Purification, Properties, and Partial Amino Acid Sequences of Thermostable Xylanases from
*Streptomyces thermoviolaceus* OPC-520

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Two types of xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) were isolated from the culture filtrate of a thermophilic actinomycete, *Streptomyces thermoviolaceus* OPC-520. The enzymes (STX-I and STX-II) were purified by chromatography with DEAE-Toyopearl 650 M, CM-Toyopearl 650 M, Sephadex G-75, Phenyl-Toyopearl 650 M, and Mono Q HR. The purified enzymes showed single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weights of STX-I and STX-II were 54,000 and 33,000, respectively. The pI of 4.2 (STX-I) and of 8.0 (STX-II). The optimum pH levels for the activity of STX-I and STX-II were pH 7.0. The optimum temperature for the activity of STX-I was 70°C, and that for the activity of STX-II was 60°C. The enzymes were completely inhibited by N-bromosuccinimide. The enzymes degraded xylan, producing xyllose and xylobiose as the predominant products, indicating that they were endoxylanases. STX-I showed high sequence homology with the endoxylanase from *Cellulomonas fimi* (47% homology), and STX-II showed high sequence homology with the xylanase from *Bacillus pumilus* (46% homology).

Xylan is one of the major components of hemicellulose and has recently received increased attention as a renewable resource in the exploitation of plant biomass. It consists of a β-1,4-linked D-xylene polymer which commonly contains side branches of α-1,3-linked D-arabinofuranose and α-1,2- linked D-glucopyranose. Xylanases are enzymes which play important roles in the breakdown of xylan and therefore in the bioconversion of biomass materials for the production of xylose, from which single-cell protein, single-cell oil, or ethanol could be produced (4, 5). Many microbial xylanases from fungi and bacteria have been purified and characterized, and the number of reports dealing with xylanases from thermostable microorganisms is increasing. Recently, we isolated many thermophilic actinomycetes. One of the most interesting was *Streptomyces thermoviolaceus* OPC-520, producing a relatively high xylanase activity, which was isolated from decayed wood collected in Osaka City, Osaka, Japan. In the present paper we report the purification and properties of xylanases from the culture filtrate, as well as the taxonomy of the OPC-520 isolate. Partial amino acid sequences of STX-I and STX-II were determined and compared with those of xylanases from other microorganisms.

MATERIALS AND METHODS

**Taxonomic studies of strain OPC-520.** Details in the methods relating to taxonomic studies of strain OPC-520 were carried out as previously described (22).

**Xylanase production.** The organism was inoculated directly into a production medium (100 ml) containing (in grams per liter) oat spelt xylan (Sigma), 1.0; yeast extract (Difco), 0.1; protease peptone (Difco), 0.5; K₂HPO₄, 0.1; and MgSO₄·7H₂O, 0.02 (pH 7.0), in a 500-ml flask and then cultured at 50°C on a reciprocal shaker for 24 h. After filtration of the culture with Toyoyoshi no. 2 filter paper (Toyofun Co., Ltd., Tokyo, Japan), the filtrate was used as crude xylanase.

**Xylanase assay.** Xylanase was assayed by mixing a 0.5-ml aliquot of appropriately diluted enzyme with 0.5 ml of 1.0% oat spelt xylan in 50 mM Tris-HCl buffer, pH 7.0. After incubation at 70°C for 10 min, the reaction was terminated by adding 2 ml of 3,5-dinitrosalicylic acid reagent (15). The mixture was heated in a boiling water bath for 5 min. Next, 5 ml of water was added and the A₅₉₀ of the sample was measured. One unit of xylanase was defined as the amount of enzyme that liberated 1 μmol of xylose equivalent in 1 min under the conditions described above.

**Purification of xylanase.** All purification steps were carried out at 4°C unless otherwise mentioned. The crude xylanase (540 ml) was dialyzed overnight against 50 mM acetic acid buffer, pH 5.0. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column (1.9 by 40 cm; Tosoh, Tokyo, Japan) equilibrated with the same buffer. Two fractions of xylanase activity were obtained. Fraction I was eluted with the buffer containing 0.3 M NaCl, while the other fraction, Fraction II, was passed through the column. Fraction I was concentrated by ultrafiltration with Q 1000 membrane (Advantec, Tokyo, Japan). The concentrated Fraction I was applied to a Sephadex G-75 column (2.6 by 100 cm; Pharmacia) equilibrated with 50 mM acetate buffer, pH 5.0, containing 0.1 M NaCl. The active fraction obtained was dialyzed overnight against 50 mM acetate buffer, pH 5.0. Ammonium sulfate (final concentration, 2 M) was added to the dialyzed enzyme solution and applied to a Phenyl-Toyopearl 650 M column (1.0 by 20.0 cm; Tosoh) equilibrated with 50 mM acetate buffer, pH 5.0, containing 2 M ammonium sulfate. The column was eluted with the buffer and then with a linear gradient of 2.0 to 0 M ammonium sulfate.

