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PURIFICATION AND CHARACTERIZATION OF A NEW XYLANASE FROM *HUMICOLA* GRISEA VAR. *THERMOIDEA*

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ABSTRACT

The thermophilic fungus *Humicola grisea* var. *thermoidea* secretes extracellular xylanase when grown on solid and in liquid media containing wheat bran and banana plant residue as substrates, respectively. At 55ºC, xylanase from the culture filtrate of *H. grisea* var. *thermoidea* grown on banana stalk retained 50% of its activity after 28 h of incubation. A xylanase (X2) was isolated from solid state cultures with wheat bran as the carbon source. It was purified to apparent homogeneity by ultrafiltration followed by ion-exchange and hydrophobic interaction chromatography on DEAE-Sepharose and Phenyl-Sepharose resins, respectively. The enzyme had an apparent molecular weight of 29 kDa, as determined by SDS-PAGE. The purified enzyme was most active at pH and temperature ranges of 4.5-6.5 and 55-60ºC, respectively. In addition, X2 showed thermostability at 60ºC with a half-life of approx. 5.5 h. The apparent Km values, using soluble and insoluble arabinoxylans as substrates, were 10.87 and 11.20 mg/ml, respectively.

Key words: *Humicola grisea*, xylan, xylanase

INTRODUCTION

Xylanases have potential applications in the food, chemical, pharmaceutical and paper industries (8,17). These enzymes are produced by several fungal species, including *Humicola grisea* var. *thermoidea*. The thermophilic fungus *H. grisea* var. *thermoidea* has attracted attention as a rich source of xylanolytic enzymes (1,13,20). Earlier studies have demonstrated that *H. grisea* var. *thermoidea* produces xylanases with different molecular masses. Some properties of these purified enzymes have been published (13,22). Solid-state cultivation on wheat bran as a substrate is an efficient procedure for xylanase production (17,23). Some of the advantages of the use of solid-state cultivation over liquid medium are described such as lower costs and the production of enzymes with higher specific activities (11). The banana plant (*Musa cavendishii*) produces a residual component named fruit stalk with holocellulose and lignin corresponding to 33% and 8.67% of the dry weight of this component, respectively (11). An earlier study reported the ability of *H. grisea* var. *thermoidea* to grow and produce xylanase activity in a medium containing banana stalk (11). In this study, we report the inductive effect of banana stalk on the xylanase activity of *H. grisea* var. *thermoidea* when grown in a liquid-state medium. The purification and some properties of a new xylanase (X2) from solid-state cultures of the fungus on wheat bran as a carbon source are also described.

MATERIALS AND METHODS

Growth conditions

For the production of xylanase in a solid-state medium containing wheat bran as the carbon source, *H. grisea* var. *thermoidea* was cultured at 42ºC for 7 days (20). After the growth procedure, the protein content of the solid-state culture flasks was extracted with 500 ml of 25 mM sodium phosphate buffer, pH 7.0 and placed on a rotary shaker (100 rpm) at room temperature for 3 h. The resulting crude extract was centrifuged for 30 min at 9,600 g and 4ºC, filtered and stored at 5ºC for

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subsequent use as source of xylanase. *H. grisea* var. *thermoidea* was also grown at 40ºC for 72 h with rotary shaking (100 rpm) in 1-liter Erlenmeyer flasks containing 300 ml of medium. Liquid cultures were inoculated with 10^7 spores ml^{-1} from a 7-day old culture. The composition of the medium (w/v) was as follows: 0.5% banana stalk, 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.06% yeast extract. The content of each flask was filtered through filter paper and hereafter called crude extract. For experiments on induction of xylanase activity, aliquots were harvested every 6 h during 16 days, and used to measure xylanase activity.

Assays

Xylanase activity was routinely determined by mixing 100 ml of enzyme solution with 200 ml of oat spelt xylan (1%, w/v) in 100 mM sodium acetate buffer, pH 5.0 at 50ºC for 30 min. The release of reducing sugar was measured using the dinitrosalicylic reagent method (12). Xylose was used as the standard. Xylanase activity was expressed as µmol of reducing sugar formed per milliliter and minute of enzyme solution, i.e., as IU ml^{-1}. Cellulase assay was carried out as described above using carboxymethylcellulose (CMC) as substrate. Protein concentration was quantified by the Bradford method (3) using bovine serum albumin as the standard. For the kinetic experiments, soluble and insoluble samples from oat spelt xylan were used as substrates in a concentration range of 1.5-25 mg/ml and 10-50 mg/ml, respectively. The substrates were prepared as described by Filho *et al*. (7). K_m and V_{max} values were estimated from the Michaelis-Menten equation with a non-linear regression data analysis program (10). The determination of optimum temperature of X2 was carried out in the temperature range of 30 to 80ºC. To determine the optimum pH of X2 activity at 50ºC, the range was from 3.0 to 8.0. The McIlvaine type buffer systems were adjusted with the same ionic strength with KCl (5). Temperature stability was determined by pre-incubating an enzyme sample at 55ºC or 60ºC. At various time periods, aliquots were withdrawn and the residual activity was measured under standard conditions. All activity values were repeated at least three times.

