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Specificity and Mode of Action of a Thermostable Xylanase from *Bacillus amyloliquefaciens*

On-line Monitoring of Hydrolysis Products

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ABSTRACT

A thermostable xylanase purified from a strain of *Bacillus amyloliquefaciens* MIR 32 was characterized with respect to its substrate specificity and mode of hydrolytic action. The enzyme was highly specific for xylans as substrate and displayed no activity toward other polysaccharides, including cellulose. The enzyme exhibited K_m and V_{max} of 4.5 mg/mL and 0.58 mmol/min/mg, respectively, with birchwood xylan as the substrate. Microdialysis sampling with anion exchange chromatography and integrated pulsed electrochemical detection were used for rapid on-line monitoring of products during hydrolysis of oat spelt and bagasse xylan, and xylooligosaccharides. Xylobiose and xylotriose were the main end products. Xylotetraose was the smallest oligosaccharide to be acted on by the xylanase. The product pattern confirmed that the enzyme was an endoxylanase.

Index Entries: Endoxylanase; *Bacillus amyloliquefaciens*; substrate specificity; on-line monitoring.

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INTRODUCTION

Microbial xylanolytic enzymes have been a subject of great interest for more than a decade now, because of their emerging use in several processes and applications, particularly in pulp and paper industry *(1–3)*. Endo-β-1,4-xylanases (EC 3.2.1.8) are the crucial components of the complex xylanolytic systems. These enzymes hydrolyze the glycosidic linkages between β-(1,4)-linked xylopyranosyl groups of the xylan backbone. A number of xylanases have been isolated so far from various fungi *(2,4–6)*, and bacteria mostly belonging to *Bacillus* species *(7–13)*. Moreover, most organisms produce multiple forms of enzyme *(14)*, which, besides differing in physicochemical characteristics, may also be distinguished on the basis of substrate specificity and end products generated on xylan hydrolysis.

For some important applications, such as bleaching of kraft pulp, and processing of plant fiber sources such as flax and hemp, the presence of cellulolytic activity in xylanase preparations is undesirable. Although most of the xylanases isolated from various organisms have been reported to be cellulase free, nonspecific xylanases from *Trichoderma, Penicillium* spp, and so on, are known to exhibit hydrolytic activity toward cellulosic substrates *(5,15,16)*. Endoxylanases may be further distinguished by their hydrolytic action on the substrates on which they act *(17)*. Hence, during screening of xylanases from new microbial sources, the evaluation of the substrate specificity and mode of action of the xylanase is a prerequisite for identifying their potential applications. Chromatographic techniques are used for monitoring of hydrolytic products, including paper chromatography, thin layer chromatography, and ion exchange chromatography in an HPLC format *(18)*. Product monitoring in HPLC has been commonly done using refractive index mode of detection, but use of pulsed amperometric detection (PAD) has also been reported *(19,20)*.

In our previous report, we described the purification and properties of xylanase from a thermophilic strain identified as *Bacillus amyloliquefaciens*, isolated from a soil sample in South America *(21)*. The enzyme showed good stability at 50°C and even under alkaline conditions. The enzyme had a mol wt of around 19,000, and an isoelectric point of 10.1, and thus seemed to fit into family G of xylanases, i.e., those having low mol wt/high p*I* *(14)*. In this paper, we report studies on substrate specificity and mode of hydrolytic action of the *B. amyloliquefaciens* MIR 32 xylanase. For the latter, use was made of anion exchange chromatography with integrated pulsed electrochemical detection (IPED) for identification of the hydrolysis products. The analysis was further combined with microdialysis to achieve *in situ* sampling, sample cleanup and selective separation, and rapid detection of hydrolysates *(22,23)*.

MATERIALS AND METHODS

Materials

Bagasse xylan was prepared as described earlier *(24)*. Oat spelt, birchwood, and larchwood xylan, arabinogalactan, carboxymethyl cellulose, levan, laminarin, and galactomannan were purchased from Sigma (St. Louis, MO). Cellulose (Avicel) and soluble starch were bought from Merck (Darmstadt, Germany); β-glucan and lichenan were from ICN (Costa Mesa, CA). Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were procured from MegaZyme (Sydney, Australia). All the other chemicals were of analytical grade. All solutions were prepared using water from a Milli-Q system (Millipore, Bedford, MA).

