Molecular Cloning, Characterization, and Expression Analysis of the \textit{xynF3} Gene from \textit{Aspergillus oryzae}

Tetsuya KIMURA,*,† Hayato SUZUKI, Hirofumi FURUHASHI, Takeshi ABURATANI, Kenji MORIMOTO, Kazuo SAKKA, and Kunio OHMIYA

Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

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The gene encoding xylanase F3 (\textit{xynF3}) was isolated from a genomic library of \textit{Aspergillus oryzae} KBN616, used for making shoyu koji. The structural part of \textit{xynF3} was found to be 1468 bp. The nucleotide sequence of cDNA amplified by RT-PCR showed that the open reading frame of \textit{xynF3} was interrupted by ten short introns and encoded 323 amino acids. Direct N-terminal amino acid sequencing showed that the precursor of \textit{XynF3} had a signal peptide of 22 amino acids. The predicted amino acid sequence of \textit{XynF3} has strong similarity to other family 10 xylanases from fungi. The \textit{xynF3} gene was successfully overexpressed in \textit{A. oryzae} and the \textit{XynF3} was purified. The molecular mass of \textit{XynF3} estimated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 32,000. This was almost the same as the molecular mass of 32,437 calculated from the deduced amino acid sequence. The purified \textit{XynF3} showed an optimum activity at pH 5.0 and 58°C. It had a Km of 6.5 mg/ml and a Vmax of 435 μmol·min⁻¹·mg⁻¹ when birch wood xylan was used as a substrate. Expression of the \textit{xynF3} gene was analyzed using an \textit{Escherichia coli} \beta-glucuronidase gene as a reporter. The result indicated that \textit{xynF3} is expressed in the medium containing wheat bran as a carbon source.

Key words: \textit{Aspergillus oryzae}; shoyu-koji; xylanase

The filamentous fungus \textit{Aspergillus oryzae} has been important in Japan for traditional food production, for example, production of soy sauce ‘shoyu’ and rice wine ‘sake’. Commercially available enzymes are also produced by \textit{A. oryzae}. \textit{A. oryzae} produces plant cell wall degrading enzymes when grown on a \textit{shoyu koji} composed of soybeans and wheat. A greater efficiency of degradation of soybean and wheat cell wall would improve the usage of raw materials and decrease the amount of pressed cake left following the press-filtration of soy sauce mash, which is difficult to dispose of. Xylan is one of the major components of these cereal plant cell walls. It is composed of polysaccharide, consisting of β-1,4-linked D-xylopyranoside residues. Endo-β-1,4-xylanase (EC 3.2.1.8), designated xylanase, degrades this backbone of β-1,4-linkages of xylose. To improve the enzyme production in \textit{A. oryzae}, we have been analyzing the expression mechanism of xylanase genes in \textit{A. oryzae}. In these studies, we have cloned two family 11 xylanase genes and one family 10 xylanase gene from \textit{A. oryzae} KBN616, which is used for shoyu-koji, and characterized the gene products.1–4) Here, we report the cloning of a new xylanase gene of \textit{A. oryzae} and characterization of its product. Also, we analyzed the expression of this gene.

Materials and Methods

Strains and media. \textit{A. oryzae} KBN 616, a shoyu koji mold, was obtained from Bio’c (Toyohashi). \textit{A. oryzae} KBN616 was cultivated in liquid medium (0.5% yeast extract, 0.1% peptone, 0.1% K2HPO4, 0.1% NaNO3, MgSO4·7H2O, pH 6.0) containing 1% carbon sources or in solid state containing steamed wheat bran. \textit{Escherichia coli} DH5α was used for DNA manipulation.

