Indian J. Plant Physiol., Vol. 7, No. 1, (N.S.) pp. 15-19 (Jan.-March, 2002)

A COMPARATIVE STUDY ON THE PHYSIOCHEMICAL PROPERTIES OF DIFFERENT TYPES OF XYLANASES FROM *AEROMONAS CAVIAE* W-61

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Received on 2 June, 2001, Revised on 1 Nov., 2001

SUMMARY

***Aeromonas caviae* W-61 produced five types of xylanases 1, 2, 3, 4, and 5 extracellularly. Xylanases 1, 2, 3, 4, and 5 have been purified to homogeneity by using ultrafiltration, salt precipitation with ammonium sulphate and column chromatographies with DEAE-Toyopearl 650M, CM-sephadex C-50 and hydroxylappatite, followed by high performance liquid chromatography with a TSK gel phenyl-5PW and DEAE-5PW column. The purified xylanases 1, 2, 3, 4, and 5 consists of single polypeptides with molecular masses of 22, 41, 58, 120 and 140Kda, respectively.**

The optimum pH and temperature of xylanases 1, 2, 3, 4, and 5 were 7.0, 5.5, 5.0, 6.0 and 6.0 and 55°C, 45°C, 50°C, 40°C and 40°C respectively. These xylanases showed no activity towards cellulose, CM-cellulose, arabinose and β-1, 3 xylan. These five types of xylanases degraded β-1, 4-xylan, producing a mixture of different types of xylo-oligosaccharides such as xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexose. Its action patterns on β-1, 4-xylan indicate that they are β-1, 4-endoxylanase.

INTRODUCTION

Xylan the major portion of the hemicellulose of plant cell walls, are heterogeneous polysaccharides. It is built from a homopolymeric backbone chain of 1, 4-linked β-D-xylopyranose units and short chains including O-acetyl, β-L-arabinofuranosyl and D-glucuronyl (Whistler 1970). Xylanases catalyze the hydrolysis of xylan to xylo-oligosaccharides and xylose. There has been increasing interest in applying xylanases in the pulp and paper industry during recent year. Viikari (1994) initially demonstrated the use of xylanases for the selective removal of hemicelluloses from kraft pulp prior to pulp bleaching. Since then, research has been performed on this subject. Possible application for xylanases in biotechnology are digestion of industrial and agricultural wastes, as well as reduction of the amount of required chemicals (chlorine) in paper pulp bleaching, thus reducing the release of polychlorinated compounds, suspected to be human carcinogens (Sunna 1997). Xylanases can also be used in increasing the brightness of pulp, improving the digestibility of animal feed, in clarification of juices, preparation of dextrans for the use as food thickeners, in poultry diets, production of fluids and juices from plant materials, in processes for the manufacture of liquid coffee, adjustment of wine characteristics and enhancement of astaxanthin extraction (Prade 1995).

Nguyen (1991) isolated a xylanases producing bacterium, *Aeromonas caviae* W-61, which secreted five xylanases of different molecular sizes, xylanases 1, 2, 3, 4, and 5 in the culture fluids of the bacterium. They also

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purified and characterized the xylanases 1, 2, and 3 from this bacteria (Nguyen 1993). Roy (2000) also purified the other two high-molecular-weight xylanases 4 and 5 from the culture fluids of the bacterium. In this study, we showed that the five xylanases have different molecular masses and different physico-chemical properties such as molecular size, optimum pH, temperature, different hydrolysis products and substrate specificities.

MATERIALS AND METHODS

The bacterial strain *A. caviae* W-61, isolated previously (Nguyen 1991) was used throughout this experiment. *A caviae* W-61 was aerobically grown in liquid medium I containing 0.5% oat spelt xylan, 0.2% yeast extract, 0.25% NaCl, 0.5% NH_4Cl, 1.5% KH_2PO_4, 3% Na_2HPO_4, and 0.025% $MgSO_4\ 7H_2O$. The bacteria was pregrown for 48 hour at 30°C. The pregrown cells were incubated into 700 ml of medium I in a 3,000 ml shaking flask at 30°C. After 48 hour of incubation, the culture fluid was obtained by centrifugation at 10,000 xg for 15 min and used as a crude enzyme preparation.

Assay for xylanase activity: Xylanase activity was measured by the amount of the reducing sugar liberated from oat spelt xylan. The reaction mixture contained 0.25 ml of the purified enzyme and 0.25 ml of 1% xylan in 50 mM sodium phosphate buffer, pH 7.0 The reaction was done at 37°C for 10 min. The reducing sugar formed was measured by Somogyi's method (Somogyi 1952) using D-xylose as a standard. One unit of xylanase was defined as the amount of enzyme which liberated 1 μmol of xylose per min.