Xylanase Production and Purification

The xylanase was purified from the culture supernatant of *B. amyloliquefaciens* MIR 32, as described elsewhere *(21)*. The procedure included treatment with ion exchange matrix for adsorption primarily of colored substances, salt precipitation of some of the contaminating proteins, and, finally, hydrophobic interaction chromatography, to yield more than 50% of the active enzyme in pure form. The product was desalted and concentrated before storing at –20°C as a stock solution containing 149.2 U/mL (0.7 mg protein/mL) in 5 m*M* Tris-HCl solution, pH 6.8.

Assay of Enzyme Activity

Xylanase activity was measured by the amount of reducing sugars released *(25)* at 50°C from birchwood xylan. The assay mixture containing 0.45 mL of 10 g/L birchwood xylan in 50 m*M* sodium phosphate buffer or Tris-HCl solution, pH 6.8, and 0.05 mL of appropriately diluted enzyme solution was incubated for 10 min at 50°C. The reaction was stopped with 0.75 mL of dinitrosalicylic acid (DNS) reagent. The tubes were then placed in a boiling water bath for 10 min, and cooled before measuring the absorbance of the solutions at 590 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of sugar (as xylose) per min.

Kinetic parameters of the xylanase were determined by measuring the enzyme activity under the above conditions, using birchwood xylan in the concentration range of 0.5–40 mg/mL.

For the determination of substrate specificity, the birchwood xylan was replaced with similar concentrations (1%) of other polymers.

Monitoring of Hydrolysis Products

Monitoring of hydrolysis products was performed on-line using the experimental setup described earlier *(22)*. The analytical system was com-

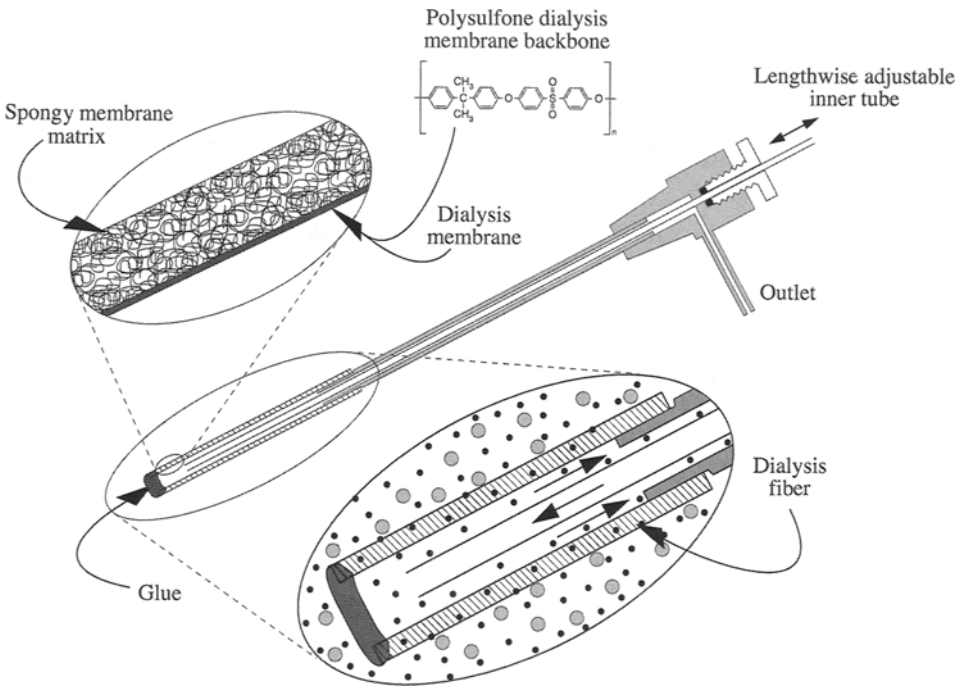

Fig. 1. A schematic representation of the in-house machined, *in situ* tunable microdialysis probe used for sampling the xylooligomers. The probe is comprised of two concentric tubes. The length of the inner tube is adjustable lengthwise, enabling control of the recovery, and the outer tube houses the hollow fiber membrane (having a total membrane thickness of about 80 μm). The thin dialysis layer located on the inner side of the hollow fiber is supported by a thicker spongious layer that is bound to it by the phase formation process during manufacture of the membrane. The perfusion liquid is pumped through the inner tube and flows back through the outer tube (as shown by the direction of arrows), carrying the sample molecules with it.

