Note

Purification and Characterization of a Family G/11 β-Xylanase from Streptomyces olivaceoviridis E-86

Satoshi Kaneko, Atsushi Kuno, Mizuho Muramatsu, Shinnosuke Iwamatsu, Isao Kusakabe, and Kiyoshi Hayashi

1National Food Research Institute, Ministry of Agriculture, Forestry, and Fisheries, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan
2Department of Material and Biological Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan
3National Institute for Advanced Interdisciplinary Research, 1-1-4 Higashi, Tsukuba, Ibaraki 305-8562, Japan
4Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennooodai, Tsukuba, Ibaraki 305-8572, Japan

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A β-xylanase (GXYN) was purified from the culture filtrate of Streptomyces olivaceoviridis E-86 by successive chromatography on DE-52, CM-Sepharose and Superose 12. The molecular mass of the xylanase was estimated to be 23 kDa, indicating that the enzyme consists of a catalytic domain only. The enzyme displayed an optimum pH of 6, a temperature optimum of 60°C, a pH stability range from 2 to 11 and thermal stability up to 40°C. The N-terminal amino acid sequence of GXYN was A-T-V-I-T-N-Q-T-G-T-N-N-G-I-Y-Y-S-F-W-, and sharing a high degree of similarity with the N-terminal sequence of xylanases B and C from Streptomyces lividans, indicating GXYN belongs to family G/11 of glycoside hydrolases. GXYN was inferior to xylanase B from Streptomyces lividans in the hydrolysis of insoluble xylan because of its lack of a xylan binding domain.

Key words: family G/11 xylanase; purification; Streptomyces olivaceoviridis; Streptomyces lividans; xylan binding domain

Xylan is a major component of the hemicelluloses in plant cell walls. It consists of a linear backbone of β-1,4-linked xylopyranose units and often has side chains composed of other sugar residues such as arabinose and glucuronic acid. β-Xylanase (EC 3.2.1.8) hydrolyses the β-1,4-glycosidic linkages within the xylan backbone to yield short chain xylooligosaccharides of varying length. On the basis of the amino acid sequences of the catalytic domains, β-xylanases have been classified into two glycanase families (F/10 and G/11). These two families of xylanases differ in their patterns of cleavage of various heteroxylans. For example, members of the F/10 family of xylanases cleave the β-1,4-linkages of arabinoxylans at the non-reducing ends of arabinofuranose branched xylopyranoses whereas members of the G/11 family do not perform this cleavage. It is therefore of interest to compare the reaction mechanisms and structure-function relationships of these different families of enzymes.

It has been reported that Streptomyces olivaceoviridis E-86 produces a xylanase (FXYN) extracellularly. This xylanase has been purified and characterized on the basis of its substrate specificity. Recently the gene of this xylanase was cloned and identified as belonging to the F/10 family of glycanases. The deduced amino acid sequence of FXYN shared a reasonably high degree of similarity (more than 90%) with xylanase A from Streptomyces lividans.

S. lividans is reported to have three kinds of xylanases; xylanases A, B and C. Xylanase A belongs to the F/10 family and xylanases B and C are classified into the family G/11 of glycanases. We therefore focussed our attention on finding xylanases other than FXYN in the culture broth of S. olivaceoviridis E-86 and we were able to locate a xylanase with a lower molecular weight than that of FXYN. Herein we report the purification and characterization of this low molecular weight xylanase from S. olivaceoviridis E-86.

S. olivaceoviridis E-86 was cultured in 1000 ml of medium (2.0% K2-xylan, 1.4% peptone, 0.1% yeast extract, 0.5% corn steep liquor, 1.0% KH2PO4, and 0.05% MgSO4·7H2O) in 5000-ml conical flasks. Cultivation was performed at 35°C for 90 h on a

