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Characterization and *N*-Terminal Amino Acid Sequences of β-(1–4)Endoxylanases from *Streptomyces roseiscleroticus:* Purification Incorporating a Bioprocessing Agent

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***Streptomyces roseiscleroticus* produces extracellular xylanases when cultured on a liquid xylan medium. Purified xylanases are used to facilitate bleaching of kraft pulps in the pulp and paper industry. Downstream processing and purification of xylanases from *S. roseiscleroticus* is difficult unless red pigments produced by the bacterium are removed. We report that the bioprocessing agent, Biocryl BPA-1000, removes these pigments allowing purification of four xylanases by HPLC employing cation exchange, hydrophobic interaction, and gel filtration. The xylanases have been named Xyl1, Xyl2, Xyl3, and Xyl4 according to their order of elution from the cation exchange column. The purified xylanases have been characterized according to their molecular weights, pH and temperature stabilities, N-terminal amino acid sequences, and hydrolysis action patterns on oat spelt xylan. The molecular weights by mass spectroscopy for Xyl1–Xyl4 are 33,647, 33,655, 21,070, and 46,855, respectively. All four xylanases exhibit pH optima between 5.0 and 7.0 and temperature optima between 50 and 60°C. The N-terminal amino acid sequences are compared to sequences from *Streptomyces lividans, Streptomyces* 36A, and a *Chainia* sp. The N-terminal amino acid sequence of Xyl1 appears to be unique, but sequences from Xyl2, 3, and 4 bear strong homology to xylanases cloned from *S. lividans*. Xyl3 is also homologous to xylanases from *Streptomyces* 36A, and a *Chainia* sp. Predominant products of arabinoxylan hydrolysis by the purified xylanases included xylotriose, tetraose, and pentaose. None of the xylanases purified from *S. roseiscleroticus* produced xylose.**

Streptomyces species commonly produce various pigments. The nature of these pigments has been of interest due to their variety of color as well as their metabolic function and biochemistry. They have been described as part of the respiratory mechanism, and for protection from solar rays (1). Many of these pigments have antibiotic activity (2–5). One species produces a nontoxic pigment with potential as a food coloring agent (6). However, in *Streptomyces roseiscleroticus,* pigments produced during culture for xylanase production must be removed. The red pigments are a nuisance, interfering with purification steps such as ultrafiltration and HPLC (7).

In recent years there has been an increasing interest in applying xylanases to pulping processes. Particularly, they have been used to facilitate the bleaching of kraft pulps (8–11) or to improve fiber properties (12,13). Endoglucanase activity is unwanted in most cases, because it degrades cellulose fibers and destroys pulp properties. However, cellulases are often present in crude extracellular broths, so they must be removed by purification (14) or inhibited (12). *S. roseiscleroticus* cultured on liquid xylan medium produces little cellulase but does produce potent xylanases able to remove color bodies and enhance bleachability of kraft pulps. However, troublesome pigments and undesirable cellulases contaminate the culture fluid and must be removed. This purification provided an excellent opportunity to address the common bioprocessing procedures of clarifica-

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tion, concentration (volume reduction), and HPLC. The four purified endoxylanases could then be evaluated for their efficacy as enzymatic prebleachers of wood pulps (15).

Our work describes an efficient method for purifying proteins from *S. roseiscleroticus* pigmented fermentation broth. Extracellular xylanases are highly purified using a three-step recovery strategy. The first step is clarification of the fermentation stream using Biocryl BPA-1000; the second step is concentration of the clarified stream by ultrafiltration; the third step is purification of the xylanases by HPLC. We have applied all or part of this three-step recovery strategy to a variety of systems where clarification of the feed stream is essential in order to ultimately recover the purified products (16–18).

MATERIALS AND METHODS

Microorganism

S. roseiscleroticus NRRL B-11019 was obtained from David P. Labeda, USDA Northern Regional Research Laboratory (Peoria, IL).

Growth Media

Yeast extract malt extract xylose (YMX) agar consisted of (g/liter): yeast extract, 4.0; malt extract, 10.0; xylose, 4.0; agar, 15.0. pH was adjusted to 7.3 prior to autoclaving. Defined xylan (DX) agar consisted of (g/liter): oat spelts xylan (Sigma Chemical Co., St. Louis, MO), 10.0; K_2HPO_4, 1.0; NaCl, 1.0; $(NH_4)_2SO_4$, 2.0; $CaCO_3$, 2.0; agar, 15. Without adjustment, the pH of the medium was 7.0. Sterilization was achieved by autoclaving. Basic formulations for these media were suggested by D. P. Labeda; we substituted xylose for glucose and oat spelts xylan for starch in YMX and DX, respectively. Trypticase soy broth (TSB, Difco) was prepared as per manufacturer's instructions. Xylanase production (XP) medium was formulated according to the method of Morosoli *et al.* (19) except that a more complete trace element solution was employed (20,21).