DNA manipulation. A part of the \textit{xynF3} fragment amplified by PCR was labelled with digoxigenin-11-dUTP (Boehringer Manheim Biochemica) and used to screen the genomic library. A genomic library of \textit{A. oryzae} KBN616 was constructed in Charomid 9-28 (Nippon Gene) as described previously.5) Nucleotide sequences were analyzed using the dideoxy chain-termination method. The sequence was analyzed by a Lioric model 4000L automated DNA sequencer using a Thermo Sequenase cycle sequencing kit (Amersham Life Science Inc). The nucleotide sequence data was analyzed with GENETYX computer software (Software Development Co. Ltd.). Sequence similarity searches in GenBank were done with a BLAST program. All of the DNA

* To whom correspondence should be addressed. Phone: +81-59-231-9606; Fax: +81-59-231-9634; E-mail: t-kimura@bio.mie-u.ac.jp

Abbreviations: PCR, polymerase chain reactions; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Table 1. Primer Sequences Used for PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>xynF3ORF-F</td>
<td>5′CTCGAGACATGGTTCATCTCAAGTCTCT3′&lt;br&gt;<strong>XhoI</strong></td>
</tr>
<tr>
<td>xynF3ORF-R</td>
<td>5′TCTAGAACGACCTACAAGCGGTTAA3′&lt;br&gt;<strong>XbaI</strong></td>
</tr>
<tr>
<td>xynF3ProF</td>
<td>5′GCGGCCGCCCATGAGGATAACCTTAAG3′&lt;br&gt;<strong>NotI</strong></td>
</tr>
<tr>
<td>xynF3ProR</td>
<td>5′GGATCCGTCTAGAGGTTGGATGTAAAAAACT3′&lt;br&gt;<strong>BamHI</strong></td>
</tr>
<tr>
<td>xynF1ORF-F</td>
<td>5′AAGCTTCGCTGAGTCTTACAAGGCGTTAAG3′&lt;br&gt;<strong>HindIII</strong></td>
</tr>
<tr>
<td>xynF1ORF-R</td>
<td>5′GGATCCGTCTAGAGGTTGGATGTAAAAAACT3′&lt;br&gt;<strong>NotI</strong></td>
</tr>
<tr>
<td>xynF1ProF</td>
<td>5′GCGGCCCGCTGGATTTACAGAGCATCGATGAT3′&lt;br&gt;<strong>NotI</strong></td>
</tr>
<tr>
<td>xynF1ProR</td>
<td>5′GGATCCTTTGAATAAAAAGGAATAGTAGACGT3′&lt;br&gt;<strong>BamHI</strong></td>
</tr>
</tbody>
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**Construction of plasmids.** In order to efficiently express the xynF3 gene in *A. oryzae*, the ORF region of the xynF3 gene was amplified by PCR with xynF3ORF-F and xynF3ORF-R primers (Table 1). The amplified fragment was subcloned between *XhoI* and *XbaI* sites in pBluescript KS (+) and sequenced. The ORF region of the xynF1 gene was also amplified by PCR with xynF1ORF-F and xynF1ORF-R primers. The amplified fragment was subcloned between *HindIII* and *NotI* sites in pBluescript KS (+) and sequenced. Then they were inserted into the pNAN8142 vector, which was kindly provided by Ozeki Corporation. The resultant plasmids were introduced into a niaD deficient *A. oryzae* strain, KBN616-ND1.7)

**Transformation of A. oryzae.** The transformation of *A. oryzae* KBN616-ND1 was done by the method of Gomi et al.8) *A. oryzae* protoplasts were prepared from mycelia grown at 30°C for 15 h using Yatalase (Takara Shuzo). Transformants were selected on Czapek-Dox medium.

**RT-PCR and cDNA cloning.** A xynF3 over-expressing transformant described below was cultivated in Czapek-Dox medium for 48 h at 30°C and mycelia were collected by filtration with Watman 3MM paper. Total RNA was extracted from the mycelia and RT-PCR was done using SuperScript One-Step RT-PCR (Gibco BRL) as in the manufacturer’s protocol. The xynF3 cDNA was amplified by PCR using the RT-PCR products as the template with xynF3ORF-F and xynF3ORF-R primers (Table 1). Five independent RT-PCR-derived cDNAs were subcloned in pBluescript KS (+) and sequenced.

