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Purification and Characterization of Two α-L-Arabinofuranosidases from *Streptomyces diastaticus*

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A nonsporulating strain of *Streptomyces diastaticus* producing α-L-arabinofuranosidase activity (EC 3.2-1.55) was isolated from soil. Two α-L-arabinosidases were purified by ion-exchange chromatography and chromatofocusing. The enzymes had molecular weights of 38,000 (C1) and 60,000 (C2) and pIs of 8.8 and 8.3, respectively. The optimum pH range of activity for both enzymes was between 4 and 7. The apparent K_m values with *p*-nitrophenyl arabinofuranoside as the substrate were 10 mM (C1) and 12.5 mM (C2). C1 retained 50% of its activity after 8 h of incubation at 25°C, while C2 retained 80% activity. After 3 h of incubation at 50°C, C1 lost 90% of its initial activity while C2 lost only 40%. The purified enzymes hydrolyzed *p*-nitrophenyl α-L-arabinofuranoside and liberated arabinose from arabinoxylan and from a debranched β-1,5-arabinan.

Hemicelluloses are polysaccharides built from different types of sugar residues, forming linear or branched polymers. Arabinose-containing hemicelluloses such as arabinoxylans, arabinans, and arabinogalactans are composed of xylose, galactose, arabinose, or glucuronic acid, forming linear or branched polymers (19). The enzyme system for the degradation of these heteropolysaccharides consists of endo- and exoenzymes which cleave the glycosidic linkages of the polymeric backbone and the various side chains (1, 7, 21). α-L-Arabinofuranosidases catalyze the cleavage of terminal α-L-arabinofuranosyl residues of arabinoxylans, arabinans, and arabinogalactans (8). These enzymes are part of the microbial xylanolytic enzyme system necessary for the complete hydrolysis of heteroxylans (1, 8, 21). α-L-Arabinofuranosidases are produced by bacteria, including actinomycetes, by fungi, and by plants. Only a few of these enzymes have been purified and characterized (4, 9–13, 16, 18). Actinomycetes are producers of a whole range of enzymes involved in the degradation of hemicelluloses such as xylanases and mannanases and in side-chain-cleaving activities (21). *Streptomyces diastaticus* ET has been selected from 130 strains of actinomycetes as the best producer of α-L-arabinofuranosidase activity (22).

In this article, the purification and characterization of two α-L-arabinofuranosidases (C1 and C2) produced by *S. diastaticus* ET are described.

MATERIALS AND METHODS

Bacterial strain. The strain ET/BW200 was isolated from soil in a vineyard near Sitges, Spain. It was identified as a nonsporulating variant of *S. diastaticus* at the Department of Biological Sciences, University of Warwick, United Kingdom (by L. Wellington).

Culture conditions. The organism was grown in shake flasks in a medium containing the following (in grams per liter): $(NH_4)_2SO_4$, 0.3; NaCl, 0.1; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCO_3$, 0.04; KH_2PO_4, 0.6; yeast extract (Difco), 1; trace element solution, 1 (6). The medium was supplemented with 20 g of mannitol per liter (Fluka) and 20 g of soybean meal per liter (Morga, Ebnat-Kappel, Switzerland) or 5 g of xylan from oat spelts per liter (Fluka or Sigma) or 5 g of arabinogalactan per liter (Janssen) or wheat bran (Migros, Switzerland) in different concentrations (1, 3, 5, or 8 g/liter). The wheat bran media also contained glucose (5 or 10 g/liter). The pH of the media was adjusted to 7.2 after autoclaving. The shake flasks were inoculated with 2 ml of an aqueous suspension of hyphae of *S. diastaticus* ET and cultivated on a rotary shaker (150 rpm) at 30°C.

Cultures were also grown in a 42-liter stirred tank reactor at 350 rpm, 30°C, and pH 7.0, with an airflow of 0.1 to 0.2 vol/vol/min. A 60-h preculture (3%) in a medium containing L broth (6) was used to inoculate 28 liters of a medium containing the following (in grams per liter): yeast extract (Difco), 1; wheat bran (Migros), 8; glucose, 5; $(NH_4)_2SO_4$, 0.5; NH_4Cl, 1.2; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; KH_2PO_4, 0.6; and trace element solution, 1 (6). After autoclaving, 20 ml of polypropylene glycol was added to the medium. The oxygen uptake rate and carbon dioxide production rate were calculated from the data of the exhaust gas analysis and the gas flow rate by using mass balancing techniques (3).

Enzyme purification. Supernatant was obtained from 24-h bioreactor cultures by centrifugation. It was concentrated under reduced pressure and lyophilized. A fast protein liquid chromatography system and columns (Pharmacia) were used for the purification.