Protein assay: Protein was measured by the method of Bradford using bovine serum albumin as a standard (Bradford 1976).

Electrophoresis: Protein from the column was measured at A 280. The protein concentrations of pooled samples was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Protein-containing fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The stacking and resolving gels were stained with coomassie brilliant blue, R-250.

Zymogram analysis: Xylanases in the test samples were differentially assayed by the zymography using Remazol brilliant blue (RBB)-stained xylan, as described previously (John 1990). Briefly, xylanases in the samples were separated by SDS-PAGE, followed by renaturation in a solution of 2.5% TritionX-100. The SDS-polyacrylamide gel was layered on a 1.3% agarose gel containing 0.5% RBB-stained xylan, and incubated at 37°C for 1-2 hour. After the incubation, the gels were immersed in an 66% (v/v) ethanol solution to remove cleaved products from RBB-stained xylan.

Purification of xylanases 1, 2, 3, 4, and 5: After concentration of culture supernatant by ultrafiltration, xylanases were precipitated with ammonium sulphate at a concentration corresponding to 75% saturation. The resultant precipitates were collected by centrifugation at 12,000 xg for 20 min, dissolved in 50 mM sodium phosphate buffer (pH 7.0). The dialyzed sample was mixed with 70 ml of the DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) and the mixture was stirred for 30 min and centrifuged at 10,000 xg for 10 min. The supernatant which contained xylanases 1, 2, and 3 was removed and purified by the method described previously. The precipitated DEAE-Toyopearl 650M resins, which absorbed the xylanases 4 and 5 were also purified as described in our previous study (Roy 2000).

Thin-layer-chromatography (TLC): Hydrolysis products produced by the action of β-1, 4-xylanases 1, 2, 3, 4, and 5 were characterized by TLC using Merch Silica Gel 60 F 254 plates (0.25 mm thickness) with the solvent system of 1-butanol-2-propanol-water-acetic acid (7:5:4:2, v/v). The sugars on the plates were detected by heating at 110°C for 30 min after spraying a mixture of aniline-diphenylalanine-acetone-85% phosphoric acid (0.4:0.3:20:3, w/w). D-xylose and xylo-oligosaccharides (X2-X6) were used as the standards.

RESULTS

Multiplicity of xylanases from *A. caviae* W-61: The detailed analysis by zymography of the extracellular culture fluid of *A. caviae* W-61 showed five xylanase activity bands with different molecular weights (Fig.1), indicating that *A. caviae* W-61 has a system of multiple extracellular xylanase enzymes.

Purifications of xylanases 1, 2, 3, 4, and 5: Xylanases 1,2,3,4 and 5 from *Aeromonas caviae* W-61 has been purified and reported described in Materials and Methods. The purified xylanases 1, 2, 3, 4, and 5 gave a single band of protein on SDS-PAGE with a different molecular masses of 22, 41, 58, 120, and 140 KDa respectively, shown inFig 2.

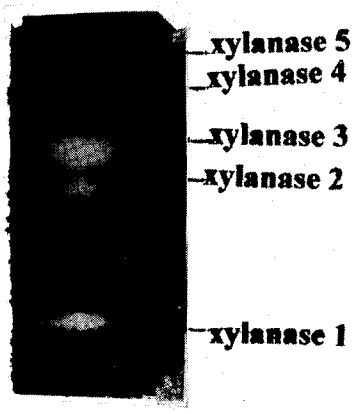

Fig. 1. Zymogram analysis of xylanases 1, 2, 3, 4 and 5 in the culture supernatant of *A. caviae* W-61

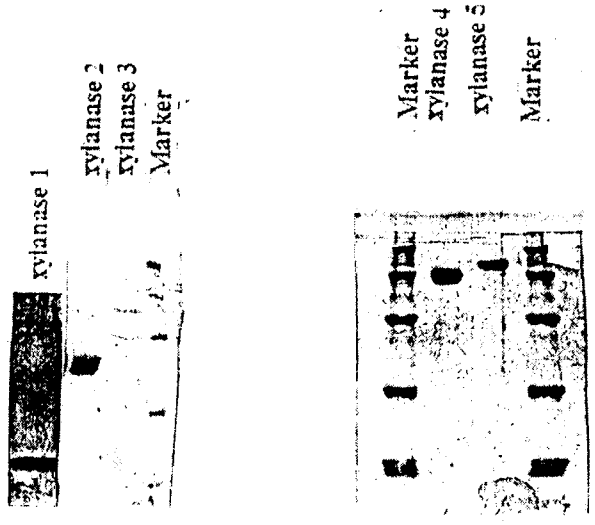

Fig. 2. SDS-PAGE pattern of purified xylanases 1, 2, 3, 4 and 5 M= Marker protein (from top) : myosin (200 Kda), β- galactosidase (116 Kda), bovine serum albumin (66 Kda), Aldolase (42 Kda), Carbonic anhydrase (30 Kda) and myoglobulin (17 Kda)