**Purification of XynF3.** One of the *A. oryzae* transformants was grown in glucose-polypeptone medium (2% glucose, 1% polypeptone, 0.5% KH2PO4, 0.5% KCl, 0.1% NH4Cl, 0.05% MgSO4, pH 6.0) for 3 days. A 150-ml sample of culture supernatant was concentrated by ammonium sulfate precipitation at 70% saturation and pelleted by centrifugation. The pellet was dissolved in 20 mM Tris-HCl (pH 7.5) buffer containing 0.15 M sodium chloride. The solution was concentrated and dialyzed against the same buffer by an Amicon ultrafiltration cell with YM10 membrane (Millipore). The resultant solution was put onto a SuperdexG75 column (Pharmacia Biotech) equilibrated with the same buffer. Active fractions were combined and used as the purified enzyme.

**Xylanase assays.** The xylanase activity was assayed by measuring the reducing groups liberated from 0.75% (w/v) birch wood xylan (Sigma) after incubation at 40°C for 10 min in 50 mM of succinate buffer (pH 6.0) by the 3,5-dinitrosalicylic acid method using xylose as standard.9) One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugars equivalent to 1 μmol of xylose per min. Protein was measured by the Bradford protein assay kit (Bio-rad Laboratories) using bovine serum albumin fraction V (Sigma) as the standard. The pH optimum of the enzyme was measured by incubating the enzyme for 10 min at 40°C in 40 mM Britton-Robinson buffer. The pH stability was measured by incubating the enzyme at various pHs (2.0 to 9.0) for 12 h at 25°C. The temperature optimum was identified by incubation at various temperatures in 50 mM succinate buffer of the optimal pH. The thermal stability was measured by incubating the enzyme at various temperatures (30°C–70°C) for 10 min at pH 6.0.

**Manipulations** were done using standard methods as described by Sambrook et al.6)
β-glucuronidase assay. Approximately 10⁶ conidiospores of A. oryzae transformants were inoculated into 30 ml of complete medium (0.5% yeast extract, 0.1% Bacto peptone, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.1% NaNO₃, pH 6.0) containing 1% fructose for 48 h at 30°C and then mycelial pellets were collected by filtration with mesh. The mycelial pellets were washed once with distilled water and transferred to a new complete medium containing each carbon source. They were cultivated for 48 h at 30°C. After cultivation, mycelia were collected by filtration with a Whatman 3MM filter. Cell-free extracts were prepared by grinding mycelia with a mortar and pestle in liquid nitrogen. Lysis buffer (50 mM Na₂HPO₄, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10 mM β-mercaptoethanol, pH 7.0) was added to the mycelial powder and mixed well. The extracts were centrifuged at 10,000 × g for 10 min and supernatants were used for the β-glucuronidase (GUS) assay. For the solid-state cultivation, 10% (w/w) of distilled water was added to the wheat bran and mixed well. Ten grams of wet wheat bran was autoclaved in a 100-ml flask. One loop of conidiospores of the transformant was cultivated at 30°C for 48 h, after which the wheat bran including the A. oryzae mycelia was disrupted with a mortar and pestle in liquid nitrogen. The powder was used to extract GUS as described above. GUS activity was measured as described by Jefferson et al. with bovine serum albumin as the standard.

Electrophoresis of proteins and N terminal amino acid sequencing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12% acrylamide gel as described by Laemmli. The gels were stained with Coomassie Brilliant Blue R 250. After electrophoresis, protein was electroblotted onto a PVDF membrane (Millipore). The protein was stained with Coomassie Brilliant Blue R250 and was cut off from the membrane. Its N-terminal amino acid sequence was analyzed on an Applied Biosystems model 476A protein sequencer.

Nucleotide sequence accession number. The sequence has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB066176.