Cultivation

Lyophilized cultures were rehydrated in sterile YMX medium without agar. For luxuriant growth, YMX agar was used. For routine maintenance and to obtain better sporulation, DX agar was employed. It was essential to subculture the organisms frequently (every 2 weeks) in order to maintain viability. Stock inocula were preserved at an early stage by cutting plugs of agar from 1-week-old YMX agar plates and freezing at −70°C in 10% (v/v) sterile glycerol (22). In order to obtain maximal xylanase production it was necessary to prime the cultures in TSB as described by Morosoli *et al.* (19).

Seven-day-old (or sporulating) slants grown on DX agar were washed with sterile TSB and 10 ml was used to inoculate 100 ml of TSB in 500-ml Erlenmeyer flasks stopped with cotton plugs. Cultures were incubated at 37 to 38°C with shaking at 240 rpm for 24 h. Primer cultures were assayed for xylanase activity and those with the highest xylanase titers were used as inocula. The full 110 ml inoculum was added to 500 ml of XP medium in a 2800-ml Fernbach flask. Cultures were incubated at 37 to 38°C with shaking at 240 rpm for 48 to 72 h. Xylanase assays were performed at 12-h intervals, and cultures were harvested when xylanase activity peaked. Duplicate cultures were used for xylanase production and the averages of titers are reported.

Assays

All sugar analyses were performed by Nelson's modification (23) of the Somogyi method (24) using either D-xylose or D-glucose as a standard.

Samples (1.0 ml) were taken every 12 h and cells were removed by centrifugation (10,000*g* for 15 min at 4°C). Supernatant solutions were decanted and assayed for xylanase activity. Cellulase activity was assayed only at peak xylanase timepoints. Xylanase and cellulase assays employed 0.25 ml of substrate 1% oat spelts xylan or carboxymethylcellulose respectively (Sigma) plus 0.25 ml of appropriately diluted enzyme in 50 mM sodium phosphate buffer, pH 7.0. Xylan was solubilized in 0.5 N NaOH and neutralized with 1.0 N HCl. Reactions were started by the addition of substrate and incubated for 10 min at 60°C. The cellulase assay temperature was reduced to 55°C (19). Reactions were stopped by the addition of Nelson Somogyi reagent C.

Enzyme Purification

Step I—Clarification. Cells were harvested by centrifugation (10,000*g* for 30 min at 4°C). The dark red supernatant was decanted and pellets were discarded. Supernatant (~1000 ml) was treated with the minimum amount of Biocryl BPA-1000 (Supelco Div., Rohm and Haas Co., Bellefonte, PA) required to precipitate the pigment(s) to approximately 20% of its initial value as measured by reduction in $A_{392\,nm}$. BPA-1000 suspension is used as supplied by the manufacturer and typically 1.5 to 2.5% (v/v) was required for clarification. After stirring with the BPA for 5 min at 4°C, a milky-grey floc was formed which was subsequently removed by centrifugation at 15,000*g* for 15 min at 4°C. The clear, light yellow supernatant was then filtered through Miracloth (Calbiochem, La Jolla, CA).

Step II—Concentration. The BPA clarified filtrate was concentrated about fivefold by ultrafiltration in a Lab 1 EX ultrafiltration system (Supelco Div., Rohm

and Haas Co.) using a 0.093-m^2 PM-1 hollow-fiber cartridge (1000 mol wt cutoff) at a transmembrane pressure of 10 psi. The retentate (200 ml) was diafiltered with 250 ml of 100 mM sodium acetate buffer, pH 5.5, followed by 500 ml of 10 mM sodium acetate, pH 5.5 (Buffer A). Final retentate volume after diafiltration was approximately 200 ml.

The retentate was transferred to a 50-ml stirred ultrafiltration cell (Amicon Div., Grace & Co., Danvers, MA) equipped with a YM-3 disc membrane (3000 mol wt cutoff) and concentrated fivefold. The retentate was diafiltered with buffer A until its pH was adjusted to 5.5 and volume was approximately 40 ml.

(Caution: do not dialyse or use cellulose-based membranes. Cellulases are present.)

Step III—HPLC purification. The YM-3 retentate was centrifuged at 15,000*g* for 15 min at 4°C. Pellets were discarded and supernatant, 40–60 mg protein/load, was applied (6 ml/min) to a 21.5 × 150-mm TSK SP-5PW column (Supelco Div., Rohm and Haas Co.). Proteins were separated using a gradient HPLC system (Beckman Instruments, Inc., Waldwick, NJ) consisting of two 114M preparative head pumps, Model 165 variable wavelength detector, and Model 210A sample injection valve. All HPLC was performed at ambient temperature. Elution buffers were buffer A and buffer A + 1.0 M NaCl (buffer B). Unbound proteins were washed from the column with 100% buffer A for 15 min. Chromatography of adsorbed proteins was achieved with a discontinuous gradient of buffers A and B as follows: 100% A for 5 min, 100% A to 50% B in 25 min, 50% A to 100% B in 2 min, 100% B for 3 min, 100% B to 100% A in 2 min, and 100% A for 50 min to equilibrate the column for the next sample. Elution was monitored at 280 nm for protein, and 6-ml fractions were collected.

