Document No: IRG/WP/1318

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

WORKING GROUP I a — Biological Problems (Flora)

Physical Properties of ß-1,4-Xylanase Produced by Postia (= Poria) Placenta: Implications for the Control of Brown Rot

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Paper prepared for the Eighteenth Annual Meeting

Honey Harbor, Ontario, Canada

17 - 22 May 1987

IRG Secretariat
Drottning Kristinas väg 47C
S - 114 28 Stockholm
SWEDEN

6 March 1987

Physical properties of ß-1,4-xylanase produced by Postia (=Poria) placenta: implications for the control of brown rot

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SUMMARY

The degradation of hemicelluloses is an early event in wood decay by brown-rot fungi. An understanding of the physical properties of hemicellulases may suggest target mechanisms for the development of new control agents. Endo-ß-1,4-xylanase was partially purified by column chromatography from wood decayed by Postia (= Poria) placenta. The enzyme was extremely resistant to denaturing conditions; no loss of activity was detected after 2 h in 9 M urea or 6 M guanidine-HC1. Boiling the enzyme for 5 min in 2.5% SDS + 0.5% ß-mercaptoethanol reduced its activity by 65%, as measured by the production of reducing sugars. The activity of α-D-galactosidase, another enzyme detected in large quantities in the decayed wood, was reduced by 98% under these conditions. Optimum pH and temperature ranges were pH 2 - 6 and 50 - 60°C, respectively. The enzyme appears to be a glycoprotein containing 50 - 60 % carbohydrate (w/w); the carbohydrate moiety may protect the enzyme from adverse environmental conditions. The control of brown rot by in situ inactivation of xylanase may not be feasible because of the enzyme's extreme stability.

Key words: brown-rot fungi, xylanase, hemicellulase, wood decay

INTRODUCTION

The control of wood decay by white- and brown-rot fungi is currently dependent on the application of' broad-spectrum biocides, many of which are being banned or restricted because of their toxicity. An understanding of the physiology of wood decay fungi could lead to the development of more specific control agents which would target specific pathways of fungal metabolism. The mechanisms involved in wood degradation by brown-rot fungi are not fully understood, but the production of cellulose- and hemicellulose-degrading enzymes are clearly essential to the process. The depolymerization and removal of hemicellulose precedes, and may be a requirment for, cellulose depolymerization in both conifers and hardwoods (Kirk and Highley, 1973; Highley, 1987) , thus the inactivation of hemicellulose-degrading enzymes could inhibit or prevent subsequent stages of decay. For this reason, the activity and stability of endo-ß-1,4-xylanase, a hemicellulase detected in large quantities in wood decayed by Postia placenta (Fr.) M. Lars. et Lomb. [= Poria placenta (Fr.) Cke.],

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were studied under a variety of denaturing conditions. Portions of this work have been previously reported (Green et al., 1987).

MATERIALS AND METHODS

Enzyme production and purification: Enzyme production and purification were conducted as previously described (Green et al., 1987) with some modifications. An aqueous extract was prepared from wood blocks decayed by P. placenta. This extract was concentrated by ultrafiltration or alcohol precipitation. In subsequent extractions, the aqueous extract was concentrated by lyophilization, which proved to be more efficient in preserving enzyme activity. The concentrated material was then partially purified by gel filtration chromatography, using Sepharose 6B (Pharmacia, Piscataway, NJ) followed by Fractogel TSK HW-55 (F) (EM Science, Gibbstown, NJ).

Enzyme assays: The activity of endo-ß-1,4-xylanase (E.C. 3.2.1.8) was detemined by the increase in reducing groups at 40°C after 24 h (Nelson, 1944). The substrate was larchwood xylan (Sigma Chemical Co., St. Louis, MO). One unit of enzyme activity was defined as the amount required to liberate reducing power equivalent to 1 µg glucose per 24 h at 40°C. The activity of α-D-galactosidase (E.C. 3.2.1.22) and ß-D-galactosidase (E.C. 3.2.1.23) was determined by the liberation of p-nitrophenol from the respective p-nitrophenyl derivatized substrates (Nelson, 1944). One unit of enzyme activity was defined as the amount of enzyme which will liberate 1 µM of p-nitrophenol per hour.

