Cloning and Heterologous Expression of Gene Encoding A Polygalacturonase from *Aspergillus awamori*

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Received December 1, 1999; Accepted April 12, 2000

A polygalacturonase gene of *Aspergillus awamori* IFO 4033 was cloned by genomic Southern hybridization with a probe of a DNA fragment synthesized by PCR. This was done using primers constructed based on the N-terminal amino acid sequence of a polygalacturonase, protopectinase-AS, produced by the strain and the consensus internal amino acid sequence of fungal polygalacturonases. The cloned polygalacturonase gene, containing an ORF, encodes 362 amino acids, including a 52-bp intron. It contains the consensus nucleotide sequence of PaeC binding sites, and its expression was appeared to be regulated by ambient pH. After the intron was excised, the cloned gene was inserted into an expression plasmid for yeast, pMA91, and introduced into *Saccharomyces cerevisiae* to be expressed. The expressed gene product was purified to a homogeneous preparation, and this confirmed that the polygalacturonase produced was the product of the cloned gene.

**Key words:** polygalacturonase gene; *A. awamori*; gene cloning; PaeC binding site

Pectin is a heteropolysaccharide consisting of a backbone of D-galacturonic acid residues which are partially esterified. As a cause of the abundant pectin in plants, for many fungal species pectin is an obstacle or primary carbon source for their infection or degradation. In fact, many fungal species are known to produce multiple molecular forms and isoenzymes of pectin-degrading enzymes, and, with some polygalacturonases (PGase) and polygalacturonate lyases, having been studied in detail. Concerning the PGases produced by Aspergilli, it has been revealed that they have multiple PGase genes so that multiple PGases attack pectic substances by synergistic action. *A. awamori* is used for preparing *koji* for *Shochu* brewing. As the materials for *Shochu*, such as barley corn, contain abundant amounts of heteropolysaccharide as well as starch, *A. awamori* is considered to produce cellulosylolytic enzymes. Indeed, Yoshikawa *et al.* have reported that *A. awamori* IFO 4033 produces at least four different kinds of PGases in submerged culture, and suggested that properties of the PGase produced changes depending on the ambient pH of fermentation. Previously, we isolated and characterized an extremely acid-stable PGase from the solid-state fermentation of the strain. In the course of the study, we purified two different kind of PGases; one is an acid-stable PGase called PPase-AS and the other is a less acid-stable isozyme called PGase-X2. However, little is known concerning either the molecular structure of the acid-stable enzyme or the regulation systems of its production of pectin degrading enzyme in microorganisms.

This paper deals with the gene cloning and heterologous expression of a less acid-stable PGase from *A. awamori* IFO 4033, which has been done as a part of the research to reveal the molecular structure of an acid-stable enzyme and the mechanism of pH-regulation of PGase production in *A. awamori* IFO 4033.

**Materials and Methods**

**Chemicals.** Restriction endonuclease and enzymes used for DNA manipulation were purchased from Takara Shuzo (Kyoto, Japan) and used according to the user’s manual. Polygalacturonic acid (PGA, average polymerization 32 degrees) was obtained from Sigma-Aldrich Japan (Tokyo, Japan), and unless otherwise stated, all chemicals were from Wako Pure Chemicals Industries (Osaka, Japan), and were

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of certified reagent grade.

