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Purification, Characterization and Gene Cloning of High-molecular-weight Xylanase-4 of *Aeromonas caviae* W-61

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Abstract: *Aeromonas caviae* W-61 produces multiple extracellular xylanases, the xylanases 1, 2, 3, 4, and 5. In this study, we purified and characterized the xylanase-4 gene of *A. caviae* W-61, and cloned it. The purified xylanase-4 consisted of a single polypeptide with molecular masses of 120 kDa. The xylanase-4 was endo- β -1,4-xylanase with optimum temperature 40°C, optimum pH 6.0 and temperature stability 40-50°C. Various xylo-oligosaccharides such as xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose were formed, and a small amount of xylose was detected as the hydrolysis products. The N-terminal amino acid sequence and several identical internal amino acid sequences of xylanase-4 were determined. From the sequence, 1.8 kbp *xyn 4* was amplified by PCR and was cloned from the genomic DNA of *A. caviae* W-61. The flanking region of xylanase-4 were sequenced and it contained a sequence corresponding a typical signal peptide consisting of 27 amino acid residues at the 5' end. Putative promoter (-35 and -10) sequences and a typical ribosome-binding sequence were present upstream the *xyn 4*.

Key words: *Aeromonas caviae* W-61, high-molecular-weight xylanase, purification, enzyme characteristics, polymerase chain reaction, gene cloning

Introduction

β -1,4-Xylans, the major components of hemicellulose, are heterogenous polysaccharides consisting of homopolymeric backbone of 1,4-linked- β -xylopyranose units and the short side chains including O-acetyl, α -L-arabinofuranosyl, and α -D-glucuronyl residues (Whistler, 1970). It has been shown that bacteria and fungi utilize β -1,4-xylan as a carbon source with the help of xylanolytic enzymes such as β -1,4-xylanases and β -xylosidases (Sunna, 1997). β -1,4-Xylanases (E.C. 3.2.1.8) are the key enzymes that hydrolyze the backbone structure of β -1,4-xylans to initiate the degradation of complex polysaccharides by microorganisms. A number of β -1,4-xylanases have been purified from a wide range of microorganisms including *Bacillus spp.*, *Clostridium spp.*, and *Streptomyces spp.* (Wong *et al.*, 1988). Several of these microorganisms produce multiple xylanases, implying the strategy to achieve effective hydrolysis of β -1,4-xylan. Each enzyme may have a specialized function in the biodegradation of complex polysaccharides, and specialized functions of individual xylanases may be useful for biotechnological applications in food and paper industries (Viikari *et al.*, 1994). We have previously isolated a β -1,4-xylan-degrading bacterium, *A. caviae* W-61, which produced five extracellular xylanases, the xylanases 1, 2, 3, 4, and 5 (Nguyen *et al.*, 1993). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by the zymography using Ramazol brilliant blue (RBB)-stained xylan showed that the xylanases 1, 2, 3, 4, and 5 had molecular masses of 22, 41, 58, 120 and 140 kDa, respectively (Nguyen *et al.*, 1988). Of these xylanases, we have purified the xylanases 1, 2, and 3 from the culture fluids of *A. caviae* W-61, and characterized their enzymatic properties. The results showed,

- (i) the xylanase 1 hydrolyzed oat spelt xylan to release xylobiose (X2), xylotriose (X3), xylotetraose (X4), and xylopentaose (X5);
- (ii) the xylanase 2 hydrolyzed oat spelt xylan to liberate X3, X4, X5, and X6 as the major products (unpublished observation);
- (iii) and the xylanase 3 produced xylo-oligosaccharides larger than xylohexaose (X6) from the same substrate.

Thus, the xylanases 1, 2, and 3 from *A. caviae* W-61 have

different enzymatic properties, implying their collaboration in the degradation of xylans by the bacterium.

In this study, we purified and characterized the 120 kDa xylanase, the xylanase-4 from the culture fluids of *A. caviae* W-61, and cloned the 1.8 kbp gene encoding the xylanase-4 (*xyn 4*).