Four peaks containing xylanase activity against oat spelt xylan were pooled separately according to activity, protein chromatogram, and SDS–PAGE determinations of purity. The four xylanase peaks (Xyl1, Xyl2, Xyl3, Xyl4) containing the highest enzyme activity and absorbance at 280 nm eluted between 300 and 400 mM NaCl. Xyl1–Xyl4 fractions from triplicate HPLC runs were pooled, concentrated, and diafiltered into buffer A using a stirred ultrafiltration cell as described above. $(NH_4)_2SO_4$ was added to each sample to a final concentration of 1.25 M. The samples were microfuged at 16,000*g* for 3 min at ambient temperature. Pellets were discarded and the supernatant was applied (1 ml/min) to a 7.5 × 75-mm TSK Phenyl-5PW column (Supelco Div., Rohm and Haas Co.). Proteins were separated using a Beckman gradient HPLC system consisting of a 126 dual analytical pump, a Model 167 variable wavelength detector, an IBM PC based data/system controller (Beckman System Gold software), and a Model 210A sample injection valve. Elution buffers were buffer A and buffer A + 1.25 M $(NH_4)_2SO_4$ (buffer C). Unbound proteins were washed from the column with 100% buffer C for 10 min. Chromatography of adsorbed proteins was achieved with a discontinuous gradient of buffers C and A as follows: 100% C for 5 min, 100% C to 35% A in 3 min, 65% C to 100% A in 17.5 min, 100% A for 4.5 min, 100% A to 100% C in 2 min, and 100% C for 15 min to equilibrate the column for the next sample. Elution was monitored at 280 nm for protein, and 1-ml fractions were collected. Xyl1 was highly purified and if high yield is not a priority, highly purified Xyl3 can be obtained after this step by careful fraction selection. Xyl2 and Xyl4 must be further purified by gel-filtration chromatography.

Xyl1–Xyl4 fractions were pooled separately according to activity, protein chromatogram, and SDS–PAGE determinations of purity as above. Pooled fractions of Xyl1–4 from the Phenyl-5PW HPLC runs were concentrated and diafiltered into 200 mM sodium phosphate, pH 7.0 (Buffer D) to a final protein concentration of approximately 5–10 mg/ml, using a Centricon-3 (Amicon Div., Grace & Co.). The xylanase was microfuged at 16,000*g* for 3 min at ambient temperature. Pellets were discarded and the supernatant, 200–600 μg protein/load, was applied (1 ml/min) to a 7.8 × 150-mm TSK QC-PAK gel-filtration column (Supelco Div., Rohm and Haas Co.). Proteins were separated using the Beckman analytical HPLC system previously described using isocratic elution with buffer D. Elution was monitored at 280 nm for protein, and 0.5-ml fractions were collected. Purified xylanases (10 mg/ml) were stored in 50 mM sodium phosphate buffer, pH 7.0, at −70°C.

Electrophoresis

Protein purity and molecular weight were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a Pharmacia Phast System (Piscataway, NJ) using 10 to 15% gradient polyacrylamide gels and a Sigma SDS-7 molecular weight standard mixture (Sigma) containing seven proteins in the 14,200 to 66,000 mol wt range. The proteins were electrophoresed for 70 V h according to Pharmacia SDS–PAGE separation technique file No. 110 and were stained with Coomassie brilliant blue G-250.

Protein

Protein concentrations were determined by the method of Lowry *et al.* (25), with bovine serum albumin, Cohn fraction V (Sigma) as standard.

(Caution: do not measure protein using Coomassie blue dye reagents. The xylanases are difficult to stain and unusually low protein estimates will result.)

Gel Filtration

Protein purity and molecular weight were determined by gel-filtration chromatography using the same TSK QC-PAK column and conditions described above in the purification protocol. Sigma standards for gel-filtration and corresponding molecular weights were: blue dextran (2,000,000), bovine serum albumin (66,000), cytochrome C (12,400), and vitamin B_{12} (1,400).

Mass Spectroscopy

Protein molecular mass was determined with a Finnigan MAT TSQ-700 triple sector quadrupole mass spectrometer equipped with an electrospray ion source. Samples were introduced with an on-line capillary HPLC. A standard water, acetonitrile, trifluoroacetic acid buffer system was used in the chromatography (26). Scans over the mass range m/z 500–2000 were taken at 5-s intervals during the course of the liquid chromatography run. Chromatograms were generated by monitoring the ion current to the mass spectrometer detector. Mass spectra were collected as centroid data.

Temperature Stability

Appropriately diluted enzyme was incubated with 50 mM phosphate buffer, pH 7.0, at desired test temperatures. At the indicated time intervals, a 0.25-ml aliquot of the enzyme was withdrawn and mixed with 0.25 ml of 2% oat spelts xylan prepared in 100 mM phosphate buffer, pH 7.0. The enzyme–substrate mixture was then incubated at 60°C for 10 min. The reducing sugars liberated were determined as described above.

pH Effect

Oat spelts xylan (2%) was prepared in 100 mM buffers at different pHs. Citrate-phosphate buffer was used for pH 4.0–7.0, phosphate buffer for pH 7.0–8.0, and Tris-HCl buffer was used for pH 8.0–9.0. The ionic strength each buffer was 100 mM. Appropriately diluted enzyme (0.25 ml) was mixed with 0.25 ml of the substrate and was incubated at 60°C for 10 min. The reducing sugars liberated were determined as described above.