Strains, plasmids, and culture conditions. A. awamori IFO 4033 was cultured in Czapec medium (0.2% NaNO3, 0.1% K2HPO4, 0.05% KCl, 0.005% MgSO4·7H2O, 0.001% FeSO4·7H2O, 2% soluble starch, pH 5.0) for the preparation of chromosomal DNA; 1 × 10⁶ spores of the fungus were inoculated in 5 ml of Czapec medium. After these were cultured at 30°C for 48 h on a shaker (102 rpm, 10-cm stroke), the culture was transferred into a shaking flask (300-ml) containing 100 ml of Czapec medium and cultured with shaking (102 rpm, 10-cm stroke), at 30°C for 120 h. Saccharomyces cerevisiae DKD-5D-H (MATa trpl leu2 his3) was used as a host for the expression of the cloned PGase gene, and the strain cultured in SD-medium [2% glucose, 0.65% Yeast Nitrogen Base w/o amino acid (Difco, Becton Dickinson, Sparks, MD, U.S.A.), 50 μg/ml each of 1-tryptophan and 1-histidine] at 30°C. Escherichia coli DH5α [supE 44 Δlac U169 (φ80d lac ZΔM15) hisdR17 recA1 endA1 gyrA96 thi-1 relA1] cultured in L-broth (0.5% peptone, 0.5% yeast extract, 1.0% NaCl) at 37°C, for 12 h was used for a plasmid preparation. Plasmid pUC118 and pCR™ II vector (TA-Cloning vector, Invitrogen, Carlsbad, CA, U.S.A.) were used for routine subcloning. Plasmids constructed in this study were pCROGP, pUCP2-1, and pUCCL1, which were obtained by screening of a full-length PGase gene in a genomic DNA library. The yeast expression plasmid pMA91 was kindly provided by Dr. A. Kingsman of Oxford University, UK.

Cloning strategy of PGase gene. Cloning of the PGase gene was done by the following 4 steps: (1) preparation of a DNA probe, (2) isolation of a DNA fragment containing a PGase gene from genomic DNA by Southern hybridization, (3) preparation of a gene library and selection of a clone containing a PGase gene, and (4) excising of the intron in a cloned PGase gene.

(1) Preparation of DNA probe. In order to obtain the DNA fragment encoding a part of PGase that was used as the probe, degenerate sense and antisense primers were synthesized. The sense primer was 5'-TCIACITGYACITTAC-3' (I, inosine; Y, C or T), constructed based on the N-terminal amino acid sequence (STCTFT) of protoprotease-AS²⁷, and the antisense primer, 5'-ARRCCARTCRTCTYGRTRTT-3' (R, A or G) constructed based on a consensus sequence [H/N]NQDCL in Aspergilli PGE genes. PCR was done in a reaction mixture (100 μl) containing 1 μg of chromosomal DNA of A. awamori IFO 4033 as a template, both primers (925 ng each), four deoxyribonucleotide triphosphates (final concentrations of 200 μM of each), 2.5 U of Ex-Taq DNA polymerase (Takara Shuzo) in a buffer containing 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.01% gelatin. The reaction was done for 40 cycles with a DNA AMPLIFIER MIR D-40 (Sanyo Medical System, Osaka, Japan). Each cycle was 1 min at 94°C (degeneration), 1 min at 40°C (annealing), and 2 min at 72°C (elongation). The DNA fragment amplified by the PCR, PCR1, was electrophoresed using 1% (w/v) agarose gel, extracted from the gel, purified using SUPREC™, and ligated to the vector pCR II by TA-Cloning, thereby generating the plasmid pCROGP. A 550-bp Eco RI fragment of pCROGP was labeled with [α-³²P] dCTP (110 TBq/mmol, Amersham Pharmacia Biotech, Tokyo) using Random Primer DNA Labeling Kit (Takara Shuzo) or with fluorecein using a Fluorescein Gene Images™ Labeling Kit (Amersham Pharmacia Biotech, Tokyo) and used as a probe to isolate the full-length PGase gene in genomic DNA. The chromosomal DNA was isolated according to the method described by Hynes et al.³¹ For DNA ligation, Ligation Kit version 2 (Takara Shuzo) was used.

(2) Isolation of DNA fragment containing PGase gene from genomic DNA by Southern hybridization. The genomic library of A. awamori IFO 4033 was screened using the probe; Genomic DNA (1 μg) of the strain was digested with a restriction endonuclease, Hin dIII, and resulting fragments were separated by 1% (w/v) agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N™, Amersham Pharmacia Biotech). Southern hybridization was done at 60°C by the method of Iguchi et al.³⁰ DNA fragment (hybridized with the probe) in the agarose gel was extracted using SUPREC-01 according to its user’s manual.