Enzyme Purification

All purification steps were carried out at 4ºC unless otherwise specified. The crude extract was concentrated and dialyzed (against 25 mM sodium phosphate buffer, pH 7.0) by ultrafiltration using an Amicon system with a 10 kDa cut-off point membrane (PM 10). The retentate was applied to a DEAE-Sepharose column (2.5 x 9.5 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.5. The column was washed with the same buffer and eluted with a linear gradient of NaCl (0-1 M). Fractions of 5.2 ml were collected at a flow rate of 90 ml/h. The fractions containing the highest xylanase activity were pooled from successive runs and submitted to hydrophobic interaction chromatography in a Phenyl-Sepharose column equilibrated with the above buffer at a flow rate of 120 ml/h. The residual protein was eluted with an ammonium sulphate descending gradient from 1 to 0 M. Fractions of 4.0 ml, corresponding to xylanase activity were pooled, concentrated by ultrafiltration and stored for later use at 4ºC.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was carried out as described by Laemmli (9) using 12% gels. After electrophoresis, the protein bands were silver stained by the method of Blum *et al*. (2). Low molecular weight standards from Sigma (USA) were used as size markers. A SDS-PAGE gel (12%) containing 1% oat spelt xylan was submitted to zymogram analysis (14). It was stained for xylanase activity in a Congo red solution (0.1%) for 30 min at room temperature and washed with 1 M NaCl to remove excess dye and fixed with 0.5% acetic acid.

Chemicals

Oat spelt xylan was purchased from Sigma Chemical Co., St. Louis, MO 63178, USA. Wheat bran was obtained from a local source. Banana stalk was kindly provided by Francides Gomes da Silva Jr. (University of São Paulo, Brasil). All other chemical were analytical grade reagents.

RESULTS AND DISCUSSION

The induction profile after growth of *H. grisea* var. *thermoidea* on fruit stalk as substrate showed that the time lag for induction of β-xylanase was about 18 h and thereafter the activity increased steadily and reached its highest value at 264 h of cultivation (results not shown). The growth profile was accompanied by several peaks of xylanase activity. Xylanase activity isolated from crude extract of *H. grisea var. thermoidea* grown on 0.5% banana stalk was most active over a temperature range of 55 to 60ºC. It dropped to 50% of its original activity after 28 h incubation at 55ºC. The influence of banana stalk on xylanase thermostability can not be discarded. Components of lignocellulosic substrates (coarse corn cobs) were found to improve the thermostability of crude xylanases from *Thermomyces lanuginosus* (21).

Procedure for purification of a new β-xylanase, X2, from *H. grisea* var. *thermoidea*, grown on wheat bran as substrate, is summarized in Table 1. The crude extract was concentrated by ultrafiltration and purified by a simple two step procedure on ion-exchange and hydrophobic interaction chromatographies. A major xylanase activity was detected in the concentrated crude extract (retentate), while a small amount of enzyme activity was found in the filtrate (permeate). The ability of xylanases to penetrate an ultrafiltration membrane has been reported before (18,19,23). It is suggested to be due to xylanase compact structure

and/or non-uniformity of membrane pore size (15,18). The retentate elution in DEAE-Sepharose chromatography resulted in the separation of two peaks of xylanase activity. X2 was eluted in the pre-gradient fractions, while the second xylanase activity was adsorbed to the ion exchange resin (Fig. 1). Further purification of X2 on Phenyl-Sepharose column showed three peaks of protein containing xylanase activity eluted after application of an ammonium sulphate descending gradient (Fig. 2). The fractions representing the third protein peak were pooled. After the Phenyl-Sepharose step, the enzyme was purified to apparent homogeneity as determined in a SDS-12% polyacrylamide gel (Fig. 3). The recovery of X2 was very low (6.40%), probably due to the presence of other xylan-degrading activities determined during the purification steps. We observed the presence of at least five xylanase activity peaks. The multiplicity of forms is commonly described for β-xylanases from fungi and bacteria as the result of differential mRNA processing and posttranslational modifications (7,19).