N-terminal Amino Acid Sequence

Xylanase N-terminal sequence was determined by gas-phase sequencing on Applied Biosystems Model 475A protein sequencer (Foster City, CA) at the University of Wisconsin Biotechnology Center using the protocols, reagents, and solvents supplied by the manufacturer.

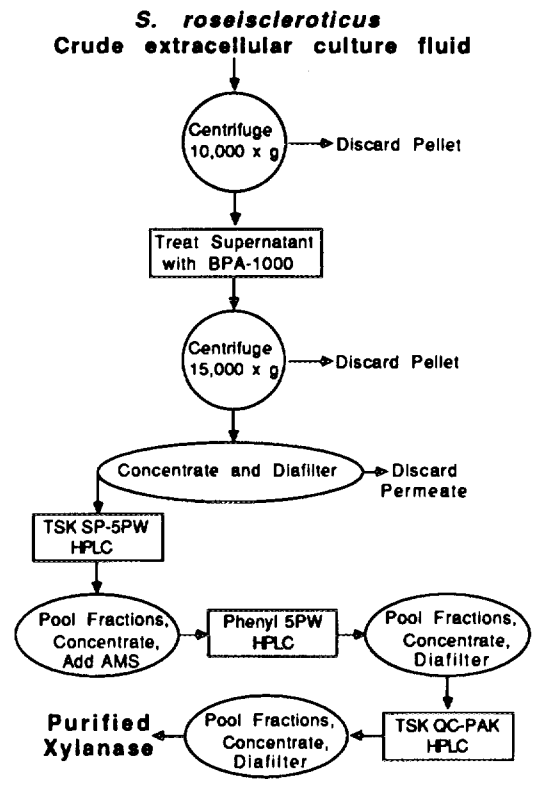

FIG. 1. Purification scheme for xylanases (Xyl1–Xyl4) isolated from *S. roseiscleroticus* cultured on a liquid xylan medium.

RESULTS AND DISCUSSION

Xylanase Purification

The scheme used to purify the xylanases of *S. roseiscleroticus* is outlined in Fig. 1. After culturing the organism the extracellular medium is characteristically a deep red color. Although the initial centrifugation step adequately removed cells and debris from the culture fluid, the soluble red pigmented material remained. Bioprocessing agent Biocryl BPA-1000 effectively removed the pigment. BPA-1000 is one of several Biocryl bioprocessing materials composed of crosslinked acrylic or styrenic polymer particles in suspension with diameters of 0.1 or 0.2 μm and basic or acidic surface functional groups. The surface functional groups on BPA-1000 are strongly basic quarternary ammonium. These charged particles adsorb oppositely charged species such as cell debris, nucleic acids, contaminant proteins and color bodies in a wide variety of fermentation broths, cell or tissue extracts, and homogenates (16).

Other bioprocessing agents such as chitosan and polyethyleneimine (PEI) have been used to remove the extracellular pigment from *S. roseiscleroticus* culture fluid (27). However, large volumes of chitosan are required in order to achieve the same degree of clarification ob-

TABLE 1

Purification of β-(1-4)Endoxylanases Isolated from *S. roseiscleroticus* Grown on a Liquid Xylan Substrate

Purification step	Total volume (ml)	Protein concentration (mg/ml)	Units per milliliter[a]	Total units[a]	Specific activity (U/mg)	Yield (%)	Fold purification
Crude culture filtrate	1000	1.59	7.8	7800	4.9	100	1.0
BPA-1000 clarification	985	1.28	7.6	7486	5.9	96	1.2
Concentration, diafiltration[b]	40	4.00	159	6400	40	82	8.1
SP 5PW HPLC Xyl1	28	0.44	46	1288	104	17[c]	21
SP 5PW HPLC Xyl2	28	0.46	20	560	43	7[c]	8.8
SP 5PW HPLC Xyl3	22	1.27	73	1606	58	21[c]	12
SP 5PW HPLC Xyl4	17	0.27	15	255	56	3[c]	11
Phenyl 5PW HPLC Xyl1	7	0.34	59	413	172	5	35
Phenyl 5PW HPLC Xyl2	5	0.37	58	290	159	4	33
Phenyl 5PW HPLC Xyl3	2	3.39	375	750	111	10	23
Phenyl 5PW HPLC Xyl4	9	0.24	33	297	139	4	28
QC-PAK HPLC Xyl2		0.60	73		122		25
QC-PAK HPLC Xyl3		0.28	46		165		34
QC-PAK HPLC Xyl4		0.43	51		119		24

[a] Determined with 1% oat spelt xylan as substrate and D-xylose as reducing sugar standard.

[b] Ultrafiltration units employed a Romicon PM-1, 1000 mol wt cutoff hollow fiber cartridge for concentration or an Amicon YM-3, 3000 mol wt cutoff disc membrane for diafiltration.