(3) Preparation of gene library and selection of clone containing PGase gene. The gene library was constructed by inserting the Hin dIII-digested fragments of genomic DNA of A. awamori (about 2.1-kbp, hybridized with the DNA probe) into the Hin dIII site of pUC118, and transformed E. coli DH5α with the recombinant plasmid. The ampicillin-resistant colonies were picked onto a nitrocellulose filter membrane and cultured on L-broth agar plates. Filter hybridization was done with the membrane at 60°C in a buffer containing 5 × SSC, 1% SDS (sodium dodecyl sulfate), 5% dextran sulfate, 5% Liquid Block (Amersham Pharmacia Biotech), and 200 ng of probe. Hybridized fragments were detected using a Fluorescein Gene Images Labeling Kit detection system (Amersham Pharmacia Biotech). The plasmids extracted from positive clone pUC118 (containing the 2.1-kbp Hin dIII fragment) was used in the following analysis.

(4) Excision of intron from PGase gene. The intron was excised from PGase gene by PCR as follows: In the first step, two sets of sense and antisense primers were synthesized. The primer A (5'-CTCAT-
TTGACATCGACTGCGA-3') was based on the sequence between the deduced promoter region and the deduced transcriptional site (from -63 to -43). The primer D (5'-GCTACACCCGACTCAACCT-3') was based on the sequence downstream of the stop codon (from +1,148 to +1,165), and primer B (5'-CCAGATGTTCCTCGCCAGAATTGGATCGCAAG-3') and C (5'-ATTCTGGCCAGAACATCTGTGGTACCAGCCGGC-3') were the sequences adjacent to the regions of the intron. PCRs were done using KOD 1 polymerase (Toyobo, Osaka, Japan) with primers A and B, or with primers C and D, using pUC118 as a template. Each cycle was 30 s at 94°C (denaturation), 10 s at 55°C (annealing), and 30 s at 74°C (elongation). When a second PCR was done with primer A and primer D, using the amplified DNA fragments at each first PCR as the template, a 1.2-kbp DNA fragment was amplified. The fragment was inserted into the Smal I site of pUC118, with a DNA Ligation Kit, to construct the recombinant plasmid (pUCP2-1), and sequenced to confirm that the intron was excised. The fragment was named pgx2".

\[\text{Construction of PGase expression vector and transformation of yeast.}\]

To express the PGase gene in S. cerevisiae DKD-5D-H, pgx2" was inserted between the PGK (phosphoglycerate kinase) -promoter and -terminator in a yeast expression plasmid, pMA91', containing the LEU2 gene as a selection marker. pMA91 was digested with Bgl II and blunted by a DNA Blunting Kit (Takara Shuzo). The 1.2-kbp Eco RI-Pst I-digested DNA fragment of pUCP2-1 was blunted and ligated with this digested pMA91, thereby generating the pgx2" expression plasmid, pMAX2. Transformation of S. cerevisiae was done by the electroporation method described by Iguchi et al." The transformants were streaked onto a SD-medium plate and incubated at 30°C for 72 h. The colonies formed on the plates were transferred onto a SD-PGA plate (0.1% PGA in SD-medium) and incubated at 30°C for 24 h.

\[\text{Nucleotide sequencing.}\]

The nucleotide sequences were analyzed by the dideoxy chain termination method using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) in an A.L.F. DNA Sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Both strands of the DNA were sequenced, and computer analysis was done using a GENETYX Mac program (SDC Software Development, Tokyo, Japan).

\[\text{Analysis of amino acid sequence.}\]