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sulfate. The active fraction eluted at a concentration of about 0.2 M ammonium sulfate was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5, and then applied to a fast protein liquid chromatography (FPLC) Mono Q column (0.5 by 5.0 cm; Pharmacia) equilibrated with the same buffer. The column was washed with the buffer, and then the xylanase was eluted with a linear gradient of 0 to 1.0 M NaCl. It was eluted at a concentration of about 0.23 M NaCl. This xylanase was named STX-I. Fraction II was dialyzed overnight against 20 mM acetate buffer, pH 4.7. The dialyzed enzyme solution was applied to a CM-Toyopearl 650 M column (1.9 by 40 cm; Tosoh) equilibrated with the same buffer. The column was washed with the buffer, and then a linear gradient of 0 to 0.5 M NaCl was applied. The xylanase activity was eluted at a concentration of about 0.2 M NaCl. This fraction was concentrated by ultrafiltration with a Q 10100 membrane and then applied to a Sephadex G-75 column (2.6 by 100 cm; Pharmacia) equilibrated with 50 mM acetate buffer, pH 5.0, containing 0.1 M NaCl. This xylanase was named STX-II.

**Isoelectric point estimation.** Isoelectric points of STX-I and STX-II were determined by isoelectric focusing in immobilized pH gradient gel (24).

**Molecular weight estimation.** Molecular weights of STX-I and STX-II were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (11). Low-molecular-weight kit E (Pharmacia) was used as a molecular weight marker.

**Protein estimation.** Protein concentration was measured by the method of Lowry et al. (14) with bovine serum albumin as a standard.

**Effects of pH and temperature on xylanase activity.** Xylanase activity was measured at pH values from 4 to 10 under the standard assay conditions with oat spelt xylan as a substrate. Buffers used were 50 mM acetate buffer (pH 4 to 6), 50 mM Tris-HCl buffer (pH 7 to 8) and 50 mM glycine-NaOH buffer (pH 9 to 10). The enzyme activities were also assayed at temperatures from 30 to 80°C at pH 7.0.

**Effects of pH and temperature on xylanase stability.** The initial activities of the enzymes were assayed under the standard assay conditions. The enzyme solution was preincubated for 30 min under various conditions without the substrate and immediately cooled down to 0°C. The remaining activities were then assayed.

**Effects of various reagents on xylanase activity.** The enzyme was preincubated with various reagents dissolved in 50 mM Tris-HCl buffer (pH 7) at 30°C for 30 min. Then, the residual activity was measured under the standard assay conditions.

**Paper chromatography of hydrolysis products.** STX-I (11.4 U) and STX-II (45.3 U) were incubated with 1% xylan in 10 mM Tris-HCl buffer, pH 7.0, at 50°C. Aliquots (20 µl) of reaction mixtures and appropriate controls were spotted on chromatography paper after various intervals between 0 and 24 h. The xylanase reaction products were chromatographed on Toyo filter paper no. 51 with a solvent system of 1-butanol–pyridine–water (6:4:3 [vol/vol/vol]) and developed with silver nitrate reagent (21).

**Amino acid analysis.** Amino acid analyses of STX-I and STX-II were performed on a Hitachi L-8500 amino acid analyzer equipped with a D-2850 chromatotriator by the method of a previous paper (23).

**Amino-terminal amino acid sequence.** The amino-terminal amino acid sequences of STX-I and STX-II were determined with an Applied Biosystems model 477 A gas-phase sequencer, and the phenylthiohydantoin derivatives of amino acids were identified by an automatic on-line analysis system.

**RESULTS**

**Taxonomic studies of strain OPC-520.** Microscopic studies, cultural characteristics, carbohydrate utilization, and cell analysis of strain OPC-520 indicated that the strain was closely related to *S. thermoviolaceus* (data not shown). Therefore, strain OPC-520 was directly compared with *S. thermoviolaceus* IFO 13905. From the results of comparison, we regard strain OPC-520 as *S. thermoviolaceus*.

