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Purification and properties of xylanase from *Aureobasidium*

Timothy D. Leathers

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL, U.S.A.*

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SUMMARY

The yeast-like fungus *Aureobasidium* is a promising source of xylanase (EC 3.2.1.8) with an exceptionally high specific activity. For enzyme production in volumes of several liters, xylose was the preferred carbon source and inducer. Xylanase in clarified cultures was concentrated by reversible adsorption to cation-exchange matrix to 5% of the initial volume, and recovered at nearly 2 million IU/l. Selective conditions permitted 97% recovery of xylanase with a 1.8-fold enrichment in specific activity, to 70% of purity. The predominant xylanase species (20 kDa) was subsequently purified to >99% of homogeneity by gel filtration chromatography. Purified enzyme exhibited an isoelectric point of 8.5, and specific activity of 2100 IU/mg under optimal conditions, determined to be pH 4.5 and 45°C. The activity of purified enzyme was specific for polymeric xylan.

INTRODUCTION

Potential industrial applications for xylanases are numerous. Xylan may comprise up to 30% of lignocellulose from grasses and hardwoods; enzymatically hydrolyzed biomass has potential for conversion to fuels and chemicals [6]. Xylanases can also be useful in the clarification of fruit juices [5], the production of wine [22], and in the bleaching of high-grade cellulose pulps (involving the selective removal of lignin and xylan) [15].

Many hyphal fungi (molds) produce xylanases, and widely-used conventional sources include *Trichoderma reesei* [19] and *Aspergillus niger* [7]. However, hyphal fungi also produce cellulases, many of which have broad substrate specificity that may include xylan [20]. These cellulases complicate basic studies of xylanases, and must be scrupulously removed for certain industrial applications [18].

While only a few species of yeast-like fungi pro-

Correspondence: T.D. Leathers, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604, U.S.A.

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duce xylanases, none of these produces cellulase activity [4,11,17]. Certain naturally occurring isolates of the yeast *Aureobasidium*, previously described as 'color variant' strains [21], overproduce an extracellular xylanase (EC 3.2.1.8) with exceptionally high specific activity [9]. Described here are methods for production of concentrated xylanase from *Aureobasidium*, and the subsequent characterization of purified enzyme.

MATERIALS AND METHODS

Carbon sources and enzyme substrates

Xylobiose was from Bachem, Inc., Torrance, CA. Microcrystalline cellulose (Avicell) was from FMC Corp., Rockland, Maine. Carboxymethylcellulose (7M8SFX) was a gift of Hercules Co., Parlin, NJ. Other carbon sources and enzyme substrates, including arabinoxylan from oat spelts, were from Sigma Chemical Co., St. Louis, MO.

Organism and cultivation

Aureobasidium strain NRRL Y-2311-1 was obtained from the USDA Agricultural Research Service Culture Collection, Peoria, IL. Basal medium was previously described [9], and was supplemented to 1.0% (w/v) with the indicated carbon sources. Analytical cultures were 2.0 ml, in loosely capped 13 × 100 mm tubes. Preparative cultures were multiples of 1 liter, in Fernbach flasks. All cultures were shaken at 200 rpm at 28°C for 3 days (New Brunswick model G-53 shaker, stroke ca. 2 inches).

Ion-exchange adsorptions

Cultures were cleared by centrifugation (20 min, 8000 rpm, Sorvall GS3). Supernatants were deionized with rechargeable mixed-bed resin (AG 501 X-8, Bio-Rad Laboratories, Richmond, CA), from initial values of 14 000 to 17 000 μmho/cm, to a final value of about 1500 μmho/cm. Deionized supernatants were adjusted to pH 4.0 with glacial acetic acid. Ion-exchanger (carboxy-methyl sephadex, CM-Sephadex, Pharmacia, Inc., Piscataway, NJ) was slowly stirred into the deionized supernatants at 500 mg/l, which were then incubated with stirring overnight at 4°C. Xylanase-depleted supernatants were decanted from settled matrix, which was then resuspended and adjusted to pH 7.0 with sodium hydroxide to release adsorbed xylanase.