Determination of thermal activity and stability: The effect of temperature on enzyme activity was determined by assaying the column-purified enzyme for activity at 25, 40°, 50°, 60° and 70°C for 24 h. The thermostability of xylanase was determined by holding the column-purified enzyme at the above temperatures for 1 h, followed by the standard assay at 40°C for 24 h. The length of time that the enzyme needed to be exposed to high temperatures in order to affect stability was also determined. Column-purified enzyme initially concentrated by lyophilization, was subjected to 60° and 80°C for 30, 60, 90 and 120 min. In a second experiment, the same preparation was heated to 60°, 65°, 70°, 75° and 80°C for 30 min.

Effect of pH on activity and stability: The effect of pH on enzyme activity was determined by assay in 1:1 (v/v) dilutions of column-purified enzyme and the following buffers: 0.05 M citrate, pH 2.0 - 6.0; 0.05 M phosphate, pH 7.0; and 0.05 M Tris-C1, pH 8.0 and 9.0. The effect of pH on enzyme stability was measured by incubating the enzyme-buffer mixtures for 24 h at 4°C, and then conducting the enzyme assay in 0.1 M citrate, pH 5.0, for 24 h at 40°C.

Stability to denaturation: Column-purified xylanase was subjected to 100°C for 5 min in 0.01 M Tris-C1, pH 7.0, with 2.5% SDS and 0.5% ß-mercaptoethanol. Column-purified xylanase was also incubated with 9 M urea and 6 M guanidine hydrochloride and incubated at 25°C for 2

h. Losses in enzyme activity were determined by the reducing sugars assay.

Stability to phenols: Lyophilized crude extract was reconstituted in 0.1 M citrate buffer, pH 5.0, at the rate of 10 mg/ml and dialyzed for 48 h in the same buffer to remove excess reducing sugars. The enzyme mixture was then added to dilutions of catechol, vanillin and tannic acid for final phenolic concentrations selected from among the following: 0.01, 0.05, 0.1, 0.5, 1 and 10%. The higher concentrations of tannic acid and catechol could not be used since they interfered with the reducing sugars assay. Stock solutions of phenolics were made in absolute ethanol and adjusted to pH 5.0 with 0.01 M HC1 or NaOH. The enzyme-phenol mixtures were incubated in the dark at 25°C for 2 h. The decrease in reducing sugars was then determined after 24 h at 40°C.

Stability to protease - Dialyzed crude enzyme and column-purified xylanase, both of which had been concentrated by alcohol precipitation or ultrafiltration, were incubated with 1 mg/ml protease (Sigma #P-7026) for 2 h at 25°C. The percent inhibition of xylanase and α - and B-D-galactosidase activities were calculated.

RESULTS

The effects of temperature and pH on the activity and stability of xylanase, when concentrated by alcohol precipitation and ultrafiltration, are presented in Fig. 1. The optimum conditions for enzyme activity were 40 - 60°C and pH 2 - 6. Xylanase was more tolerant of extreme conditions when concentrated by alcohol precipitation than by ultrafiltration. The enzyme was most stable at 20 - 40°C and pH 4 - 6. The alcohol-precipitated xylanase was more stable at higher temperatures than the ultrafiltered enzyme. The length of time that the enzyme needed to be exposed to high temperatures to affect activity is shown in Fig. 2. The stability of the column-purified enzyme, which was initially concentrated by lyophilization, was not altered after 2 h at 60°C but was greatly inhibited after 30 min at 80°C. The same preparation lost 30% of its activity after 30 min at 65° and 70°C, and 80 - 90% of its activity at 75° and 80°C (Fig. 3).