Results

Isolation and characterization of the A. oryzae xynF3 gene

Previously, we reported the cloning of the xynF3 gene encoding a major family 10 xylanase secreted from A. oryzae KBN616 when it grows in a medium containing xylan as a carbon source. In that research, we cloned four different PCR fragments, which encode a portion of family 10 xylanases. This suggests that A. oryzae has at least four family 10 xylanase genes. To clone the full length of the xynF3 gene, a genomic library of A. oryzae KBN616 constructed in Charomid 9–28 was screened with the 600 bp of a PCR-amplified xynF3 fragment as a probe. Of 4 × 10⁵ colonies screened, 10 positive clones were obtained. They contained 2.0 and 5.5 kb fragments which were hybridized to a probe on Southern blot analysis and further characterized in detail (Fig. 1). The xynF3 gene was sequenced in both directions (Fig. 2). Although the exact translation-initiation site was not identified, the structural part of xynF3 appeared to be 1468 bp long since no other translation initiation codons (Met) were present in this ORF. Sequencing of the xynF3 cDNA indicated that the coding sequence was interrupted by ten introns ranging in size from 46 to 55 bp. The intron/exon junction, which followed the GT-AG rule, was present with the exception of intron eight. The size of the intron resembled those of other filamentous fungal introns. Moreover, the intron occurs at the same position as those of other family 10 xylanase genes of filamentous fungi. The open reading frame of xynF3 encodes 323 amino-acid residues. Comparison of the deduced amino acid sequence of XynF3 with other fungal xylanases suggests that the precursor of XynF3 contains the signal sequence of 22 amino acids in its N-terminal. This was confirmed by direct amino acid sequencing of N-terminal amino acid of purified XynF3 from a XynF3 over-producing transformant as described later. The secretory precursor was processed at a specific cleavage site between the Gln22 and Gln23 residues, resulting in the formation of a mature enzyme composed of 301 amino acids with a predicted molecular mass of 32,437. However, signal peptidase usually recognizes amino acids with small side chains such as Ala, Gly, or Ser of the C-terminal of signal peptide. Therefore, XynF3 would have been proteolytically processed after cleavage by a signal peptidase.

Sequence similarity searches in GenBank were done with a BLAST program. The deduced amino acid sequence of XynF3 was compared to the published sequences for other fungal xylanases. The amino acid identities of XynF3 to A. sojae xylanase
Fig. 2. Nucleotide and Deduced Amino Acid Sequence of the xynF3 Gene.

Numbers on the left refer to nucleotide sequence (negative numbers refer to nucleotides upstream from the xynF3 ATG) and amino acid sequence (negative numbers refer to the amino acid upstream from the cleavage site of the signal sequence). The TATA consensus sequence is underlined. Intron sequences within the ORF of xynF3 are in lower-case letters. The amino acid identified by sequencing of the purified XynF3 is double underlined in the deduced amino acid sequence.

T. KIMURA et al. (GenBank accession no. AB040414), A. oryzae xylanase (A62445), A. aculeatus xylanase (AB013110), A. kawachii XynA,4,12 and A. niveus XylB was 97.7, 75.7, 74.7, 73.7, and 70.7, respectively. Surprisingly, the primary structure of XynF3 was very similar to that of the xylanase from A. sojae. Although other xylanase sequences from this A. sojae strain are not available, this suggests that A. oryzae is closely related to A. sojae taxonomically.

The 5' non-coding region of the sequence was screened for various consensus sequences. A typical eukaryotic promoter with a TATA box (TAATATTTATG) was detected 102 bp upstream from the translation initiation site. No 5' GGCTAAA 3' sequences as the binding site for XlnR, a transcriptional activator of the xylanolytic system in A. niger, were found within the region upstream of the translation initiation site. This results suggest that a different transcriptional system drives the expression of the xynF3 gene. In common with some other fungal genes, the typical polyadenylation signal, AATAAA, was not present within the 3' non-coding region.