[c] Low % yield after SP 5PW HPLC is attributed to selection of the single major xylanase peak and removal of three other protein peaks each with xylanase activity.

tained with BPA-1000. PEI leaves 20% more pigment in solution than BPA-1000. Another advantage to BPA-1000 as a bioprocessing agent is its particulate nature. Unlike the water soluble polymers PEI and chitosan, the BPA particles, along with the adsorbed materials, are easily removed by centrifugation or membrane filtration. Soluble PEI and chitosan are more difficult to separate from the sample and dialysis, diafiltration or an additional chromatography step is required. For these reasons, BPA-1000 was the bioprocessing agent of choice.

When increasing amounts of BPA-1000 were added to the *S. roseiscleroticus* culture fluid, more pigment was removed. The pigment formed a red–brown thread-like floc that was dense enough to settle without centrifugation and was completely pelleted by centrifugation. The color difference in the culture fluid before and after BPA treatment is quite dramatic and is analogous to the color of a burgundy wine before treatment and a chardonnay after treatment. Pigment removals of 80–90% (based on pigment maximum absorbance at 392 nm) have been achieved with greater than 95% recoveries of xylanase activity. The BPA-1000 clarification is essential in order to facilitate the remaining purification steps of concentration and HPLC.

The results of a typical purification of *S. roseiscleroticus* xylanases are shown in Table 1. The BPA-1000 treatment and subsequent concentration and diafiltration of the clarified culture fluid was rapid and nondestructive of xylanase activity. The eightfold increase in purity was likely due to removal of low molecular weight compounds and pigment that inhibited the enzymes or interfered with the reducing sugar assay. The majority of the purification was achieved through several HPLC steps. SP 5PW cation-exchange HPLC (Fig. 2A) gives good separation of the four xylanases. The active peaks eluted between 300 and 400 mM NaCl and were designated Xyl1, Xyl2, Xyl3, and Xyl4 according to their order of elution from the SP column. Reducing sugar assays of the column fractions revealed synergistic activity between Xyl1 and Xyl2 (Fig. 2A). Activity synergism between the nonadjacently eluting xylanases has not yet been determined.

Cation exchange chromatography resolved Xyl1–Xyl4 allowing each to be pooled separately. The total recovery after this pooling is only 48%. However, due to synergistic activity in the crude extract, yield data beyond the concentration and diafiltration step are inherently low. The four Xyl pools were each subjected to phenyl 5PW hydrophobic-interaction and QC-PAK gel-filtration HPLC. Examples of each of these separations are shown with Xyl3, the most abundant xylanase in this preparation (Figs. 2B and 2C). The series of HPLC steps was very effective and combined to give overall purifications of 35-fold for Xyl1, 25-fold for Xyl2, 34-fold for Xyl3, and 24-fold for Xyl4. The specific activities of Xyl2 and Xyl4 are lower after the final purification step by QC-PAK HPLC than after the phenyl 5PW separation. That decrease could be attributed to selection of the homogeneous xylanase fraction (i.e., Xyl2 or