prised of a Dionex 500 chromatographic system (Dionex, Sunnyvale, CA) with a Carbo Pac PA 1 pre- and analytical column. Samples were eluted at 1 mL/min with a mobile phase of 150 m*M* sodium hydroxide (eluent A, 80%) prepared from 50% w/w stock solution (J. T. Baker, Deventer, Netherlands) and 250 m*M* sodium acetate (Merck), prepared in 150 m*M* sodium hydroxide (eluent B, 20%). The eluents were sparged with helium and continuously kept under a helium atmosphere. The eluted xylooligomers were detected at a gold electrode using the IPED, also from Dionex, using the waveform *(23)*, E_1 = 0.10 V (t_d = 0.20 s, t_1 = 0.20 s), E_2 = 0.70 V (t_2 = 0.19 s) and E_3 = –0.75 V (t_3 = 0.39 s).

An in-house machined *in situ* tunable microdialysis probe *(26)*, fitted with a 5 mm SPS 4005 microdialysis membrane (5 kDa MWCO) (Fig. 1), supplied by Thomas Laurell (Department of Electrical Measurements, Lund University, Sweden), was inserted into a 10-mL reactor containing

Table 1
Substrate Specificity of Xylanase from
B. amyloliquefaciens MIR 32

Substrate	Relative activity (%)
Oat spelt xylan	100
Birchwood xylan	96.7
Larchwood xylan	82.3
Bagasse xylan	72.1
Arabinogalactan (from larchwood)	0
Cellulose (Avicel)	0
CM-cellulose	0
ß-Glucan (from barley)	0
Lichenan (from *Cetraria islandica*)	0
Starch (soluble)	0
Levan (from *Erwinia herbicola*)	0
Laminarin (from *Laminaria digitata*)	0
Galactomannan (from *Ceratonia siligua*)	0

10 mg/mL xylan (previously dialyzed against 5 m*M* sodium phosphate buffer, pH 6.8) or 5 mg/L xylooligomers in the same buffer. The hydrolysis of the substrates was performed at 50°C in the presence of 10 µL xylanase stock solution (1.5 U). Perfusion liquid (pure water) was delivered to the microdialysis probe at a rate of 2 µL/min by a CMA/100 microinjection pump (CMA/Microdialysis, Stockholm), and the dialysate (20 µL) was injected into the chromatographic system using a CMA/160 on-line injector (CMA/Microdialysis). The entire system was computer controlled using a PeakNet™ software.

RESULTS AND DISCUSSION

The activity of *B. amyloliquefaciens* xylanase toward various polysaccharides at 50°C is shown in Table 1. The enzyme was able to break down all types of xylans, represented from hardwoods, softwoods, and grasses. The maximum activity was found with insoluble oat spelt xylan; the least attacked substrate was bagasse xylan, which was used as the carbon source during cultivation of the organism *(21)*. However, the differences in activity observed in Table 1 are not very significant, considering the variations in composition of the xylans, and even heterogeneity from batch to batch of the polymer isolated from the same source. The enzyme did not show activity toward any other polysaccharide, including structurally similar