Materials and Methods

Bacterial strains and plasmids: *A. caviae* W-61 and *Escherichia coli* DH 5 α were used. Plasmid pUC 119 was obtained from TaKaRa (Kyoto, Japan)

Growth of *A. caviae* W-61 for xylanase-4 production: *A. caviae* W-61 was aerobically grown at 26°C for 36 h in a 3-liter flask containing 600 ml of the liquid medium I (0.5% oat spelt xylan, 0.2% yeast extract, 0.25% NaCl, 0.5% NH₄Cl, 1.5% KH₂PO₄, 3% Na₂HPO₄, and 0.025% MgSO₄ · 7H₂O). After the cultivation, culture supernatants were collected by the centrifugation at 10,000xg at 4°C for 15 min.

Assay for xylanase activity: Unless otherwise stated, xylanase activity was assayed by measuring the amount of reducing sugars liberated from oat spelt xylan in 50 mM sodium phosphate buffers, pH 7.0 at 37°C, as described previously (Nguyen *et al.*, 1988). The reducing sugars formed were measured by the Somogyi-Nelson method, using D-xylose as a standard (Somogyi, 1952). One unit of xylanase was defined as the amount of enzyme which liberated 1 μ mol of xylose per min.

Zymographic assay for xylanase: Xylanase in the test samples were differentially assayed by the zymography using Ramazol brilliant blue (RBB)-stained xylan. Briefly, xylanase in the samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by renaturation in a solution of 2.5% Triton X-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 h. After the incubation, the gels were immersed in an 66% (v/v) ethanol solution to remove cleaved products from RBB-stained xylan.

Protein assay: Protein was measured using bovine serum

albumin as a standard (Bradford, 1976).

Purification of xylanase-4: All purification steps except for the high performance liquid chromatography were carried out at 0-4°C. Culture supernatants of *A. caviae* W-61 were concentrated approximately 20-fold by ultrafiltration using a Model Militan Plate 4/PK (Millipore Co., Bedford, MA, USA). Xylanase was precipitated with ammonium sulphate at a concentration corresponding to 75% saturation. The resultant precipitates were collected by centrifugation at 12,000xg for 20 min, dissolved in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer. The dialyzed sample was mixed with 70 ml of the DEAE-Toyopearl 650M (Tosoh; Tokyo, Japan) previously equilibrated with the same buffer, and the mixture was stirred for 30 min and centrifuged at 1000xg for 10 min. The supernatant, which contained the xylanases 1, 2, and 3 was removed. The precipitated DEAE-Toyopearl 650 M resins, which adsorbed the xylanase-4, was washed with the same buffer, and packed into a column (diameter 3 cm, height 10 cm). The adsorbed xylanase was eluted with 0.16 M NaCl, and dialyzed against 5 mM sodium phosphate buffer, pH 7.0. The dialyzed enzyme solution was loaded onto a hydroxylapatite column (diameter 2.2 cm, height 8 cm) previously equilibrated with the same buffer. Adsorbed proteins were eluted with a linear gradient of 5-100 mM sodium phosphate buffer (pH 7.0). Active fractions were combined, mixed with the same volume of 3 M ammonium sulphate, and loaded onto a TSK gel phenyl-5PW column (Tosoh; diameter 0.75 cm; height 7.5 cm), which was previously equilibrated with 25 mM sodium phosphate buffer (pH 6.8) containing 1.5 M ammonium sulphate. Adsorbed proteins were eluted with a descending linear gradient of ammonium sulphate (from 1.5 to 0). The xylanase-4 was eluted with 0.2-0 M ammonium sulphate. The active fractions were combined, dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and loaded onto a TSK gel DEAE-5PW column (Tosoh; diameter 0.75 cm, height 7.5 cm). Adsorbed xylanase-4 was eluted with a constant concentration of NaCl (165 mM). Eluted xylanase-4 was tested by SDS-PAGE and SDS-PAGE/zymography.