(i) Step 1. Anion-exchange chromatography. The lyophilized extracellular proteins (corresponding to 200 ml of supernatant) were dissolved in deionized water and passed over a PD 10 gel filtration column (Bio-Rad, Richmond, Calif.), previously equilibrated with 10 mM bis-Tris-HCl (pH 6.2). The enzyme solution was then applied to a Mono-Q column equilibrated with 10 mM bis-Tris-HCl (pH 6.2), the column was washed with the same buffer, and then the proteins were eluted with a gradient of 0 to 0.5 M NaCl in 10 mM bis-Tris-HCl (pH 6.2).

(ii) Step 2. Cation-exchange chromatography. The active fractions from Mono-Q (10 ml) were pooled, and the buffer was changed to 50 mM morpholineethanesulfonic acid (MES)-HCl (pH 5.5) by using a PD 10 column. The enzyme solution was applied to a Mono-S column, previously equilibrated with 50 mM MES-HCl (pH 5.5), the column was washed with the same buffer, and the proteins were eluted with an NaCl gradient of from 0 to 0.3 M in 50 mM MES-HCl (pH 5.5).

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(iii) **Step 3. Chromatofocusing.** Active fractions from the Mono-S column (8 ml) were pooled, and the buffer was changed to 0.075 M Tris-CH_3COOH (pH 9.3) by using a PD 10 column. The proteins were bound to a Mono-P column previously equilibrated with 0.075 M Tris-CH_3COOH (pH 9.3), the column was washed with the same buffer, and then the proteins were eluted by applying a gradient in the range of pH 9 to 6 by using Polybuffer 96 (Pharmacia).

Protein concentrations. The protein concentrations were measured by the method of Bradford (2), with crystalline bovine serum albumin as the standard.

Enzyme assay. *p*-Nitrophenyl-α-L-arabinofuranoside (7 mM) (Sigma) in 0.1 M potassium phosphate buffer (pH 7.0) was used as the substrate to measure α-L-arabinofuranosidase activity. Forty microliters of enzyme solution was incubated with 360 μl of substrate solution for 5 min at 50°C. The reaction was stopped by the addition of 500 μl of 0.1 M 2-amino-2-methyl-1-propanol buffer (pH 11). The A_{405} of liberated *p*-nitrophenol was measured. One unit of arabinofuranosidase activity was defined as the amount of enzyme that liberated 1.0 μmol of *p*-nitrophenol per min.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Schägger et al. (17). A low-molecular-weight protein calibration kit (Sigma) was used as the standard. Proteins were silver stained by the method of O'Farrell (14).

The proteins were also separated in an isoelectric focusing (IEF) gel (pH 4 to 10) (15) and silver stained by the method of Vesterberg and Graneslüp (20). IEF protein standards with pIs ranging from 4.6 to 9.6 (Bio-Rad) were used.

Enzyme characterization. The effect of pH on α-L-arabinofuranosidase activity was determined over the pH ranges of 4 to 6 (0.1 M citrate-phosphate buffer), 6.5 to 7.5 (0.1 M potassium phosphate buffer), and 8 to 9 (0.1 M Tris-HCl buffer) by using the standard assay conditions.

To determine the temperature stability of the α-L-arabinofuranosidases, the purified enzymes were incubated for 8 h at temperatures from 25 to 50°C and then the residual activity was determined.

The substrate specificity of the purified α-L-arabinofuranosidases was determined by using a debranched carboxymethyl-arabinan from sugar beets (MegaZyme, Australia) and arabinoxylan (Sigma). The substrate concentration was 0.2% for carboxymethyl-arabinan and 1% for arabinoxylan. The hydrolysis was performed in 0.1 M citrate-buffer (pH 6.0) at 50°C for 2 h. The products were analyzed by thin-layer chromatography (5) and by high-performance liquid chromatography (HPLC) with a pulsed amperometric detector (Dionex, Sunnyvale, Calif.). The carbohydrates were separated on a CarboPac PA1 column (Dionex) by using an aqueous NaOH solution (22 mM) as the eluent.

RESULTS

Production of α-L-arabinofuranosidase activity. *S. diastaticus* ET produced α-L-arabinofuranosidase activity in shake flask cultures containing mannitol-soybean meal, xylan, or wheat bran. No activity was detected when arabinogalactan was used as the substrate. The highest α-L-arabinofuranosidase activity (260 mU ml^{-1}) was measured when growing the strain in a medium containing 8 g of wheat bran $liter^{-1}$ and 5 g of glucose $liter^{-1}$.

A culture of 28 liters was grown in batch cultivation in a medium containing wheat bran. After a lag phase of 4 h, an exponential growth phase of 9 h was observed, as indicated by the oxygen uptake rate, followed by a linear growth phase. After 24 h of cultivation, an α-L-arabinofuranosidase activity of 600 mU ml^{-1} was detected.