Effect of pH and temperature on the xylanases activities: To identify the optimal pH of the enzyme activities, 50 mM sodium acetate buffer (pH 4.0 to 5.5), 50 mM sodium phosphate buffer (pH 6.0 to 8.0) and 50 mM Tris-hydrochloride buffer (pH 8.5 to 9.0) were used. The optimal pH for the enzyme activities of xylanases 1, 2, 3, 4 and 5 were 7.0, 5.5, 5.0, 6.0, and 6.0 respectively when the activities were measured over a pH range from 4.0 to 9.0 (Fig 3). The optimal temperatures for activities

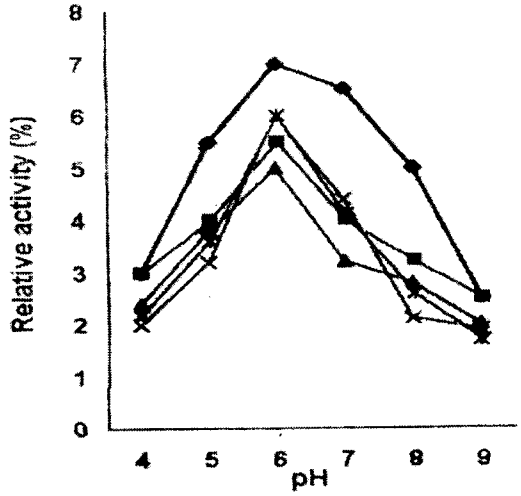

Fig. 3. Effects of pH on the xylanases 1, 2, 3, 4 and 5 activity
• Xylanase 1 ■ Xylanase 2 ▲ Xylanase 3
✖ Xylanase 4 ✱ Xylanase 5

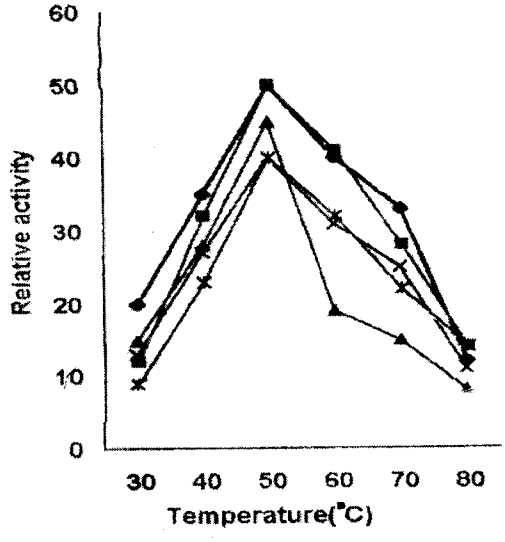

Fig. 4. Effects of temperature on the xylanases 1, 2, 3, 4 and 5 activity
• Xylanase 1 ■ Xylanase 2 ▲ Xylanase 3
✖ Xylanase 4 ✱ Xylanase 5

of xylanases 1, 2, 3, 4, and 5 were 55°C, 45°C, 50°C, 40°C and 40°C, when the enzyme activities were examined at various temperatures from 30-80°C (Fig 4).

Substrate specificity of xylanases 1, 2, 3, 4 and 5: Substrate specificities of the xylnases 1, 2, 3, 4, and 5 were studied using various polysaccharides as the substrates, and the results obtained summarized in Table 1. The xylanases 1, 2, 3, 4, and 5 hyhdrolyzed water-soluble xylan, birch wood xylan and beech wood xylan efficiently. The xylanases 1, 2, 3, 4, and 5 showed no hydrolytic activity towards β-1, 3-xylan, cellulose and carboxymethyl-cellulose.

Analysis for the products from the oat spelt xylan cleaved by the xylanases 1, 2, 3, 4 and 5: Oat spelt xylan (1mg) was incubated with the xylanases 1, 2, 3, 4 and 5 in 50 mM sodium phosphate buffer (pH 7.0), at 37°C for 0-240 min, and the products were analyzed on a thin layer chromatography. As shown inFig 5 the xylanases 1, 2, 3,