Protein sequencing for the intact xylanase-4 and for the fragments obtained from the cyanogens bromide (CNBr)-cleaved: The N-terminal amino acid sequence was analyzed for the protein bands blotted onto polyvinylidene difluoride sheets using a gas phase protein sequencer (Model PSQ-1; Shimadzu, Kyoto). Cleavage of the xylanases by CNBr was done as described previously (Okai *et al.*, 1998).

Analysis for the products from the oat spelt xylan cleaved by the xylanase-4: Oat spelt xylan (1mg) was incubated with the xylanase 5 in 200 μ l of 50 mM sodium phosphate buffer (pH 7.0), at 37°C for 0-4 h. The reaction mixture was deionized, lyophilized, and spotted on a silica gel plate (Merck Silica Gel 60F 254; E. Merck, Darmstadt, Germany). The thin layer chromatography (TLC) was developed with a solvent of 1-butanol/2-propanol/water/acetic acid (7/5/ 4/2, v/v), and the silica gel plate was sprayed with aniline/diphenylamine/acetone/ 85% phosphoric acid (0.4/0.4/20/3) and heated at 110°C for 15 min. D-xylose and xylo-oligosaccharides (X2-X6) were used as the standards.

Amplification of a part of the xylanase-4 gene (*xyn 4*) by PCR: Chromosomal DNA was isolated from the cleared lysate of *A. caviae* W-61 and purified as described previously (Okai *et al.*, 1998). The purified chromosomal DNA from *A. caviae* W-61

was used as a template for the PCR of a part of the *xyn 4*. The oligonucleotide primers F and R were synthesized according to the amino acid sequences, which were found in N-terminal region of the xylanase-4 and in the fragment from the CNBr-cleaved xylanase-4. The PCR was done 30 cycles with TaKaRa Ex Taq (TaKaRa; Kyoto) using the following temperature profile: 94°C, 1 min; 44°C, 1 min; 72°C, 2 min. An approximately 1.8 Kbp fragment was amplified by PCR, and the amplified fragment was purified by an agarose gel electrophoresis. The purified 1.8 Kbp fragment was inserted into the *Hinc* II site of pUC 119. The pUC 119 vector thus obtained was used to transform *E. coli* DH 5 α . The inserted 1.8 Kbp fragment was confirmed to contain the DNA sequences corresponding to N-terminal amino acid sequences of intact and internal xylanase-4.

DNA hybridization: The cloned 1.8 Kbp fragment was digested with *Hind* III, and the resultant 0.6 Kbp fragment was purified and labeled with ECL random prime labeling system (Amersham International, Buckinghamshire, UK). Hybridization was done at 45°C overnight with the labeled probe, and the hybridized DNA fragments and the hybridized colonies were detected fluorographically.

Genomic cloning of the *xyn 4*: Chromosomal DNA from *A. caviae* W-61 was digested with various restriction endonucleases according to the protocols of manufacture. The digested DNA fragments were electrophoresed in a 0.7% agarose gel, blotted onto a Hybond-N⁺ nylon sheet (Amersham International). DNA hybridization for the blotted DNA fragments was done with the labeled 0.6 kbp probe as described above. The 4 kbp *Eco*RI fragment and the 2.6 Kbp *Kpn*I fragment, which were hybridized with the probe, were extracted from the gel, and inserted into the *Eco*RI and the *Kpn*I sites of pUC 119 vector, respectively. *E. coli* DH5 α . was transformed with the pUC 119 vectors carrying the inserts, and the recombinant *E. coli* DH5 α were selected by colony hybridization using the same probe.

DNA sequencing: DNA sequencing was done using the SequiTherm Long read cycle sequencing kit (Epicenter Technologies, Madison, WI, USA) and M13 forward and reverse IR-dyeprimers, as described previously (Okai *et al.*, 1998). The resultant sequences were analyzed with the GENETYX software package (Software Development, Tokyo).