Table 1. Summary of the purification of xylanase (X2) from *H. grisea* var. *thermoidea*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
Culture filtrate	204.74	318.63	1.56	100	1
DEAE Sepharose	20.92	85.00	4.07	26.66	2.61
Phenyl Sepharose	4.58	32.60	10.23	6.40	4.55

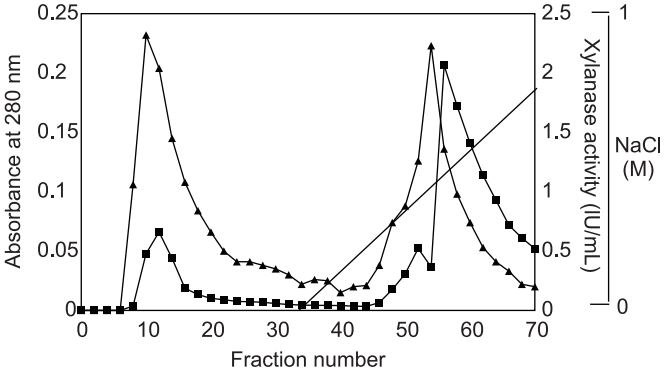

Figure 1. Fractionation on DEAE-Sepharose of *Humicola grisea* var. *thermoidea* crude extract., - ■ - absorbance at 280 nm; -▲- xylanase activity.

Since these enzymes act synergistically for the complete hydrolysis of xylan, the yield and fold values were probably underestimated. Furthermore, a small level of xylanase was present in the ultrafiltrate. Ultrafiltration procedure is reported to reduce xylanase recovery yields (15). Low purification yields for xylanase activities are often reported in the literature (7,15,18,22,23).

The enzyme migrated as a single 29 kDa band on SDS-PAGE (Fig. 3). The molecular weight found for X2 was within the range

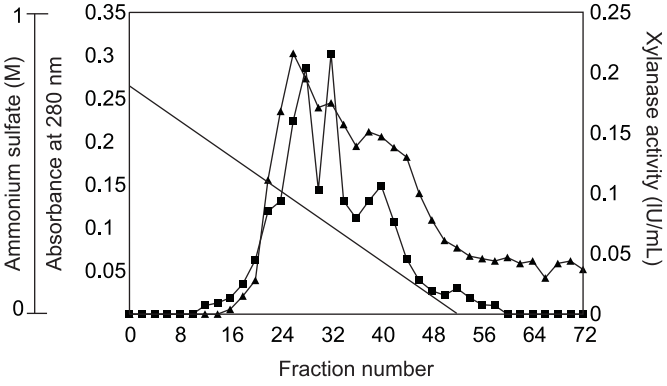

Figure 2. Hydrophobic interaction chromatography of β-xylanase activity on Phenyl-Sepharose, - ■ - absorbance at 280 nm; -▲- xylanase activity.

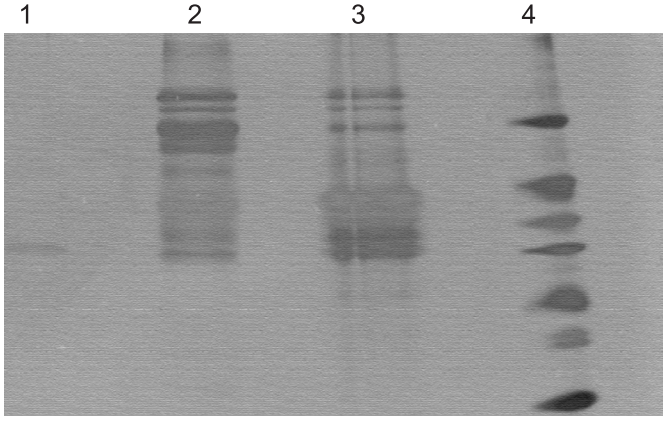

Figure 3. SDS-PAGE of X2 from *Humicola grisea* var. *thermoidea*. Lane 1, X2; lane 2, first xylanase peak from Phenyl Sepharose chromatography; lane 3, second xylanase peak from Phenyl Sepharose chromatography; lane 4; molecular weight standards (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), lactalbumin (14.2 kDa), aprotinin (6.5 kDa).

detected for xylanases belonging to the family G/11 (16). Smaller xylanases are able to penetrate further the fiber wall structure and modify more efficiently pulp properties (16). The molecular weight of X2 differed from the ones purified from crude extract samples of the same fungus strain, which were 13, 23, 25.5 and 95 kDa (13,22). The zymogram analysis was performed by renaturing X2 after SDS-PAGE. It showed enzyme activity that was visualized by staining with Congo red corresponded to the protein band (result not shown). The xylanase zymogram of the second protein peak from DEAE Sepharose chromatography revealed two xylanase-active bands.