Overexpression, purification, and characterization of XynF3

The xynF3 gene was expressed under the control of P-No8142 promoter since several fungal proteins
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**Fig. 3.** SDS-PAGE of the Proteins in the Culture Supernatant of *A. oryzae* KBN616 and Purified XynF3.

Left panel; *A. oryzae* KBN616 was cultivated in the medium containing 1% oat spelt xylan (lane 1) or Birchwood xylan (lane 2) for 3 days. The *xynF1* overexpressing transformant (lane 3) and the *xynF3* overexpressing transformant (lane 4) were also cultivated in glucose-polypeptone medium for 3 days. Proteins in one ml of each culture supernatant were precipitated with trichloroacetic acid, separated by SDS-PAGE on a 12.5% gel, and stained with Coomassie Brilliant Blue. Right panel; Purified XynF3 was also electrophoresed.

**Fig. 4.** Schematic Diagram of Plasmid pF1 and pF3.

Promoter analysis of the *xynF3* gene

Construction of plasmids for promoter analysis was done as described in Fig. 4. The promoter region of pNAN8142 was removed by ligating *Xho*I and *Pst*I sites after cutting with both enzymes and blunting with T4 polymerase. The resultant plasmid was designated pNAN-d. From the 5′-upstream region, 1315 bp of *xynF3* and 904 bp of the 5′-upstream region of *xynF1* were amplified by PCR using the *xynF3* gene and the *xynF1* gene as the template, respectively. The oligonucleotide primers *xynF3ProF* and *xynF3ProR* were used to amplify *xynF3* promoter and *xynF1ProF* and *xynF1ProR* were used to amplify *xynF1* promoter (Table 1). They were placed upstream of the open reading frame of the *E. coli uidA* gene in pGUS-T. After the nucleotide se-
quences were confirmed, each of the promoter-uidA-terminator cassette was excised with NotI and XhoI and was cloned between the NotI and Sall sites in pNAN-d. The resultant plasmids containing xynF1 promoter-uidA-terminator and xynF3 promoter-uidA-terminator were designated as pF1 and pF3, respectively. For the control experiment without any promoters in the upstream of uidA, the uidA-terminator cassette was also subcloned into pNAN-d. The plasmids were linearized by digesting them with EcoRI and used for transformation of A. oryzae KBN616-ND1. To compare the GUS activity in A. oryzae transformant carrying pF1 or pF3, the gene dosage effect should be considered. The copy number of the integrated plasmid in the transformant was measured. The same amount of total DNA extracted from each transformant was digested with HindIII and NotI for the transformants. Hybridization was done with the niaD gene as a probe. The integrated xynF1 promoter-uidA and xynF3 promoter-uidA fusion gene gave a 11-kb band and the native niaD gene gave a 4.5-kb band. Densitometry was used to estimate the copy number of the integrated gene. From comparison of the hybridization intensity of the integrated gene with that of the native niaD gene, the integrated copy number of the plasmid was evaluated. Integration of uidA was also confirmed by Southern blot analysis with the uidA gene as a probe. Three independent transformants that harbored a single copy of the plasmid at the native niaD locus were selected and used for further analysis.

The transformants were assayed for the amount of GUS activity. Quantitative GUS assays were done using mycelial extracts obtained from each transformant. The inductive effect of xylan on the expression of the uidA gene was examined after 48-h cultivation in each medium (Table 2). GUS activity of each F1 transformant in the medium composed of xylan was shown to be much higher than that in the medium composed of glucose. GUS activity extracted from the mycelia cultivated in the xylan medium was linearly increased from 24 h to 48 h of cultivation. Then it was gradually increased until 72 h of cultivation. In contrast, GUS activity of each F3 transformant in the medium composed of xylan was not induced. It was at almost the same level as in the glucose medium. We confirmed that GUS activity was not observed in the mycelial extract of the transformants harboring the control plasmid. The results presented here suggest that expression of the xynF3 gene is not induced by xylan in A. oryzae KBN616.