Gel filtration chromatograpy

An Econo-column (2.5 × 75 cm, Bio-Rad) packed with Sephadex G-75 (Pharmacia) was used for gel filtration chromatography. Samples (2.5 ml) of xylanase (concentrated as described above, containing approximately 3.0 mg of protein, and adjusted to 10% glycerol) were applied to the column and eluted with 12.5 mM Tris-HCl pH 7.3, 0.02% sodium azide at a flow rate of 0.6 ml/min. Four-ml fractions were collected and analyzed for protein and xylanase activity.

Electrophoresis of proteins

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 3.0% stacking, 12.35% resolving gels) was performed according to the method of Laemmli [8]. Molecular weight standards were from Bethesda Research Laboratories, Gaithersburg, MD. The M_r values of unknowns were estimated by the method of Shapiro [16]. Two-dimensional gel electrophoresis was by the non-equilibrium method of O'Farrell et al. [14], modified by the use of agarose in the first dimension (Isogel agarose, FMC Corp.). Ampholytes were the 3.5–9.5 range set, isoelectric point standards were the 3.6–10.2 range set (FMC Corp.). Gels were silver stained by the method of Wray et al. [23].

Enzyme and protein assays

Hydrolysis of xylan and other polysaccharides was assayed by the dinitrosalicylic acid method [13] (DNSA, Aldrich Chemical Co., Milwaukee, WI), modified as previously described [9]. One unit of xylanase activity is defined as that releasing 1 μmol of xylose equivalents per min (IU, international units) under optimal conditions. Xylosidase and glucosidase assays were as previously described [11]. Protein assays were performed by the method of Lowry et al. [12], with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Selection of growth medium

Natural small molecule inducers of xylanase from *Aureobasidium* were previously shown to include xylose, xylobiose, and arabinose [10]. Xylobiose alone is the natural inducer of the well-characterized xylanases from the yeasts *Cryptococcus albidus* and *Trichosporon beigelii* [2,17]. Our previous analytical studies employed cultures of *Aureobasidium* grown on purified xylan, from which the highest enzyme yields were obtained [1,9].

For larger-scale production of xylanase (at the level of several liters), we wished to use a carbon source and inducer other than purified xylan. Commercially available xylan varies considerably from lot to lot (particularly, we believe, in lignin content). Further, purified xylan is relatively costly (over $ 400.00/kg at current prices), and is of limited availability. Finally, xylan has poor handling characteristics, and is not truly water-soluble.

Table 1 summarizes xylanase production levels from cultures grown on xylan, arabinose, xylobiose, and xylose. Although xylobiose is hydrolyzed intracellularly to xylose, previous studies suggested that it serves as an independent inducer [10]. The highest enzyme levels, of 725 000 IU/l, were produced by xylan-grown cultures, with specific activity in excess of 1000 IU/mg protein. Xylose and xylobiose were equivalent as inducers of xylanase, producing yields of at least 100 000 IU/l. In spite of these reduced yields, specific activities of xylanase from xylose- or xylobiose-grown cultures compared favorably to that from xylan-grown cultures. Arabinose-grown cultures produced a relatively low yield of about 20 000 IU/l xylanase, with relatively low specific activity.

Protein patterns of xylanase-producing crude culture supernatants were examined (Fig. 1, lanes A–D). Partial purifications previously demonstrated that xylanase activity was exclusively associated with two or more protein species, apparently isozymes, and estimated to be 21 kDa and 20 kDa [9]. Potential relationships among these putative isozymes are unconfirmed; proteins occurred as native monomers, accumulated coordinately during xylanase production, and were not glycosylated as judged by periodic-acid-Schiff's staining [9].