1 To whom correspondence should be addressed. Fax: +81-298-38-8122; E-mail: sakaneko@nfri.affrc.go.jp

Abbreviations: FXYN, family F/10 β-xylanase from Streptomyces olivaceoviridis E 86; GXYN, family G/11 β-xylanase from Streptomyces olivaceoviridis E-86; NynB, xylanase B from Streptomyces lividans; NBD, xylan binding domain
reciprocal shaker (125 r.p.m). The culture broth was then filtered through filter paper and the filtrate was dialyzed against deionized water. The pH of the dialyzed enzyme solution was adjusted to pH 5.7 and the solution was loaded onto a CM-Sepharose FF column (25 x 200 mm) connected to a pre-column of DE-52 (50 x 100 mm) pre-equilibrated with 50 mM phosphate buffer (pH 5.7). The columns were washed with the same buffer and the DE-52 column was then removed. The adsorbed materials on the CM-Sepharose FF were eluted with a linear gradient of NaCl solution (0 to 0.5 M; total volume 1 liter) at a flow rate of 100 ml per h. The eluate was fractionated into 10-ml aliquots. The fractions containing the xylanase activity were pooled, concentrated by ultrafiltration, and then loaded onto a Superose 12 column (16 x 500 mm) which was equilibrated with 0.15 M NaCl in 50 mM phosphate buffer (pH 6.5) at a flow rate of 0.7 ml per min. The eluate was fractionated into 2.5-ml aliquots. A single peak of xylanase activity was detected and the corresponding fractions were pooled and dialyzed against deionized water. The resulting enzyme solution was used as purified GXYN. Purified GXYN could be resolved as a single band on SDS-PAGE\(^{15}\) when visualized by Coomassie Brilliant Blue R-250-staining (Fig. 1).

The steps for the purification of GXYN from \textit{S. olivaceoviridis} E-86 are summarized in Table 1. Almost all of the xylanase activity came through in the initial phosphate buffer wash off the CM-Sepharose column indicating that FXYN present in the culture filtrate did not bind to the DE-52 and CM-Sepharose columns. The recovery of GXYN on the final purification step was very low at 0.88\% compared to a 31\% recovery of FXYN from the culture filtrate of \textit{S. olivaceoviridis} E-86.\(^{16}\) These findings indicate that \textit{S. olivaceoviridis} E-86 produces much more FXYN than GXYN. These results agreed with the earlier report where more than 94\% of the xylanase activity was present as FXYN.\(^9\) This is the reason why GXYN was not found at this stage.

The Mw of GXYN was estimated to be 23,000 which is smaller than FXYN (45,000).\(^{9}\) The Mws of the xylanases isolated from \textit{S. lividans} are 45,000 (xylanase A), 31,000 (xylanase B) and 22,000 (xylanase C).\(^{11}\) Xylanases A and B consist of a catalytic domain and a substrate binding domain, however xylanase C consists only of a catalytic domain. The Mw of GXYN is closest to that of xylanase C, indicating that the enzyme consists of a catalytic domain only. The N-terminal amino acid sequence of GXYN was A-T-V-I-T-T-N-Q-T-G-T-N-N-G-I-Y-Y-S-F-W-, similar to other xylanases belonging to the family G/11 glycanases (Fig. 2). From this N-terminal amino acid sequence, it is apparent that GXYN is a member of the G/11 family of glycanases. The enzymatic properties of GXYN were investigated. GXYN showed maximal activity at pH 6.0, and stability over the pH range of 2-11 after 1 h of incubation at 30°C. The optimal temperature for enzyme activity was identified as being 60°C, however, a reduction in this activity was observed after 1 h of incubation at temperatures above 40°C.

![Fig. 1. SDS-PAGE of Purified GXYN.](image)

Lane 1 and 3: 10 kDa ladder standard proteins (2 \(\mu\)g); lane 2: purified GXYN (2 \(\mu\)g). SDS-PAGE was carried out in 14% gel by the method of Laemmli.\(^{15}\) The protein was stained with Coomassie Brilliant Blue (CBB) R-250 and then destained with 10% acetic acid in 30% methanol. The molecular weight of the enzyme was measured by SDS-PAGE using molecular weight markers as a reference (10 kDa-protein ladder, GIBCO BRL).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>432000</td>
<td>18000</td>
<td>24</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>9976</td>
<td>14</td>
<td>713</td>
<td>2.3</td>
<td>30</td>
</tr>
<tr>
<td>Superose 12</td>
<td>3803</td>
<td>1.4</td>
<td>2716</td>
<td>0.88</td>
<td>113</td>
</tr>
</tbody>
</table>