A. oryzae for Japanese traditional brewing is usually grown in a solid state culture that results in a high level of enzymes. To find whether the transformants grown in solid state could produce more GUS than that in liquid culture, both transformants were grown on wheat bran and GUS activity was examined. GUS activity of F1 transformants was higher than that of F3 transformants. However, the difference of GUS activity between F1 and F3 transformants was less than that in the liquid culture containing xylan as a sole carbon source. Expression of GUS in F3 transformants cultivated on solid-state culture (32.7 ± 9.12) was much higher than that in the liquid culture of xylan-containing medium (5.18 ± 0.220). To find whether the expression of GUS in F3 transformants depends on solid-state culture or wheat bran, we analyzed GUS activity of F1 and F3 transformants cultivated in liquid culture containing wheat bran. Interestingly, GUS activity of F3 transformants, was much higher than that of F1 transformants and its level was slightly higher than that in solid-state culture.

**Discussion**

To date, many xylanase genes have been cloned from fungi. They can be grouped into two families, family 10 and family 11 glycosyl hydrolases. Most fungi have been shown to harbor two family 11 xylanases and one family 10 xylanase. We have also reported the cloning of two family 11 xylanases and one family 10 xylanase from A. oryzae. In these studies, we found that A. oryzae KBN616 for Japanese soy sauce production has at least four family 10 xylanase genes. Genomic Southern blot analysis showed that other A. oryzae strains used for industrial purpose such as shoyu, miso and sake brewing also have at least four family 10 xylanase genes. However, the reason for the presence of multiple xylanase genes in A. oryzae was unknown. In this study, we cloned the xynF3 gene, characterized its product, and analyzed the expression of the xynF3 gene. The enzymatic characteristics of XynF3 were similar to those of XynF1 of A. oryzae. Promoter analysis of xynF1 showed that the expression of xynF1 was induced by xylan and repressed in the presence of glucose in the liquid medium. This result showed good accordance with the facts that the xynF1 gene has Xnr binding consensus and CreA binding consensus sequences in the 5'-upstream sequence. The xynF1 gene would be expressed by the same mechanism as described for xylan-inducible-genes in other Aspergillus. On the contrary, expression of the xynF3 gene indicated that xynF3 is actively transcribed in the wheat-bran-

| Table 2. GUS Activity of the Cell Extract from Each Transformant |
|-----------------|-----------------|
| Culture medium | xynF1-GUS | xynF3-GUS |
| Glucose (1%) | 0.033 ± 0.011 | 8.24 ± 0.800 |
| xylan (1%) | 862 ± 287 | 5.18 ± 0.220 |
| Wheat bran (6% liquid) | 2.19 ± 0.727 | 56.0 ± 6.50 |
| Wheat bran (solid) | 90.05 ± 9.26 | 32.7 ± 9.12 |

GUS activity: units/mg protein
containing medium. This expression was not induced by xylan but component(s) in wheat bran. The induction was not repressed in the presence of glucose, starch, xylan, and cellulose in liquid medium (data not shown). None of the XlnR binding consensus sequences was observed in the 5'-upstream sequence of the xynF3 gene. The expression mechanism of the xynF3 gene would depend on a novel system.

Our results presented in this study suggest that A. oryzae produces multiple isoymes for efficient degradation of natural substrates such as wheat bran. Nakata et al. reported A. oryzae, when grown on a shoyu koji composed of soybean and wheat, secreted six xylanases. At least three of them were family 10 xylanases since their molecular weights were estimated to be 34,000, 35,000, and 32,000. This result strongly supports the idea that A. oryzae have multiple family 10 xylanase genes and these genes are expressed when A. oryzae grows on natural substrates such as wheat bran.

In the case of xylanases from A. sojae, two family 10 xylanases, X-I and X-II-B, were purified from a shoyu koji. A. sojae produces multiple isozymes for efficient degradation of natural substrates such as wheat bran. The activity of the Taka-amylase gene and the phos- 


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