Chemicals: Oat spelt xylan and RBB-stained oat spelt xylan were purchased from Nacalai Tesque Inc. (Kyoto, Japan). D-xylose, carboxymethyl-cellulose, and hydroxylapatite were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). DEAE-Toyopearl 650M was from Tosoh (Tokyo, Japan). Molecular standards for SDS-PAGE were purchased from Dai-ichi Chemicals Co., (Tokyo, Japan). Restriction endonucleases and the other enzymes used in gene cloning were purchased from TaKaRa (Kyoto, Japan). Unless otherwise stated, chemicals used in this study were of the highest grade commercially available.

Results and Discussion

Purification of xylanase-4 from culture fluids of *A. caviae* W-61: Xylanase-4 was purified to homogeneity by the DEAE-5PW column chromatography (Fig. 1) and the purity was checked by SDS-PAGE and zymography. Xylanase-4 gave single band of protein in both SDS-PAGE and zymography. Molecular mass of the purified xylanase-4 was estimated to be 120 kDa, on SDS-PAGE under reducing conditions (Fig. 2A),

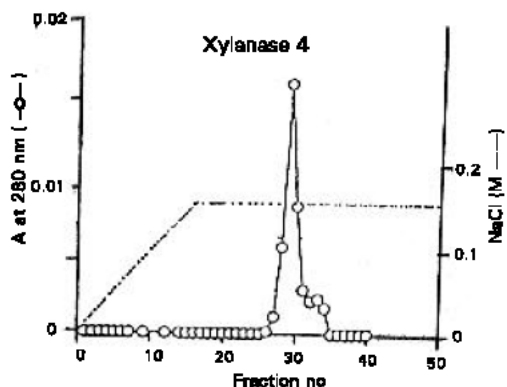


Fig. 1: Elution profile of xylanase-4 from DEAD-5PW column. Symbols, --O-- A280nm and NaCl concentration.

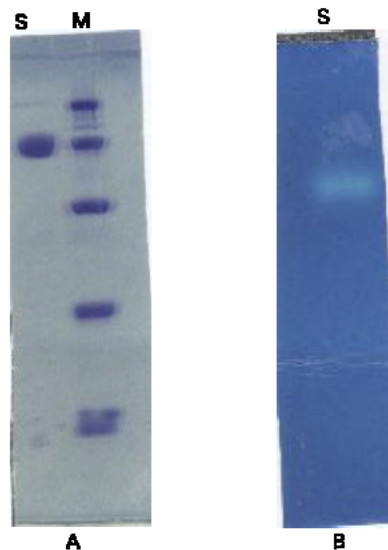


Fig. 2: SDS-PAGE (A) pattern and Zymogram analysis (B) of purified xylanase-4. Lane S indicated purified xylanase-4. M, standard protein (from top): myosin (200 kDa), galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa) and myoglobin (17 kDa).

and the purity and the molecular mass of the xylanase-4 was confirmed by the zymographic results (Fig. 2B).

N-terminal and internal amino acid sequences of the xylanase-4:

Protein sequencing for the purified xylanase-4 showed 17 N-terminal amino acid residues: Val-Ser-Ala-Ala-Asp-Pro-Glu-Ala-Ser-Asn-Ser-Gln-Ile-Val-Tyr-Glu-Glu-. The xylanase-4 was cleaved by the treatment with CNBr, and the resultant fragments were electrophoresed on SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride sheet. The CNBr-treated xylanase-4 gave four major bands (i. e., the fragments 1-4) on SDS-PAGE. The N-terminal amino acid sequences for the 4 fragments were: Asp-Val-Asn-Pro-Ser-Ala-Ala-Gly-Lys-Ala-Val-Leu-Glu-Val-Val-Thr-Asn-Lys-Gly-Val-Asp-Gly-Ser-Glu-Ala-Tyr-Asn- for the fragment 1, Ala-Pro-Gly-Glu-Ala-Pro-Thr-Thr-Leu-Arg-Ile-Ser-Val-Gln-Tyr-Ala-Glu-Ser-Gly-Tyr-Ala-Asn-Val-Ser-Gln-Asn-Ala-Thr-Val- for the fragment