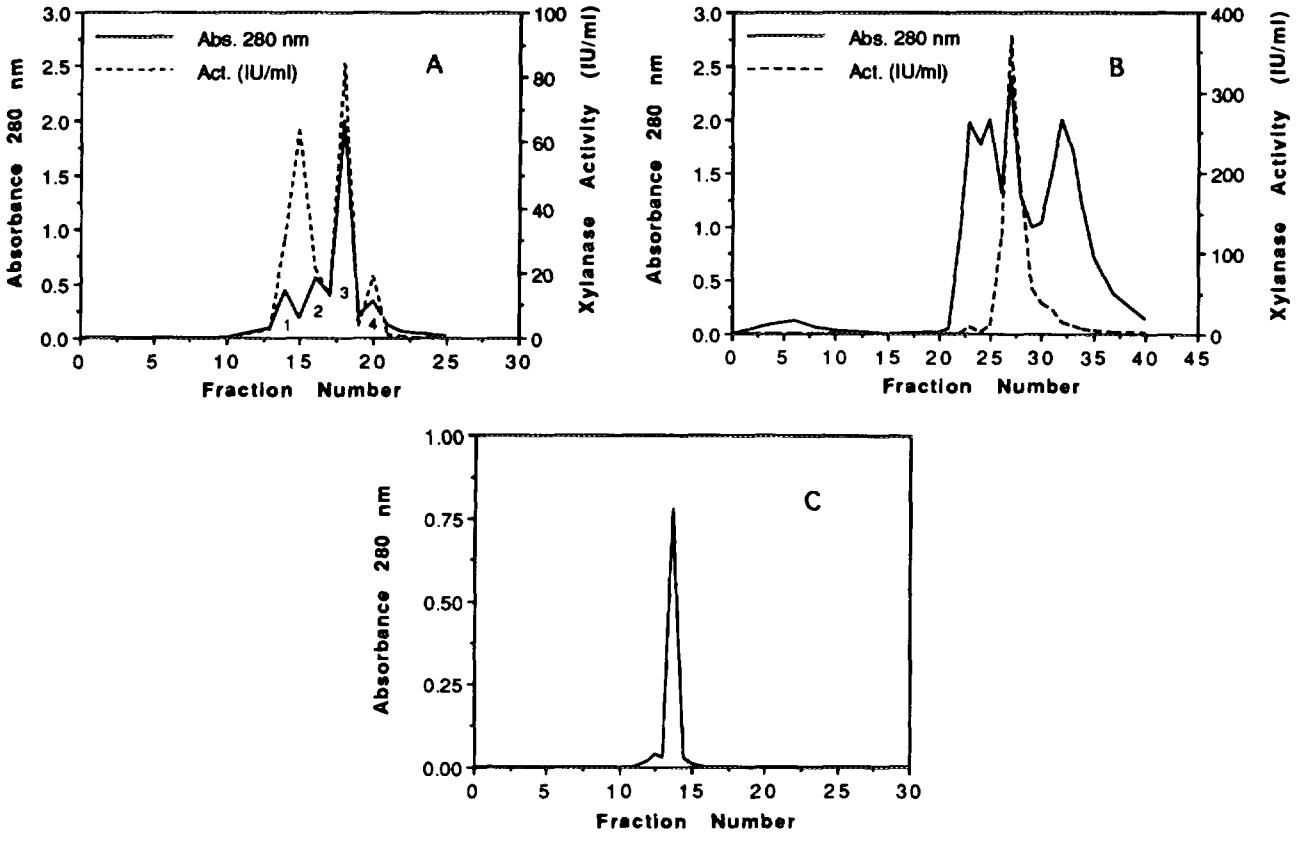

FIG. 2. (A) Cation exchange HPLC of BPA-1000 clarified, concentrated, and diafiltered culture fluid from *Streptomyces roseiscleroticus*. Xylanases (Xyl1–Xyl4) peaks are indicated 1, 2, 3, and 4, respectively. (B) Representative hydrophobic interaction HPLC for Xyl3 fractions pooled from the previous cation exchange HPLC separation (A). Xyl1, Xyl2, and Xyl4 were also separated using this procedure. (C) Representative gel-filtration HPLC for Xyl3 fractions pooled from the previous hydrophobic interaction HPLC separation (B). Xyl2 and Xyl4 were also separated using this procedure.

Xyl4) with the resultant removal of other synergistic xylanase species. This selection amounted to the removal of approximately 46 and 54% contaminating protein from the Xyl2 and Xyl4 fractions, respectively. Other factors contributing to the lower specific activities of Xyl2 and Xyl4 are losses due to temperature, pH, and storage effects prior to determining the activities. These xylanases are less stable than Xyl1 or Xyl3 and may have lost activity.

The protein concentration throughout the purification was measured using the method of Lowry *et al.* (25). It is important not to measure protein concentration using a Coomassie blue staining reagent. We have measured protein using a commercial Coomassie blue reagent (Bio-Rad, Richmond, CA) and compared resultant protein concentrations with those obtained by absorbance at 280 nm and by the Lowry method. The Lowry and absorbance 280-nm values were very similar, but values obtained by dye binding of Coomassie blue were ~94% less (data not shown) than each of these methods, respectively.

The SDS–PAGE of concentrated, BPA-1000-treated, extracellular culture fluid and the final purified Xyl1–Xyl4 proteins is shown in Fig. 3. At least seven other proteins as well as the four xylanases can be identified in the clarified crude sample. These contaminating proteins were removed by the purification scheme to yield highly purified preparations of Xyl1–Xyl4. The importance of the BPA-1000 clarification step in this purification scheme must be emphasized. Previous purifications of *S. roseiscleroticus* Xyl3 without the use of BPA-1000 were hampered by the soluble red pigments present in the culture fluid. The pigments are a nuisance and restrict downstream processing steps, especially ultrafiltration and chromatography (7). BPA-1000 treatment of the feedstream removes the pigments allowing uninhibited ultrafiltration and HPLC purification of the xylanases from *S. roseiscleroticus*.

Physical Characteristics

Physiochemical properties of *S. roseiscleroticus* Xyl1–Xyl4 are summarized in Table 2. The molecular weights

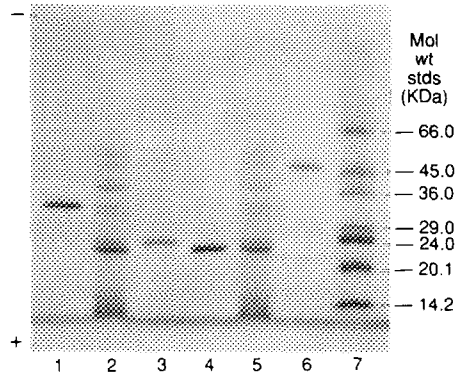

FIG. 3. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis stained with Coomassie brilliant blue. Fractions following purification of Xyl1–Xyl4 from *S. roseiscleroticus:* lanes 1, Xyl1; 2, BPA-1000 clarified, concentrated, and diafiltered culture fluid; 3, Xyl2; 4, Xyl3; 5, BPA-1000 clarified, concentrated, and diafiltered culture fluid; 6, Xyl4; 7, Sigma SDS-7 molecular weight protein standards (approximate mol wt of standards are indicated on the right edge). During electrophoresis the anode (+) was located at the bottom of the gel.