**Purification of xylanases.** *S. thermoviolaceus* OPC-520 produced and secreted two main and additional xylanases (2 to 5% of total) in the presence of xylan. In the purification procedure described above, STX-I and STX-II were purified about 15.6- and 27.8-fold, respectively. The recoveries of their activities were about 8.8 and 11.9% of culture filtrate, respectively.

**Isoelectric point estimation.** Isoelectric points of STX-I and STX-II were 4.2 and 8.0, respectively (data not shown).

**Molecular weight estimation.** The molecular weights of STX-I and STX-II were determined by SDS-PAGE (Fig. 1). They were estimated to be approximately 54,000 and 33,000, respectively.

**Effects of pH and temperature on xylanase activity.** The effects of pH and temperature on the enzyme activities are shown in Fig. 2 and 3. The optimum pH levels of STX-I and STX-II were pH 7 in 50 mM Tris-HCl buffer. The optimum temperatures of STX-I and STX-II were 70 and 60°C, respectively.

**Effects of pH and temperature on xylanase stability.** The effects of pH and temperature on xylanase stability are shown in Fig. 4. STX-I was stable in the range of pH 5 to 9 up to 50°C, and STX-II was stable in the range of pH 5 to 9 up to 60°C. The remaining activity of STX-I at pH 3 to 11 was completely absent at 40°C. On the other hand, STX-II
Effects of various reagents on xylanase activity. The effects of metal ions, p-chloromercuribenzoate, SDS, N-bromosuccinimide, monooiodoacetic acid (MIA), and EDTA on xylanase were examined (data not shown). STX-I and STX-II were completely inhibited by N-bromosuccinimide, but p-chloromercuribenzoate and monooiodoacetic acid did not influence the activity of these two enzymes. STX-I was completely inhibited by Fe^{2+}. Other metal ions had no remarkable effect on the enzymes.

Paper chromatography of hydrolysis products. Paper chromatography of xylan hydrolysates with STX-I and STX-II is shown in Fig. 5. Xylo-oligosaccharides were released by these two enzymes in the course of xylan hydrolysis. The hydrolysis mechanisms of STX-I and STX-II seemed to be somewhat different. When the reaction time was increased, STX-I hydrolyzed xylan to give xylose and xylobiose as predominant products. On the other hand, STX-II accumulated xylose, xylobiose, and xylotriose as predominant products. The spots between xylose and xylobiose were not identified.

Amino acid analysis. The amino acid compositions of STX-I and STX-II are shown in Table 1. STX-I and STX-II contained Cys residues. Histidine and methionine residues showed a low molar ratio (percentage) in these two enzymes.

Amino-terminal amino acid sequence. The amino-terminal 30 amino acid residues of STX-I were sequenced. This enzyme showed high sequence homology (47%) with exoglucanase from Cellulomonas fimi (17) as shown in Fig. 6. On the other hand, the amino-terminal 29 amino acid residues of STX-II were also sequenced and compared with those of other microbial xylanases. These results are shown in Fig. 7 with literature values for xylanases from an Aureobasidium sp. (13), Bacillus subtilis (18), Schizosphillum commune (19), and Bacillus pumilus (6). Particularly, STX-II showed high sequence homology (46%) with xylanase from B. pumilus.

DISCUSSION

S. thermoviolaceus OPC-520 produced relatively high xylanase activity when the microorganism was grown in a medium containing xylan as a carbon source. Two types of xylanases, designated STX-I and STX-II, were purified from the culture filtrate. Additional xylanases were also present; however, the amount was so little as to be negligible.

It was reported that color variants of Aureobasidium pullulans (12) produced xylanase with an extremely high specific activity (2,000 U/mg of protein). STX-I (1,460 U/mg of protein) and STX-II (1,405 U/mg of protein) also exhibited exceptionally high specific activity in comparison with the other xylanases. These two enzymes showed about two-thirds of the specific activity of xylanase from A. pullulans.

Various thermostable xylanases have been isolated from...
cultures of *Thermoascus aurantiacus* (10), *Melanocarpus albomyces* (2), *Talaromyces byssolchlamydoideus* (25), a thermotolerant *Streptomyces* sp. (9), *Clostridium stercorarium* (1), and *Bacillus* spp. (16). However, this paper reports the first purification and characterization of xylanase from *S. thermoviolaceus*. The thermostability of STX-I and STX-II could be compared with that of other xylanases purified from thermophilic microorganisms. STX-I was stable in the range of pH 5 to 9 at 50°C for 30 min, while STX-II was stable in the range of pH 5 to 9 at 60°C for 30 min. Furthermore, these two enzymes retained their original activity on incubation at pH 7.0 for 6 days at 50°C (data not shown).