These xylanase-associated proteins were clearly abundant in cultures grown on xylan, xylobiose or xylose (Fig. 1, lanes A, C and D, respectively). Further, the relative abundance of xylanase-associated proteins appeared similar among these preparations, consistent with their similar specific activities. Since xylobiose was merely the equivalent of xylose with respect to xylanase characteristics, its limited commercial availability and high cost made it a poor choice as an alternative to xylan. Interestingly, arabinose-grown cultures totally lacked the 21 kDa protein species produced on the other growth substrates (Fig. 1, lane B). Despite this potential advantage for subsequent purification of the major xy-

Table 1

Xylanase production and purification

Preparation	Xylanase, IU/l	Specific activity, IU/mg protein	Fold purification	Xylanase purity	Yield
Xylan-grown culture supernatant	725 000	1100	1.0	52%	100%
Arabinose-grown culture supernatant	20 750	123	1.0	6%	100%
Xylobiose-grown culture supernatant	100 000	850	1.0	40%	100%
Xylose-grown culture supernatant	100 800	816	1.0	39%	100%
CM-Sephadex-concentrated supernatant	1 944 000	1480	1.8	70%	97%
CM-Sephadex-depleted supernatant	3 400	58	<0.1	3%	3%
G75-Sephadex-fractionated concentrate	194 000	2100	2.6	>99%	45%

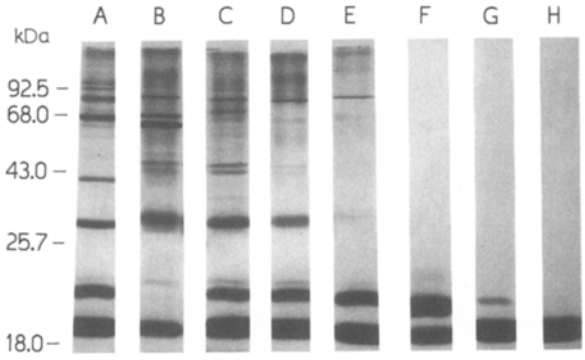

Fig. 1. SDS-PAGE of proteins from clarified culture supernatants of *Aureobasidium* strain NRRL Y-2311-1 and subsequent purification fractions, corresponding to preparations shown in Table 1. Protein samples were normalized to 10 μg/lane and resolved on 12.5% SDS-polyacrylamide gels and silver-stained. Lanes A–D show total extracellular proteins from cultures grown on different carbon sources: A, xylan; B, arabinose; C, xylobiose; D, xylose. Lane E, CM-Sephadex-concentrated xylanase. Lanes F–H show fractions from G75-Sephadex filtration of concentrated xylanase: F, fraction 30; G, fraction 40; H, fraction 42.

lanase species, overall relatively low xylanase yields, and particularly specific activity, make arabinose an undesirable alternative to xylan.

Based on the above considerations, xylose was chosen as the preferred alternative to xylan for scale-up production of xylanase. It should be noted that inexpensive biomass sources of crude xylan may ultimately be proven superior for bulk enzyme production.

Concentration of xylanase from culture supernatants

Previous attempts to concentrate xylanase by ultrafiltration were frustrated by partial loss of activity, not apparently due to aggregation of the enzyme [9]. As an alternative method of enzyme concentration, adsorption to ion-exchangers was tested. Xylanase in low ionic strength buffer bound either anionic or cationic matrices under pH conditions expected for a protein of slightly alkaline isoelectric point (unpublished results). Xylanase binding appeared most selective (as judged by SDS-PAGE) to CM-Sephadex at pH 4. Xylose-grown culture supernatants were consequently batch deionized, and proteins concentrated by adsorption to CM-Sephadex, as detailed in Materials and Methods.

As summarized in Table 1, this method of enzyme concentration was both efficient and selective. Supernatants were concentrated to 5% of initial volume, with an enzyme recovery of 97%. A 1.8-fold purification was also achieved at this step, enhancing xylanase purity to 70% of homogeneity. Concentrated xylanase contained nearly 2 million IU per liter. A slightly more complicated process (involving ultrafiltration) was recently described for production of cellulase-free concentrated xylanase from *Trichoderma harzianum* [18]; although purification to apparent homogeneity was required to remove cellulases, xylanase activity yields were about five-fold lower than those of concentrated xylanase from *Aureobasidium*.

Analysis of concentrated xylanase by SDS-PAGE is shown in Fig. 1, lane E. Abundant 20–21 kDa proteins associated with xylanase were significantly enriched in the concentrate. The most apparent contaminants of the concentrated preparations were high molecular weight species, particularly one of approximately 85 kDa.