N-terminal amino acid sequence was analyzed with enzyme adsorbed on a PVDF membrane (Nippon Bio-Rad Laboratories, Tokyo, Japan). Around 200 pmol of purified enzyme was adsorbed on the PVDF membrane (25 mm² square, previously incubated in methanol for 10 min and then in water for 10 min at room temperature), washed thoroughly with water, and dried at room temperature. The enzyme adsorbed on the PVDF membrane was put into a protein sequencer PSQ-1 (Simadzu, Kyoto, Japan).

\[\text{Enzyme assays.}\]

PGase and protease activities were measured as described by Iguchi et al." The purification of recombinant PGase. Recombinant PGase was prepared from a culture filtrate of the S. cerevisiae transformants cultured in SD-medium, with shaking (102 rpm, 10-cm stroke), at 30°C for 24 h, and purified by column chromatographies on CM-Toyopearl 650M (Tosoh, Tokyo, Japan) and Superdex 75 (Amersham Pharmacia Biotech), according to the method of Iguchi et al." The protein concentration was measured by the method of Lowry et al.», using bovine serum albumin as the standard.

\[\text{Northern hybridization.}\]

A. awamori IFO 4033 was grown in soybean medium (1.5% defatted soybean flour, 0.1% peptone, 0.1% NaNO₃, pHs 2.0, 3.0, 4.0, and 5.0) at 30°C for 30 h. The mycelia were harvested by filtration and washing with distilled water, then frozen immediately in liquid nitrogen. After the frozen mycelia were vortexed with glass beads (6 0.5 mm), total RNA was extracted by the method of Cathala et al." The RNA preparation (10 μg) was treated with formamide, separated on 1.0% (w/v) agarose gels electrophoresis, and transferred onto a Hybond-N° membrane (Amersham Pharmacia Biotech) by the method of Sambrook et al." Northern hybridization analysis was done with the membrane bound RNA using 32P-labeled pgx2" as the probe, at 60°C, by the method of Sambrook et al." After the hybridization, the membrane was washed for 20 min each of 0.05% sodium pyrophosphate in 6 x SSC, 1 x SSC, and 0.1 x SSC, in this order (at 60°C), and the membrane was exposed to Fuji-RX film at -80°C.
Results and Discussion

Isolation of PGase gene

In order to generate a probe to allow cloning of the PGase gene in A. awamori IFO 4033, the N-terminal and consensus amino acid sequences in previously cloned PGase genes were determined. Based on the amino acid sequence data, we designed several sets of deoxynucleotide mixtures. When PCR (40 cycles) was done using one set of deoxynucleotide mixtures (5'-TCACGCAACATTTATCCAGGTTTCGCGCGCTGCTGCTGAAACCCGAGGGAATCGCTACT and 5'-ARRCACTCAGTGTGCTT-3') as primers, a 550-bp fragment (PCR1) was amplified. The nucleotide sequence of the fragment was analyzed, as shown in Fig. 1. No fragments were amplified by using other primers.

By using PCR1 as probe, the PGase gene was searched for in A. awamori genomic DNA digested with Bam H1, Eco RI, and Hin dIII, by a combination of electrophoresis and Southern blotting hybridization analysis. As a result, four genomic DNA fragments hybridized with the PCR1 were found (Fig. 2). However, hybridization degrees varied depending on the fragment: one fragment hybridized strongly but others weakly. Previously, Yoshikawa et al. demonstrated that A. awamori produces at least four kinds of PGases. These results possibly show that A. awamori has multiple PGase genes, like A. niger N400 (a microorganism closely related with our strain).

A Hin dIII-digested DNA fragments (about 2.1 kbp), strongly hybridized with PCR1, were recovered from the gel and sub-cloned in a plasmid, pUC118, to construct a genomic library. The colony hybridization using PCR1 as a probe was done and two positive colonies, containing the same recombinant plasmid (pUCCIR), were selected from 15,000 transformants. The DNA fragment inserted in pUCCIR consisted of 2.1 kbp. The nucleotide and deduced amino acid sequences of a DNA fragment inserted into pUCCIR were analyzed for both strands of DNA (Fig. 3). One open reading frame (ORF-PGX2) probably encoding a PGase and containing a 52-bp deduced intron, was found. Only a part of sense primer was identified, as shown in Fig. 3. Moreover, the nucleotide sequence of ORF-PGX2 was partially different from that of PCR1, although it hybridized most strongly with PCR1 in Southern hybridization. This may indicate that our strain has multiple forms of the PGase gene that are fairly similar, and ORF-PGX2 encodes a PGase of these. ORF-PGX2 encoded a protein of 362 amino acids, that has a high