for Xyl1–Xyl4 were determined by three separate methods. Unusual results were seen in the gel-filtration experiments, with each of the proteins exhibiting extremely low apparent molecular weights. Xyl1, Xyl2, Xyl3, and Xyl4 eluted as if they were 9800, 4900, 2500, and 1200 molecular weight proteins, respectively. These native molecular weights were very low but the proteins demonstrated this property by their ability to penetrate ultrafiltration membranes with molecular weight cutoffs of 10,000 (Amicon YM 10 and Romicon PM10). For this reason it was necessary to concentrate and diafilter the xylanases using membranes with cutoffs below 10,000 (Amicon YM-3 and Romicon PM1). We subsequently determined the molecular weights using SDS–PAGE and mass spectroscopy. These two methods gave more definitive results with mass spectroscopy as the most reliable. The molecular weights by SDS–PAGE and mass spectroscopy, respectively, were Xyl1 32,000, 33,647; Xyl2 24,000, 33,655; Xyl3 21,000, 21,070; and Xyl4 48,000, 46,855. These molecular weights corresponded closely with the molecular weights of xylanases purified from *S. lividans* 66, *Streptomyces* EC10, and *Chainia* strain NCL 82-5-1 (28–31).

The low apparent molecular weights by gel filtration may be explained by the structure of the native proteins. The crystal structure of xylanase from *Bacillus pumulis* is characterized by three large β-pleated sheets giving the protein a spade-like V shape 40 × 35 × 35 Å (32). Similarly the structure of the cellulose binding domain of cellobiohydrolase I from *Trichoderma reesei,* as determined by nuclear magnetic resonance spectroscopy, is that of a compact wedge formed by three main hydrogen-bonded, antiparallel, β-pleated sheets. This structure may enable the protein to pry apart individual cellulose microfibrils from cellulose fibers (33). The ability of a xylanase to penetrate a substrate would also be facilitated by such a wedge-like structure. For example, the organism would have greater access to the xylan component in the small pore sized hemicellulose–lignin–cellulose matrix of wood. However, crystal structures of Xyl1–Xyl4 have yet to be determined, and these data are necessary to answer questions on the apparent very low molecular weight of these proteins.

Thermal stability of the four xylanases at 50, 60, and 70°C and pH effects from pH 4 to 9 have been determined (Table 2). At 70°C each of the four isoenzymes lose activity rapidly, Xyl3 being the most sensitive. At 60°C, Xyl1 has a half-life of about 100 min whereas Xyl3 has a half-life of 60 min, Xyl2 and Xyl4 were comparatively less stable. However, at 50°C the four isoenzymes retained more than 80% activity up to 200 min. The pH optima of Xyl1–Xyl4 were found to lie in the range of pH 5.0 to 7.0, but a substantial amount of activity remains at pH 8.0 to 9.0. Similar to the β-endoxylanases isolated from other *Streptomyces* sp., the xylanases of *S. roseiscleroticus* are most active near neutrality but have good tolerance to alkaline pH conditions. At pH 8.0 all four xylanases maintained over 60% of their activity

TABLE 2

Physiochemical Properties of Xylanases from *S. roseiscleroticus*

Parameter	Values			
	Xyl1	Xyl2	Xyl3	Xyl4
Mol wt by:				
QC-PAK gel-filtration	9,800	4,900	2,500	1,200
SDS–PAGE	32,000	24,000	21,000	48,000
Mass spectroscopy	33,647	33,655	21,070	46,855
Optimum pH	6.5	6.0	5.5	6.0
Relative pH effect (%)[a]				
pH 4.0	14	25	49	7
pH 5.0	100	52	100	71
pH 6.0	100	100	100	100
pH 7.0	96	75	79	91
pH 8.0	74	62	65	73
pH 9.0	44	31	37	59
Optimum temperature range (°C)	50–60	50–60	50–60	50–60
Thermal half-life (min)				
50°C	750	469	1,250	375
60°C	100	10	62	30
70°C	<10	<10	<10	<10
Action pattern[b]	Endo-1	Endo-2	Endo-1	Endo-2

[a] Normalized to the pH optimum for each xylanase in the 10-min NS assay.

[b] Data used with permission from Ref. (15).