Some of the properties of X2 are shown in Table 2. The purified enzyme gave the highest activity at a temperature range of 55-60ºC. This result was higher than the values reported for some xylanases from *Acrophialophora nainiana*, *Trichoderma harzianum* and *Penicillium capsulatum* (7,18,23). It was lesser than those of xylanase forms 1 and 2 from the same fungus (13) and similar to Xyl1 and Xyl2 from *Humicola insolens* (6). It is noteworthy that X2 was subjected to 30-min assay, while xylanase forms 1 and 2 had an incubation time of only 15 min. On the other hand, X2 showed better stability at 60ºC. After 5.5 h incubation at 60ºC, the enzyme retained 50% of its original activity, while xylanase form 2 had a half life of only 20 min at the same temperature. Xyl2 from *H. insolens* lost its activity completely after 45 min at 60ºC (6). The degree of thermostability of X2 was also higher than those found for specific and non specific xylanases from *P. capsulatum* (7) and XynIII of *A. nainiana* (4). In contrast to the xylanase forms 1 and 2, Xyl1 and Xyl2, X2 was optimal at a broader pH range of 4.5-6.5. Furthermore, X2 only showed a residual activity against CMC. The xylanases from family G/11 are reported to be cellulase-free (16).

The kinetic parameters of X2 were investigated using soluble and insoluble forms of oat spelt xylan as substrate (Table 2). The enzyme showed higher affinity for the branched xylan (soluble form). The hydrolysis of soluble oat spelt xylan was more effective than when the enzyme was incubated with the insoluble xylan, suggesting that the presence of a particular type of substituent (arabinofuranosyl group) in the vicinity would be a requirement for the action of X2 (19). In this case, the substituent is required for the proper orientation of the substrate in the catalytic site (18). However, the hypothesis that the substituents are probably located in regions of the substrate distant from the unsubstituted portions can not be discarded (18). Xyl 1 and Xyl 2 from *H. insolens* require a sequence of at least three unsubstituted xylose residues for their active binding (23).

Table 2. Some properties of purified X2 from *Humicola grisea* var. *thermoidea*.

Molecular weight (SDS-PAGE)	29 kDa
Highest pH	4.5-6.5
Highest temperature	55-65ºC
Thermostability at 60ºC (half-life)	5.5 h
Km (soluble xylan)	10.87 mg/ml
Km (insoluble xylan)	11.20 mg/ml

In conclusion, for the bioconversion of lignocellulose residues, it is desirable to have a microorganism capable of producing a range of enzyme activities that interact synergistically (10). In our case, *H. grisea* var. *thermoidea* produced a xylanase activity when grown in the presence of banana stalk or wheat bran as the carbon source. Since xylanases with useful pH optima and high temperature stabilities are of commercial importance, the wide pH profile and good thermostability of X2 make it a valuable tool for application in the process of prebleaching cellulose pulps. Based on its small molecular weight, X2 is suggested to belong to family G/11.

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RESUMO

Produção, purificação e caracterização de uma nova Xilanase de *Humicola grisea* var. *Thermoidea*

O fungo termofílico *Humicola grisea* var. secreta xilanase extracelular quando cultivado em meios sólidos e líquidos contendo farelo de trigo e engaço de bananeira como substratos, respectivamente. À temperatura de 55ºC, xilanase do filtrado de meio de cultura de *H. grisea* var. *thermoidea* cultivado em engaço de bananeira reteve 50% de sua atividade após 28 de incubação. Uma xilanase (X2) foi isolada de culturas de estado sólido contendo farelo de trigo como fonte de carbono. X2 foi purificada por ultrafiltração, seguido por cromatografias de interação hidrofóbica e troca iônica em resinas de Phenyl-Sepharose e DEAE-Sepharose, respectivamente. A enzima apresentou peso molecular de 29 kDa, como determinado por SDS-PAGE. A enzima purificada foi mais ativa em intervalos de pH e temperatura de 4,5-6,5 and 55-60ºC, respectivamente. Além disso, X2 mostrou termoestabilidade a 60ºC com meia vida de aproximadamente 5,5 h. Os valores de Km aparente, utilizando arabinoxilanas solúveis e insolúveis, foram 10,87 and 11,20 mg/ml, respectivamente.

Palavras-chave: *Humicola grisea*, xilana, xilanase

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