The method of Somogyi\(^{12}\) was used for the determination of enzyme units. One unit of enzyme activity was defined as the amount of enzyme which released 1 \(\mu\)mole of xylose from birchwood xylan, expressed as reducing power per min at pH 5.7 and 56°C with a 30 min reaction time. The protein concentration was determined by the method described by Lowry \textit{et al.}\(^{14}\) with bovine serum albumin as a standard.
Streptomyces olivaceoviridis E-86 GXYN 1 ATVIERTNQGNNQKNCN 20
Streptomyces sp. xyll (X98519) 40 ATDIERTNQGNNQKNCN 58
Streptomyces lividans Xln B (P26515) 43 ATDIERTNQGNNQKNCN 61
Streptomyces lividans Xln C (A25307) 10 ATDIERTNQGNNQKNCN 28
Streptomyces thermoviolaceus stxII (D85897) 40 ATDIERTNQGNNQKNCN 59
Bacillus pumilus xylanase II (B43937) 3 LSSQNCGNGR 19
Chaetomium gracile cpkB (D49851) 33 TSSQNCGNGR 49
Streptomyces roseisceraticularis xyn 2 (B57001) 2 XTQVITNQGNNQKNCN 19
Streptomyces sp. (S47512) 50 ATDIERTNQGNNQKNCN 68
Streptomyces roseisceraticularis xyn 3 (A57001) 1 ATDIERTNQGNNQKNCN 19
P. fluorescens ssp. cellulosa (Z49827) 27 AQTLENSGGNGKNCN 46
Cellulomonas fimi XYLID (P54865) 41 AAVSNCGNGGKNCN 60
Cellulibrio mixtus (Z49825) 27 AQTLENSGGNGKNCN 46
Nocardiopsis dassonvillei II (PQ0202) 3 LSSQNCGNGR 36
Thermomonospora fusca YX (U01242) 42 AAVSNCGNGGKNCN 60
Aspergillus niger XynB (P55330) 39 TPSQNCGNGR 54

Fig. 2. Comparison of N-Terminal Amino Acid Sequences of GXYN with the Other Xylanases of the Family G/11.
The similarity searches of N-terminal amino acid sequences of GXYN were performed with MPsearch (mpsearch@dna.affrc.go.jp). The amino acid residues identical in ten sequences are framed by a shaded box (■). The amino acid residues completely conserved are framed by a shaded box (■).

Fig. 3. HPAEC Analysis of the Hydrolysis Products of Soluble Oat Spelts Xylan by GXYN.
X₁: xylose, X₂: xylobiose, X₃: xylotriose, X₄: xylotetraose, X₅: xylopentaose, X₆: xylohexaose, X₇: xyloheptaose. The reaction mixture contained 0.1 ml of GXYN or XynB solution (25 µg/ml), McIlvaine buffer (pH 7.0, 0.3 ml), 1% BSA (0.1 ml) and 1% substrate (0.5 ml). After 180 min of incubation at 30°C, the mixture was diluted 50-fold then 25 µl of the resulting solution was applied to a Carboxyl PA-1 column to characterize the hydrolysis products. The full length of the xylanase B gene (xynB) and catalytic domain of the xylanase B gene (xynJ) were amplified from total genomic DNA of S. lividans 66 by using the sense and antisense primers as follows: sense primers, 5’-CCATGGACCTGCTCGTCCAGCCGA-3’ (xynB); and antisense primers, 5’-GGATCCGCCGCGCTGAGGAGACAG-3’ (xynB) and 5’-GGATCCGCCGCGATCGTGCGAGCTGAG-3’ (xynJ). The amplified DNA fragment was inserted into a pQE 60 vector and expressed by using the QIAexpress system (QIAGEN GmbH, Hilden, Germany). Methods described in a previous paper[10] were used for the production and purification of the enzymes.

These properties are similar to the xylanases B and C from S. lividans.11

It has been reported that xylanase B (XynB) consists of a family G/11 catalytic domain and a family IIb cellulose (xylan) binding domain (XBD).10 The binding domain has been shown to have affinity for insoluble xylan.17 The substrate specificity of GXYN was then investigated along with XynB from S. lividans. When GXYN acted on soluble oat spelt xylan, it produced a range of products from xylobiose to xyloheptaose (Fig. 3). Xylotriase and xylotetraose were the main hydrolysis products and xylose was produced at very low levels. Significant differences in the hydrolysis products between GXYN and XynB were not observed despite XynB possessing a XBD. This indicated that the presence
of the XBD did not have an effect on the substrate specificity of the enzyme. Subsequently, the activity of GXYN and XynB on soluble and insoluble xylan was examined (Fig. 4). Both enzymes acted on soluble xylan but no differences in the hydrolysis rates between GXYN and XynB were observed (Fig. 4A). In contrast, GXYN hydrolyzed insoluble xylan less effectively than XynB (Fig. 4B). This indicate that the absence of XBD in GXYN have disadvantage for the hydrolysis of insoluble xylan. It is also apparent from the results of the insoluble xylan hydrolysis by XynB (Fig. 4B). After removal of the XBD from XynB, the hydrolysis rate decreased to a similar level to that of GXYN (Fig. 4B). These results also suggest that the XBD has no effect on the hydrolysis of soluble xylan (in terms of both the rate and the mode of hydrolysis) and only increases the hydrolysis of insoluble xylan by increasing the substrate concentration around the enzyme.

In this study, we isolated and characterized a family G/11 xylanase from S. olivaceoviridis E-86. As part of our on-going research interests in the area, we would like to provide additional information in the near future which should be of use in further determinations of structure-function relationships of xylanase enzymes.

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References