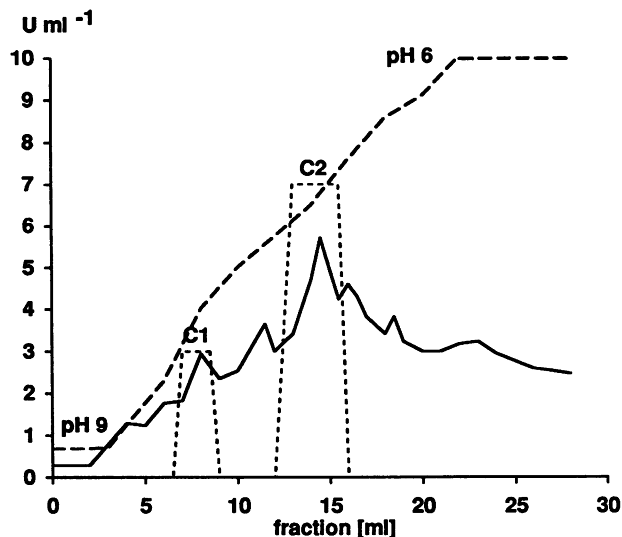

FIG. 1. Chromatofocusing of the partially purified α-L-arabinofuranosidases from *S. diastaticus* ET on Mono-P by using a pH gradient from 9 to 6. Peaks C1 and C2 represent the purified α-L-arabinofuranosidases. ---, α-L-arabinofuranosidase activity (units per milliliter); ——, proteins detected at 280 nm; ——, pH gradient.

Purification of two α-L-arabinofuranosidases. About 10% of the initial α-L-arabinofuranosidase activity did not adsorb to the anion-exchange chromatography column. This fraction contained two α-L-arabinofuranosidases, with pIs of 6.5 and 7.1, which were not further purified. Most of the α-L-arabinofuranosidase activity was bound to the anion-exchange chromatography column and was eluted in one peak at an NaCl concentration of 0.15 M. When the active fractions were applied to the cation-exchange column, most of the contaminating proteins passed through and the α-L-arabinofuranosidase activity could be eluted as a single peak at an NaCl concentration of 0.15 M. As shown in Fig. 1, the α-L-arabinofuranosidase activity was finally separated into two peaks (C1 and C2) by chromatofocusing by using a pH gradient from 9 to 6.

A summary of the purification procedure for the α-L-arabinofuranosidases C1 and C2 is shown in Table 1. The purification steps yielded an enzyme preparation which appeared to be homogeneous as judged by silver-stained single bands detected in SDS-PAGE and IEF gels (Fig. 2 and 3).

Properties of the purified enzymes. The molecular weights of the α-L-arabinofuranosidases determined by SDS-PAGE were 38,000 and 60,000 for C1 and C2, respectively (Fig. 2). The pIs determined by IEF were 8.8 for C1 and 8.3 for C2 (Fig. 3). K_m values of 10 mM for C1 and 12.5 mM for C2 with *p*-nitrophenyl α-L-arabinofuranoside as the substrate was determined by using Lineweaver-Burk plots. The V_{max} values were 0.4 μmol min^{-1} and 1.25 μmol min^{-1} for C1 and C2, respectively.

The effect of pH on the activity was examined. The apparent optimum for activity for both enzymes was found between pH 5 and 6.5. The enzymes showed 90% activity in the pH range from 3.5 to 7.5.

The effect of temperature on the stability of the enzymes

TABLE 1. Purification of α-L-arabinofuranosidases C1 and C2 from *S. diastaticus* ET

Purification step	Total protein (mg)	Total activity (U)	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
Culture supernatant	29.04	103.04	3.55	100	
Anion-exchange chromatography	4.74	55.3	11.66	53.7	3.3
Cation-exchange chromatography	0.43	37.23	86.16	36.13	24.3
Chromatofocusing:					
C1	0.041	4.33	105.6	1.25	29.7
C2	0.055	7.22	131.2	11.02	37.0

was also determined. The temperature stability of the two purified α-L-arabinofuranosidases was different. C1 lost 50% of its initial activity after 8 h of incubation at 25°C, while C2 lost only 20%. After 3 h of incubation at 50°C, C1 lost 90%, while C2 lost only 40% of its initial activity.

The substrate specificity of the purified enzymes was tested with several arabinose-containing hemicellulose preparations. Thin-layer chromatography and HPLC analysis of the reaction products showed that both enzymes liberated arabinose from oat spelts xylan, which is an arabinoxylan containing β-1,3-linked arabinose residues as side chains. Arabinose was also released from a chemically and enzymatically modified debranched β-1,5-arabinan from sugar beets (carboxymethyl-arabinan).