Purification of xylanase

We wished to purify and characterize the predominant xylanase species (20 kDa), for subsequent studies involving amino acid characterization and antibody generation. Concentrated xylanase was subjected to gel filtration chromatography, as detailed in Materials and Methods. Fig. 2 shows protein and activity analyses of Sephadex G75 column fractions. Xylanase activity from concentrated preparations eluted as a single peak coincidental with the bulk of total protein. Column calibration indicated that native xylanase subunits were exclusively monomeric.

Fractions from the xylanase activity peak were subsequently analyzed by SDS-PAGE (Fig. 1, lanes F–H). Early peak fractions (Fig. 1, lane F) were enriched for 21 kDa species, but without exception also contained the major 20 kDa species. Mid-peak fraction 40 contained only a trace of 21 kDa species (Fig. 1, lane G), and fractions 42–60 contained only 20 kDa protein (Fig. 1, lane H). Yield of xylanase

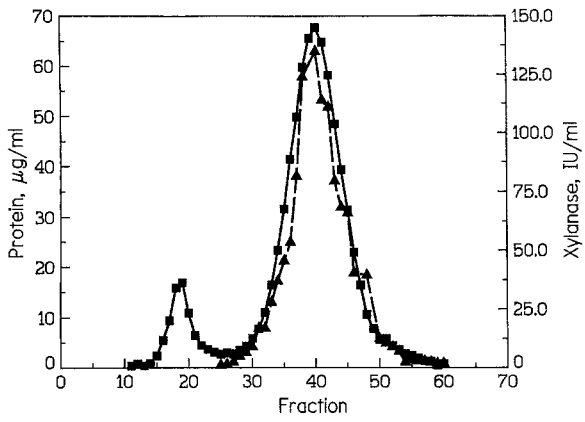

Fig. 2. G75 Sephadex column filtration of concentrated xylanase from *Aureobasidium* strain NRRL Y-2311-1. Symbols: ■, protein (in micrograms per millileter); ▲, xylanase activity (in international units per milliliter).

activity in these latter fractions, containing purified major species, totalled 45% of initial, while specific activity was approximately 2100 IU/mg (Table 1). As suggested by the symmetry of the protein and activity curves, specific activity was constant through the xylanase peak. The 21 kDa protein species thus appears to be a minor isozyme of xylanase with a similar specific activity.

Purity of xylanase pooled from peak fractions 42–60, was assessed by two-dimensional gel electrophoresis. Purified xylanase migrated with an apparent isoelectric point of 8.5 (Fig. 3). In some heavily loaded gels (containing approximately 10 μg of purified xylanase), apparent charge isomers were visible at positions corresponding to isoelectric points of 6.8 and 10.1 (Fig. 3). These species constitute less than 1.0% of total purified xylanase, based on sensitivity of silver-staining.

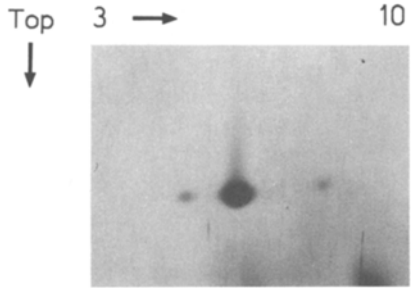

Fig. 3. Two-dimensional gel electrophoresis of purified 20 kDa xylanase (pooled fractions 42–60 from G75 column). Isoelectric focusing is horizontal dimension as presented, pH 3 to 10 from left to right. SDS-PAGE is vertical dimension as presented, from top to bottom of gel. Irregular staining regions at bottom of gel represent ampholytes.

Xylanase activity optima

Optimal conditions were determined for purified xylanase activity. Fig. 4 shows that optimal pH and temperature condition were 4.5 and 45°C, respectively. The useful temperature range was particularly broad, since at least 80% of maximal activity was found between 30° and 65°C. Similar pH and temperature optima were previously determined for crude enzyme [9] and partially purified xylanase [1].

Substrate specificity of purified xylanase

Xylanase-containing culture supernatants from *Aureobasidium* were previously reported to lack any significant cellulase or xylosidase activities [11]. Highly purified xylanase was tested with a variety of natural polysaccharides, as well as with the synthetic substrates *p*-nitrophenyl-xyloside and *p*-nitrophenyl-glucoside (Table 2). Purified enzyme exhibited extremely high specificity for xylan. Low but reproducible activites were found on chitin and starch. Other substrates were not measurably attacked.