Roiling xylanase for 5 min in 2.5% SDS + 0.5% ß-mercaptoethanol reduced its activity by 66%. The activity of α-D-galactosidase, another enzyme found in large quantities in the decayed wood, was reduced by 98% under these conditions. Lyophilization of the crude enzyme extract resulted in a 38 - 58% loss of xylanase activity and a 72 - 80% decrease in the activity of α-D-galactosidase. Xylanase activity was not. affected by 2 h incubations in 9 M urea or 6 M guanidine-HCl.

Column-purified xylanase was not degraded by a commercial protease (Table 1). Xylanase activity in the crude enzyme extract was significantly decreased in the ultrafiltered material but was not affected in the alcohol-precipitated preparation. The activity of

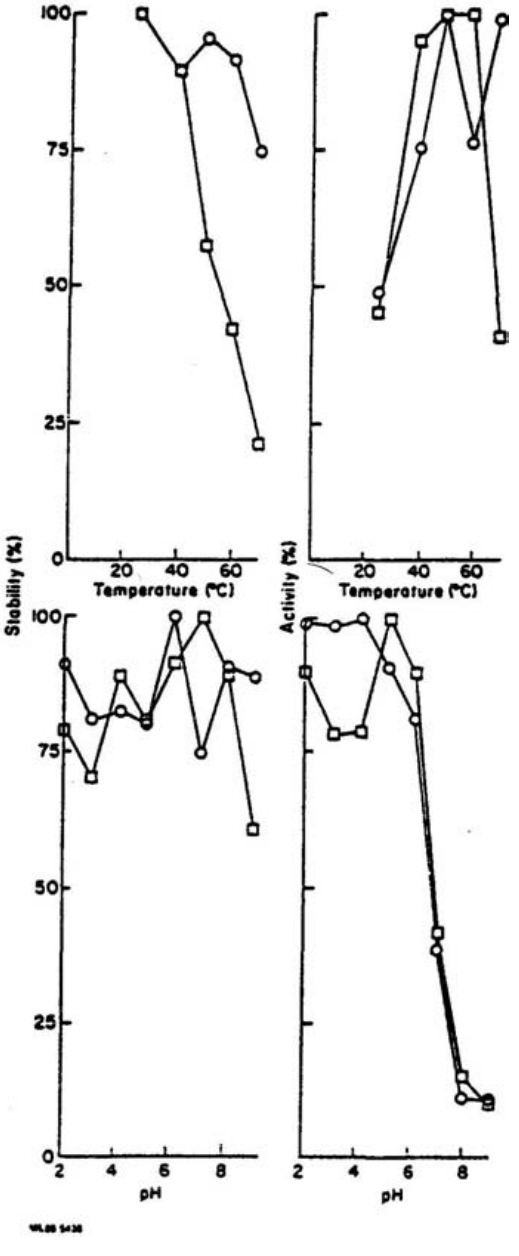

Figure 1 Effect of temperature and pH on activity and stability of xylanase fraction of P. placenta following gel filtration on Fractogel HW-55. Activity or stability values represent percent of highest value determined. □ = ultrafiltered, ○= ETOH precipitate