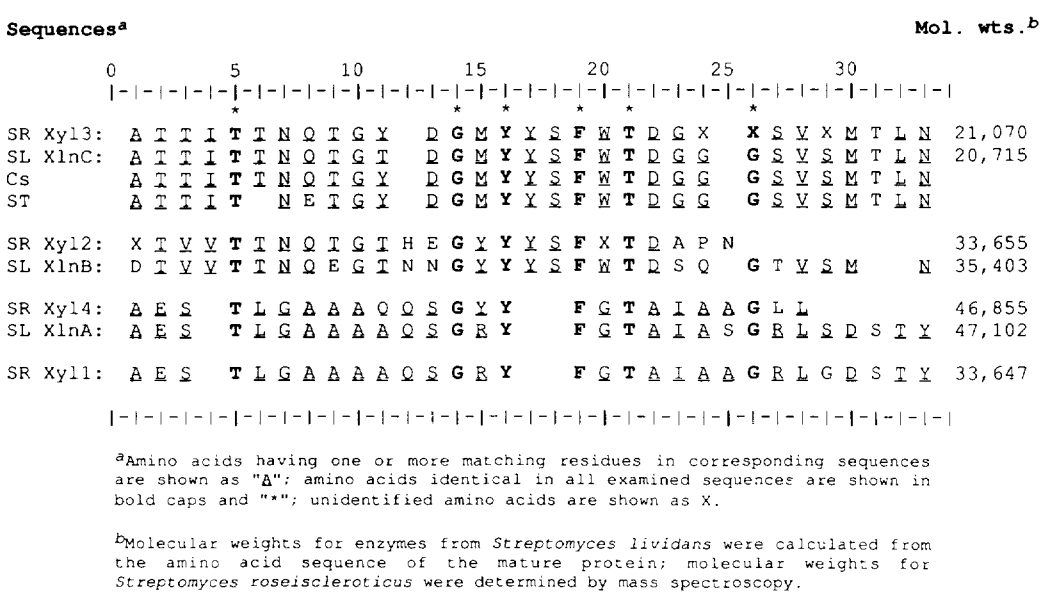

FIG. 4. Optimal alignment of N-terminal amino acid sequences Xyl1, Xyl2, Xyl3, and Xyl4 from SR, *Streptomyces roseiscleroticus* with amino acid sequences of similar xylanases from St, *Streptomyces* sp. No. 36A; SL, *Streptomyces lividans*, and Cs, *Chainia* sp.

during the 10 min assay and at pH 9.0 over 30% of their activities were maintained. The pH optima of the crude preparation is not significantly different from that of the pure xylanases, but the mixture of enzymes is less sensitive to pH change.

Several other streptomycete xylanases either have been purified sufficiently to enable N-terminal amino acid determinations or the enzymes have been cloned. Sharek *et al.* (34) have determined the nucleotide sequences of three genes (*xlnA*, *xlnB*, and *xlnC*) coding for three distinct xylanases (Xln) from *Streptomyces lividans*. Xylanases from *Streptomyces* 36A and *Chainia* sp. have also been determined (35,31).

The N-terminal regions of the mature secreted proteins from *S. lividans* are aligned with the N-terminal regions of the enzymes described here for *S. roseiscleroticus* (Fig. 4). XlnA, XlnB, and XlnC from *S. lividans* are highly similar to Xyl4, Xyl2, and Xyl3, respectively, from *S. roseiscleroticus*, as determined by both the N-terminal amino acid sequence and the molecular weights of the enzymes. Xyl3 is also very similar in sequence to xylanases sequenced from *Streptomyces* 36A and *Chainia* sp. Xyl1 is highly similar to XlnA in its N-terminal amino acid sequence, but because it has a substantially different molecular weight it appears to be a different enzyme. Xyl1 is not a proteolytic degradation product of Xyl4 because it differs from Xyl4 by three amino acids in the region shown here. However, based on N-terminal amino acid sequences, the xylanases of *S. roseiscleroticus* can be separated into two groups. Xyl1 and Xyl4 show strong sequence homology and Xyl2 and Xyl3 sequences are also very similar. These two groupings are distinct showing relatively little similarity to one another.

Even though the enzyme complexes of *S. roseiscleroticus* and *S. lividans* appear to be similar, they exhibit some important differences. First, the ratios of the various isoenzymes are different in the two organisms. *S. roseiscleroticus* produces predominantly Xyl3 under our culture conditions while in *S. lividans* XlnC is the least abundant (28). Second, while *S. lividans* produces three xylanases, *S. roseiscleroticus* produces four. Third, none of the xylanases purified from *S. roseiscleroticus* produces significant quantities of xylose during hydrolysis of xylan, whereas both XlnA and XlnB exhibit this property (19,29).

The enzymes, Xyl1–Xyl4, can be divided into two groups based on their hydrolytic action pattern on arabinoxylan. All four proteins are endo xylanases, but Xyl1 and Xyl3 tend to form xylotriose (endo-1) as the principal product from oat spelts xylan and leave less residual xylan, whereas Xyl2 and Xyl4 tend to form equimolar amounts of xylotriose, tetraose, and pentaose (endo-2), and leave a larger amount of residual xylan (15). Interestingly, these groupings based on hydrolytic action pattern on arabinoxylan do not match the groupings based on the N-terminal amino acid sequences. As briefly noted above, the strongly endo action patterns of *S. roseiscleroticus* xylanases differ slightly from the action patterns observed with *S. lividans* xylanases. The latter all produce significant amounts of xylose (19,28,29), but the former do not produce detectable quantities of xylose. The relationship between substrate specificity and hydrolytic product

profile is thought to depend upon the natures of the substrate binding region and the catalytic pocket. Substrate binding is determined largely by a highly conserved sequence near the carboxy terminus (see, for example, 34 and 36). The difference in hydrolysis products may be due to the length of incubation, amount of enzyme used, or the substrate employed.

Other differences have been noted in the relative abilities of these four *S. roseiscleroticus* enzymes to release chromophores and to reduce the kappa number of kraft pulps (15). Xyl3 tends to release more chromophores, and Xyl4 tends to facilitate the reduction of kappa number to a greater extent. The significance of these differences as well as the cloning of the enzymes are currently under study in our laboratory.

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