The molecular weights and isoelectric points of STX-I and STX-II were determined. These values fell within the range of molecular weights and pI values reported for microbial xylanases.

Endoglucanase activity is unwanted in most cases because it degrades cellulose fibers and destroys pulp properties (7). Some of the xylanases attack not only xylan but also cellulose. Such enzymes have been obtained from *Aspergillus niger* (8), *Trichoderma viride* (20), and *Ceratocystis paradoxa* (3). STX-I and STX-II showed no cellulase activity when carboxymethyl cellulose and crystalline cellulose were used as substrates and also were not accompanied by β-xylanase activity in the culture filtrate (data not shown).

Xylose and xylobiose were detected by paper chromatography of enzymatic digests. These results indicated that STX-I and STX-II were deduced to hydrolyze xylan by an endo-activity mechanism.

Keskar et al. reported that xylanase from a thermophilic *Streptomyces* sp. was completely inhibited by *N*-bromo-succinimide, 2-hydroxy-3-nitrobenzyl bromide and *p*-hydroxymercuribenzoate, and thus tryptophan and cysteine residues of the enzyme were essential for the activity (9). STX-I and STX-II were also completely inhibited by *N*-bromosuccinimide, and xylan completely protected the enzyme activity from inactivation by *N*-bromosuccinimide (data not shown). Our results were in accord with the reports of Keskar et al. with the exception that *p*-chloromercuribenzoate had no effect on STX-I and STX-II (9). These results suggest that the presence of tryptophan is essential for the activity.

There were no remarkable differences between the amino acid composition of STX-I and that of STX-II. However, partial amino-terminal sequences of these two enzymes were clearly different from each other. STX-II showed high sequence homology with xylanases from *B. pumilus* (6) and *S. commune* (19). On the other hand, STX-I showed no apparent sequence homology to other microbial xylanases. Surprisingly, this enzyme showed striking homology with exoglucanase from *Cellulomonas fimii*. STX-I hydrolyzed *p*-nitrophenyl-β-D-cellobioside as a substrate of exoglucanase as expected, while STX-II failed to hydrolyze the

### TABLE 1. Amino acid compositions of STX-I and STX-II

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<tr>
<th>Amino acid</th>
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<tr>
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<tr>
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<td>28-29</td>
</tr>
<tr>
<td>Trp</td>
<td>ND</td>
</tr>
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</table>

Total 511-528 302-326

*ND, not determined.*

FIG. 5. Paper chromatography of oat spelt xylan hydrolysates by two purified xylanases. Paper chromatography of oligosaccharides released when each of two purified xylanases, STX-I (left) and STX-II (right), was incubated with xylan for 0 and 10 min and 1, 6, and 24 h (lanes 1 to 5, respectively). Lanes 6 and 7 were standard xylose (X) and xylobiose (X2) (Sigma), respectively, and the position of xylotriose (X3) was calculated by using the standard log10(degree of polymerization) versus mobility relationship.

FIG. 6. Comparison of the amino-terminal sequence of STX-I with that of *Cellulomonas* exoglucanase. Identical amino acids between STX-I and *Cellulomonas* exoglucanase (CEX) are boxed. Dashed lines represent spacers inserted into sequences for alignment. The numbering below the sequences refers to STX-I from *S. thermoviolaceus* OPC-520.
AX

B.

VOL.

those of other microbial xylanases. AX, BSX, SCX, and BPX, with hydrolyze xylan. STX-II sequences substrate AX

Ala-Gly-----Pro-Gly Ile-Ile-Leu Gly-Pro-Tyr Tyr S

Ala-Ser----Thr-Asp Arg-Gly------Pro-Gly Ser-Asn-Pro-Setr-Ser

BPX

SCX

FIG. 7. Comparison of the amino-terminal sequence of STX-II with those of other microbial xylanases. AX, BSX, SCX, and BPX, xylanases from an *Aureobasidium* sp., *B. subtilis*, *S. commune*, and *B. pumilus*, respectively. Identical amino acids between STX-II and others are boxed. Dashed lines represent spacers inserted into sequences for alignment. The numbering below the sequences refers to STX-II from *S. thermoviolaceus* OPC-520.

substrate (data not shown). However, the ability to hydrolyze the substrate was very weak in comparison with that to hydrolyze xylan.

REFERENCES