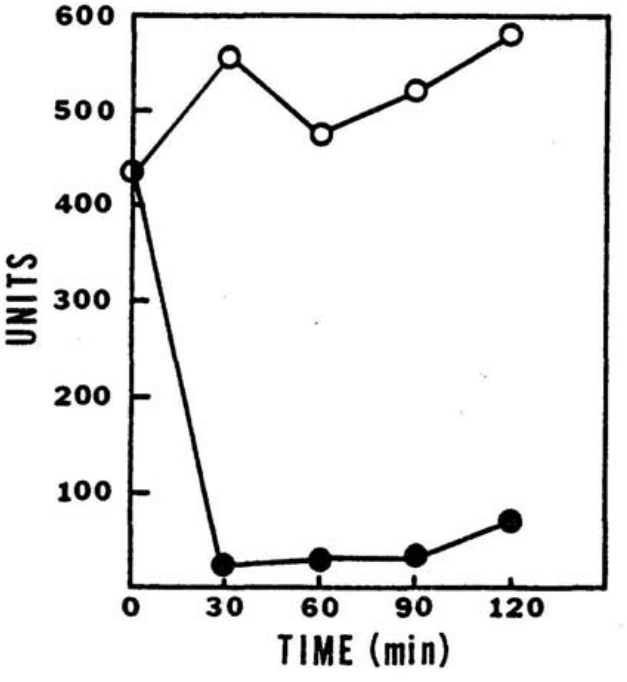

Figure 2 Effect of time on the activity of xylanase at 60° (0) and 80° (●) C

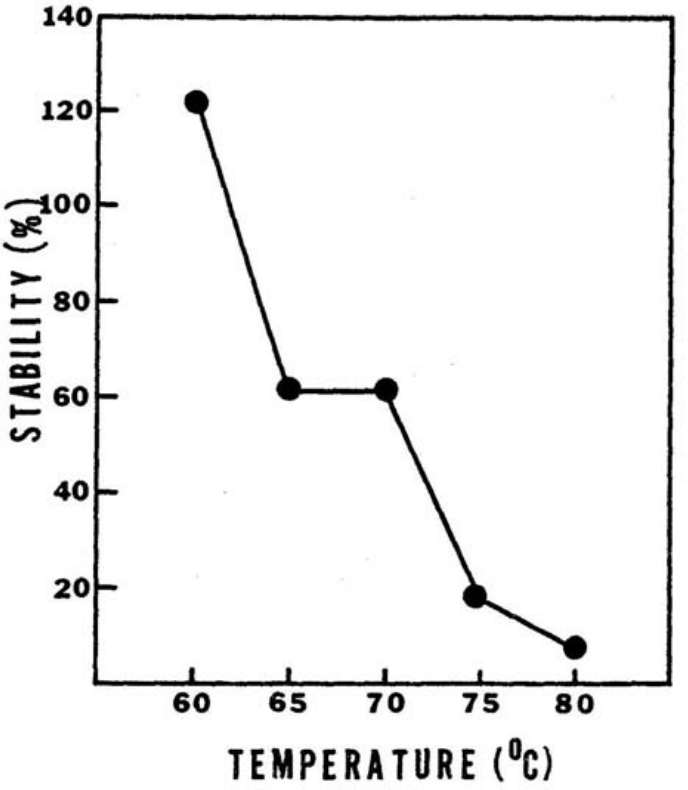

Figure 3 Effect of elevated temperatures for 30 min on percent stability of xylanase

Table 1 Influence of protease on the activity of the crude enzyme preparation and column-purified xylanase

Enzyme	Preparation	% Inhibition of Activity		
		Xylanase	α - D-galactosidase	ß–D–galactosidase
Crude	ultrafiltered	18.0	0	63.3
	alcohol-precipitated	1.2	0	1.2
Purified	ultrafiltered	0	-	-
	alcohol-precipitated	0	-	-

ß-D-galactosidase was significantly reduced in the ultrafiltered material but was not influenced in the alcohol-precipitated sample. The activity of α-D-galactosidase was not affected by the presence of pro tease,

Relatively high concentrations of the three phenolics were required to inhibit the activity of xylanase (Table 2). Catechol was the most toxic substance. A catechol concentration of 0.1% (9 mM) totally inhibited xylanase activity. Tannic acid was less toxic; a concentration of 0.5% (15 mM) was required for total inhibition. The enzyme was most resistant to vanillin; a concentration of 1% (31 mM) resulted in 40% inhibition. Low concentrations of the phenolics appeared to stimulate enzyme activity.