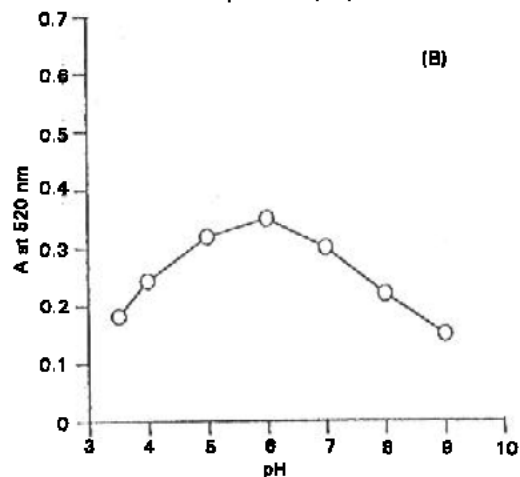
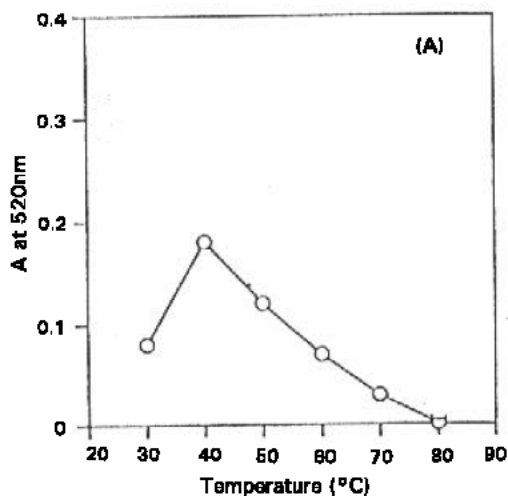


Fig. 3: Effect of temperature (A) and pH (B) on xylanase activity. The reaction was done for 10 min at various temperature and pHs. Symbol --O--, xylanase-4.

2, Lys-Pro-Asp-Tyr-Ala-Tyr-Asn-Ala-Asp-Arg-Gln-Phe-Asp-Phe-Thr-Ala-Glu-Asp-Ala-Leu-Val-Gln-Arg-Val-Arg-Asp-Ala-Gly-Leu-Asp-Leu-His- for the fragment 3 and Ala-Gly-Thr-Asn-Ser-Ala-Ile-Thr-Glu-Lys-Glu-Ala-Lys-Gln-Gln- for the fragment 4.

Effect of Temperature and pH on the xylanase-4:

Xylanolytic activity of the xylanase-4 towards oat spelt xylan was assayed at different temperatures between 0 and 80°C for 10 min. The xylanase-4 showed the highest activity at 40°C (Fig. 3A). Heat stability of xylanase-4 was assessed by measuring the residual xylanolytic activities at 37°C for 10 min after pre-incubation of the enzymes at different temperatures between 20 and 80°C for 80 min. The xylanase-4 had significant xylanolytic activities over the range between pH 3 and 9, and the enzyme had the highest activity at pH 6 (Fig. 3B). As shown in Fig. 4A, the xylanase-4 was stable at temperatures of \leq 40°C. Xylanase-4 was stable at neutral pHs of between pH 5 and pH 7, and was stable at acidic pHs (Fig. 4B).

Xylo-oligosaccharide products from oat spelt xylan by the action of xylanase-4:

Oat spelt xylan was treated with the

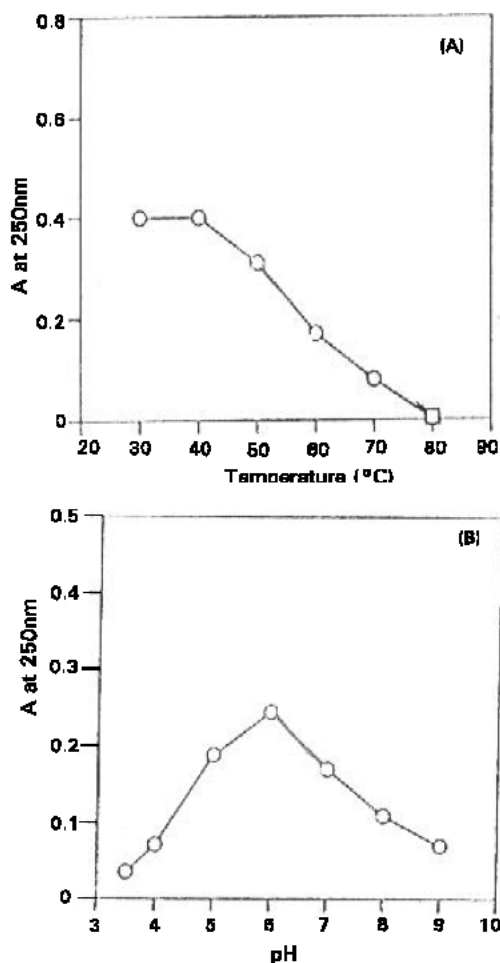


Fig. 4: Effect of temperature (A) and pH (B) on xylanase stability. The enzymes were incubated for 1 h at various temperatures and the residual activities were measured at 37°C symbols: -O-, xylanase-4.

Table 1: Hydrolysis of various polysaccharides by the xylanase-4 from *A. caviae* W-61

Substrates	Xylanase-4
Oat spelt xylan	100
Water soluble xylan	110
Water insoluble xylan	38
Birch wood xylan	200
Beech wood xylan	161
Cellulose	0
Carboxymethyl cellulose	0
β -1,3 xylan	0

xylanase-4 at 37°C for 15-240 min, and the hydrolytic products were analyzed on a thin layer chromatography. As shown in Fig. 5, the xylanase-4 hydrolyzed oat spelt xylan to liberate X2, X3, X4, X5, and X6 as the major products and a trace amount of xylose. These results indicated that the xylanase-4 was endo- β -1,4-xylanase.

Substrate specificities of xylanase-4: Substrate specificities of

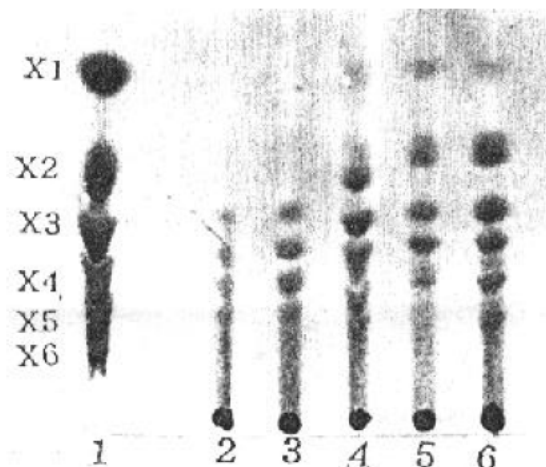


Fig. 5: TLC of hydrolyzates of β -1,4-xylan by the purified xylanase-4. A reaction mixture containing 1.0 mg of oat spelt xylan with 0.1 unit of xylanase-4 was incubated at 40 °C for 15 min (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5) and 240 min (lane 6). Lane 1 represents xylose (X1) and xylooligosaccharide (X2, X3, X4, X5 and X6) standards.

xylanase-4 were studied using various polysaccharides as the substrates, and the results obtained were summarized in Table 1. The xylanase-4 hydrolyzed water-soluble xylan, birch wood xylan and beech wood xylan efficiently. The xylanase-4 showed no hydrolytic activity towards β -1,3-xylan, cellulose and carboxymethyl-cellulose.

Cloning of the 1.8 kbp gene encoding the xylanase-4 (*xyn 4*) of *A. caviae* W-61:

We tried to amplify a part of the *xyn 4* of *A. caviae* W-61 by a polymerase chain reaction (PCR) using chromosome DNA of the bacterium as a template and the following oligo-nucleotide as the primers: 5'-CARATHGHTHTAYCARGAR-3' as the forward primer and 5'-RACRAANCGRAGRAARAG-3' as the reverse primer, where R, Y, H, and N indicates A/G, T/C, A/T/C and A/T/C/G, respectively. The oligonucleotide primers were synthesized according to the amino acid sequences which were found in N-terminal regions of the intact xylanase-5 and of the fragment 4 from the CNBr-cleaved xylanase-4: Val-Ser-Ala-Ala-Asp-Pro-Glu-Ala-Ser-Asn-Ser-Gln-Ile-Val-Tyr-Glu-Glu- of the intact xylanase 5 and Ala-Gly-Thr-Asn-Ser-Ala-Ile-Thr-Glu-Lys-Glu-ala-Lys-Gln-Gln- of the fragment 4, where the underlines indicate the amino acid sequences used for the synthesis of forward and the reverse primers, respectively. A 1.8 kbp fragment was amplified by the PCR under the conditions described in Materials and Methods, and the amplified 1.8 kbp fragment was inserted into the *Hinc* II site of pUC 119 and cloned in *E. coli* DH 5A. DNA sequencing confirmed that the 1.8 kbp fragment contained the nucleotide sequences corresponding to the N-terminal amino acid sequences of the xylanase-4 and of the fragments 1-4 (Fig. 6). So the cloned 1.8 kbp fragment was digested with *Eco*RI and *Hind* III, and the resultant 0.6 kbp fragment, which corresponded to the N-terminal region of the xylanase-4, was labeled and used as a probe for the genomic cloning of the *xyn 4*.