The properties of the two purified α-L-arabinofuranosidases are summarized in Table 2.

DISCUSSION

Wheat bran was found to be the best inducer for the production of this enzyme by *S. diastaticus* ET. When this medium was used, 260 mU of arabinofuranosidase activity ml^{-1} was obtained in shake flask cultures and about 650 mU ml^{-1} was obtained in a batch cultivation. The largest amount of enzyme was produced by *S. diastaticus* ET during the stationary growth phase and under oxygen-limited conditions. *S. diastaticus* ET yielded higher levels of α-L-arabinofuranosidase activity than other *Streptomyces* and *Bacillus* species (10, 12).

S. diastaticus ET produced more than one type of α-L-arabinofuranosidase which showed different physicochemical properties. Multiple forms of α-L-arabinofuranosidases have also been detected in *Streptomyces purpurascens* and in *Streptomyces diastatochromogenes* (13, 21).

Two α-L-arabinofuranosidases, C1 and C2, from *S. diastaticus* ET were purified from the culture supernatant and characterized. The specific activity of the two enzymes (105 and 131 U mg^{-1}, respectively) were higher than those of the purified α-L-arabinofuranosidases from *Streptomyces* sp. strain 17-1 (3.4 U mg^{-1}) (10) and *Bacillus subtilis* F-11 (48.8 U mg^{-1}) (9) but lower than those of the α-L-arabinofuranosidases from *Aspergillus niger* (366.6 U mg^{-1}) (11) and *Ruminococcus albus* 8 (655.47 U mg^{-1}) (4).

Plant and fungal α-L-arabinofuranosidases are active at pH values from 2 to 6 (8, 18), while the α-L-arabinofuranosidases from *S. diastaticus* ET retained more than 90% of their activity over a broad pH range of between 3.5 and 7.5. Interestingly, the two enzymes showed considerable differences in their thermal stabilities. While C2 retained 60% of its activity after 3 h of incubation at 50°C, C1 was almost completely inactivated by this treatment.

Both enzymes hydrolyzed *p*-nitrophenyl α-L-arabinofuran-

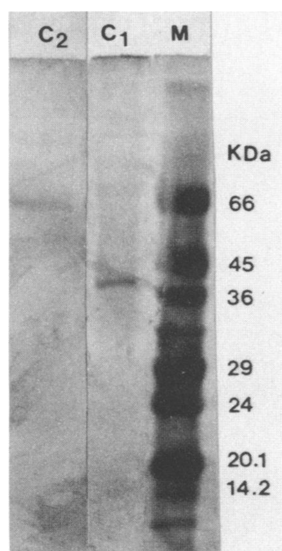

FIG. 2. SDS-PAGE of the purified α-L-arabinofuranosidases C1 and C2 from *S. diastaticus* ET. M, low-molecular-weight protein standards.

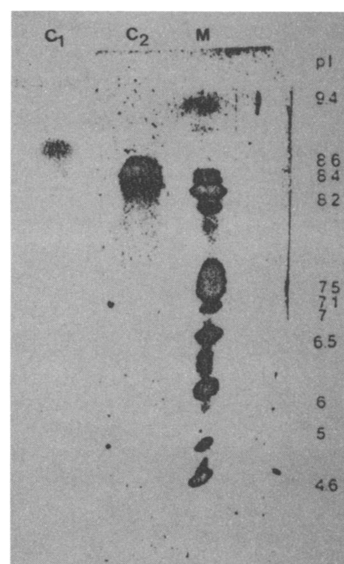

FIG. 3. IEF gel of the purified α-L-arabinofuranosidases C1 and C2. M, protein standards with pIs ranging from 4.6 to 9.6.

TABLE 2. Properties of the purified α-L-arabinofuranosidases C1 and C2 from *S. diastaticus* ET

α-L-Arabino-furanosidase	Molecular mass (kDa)	pI	pH optimum	K_m (mM)[a]	V_{max} (μmol min^{-1})[a]	Temp stability (% initial activity) after:	
						8 h at 25°C	3 h at 50°C
C1	38	8.8	4–7	10	0.4	50	10
C2	60	8.3	4–7	12.5	1.25	80	60

[a] With *p*-nitrophenyl-α-L-arabinofuranoside as substrate.

oside. In contrast to the α-L-arabinofuranosidase from *A. niger* (11) and *S. purpurascens* IFO 3389 (13), which hydrolyze either 1,5-arabinosyl linkages or 1,3-arabinosyl linkages, both α-L-arabinofuranosidases from *S. diastaticus* ET released arabinose from β-1,3-arabinoxylan and also from a debranched β-1,5-arabinan. These properties are similar to the substrate specificity of the α-L-arabinofuranosidase from *Streptomyces* sp. strain 17-1 (10).

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