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ISOLATION AND CHARACTERIZATION OF AN EXTRACELLULAR XYLANASE FROM *BACILLUS CIRCULANS*

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A natural wild type xylanase was isolated from *Bacillus circulans* culture filtrates by CM Bio-gel A and DEAE Bio-gel A column chromatography and Toyopearl HW50S gel filtration. This enzyme had the same MW (22,000), pI(9.0), optimum pH(6.0) and temperature (53°C), pH stability (3.5-6.0), temperature stability (below 40°C), Km(1.5 mg/ml as larchwood xylan) and Vmax (103.70 µmol xylose/mg protein/min) as those of the wild type xylanase cloned and expressed in *E. coli*. The amino acid composition and N-terminal amino acid sequence of this enzyme were the same as those of *Bacillus circulans* xylanase and *Bacillus subtilis* xylanase which were cloned and expressed in *E. coli*.

Une xylanase naturelle de type sauvage a été isolée d'un filtrat de culture de *Bacillus circulans* par chromatographie sur CM Bio-gel A et sur DEAE Bio-gel A et par filtration sur gel HW50S de Toyopearl. Cette enzyme possédait la même masse moléculaire (22,000), le même pI (9,0), le même pH optimal (6,0), la même température optimale (53°C), le même stabilité de pH (3,5 - 6,0), la même température de stabilité (moins de 40°C), le même Km (1,5 mg/mL en xylane de mélèze) et le même Vmax (103,70 umol de xylose/mg de protéine/min) que la xylanase de type sauvage clonée et exprimée chez *E. coli*. La composition en acides aminés et la séquence des acides aminés déterminée à partir des extrémités N-terminales étaient les mêmes que la xylanase de *Bacillus circulans* et que la xylanase de *Bacillus subtilis* qui ont été clonées et exprimées chez *E. coli*.

INTRODUCTION

A 22k xylanase from *Bacillus circulans* has been cloned and expressed in *E. coli* by Yang *et al.*[1]. Esteban *et al.*[2] described a low molecular weight xylanase (15K by gel filtration) from *B. circulans* culture filtrate. We have been investigating the similarity among low molecular weight xylanases (around 20K to 22K) from bacteria and fungi, comparing the enzymatic properties of these xylanases, and studying the relationship of enzyme structure-function. Thus, we report here a comparison of sequence and enzyme characterization of *B. circulans* xylanase cloned in *E. coli*, and that isolated from the *B. circulans* culture filtrates. We conclude that two enzymes are the same functionally and structurally.

MATERIALS AND METHODS

Growth of microorganism. *Bacillus circulans* (NRC No. 9024) was grown in a medium containing 6.0 Na_2HPO_4, 3.0 KH_2PO_4, 0.5 g NaCl, 110 µl of 1 M $MgSO_4$, 0.1 µl of 1 M $CaCl_2$ 19 yeast extract, 2.5 g urea and 2g xylan per Litre cultures were incubated for 24h at 30°C, 200 rpm.

Chemicals. Xylan was obtained from Sigma Chemical Co. DEAE Bio-gel A and CM Bio-gel A were purchased from Bio-Rad Laboratories. Toyopearl HW50S was from Toyo Soda, Japan. All other chemicals were commercials products of analytical grade.

Enzyme assay method. Xylanase activity was assayed as follows. The reaction mixture contained 0.05 ml of enzyme solution, 0.2 ml of 0.1 M acetate buffer (pH 6.0) and 0.25 ml of 1% xylan solution. After incubation at 40°C for an appropriate period, the reducing sugar produced was measured by the method of Somogyi and Nelson. One unit of enzyme activity was defined as that amount of enzyme that produced one µmol of reducing sugar in one minute under the assay conditions.

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Analytical methods. Protein content was measured by the method of Bradford using Bio-Rad Protein Assay with bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring protein in column effluents. The amount of carbohydrate was measured by the phenol-sulfuric acid method with D-glucose as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis and isoelectric focusing were performed by the standard method using a PhastSystem (Pharmacia) with 10-15% polyacrylamide gradient gels and pH 3-9 gels, respectively.

Amino acid composition and N-terminal sequence analysis. Amino acid composition analysis was done with a Durrum 500 Amino Acid Analyzer and N-terminal sequence was done with a Beckman Model 890 M Sequencer.

RESULTS AND DISCUSSION

Bacillus circulans (NRC No. 9024) was incubated under the conditions decribed in materials and methods. 10 Liters of the culture supernatant were collected and used for xylanase purification.

As previously described by Esteban *et al.*[2], the low molecular weight xylanase fraction was adsorbed on CM bio-gel A equilibrated in 10 mM phosphate buffer, pH 6.0, and eluted with a linear sodium chloride gradient of 0 to 0.5 M (Fig. 1). The fractions with xylanase activity were pooled, concentrated and applied to a DEAE Bio-gel A column (2.4 by 45 cm) equilibrated in the same buffer. Unadsorbed xylanase fractions were collected, concentrated, and applied to a Toyopearl HW50S gel filtration column (2.4 by 90 cm). The enzyme was eluted with 10 mM phosphate buffer pH 6.0, containing 0.1 M sodium chloride and showed a Ve/Vo ration which corresponded to a molecular weight of 7,000 ± 500.