Chromosomal DNA from *A. caviae* W-61 was digested with

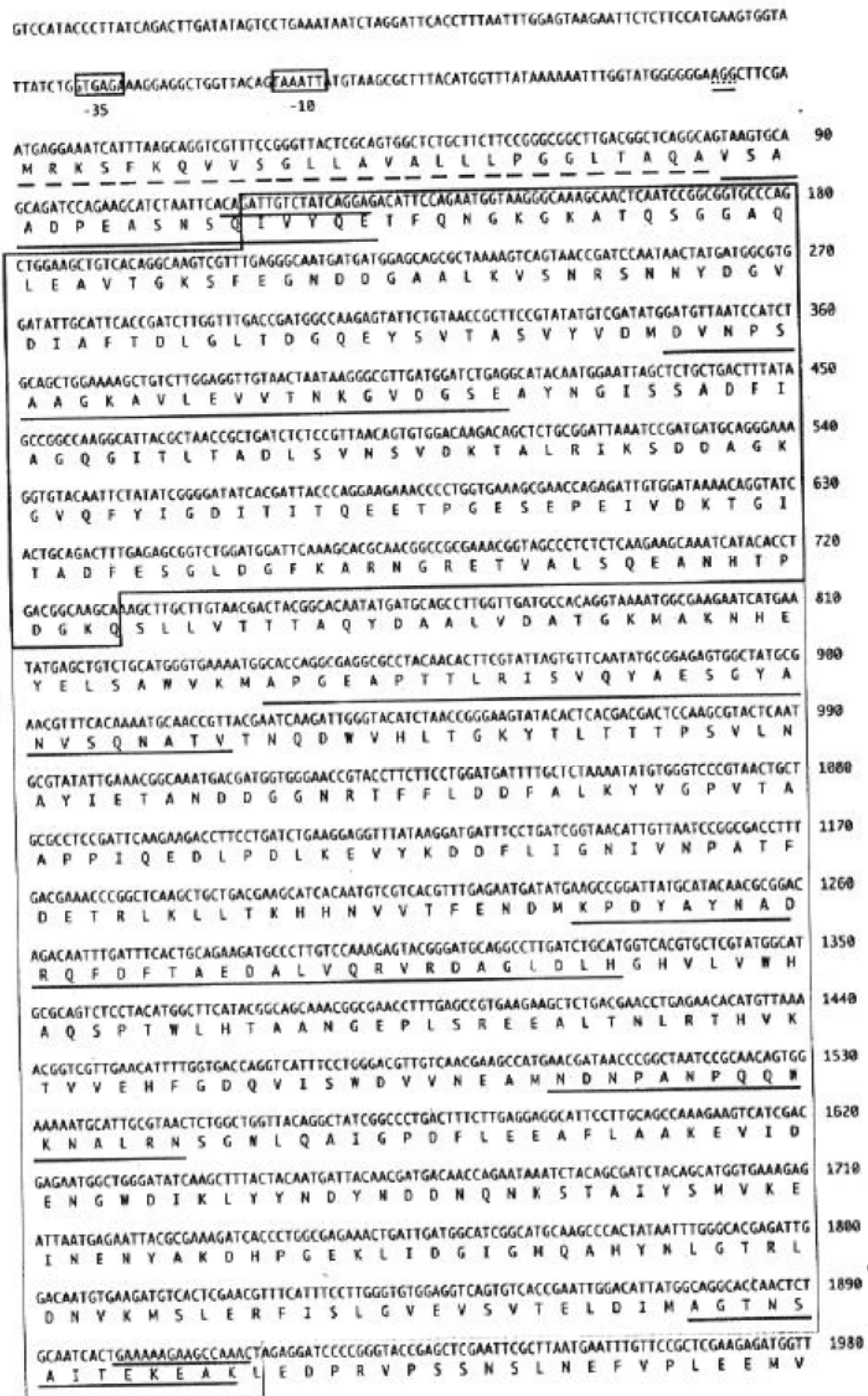


Fig. 6: The partial nucleotide sequence of the xylanase-4 gene and its flanking region and deduced amino acid sequence. The strand shown is 5' to 3' direction. The putative -10 and -35 regions of the promoter site, the putative ribosomal binding site, and the putative signal peptides sequence are indicated in boxes, underline, and broken-underline, respectively. The amino acid sequences of xylanase-4 which are determined chemically (indicated by underlines).

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various restriction endonucleases, and the digested DNA fragments were used for the Southern hybridization using 0.6 kbp probe. The DNA hybridization showed that *EcoRI* fragment and *Kpn1* fragment were hybridized with the probe. The *EcoRI* fragment was inserted into pUC 119 vector, and cloned in *E. coli* DH 5A. DNA sequencing indicated that the *EcoRI* fragment contained (i) the nucleotide sequence corresponding to the forward primer at its 5'-end, (ii) the nucleotide sequences corresponding to fragments 1-4 from the CNBr-cleaved xylanase-4, and (iii) a *Hind* III site 0.6 kbp downstream of the 5'-end. To clone the 5'-upstream the xylanase-4, we used the 2.6 kbp *Kpn1* fragment, because the *EcoRI* fragment contained a single restriction site for *Kpn1* endonuclease 1.8 kbp downstream of the 5' end (Fig. 6). The *Kpn1* fragment was inserted into the pUC 119 vector, and cloned in *E. coli* DH 5A. The partial nucleotide sequence of the *xyn 5* and its flanking region and the deduced amino acid sequence is shown in Fig. 6. It contained the nucleotide sequences corresponding to the N-terminal amino acid sequence and the internal amino acid sequences (i. e., the fragments 1-4) of the purified xylanase-4 (Fig. 6). Putative -35 (TTTACA) and -10 (TGGTAT) sequences were found at 44 and 22 Kbp upstream of the translation initiation codon of the *xyn 4*, respectively, and a typical Shine-Dargarno (GAAGG) sequence was also found upstream of the gene (Fig. 6). The nucleotide sequence corresponding to a typical signal peptide comprising 27 amino acid residues was found at the 5' end of the *xyn 4*. In this study, we purified and characterized the xylanase-4 from the culture fluids of *A. caviae* W-61. The purified xylanase was a protein with a molecular mass of 120 kDa by SDS-PAGE. Xylanase-4 from *A. caviae* W-61 was endoxylanase and cleaved oat spelt xylan randomly to form xylobiose, xylotriose, xyloetraose, xylopentaose and xylohexaose. The xylanase-4 had no activity on cellulose, carboxymethyl cellulose, thus, it is true xylanase. We also cloned a part of xylanase-4 gene. Now we are trying to clone

the whole gene of xylanase-4. It should be interesting to study the whole structural features of xylanase-4 gene and in its catalytic and non-catalytic domain.

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