the enzyme from *C. acetobutylicum* is able to partially hydrolyze acid-swollen cellulose to cellooligosaccharides in an endo- manner. The cross-specificity of endoxylanases for endoglucanase substrates is not entirely surprising because of the very similar structures of D-xylopyranose and D-glucopyranose. Like endoxylanases A and B from *C. acetobutylicum*, endoxylanase 2 from *F. succinogenes* showed low activity on lichenan, indicating a broad specificity for β-(1,4), but not β-(1,3) linkages.

The mode of action of endoxylanases 1 and 2 on water-soluble oat spelts xylan and rye flour arabinoxylan suggested that both enzymes are indeed endo-acting, releasing longer chain xylooligosaccharides before further hydrolyzing these to the terminal products. The release of only a small quantity of xylose and xylobiose by endoxylanase 2 acting in rye flour arabinoxylan indicated that this enzyme requires xylose residues devoid of arabinose substituents to effect hydrolysis to short-chain oligosaccharides. An enzyme with a similar characteristic acting on corncob arabinoxylan has been purified from *Streptomyces* sp. E-86 (28). Although endoxylanase 2 released xylose from both xylan and xylooligosaccharides, the xylose appeared to be produced more slowly than the longer oligosaccharides during xylan hydrolysis. Some bacterial and fungal endoxylanases produce xylose as a terminal product of xylan hydrolysis while others do not (51). Furthermore, endoxylanase 2 did not hydrolyze xylobiose, nor did it show activity on aryl-β-D-xylopyranoside substrates and thus did not show xylobiase or β-D-xylosidase activities. The mode of action of the two enzymes on individual xylooligosaccharides in the range xylobiose through xylohexaose was distinctly different. These results indicate a difference in the organization of the catalytic and binding subsites between the two enzymes and offers additional evidence indicating their distinct natures.

Arabinoxylans are a common feature of endospermic and nonendospermic tissues of the members of the family Gramineae (50). Arabinose substituents present in forage xylans have been shown to inhibit the action of xylanases from cell-free rumen fluid (11), cell-free extracts of rumen bacteria (23), as well as during incubation in the presence of rumen microorganisms (18). The ability of *F. succinogenes* to synthesize an enzyme capable of both debranching as well as hydrolyzing the xylan backbone helps explain the high

hemicellulose-degrading activity observed for this bacterium.

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