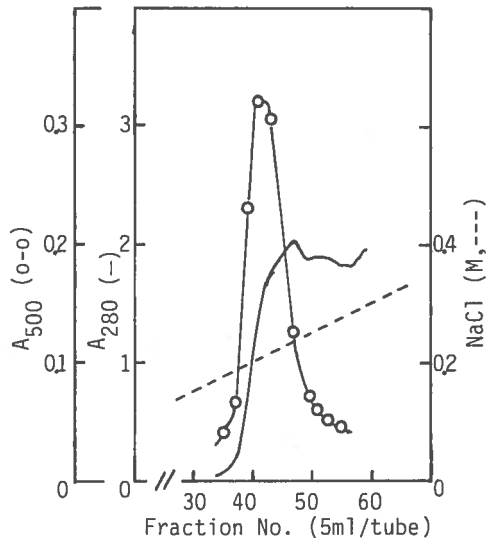

Fig. 1. CM Bio-Gel A Column Chromatography 0-0; xylanase activity, -; protein, ---; NaCl (M).

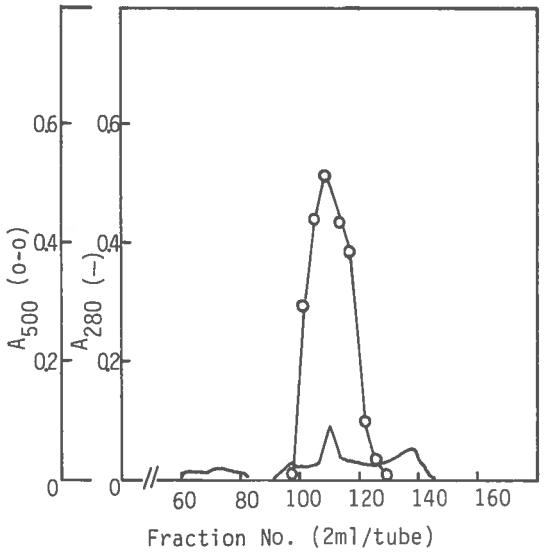

Fig. 2. Toyopearl HW50S Gel Filtration o-o; xylanase activity, -; protein.

The enzyme purified by gel filtration displayed homogeneity on SDS-PAGE (10-15% gradient gel) and isoelectric focusing (pH 3-9), with values of 22,000 obtained for the molecular weight (Fig. 3) and over 9.6 for the isoelectric point.

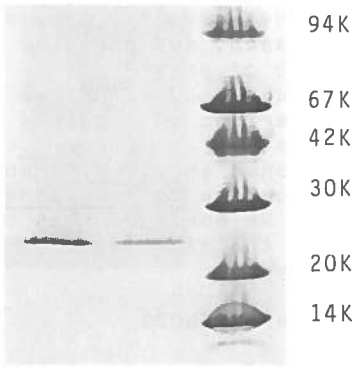

Fig. 3. SDS-PAGE.

Certain enzymatic properties were examined and compared with those of xylanases cloned and expressed in *E. coli*[11] and purified from the culture filtrate both in this work and previously[2] (Table 1).

Since Yang *et al.*[5] and Esteban *et al.*[2] identified and described two distinct xylanases from *B. circulans*, the culture filtrate must, therefore contain a second xylanase different from that which we isolated.

Table 1. Comparison of Xylanases from *B. circulans*

properties	xylanase B[2]	xylanase 22K**	cloned in E.coli**
Molecular weight (SDS-PAGE)	15,000*	22,000	22,000
pI	9.1	9.6	9.6
Sugar content (%)	ND	0	0
Optimum pH	5.5-7	6.0	6.0
Stable pH	ND	3.5-6.0	3.5-6.0
Optimum temp.(°C)	ND	53	53
Stable temp.(°C)	ND	below 40	below 40
Km(mg/ml)	4	1.58	1.50
Vmax(U/mg)	ND	103.70	108.00
Products from xylan	X_2,X_3,X_4	X_2,X_3	X_2,X_3

*, gel filtration; **, larchwood as substrate on the analyses.

Esteban *et al.*[2] did not do molecular weight determinations by SDS-PAGE and so we have no evidence to indicate that both xylanases are the same enzyme with identifical molecular weights. However, the two enzymes display similarities in their enzymatic properties, and show similar behavior in column chromatography during their purification. Consequently, we believe that the two xylanases are the same enzyme.

The enzymatic properties of the 22K xylanase from *B. circulans*, cloned and expressed in *E. coli* by Yang *et al.*[11], have been examined. As shown in Fig 3, each xylanase, cloned or purified, had the same mobility on SDS-PAGE. The two xylanases were also similar in most of their enzymatic properties (Table 1). Moreover, the N-terminal amino acid sequence suggests that the two xylanases are the same protein (Fig. 4).

(a),(b);

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 1                                  10
Ala-Ser-Thr-Asp-Tyr-Trp-Gln-Asn-Trp-Thr-

 11                                 20
Asp-Gly-Gly-Gly-Ile-Val-Asn-Ala-Val-Asn-

 21                                 30
Gly-Ser-Gly-Gly-Asn-Tyr-Ser-Val-Asn-Trp-
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Fig. 4. Identical N-terminal Amino Acid Sequence of (a), *B. circulans* extracellular purified xylanase; (b), *B. circulans* cloned xylanase.

The amino acid sequences deduced from the nucleotide sequences of the genes of the 22K xylanases from *B. circulans*[1] and *B. subtilis*[3] show that the two xylanases have an identical amino acid sequence. Paice *et al.*[3] also describe that strong evidence for homology was found when the *B. subtilis* xylanase amino acid sequence was compared to a xylanase from *B. pumilus*[6].

We are now comparing the primary structures of the low molecular weight xylanases from fungi as well as bacteria[8]. These xylanases seem to be quite similar both structurally and functionally[7],[8]. Two of these xylanases have been crystallized[9],[10], and it is hoped that the three-dimensional structure of the enzymes will become available in the near future. In the current research field of protein engineering, these xylanases may provide unique and interesting information with regard to enzyme structure-function studies[7].

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