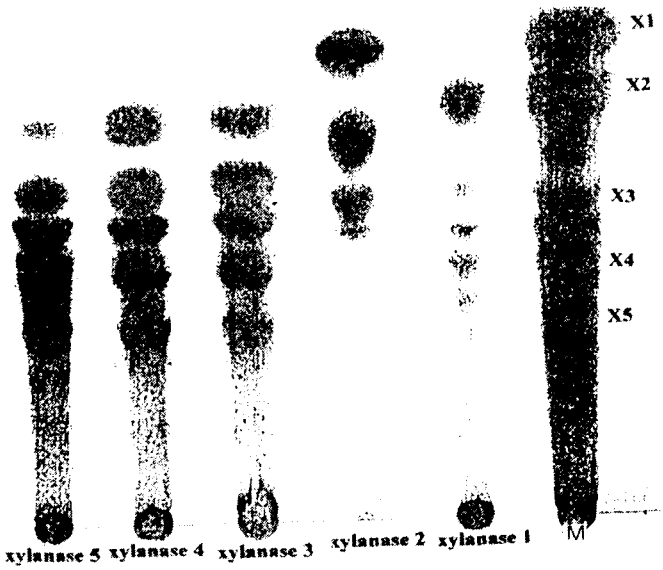

Fig. 5. TLC of hydrolysates of oat spelt xylan by the purified xylanases 1, 2, 3, 4 and 5

Table 1. Substrate specificities of xylanases 1, 2, 3, 4, and 5 from *A. caviae* W-61

Substrate	Reltive hydrolysis activities (%)				
	Xylanase 1	Xylanase 2	Xylanase 3	Xylanase 4	Xylanase 5
Oat spelt xylan	100	100	100	100	100
Birchwood xylan	-	176	675	200	220
H_2O soluble xylan	-	165	500	110	120
H_2O insoluble xylan	-	38	10	38	35
Cellulose	0	0	0	0	0
CM-Cellulose	0	0	0	0	0
Starch	0	0	0	0	0
β-1, 3-xylan	0	0	0	0	0

Table 2. Properties of various β-1, 4-xylanases from *Aeromonas* spp.

Species and Strain	Enzyme	PI	Molecular weight	Optimal pH	Optimal Temp (°C)	Hydrolytic products
A. caviae W-61	Xylanase 1	9.2	22,000	7.0	55	X2, X3, X4,
	Xylanase 2	11.5	41.000	5.5	45	X2, X3, X4,
	Xylanase 3	2.5	58.000	5.0	50	X2, X3, X4
	Xylanase 4		120,000	6.0	40	X2, X3, X4
	Xylanase 5		140,000	6.0	40	X2, X3, X4
A. caviae ME-1	Xylanase 1		20.000	7.0	50	X3, X4, X5
A. sp. Strain No. 212	Xylanase S		23,000	5-7	60	X2, X3, X4
	Xylanase M		37,000	6-8	50	X2, X3, X4
	Xylanase L		145,000	7-8	50	X2, X3, X4

4, and 5 hydrolyzed oat spelt xylan to liberate X2, X3, X5, and X6.

Table 2 shows some properties of endoxylanases 1, 2, 3, 4, and 5 from *A. caviae* W-61 and those from *Aeromonas spp.*, including molecular mass and heat stability. The distinct differences among xylanases 1, 2, 3, 4, and 5 of *A. caviae* W-61 were molecular weight, optimal temperature and pH for their activities. From the table it is seen that some differences were also found with molecular weight, optimal temperature, and pH for the enzyme activity reported previously.

DISCUSSION

As seen in this study, *A. caviae* W-61 produced an enzyme system of multiple xylanases. The cooperative actions of xylanases in this system may be effective for overall hydrolysis of xylan complexes as was found with some other microorganisms (Wong 1988).

Xylanases 1, 2, 3, 4, and 5 of *A. caviae* W-61 hydrolyzed β-1,4-xylan but not β-1,3-xylan, thus they belong to the β-1, 4-xylanase group. Xylanases 1, 2, 3, 4, and 5 of *A. caviae* W-61 did not hydroloyze arabinose substituents from heteroxylans. Hence they are non-debranching endoxylanases according to the classification of endoxylanase enzyme proposed by Dekker (1976). The xylanases 1, 2, 3, 4, and 5 from *A. caviae* W-61 had no activity on cellulose, carboxymethyl cellulose, or soluble starch and thus are true xylanases. These properties were also found similar with the xylanases from *B. pumillus* but different from xylanase A of *C. acetobutylicum ATCC* 824 (Lee 1987). These five types of xylanases show no β-xylosidase activity using p-nitrophenyl-β-D-xylopyranoside as a substrate (results not shown). The five types of xylanases cleaved oat spelt xylan randomly to form xylobiose and higher oligosaccharides. These results indicated that the five xylanases were deduced to hydroloyze xylan by endoactivity mechanism. These properties are similar to those of xylanase of most of the other bacterial xylanases (Honda 1985).

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