DISCISSION

The endo-ß-1,4-xylanase, which was partially purified from wood decayed by P. placenta, was extremely stable and survived conditions which typically denature enzymes, such as low pH, boiling in SDS, and exposure to 9 M urea, 6 M guanidine hydrochloride, protease and phenolics. Highley and Wolter (1982) also reported that the enzyme, as part of a multienzyme complex, was very stable in the presence of chelators, sulfhydryl inhibitors, and most heavy metals (with the exception of mercury and silver). One possible reason for this great stability is the large amount of carbohydrate associated with the enzyme. Green et al. (1987) determined that the column-purified xylanase contained 59% carbohydrate (w/w) when concentrated by alcohol precipitation and 48% carbohydrate (w/w) when concentrated by ultrafiltration. It is uncertain whether this carbohydrate is covalently bound to the protein, forming a true glycoprotein, or whether a weaker association is involved. The fact that the carbohydrate content varied with the concentration procedure suggests that at least a portion of the carbohydrate is artifactual. Multiple passages of the enzyme down a gel filtration column did not significantly reduce its carbohydrate content. (Green et al., 1987), as occurred with the weakly associated carbohydrate on the cellulases of Trichoderma viride (Alurralde and Ellenrieder, 1984). These data suggest that the enzyme is a glycoprotein.

The biological roles of carbohydrate moieties on glycoproteins are quite diverse and include the stabilization of protein conformation and resistance to proteolysis (Sharon and Lis, 1982). The larger amount of Carbohydrate contained in the alcohol-precipitated material helps to explain the greater Stability of this preparation to pH and temperature extremes. Column-purified xylanase was highly resistant to protease, although some degradation was observed in the crude material. It is possible that the protease was acting in concert with other contaminating substances to break down the xylanase.

One exception to the enzyme's extreme stability is its high susceptibility to basic pH (> 8.0). This information corresponds to Highley's (1973) observation that treatments which increase the pH of wood can provide protection against decay by brown-rot fungi.

Table 2 Inhibiton of xylanase by phenolics[a]

Compound	Concentration (%)	Enzyme Activity units/ml	Percent of Activity Control
Vanillin	0.00	100	100
	0.01	140	140
	0.10	160	160
	1.00	60	60
	10.00	0	0
Catechol	0.00	80	100
	0.01	100	125
	0.05	80	100
	0.10	0	0
	0.50	0	0
Tannic acid	0.00	80	100
	0.01	80	100
	0.05	120	150
	0.10	100	125
	0.50	0	0

[a] Average of three replications

Phenolics, which may be formed during the synthesis and degradation of lignin in woody tissues, are inhibitory to many enzymes. Their toxicity is based on their ability to bind to and inactivate proteins (Haslam, 1974). Xylanase was relatively resistant to vanillin and tannic acid but was more susceptible to catechol. These data correspond with those of Sharma et al. (1985), who found that most phenolics, with the exception of guaiacol and caffeic acid, did not affect the xylanase activity of Aspergillus japonicus at concentrations of 0.1%; catechol was not included in their study. Highley (1973) reported that only the oxidized forms of catechol and vanillin inhibited the soluble cellulases of brown-rot fungi; nonoxidized compounds did not affect cellulase activity. The growth of P. placenta in culture is totally inhibited by several phenolics at lower concentrations than inhibited xylanase (Highley, unpublished data). The compounds are evidently interfering with some other aspect of metabolism. The carbohydrate moiety associated with the xylanase may be protecting the enzyme from the phenolic material. Glycoproteins present on the spores of Colletotrichum graminicola have a high affinity for purified condensed tannins and may protect the fungus from the toxic phenolics present in plant tissue (Nicholson et a1., 1986). The presence of phenolics in low concentrations appeared to increase the activity of the xylanase; certain glycoprotein-phenolic interactions may actually be beneficial for the fungus.

The extreme stability of xylanase under denaturing conditions suggests that the inactivation of this enzyme, as a means for the control of wood decay, may be difficult. One exception is the enzyme's susceptibility to high pH; this may be exploited by using treatments which increase the pH of wood. Our research into the production, secretion and distribution of this enzyme is continuing in the hopes of finding other physiological targets for control agents.

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