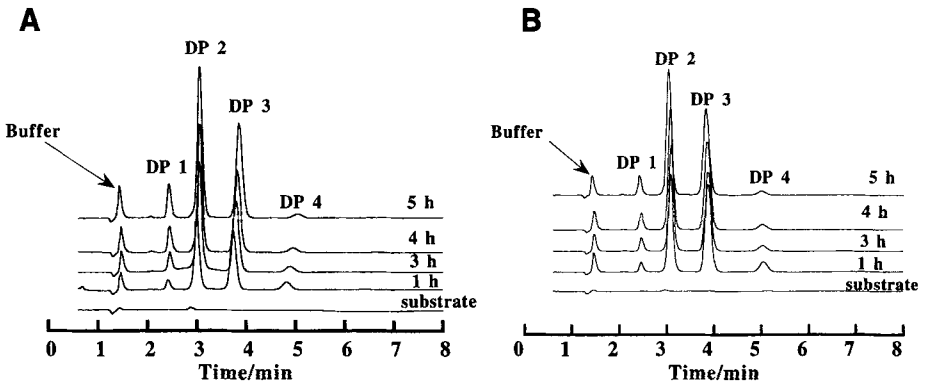

Fig. 2. Product profile of *B. amyloliquefaciens* MIR 32 xylanase catalyzed hydrolysis of **(A)** oat spelt and **(B)** bagasse xylan, monitored on-line at different time periods, using microdialysis sampling with anion exchange chromatography and IPED. Experimental details are given in the text.

cellulose and β-glucan. These characteristics make the enzyme an attractive candidate for applications requiring high specificity in xylanases. Birchwood xylan was used as the substrate for determination of kinetic parameters. Using Lineweaver-Burk plot, the values of K_m of 4.54 mg/mL and V_{max} of 0.58 mmoL/min/mg were obtained for the enzyme.

The hydrolysis products of xylans and oligomers by the xylanase MIR 32 were monitored on-line using microdialysis coupled to anion exchange chromatography with IPED. Use of this hyphenation of techniques reduces the analysis time and increases the sensitivity. Microdialysis sampling enables sample cleanup and on-line dilution to be achieved, hence eliminating sample work up, which is not always error-free, especially at very low analyte concentrations. In the present system, microdialysis offers a low resolution separation means for recovering xylooligomers from the bulk reaction into a chromatographically clean dialysate, which is injected into the analytical column. The xylooligomers were separated in their enolate form at pH 13.0 using anion exchange chromatography, and were detected quantitatively with high sensitivity by IPED *(23)*. Use of high pH anion exchange chromatography provides control over the analytes elution time, because a strong anion (e.g., acetate) can be added to the mobile phase to facilitate the chromatography. Monitoring by IPED is much more sensitive and solvent-compatible compared to refractive index and UV-visible mode, hence enabling detection of analytes at very low concentrations. IPED offers higher sensitivity, even when compared with PAD. In the PAD mode, the signal is obtained by sampling the current at a chosen time (that will be t_1 on the detection wave form), giving the resultant output in amperes. However, for IPED, the signal at the chosen time is sampled for a long period (20 ms in the present case), and the resultant signal

is the integrated area, and hence the signal output is in Coulombs (current × time). This improves significantly the signal-to-noise ratio. Thus, rapid controlled sampling and analysis with great sensitivity are some of the properties that make this integrated system very attractive for monitoring of hydrolysates. Furthermore, this system allows kinetic investigations.

Hydrolysis of oat spelt and bagasse xylan by xylanase from *B. amyloliquefaciens* MIR 32 was monitored for a period of 5 h (Fig. 2). Xylooligomers of degree of polymerization DP 2 and DP 3 were found to be the main products with small amounts of DP 1 and traces of DP 4. Relatively lower product peaks were noted during the initial period of hydrolysis (1 h) of oat spelt xylan, which increased with time and became higher than those of bagasse, in accordance with the results of Table 1. The differences observed during the initial phase could possibly be caused by varying amounts of higher oligomeric products (not observed in the product profile) formed as primary products of hydrolysis, which are subsequently degraded into final products.

The product profile indicated that the enzyme was an endoxylanase, and that DP 4 could be the smallest possible substrate hydrolyzed by the enzyme. Similar product profiles have been reported for xylanases from *B. stearothermophilus (27)* and *Aspergillus awamori (28)*, both having low p*I* in contrast to the *B. amyloliquefaciens* enzyme. On the other hand, xylanases with low mol wt/high p*I* have shown varying hydrolytic patterns; those produced by various *Bacillus* strains generally led to the formation of xylobiose and a number of higher oligomers *(7,9,29,30)*; the predominant end products produced by the enzyme from a fungus *Aureobasidium pullulans* were xylose and xylobiose *(4)*.