Partially purified xylanase from *Aureobasidium* has been shown to progressively reduce polymeric xylan to oligosaccharides, characteristic of endo-xylanases [1]. Absence of xylosidase or glucosidase activities (Table 2) is also consistent with a strictly endo mode of action [5], [22]. As a further test of the endo-specificity of xylanase, we measured activity using a partially-dyed xylan (remazol-brilliant blue xylan, RBB-xylan). This assay measures the liberation of dyed oligosaccharides from polymeric xylan (specifically, their solubilization into ethanol) [3]. Exo-xylanases should have little activity on RBB-xylan, since progressive liberation of end residues would release relatively few dyed residues. Highly purified xylanase from *Aureobasidium* was quite active on RBB-xylan. In short-term (15 min) assays, RBB-xylan assays were over three times as sensitive as reducing sugar assays.

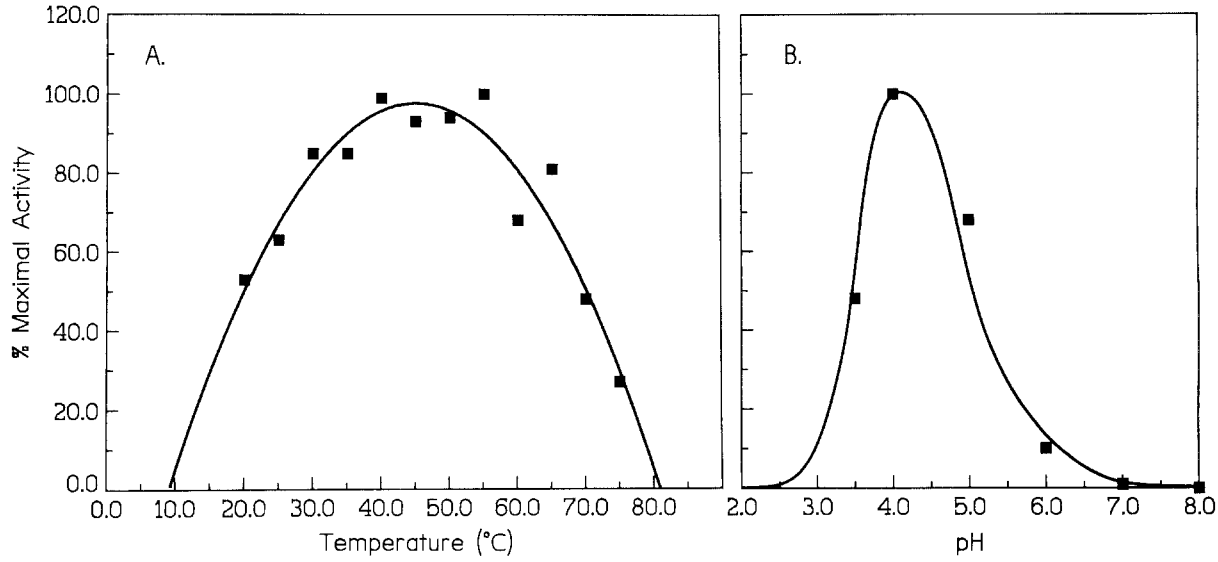

Fig. 4. pH and temperature optima of purified 20 kDa xylanase from *Aureobasidium* strain NRRL Y-2311-1. For studies of pH optima, substrate buffer was titrated to the desired test pH with acetic acid or sodium hydroxide before digestion, then returned to pH 5.0 before assays were developed, since the DNSA assay was pH sensitive.

Table 2

Substrate specificity of purified xylanase

Substrate	Specific activity, IU/mg protein
Arabinogalactan	<10
Arabinoxylan	2100
Carboxymethylcellulose	<10
Chitin	63
Lichenan	<10
Locust Bean Gum	<10
Microcrystalline cellulose	<10
p-Nitrophenylxyloside	<10
p-Nitrophenylglucoside	<10
Polygalacturonic acid	<10
Pullulan	<10
Starch	55

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