To confirm the endowise action of the xylanase, its hydrolytic properties were investigated using linear xylooligomers as substrates. The reaction was performed for several hours, and the products were monitored at different time intervals (Fig. 3). It may be noted in the figure that the xylooligomer substrates often presented a slight decrease in retention time while undergoing enzymatic treatment. This was caused by the difference in composition of the substrate and enzyme solutions, the former being in water and the latter in buffer. The buffer anions in the enzyme solution compete with those of sugar on the anion exchange sites of the stationary phase, consequently giving a shorter retention time. Xylopentaose constituted a very good substrate for the enzyme, and the profile analysis showed that DP 2 and DP 3 were the main hydrolysis products, along with lower amounts of DP 1. A small peak was observed at 6.5 min retention time, which corresponded to an xylooligomer with a DP higher than 5, but could not be identified because of the lack of standard oligomers. This may, however, suggest possible transxylosidation activity in the enzyme, as has been previously reported for some other xylanases *(5,9,10,31)*. The main product during the hydrolysis of xylotetraose

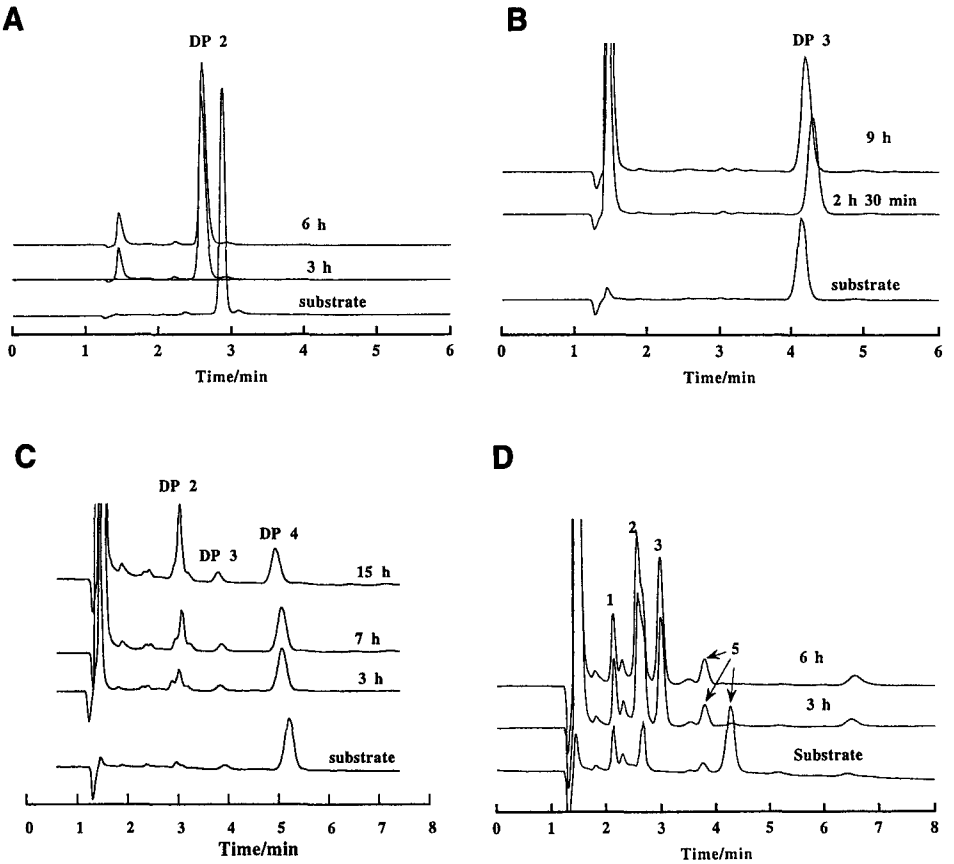

Fig. 3. On-line monitoring of *B. amyloliquefaciens* MIR 32 xylanase catalyzed hydrolysis of linear xylooligomers, using microdialysis sampling with anion exchange chromatography and IPED. Experimental details are given in the text. The oligomer substrates used are **(A)** xylobiose, **(B)** xylotriose, **(C)** xylotetraose, and **(D)** xylopentaose.

was DP 2, although a significant amount of the substrate remained unhydrolyzed. The xylanase MIR 32 did not hydrolyze xylotriose and xylobiose.

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