![Fig. 1. Nucleotide Sequences of PCR1 and pgaII.](image)

Identical nucleotides are asterisked. Two primer sequences are underlined.
two steps PCRs were done, a 1.2-kbp fragment was amplified. The fragment was sub-cloned to pUC118 and sequenced. It was then confirmed that the intron was excised, and the fragment was named pgx2'. Inserting pgx2' under the PGK promoter of pMA91, we constructed a new plasmid, pMAX2, as shown in Fig. 4-A.

Expression of pgx2' in Yeast
S. cerevisiae DKD-5D-H was transformed with pMAX2, and the transformants that recovered leucine synthesis (grown on SD-medium) were selected. The transformants were cultured on SD-PGA agar plates at 30°C for 24 h, and the PGase production was detected by forming a transparent halo by flooding the plate with 0.1 N HCl (Fig. 4-B).

A transformant, strain PX, was cultured aerobically in 100 ml of SD-medium, at 30°C for 24 h, and 63 U/ml of PGase was produced in the culture filtrate. The PGase was purified and its molecular mass was found to be approximately 41 kDa, by SDS-PAGE (Fig. 5).

It was also found that the PGase was stained with Schiff reagent so as to indicate that it contained sugar molecules. The molecular mass of the PGase treated with Endoglycosidase H was calculated to be 38 kDa by SDS-PAGE (Fig. 5). These facts indicate that the molecular mass of the recombinant PGase coincides roughly with that of the putative product of pgx2' (named PGase-X2) estimated based on the deduced amino acid sequence and that the glycosylation mechanism in the secretion pathway of S. cerevisiae has a function in fungal gene expression.

The N-terminal amino acid sequence of recombinant PGase-X2 was analyzed. Although the preparation showed a clear single band on SDS-PAGE, as shown in Fig. 5, it was found that purified recombinant PGase-X2 preparation contained two proteins. One of these proteins had the same N-terminal amino acid sequence as putative pgx2' product, and the other one was attached to a hexapeptide at the N-terminus of the former, as shown in Fig. 6. Regarding why the transformant produces two different PGases, we assume the following mechanism: The first 27 amino acid residues of the putative pgx2' product are homologous to a processing peptide of Aspergilli PGases,4,5,18-21,23 This may suggest that PGase-X2 is a pre-pro type protein, like the other Aspergilli PGases. In the upstream region of the N-terminus of the recombinant enzyme, the first 17 of the 27 amino acid residues are hydrophobic and are probably a signal peptide, involved in the secretion of PGase-X2 (Fig. 6). The dipeptides Ala-21 and Ser-22 are similar to the processing site of signal peptidase in S. cerevisiae, suggesting that the six amino acid residues from Ser-22 to Arg-27 are processed by a KEX2 protease in S. cerevisiae.

There is a hypothesis that the removal of the leader
peptide of PGI1 of A. niger is a two-step process\(^4\), and such a heterologous processing between A. awamori and S. cerevisiae has been reported as a glucosamylase precursor.\(^2\) Considering these facts, we assumed that pxx2 product has a pre-pro structure and because the cleavage site of the processing
enzyme of \textit{S. cerevisiae} (KEX2) is different from that of \textit{A. awamori}, a part of PGase-X2 (approx. 40\%) was secreted without complete processing.

**Fig. 5.** SDS-PAGE of the Purified Recombinant PGase-X2.
The purified enzyme preparations (5 \( \mu \text{g} \) or 1 \( \mu \text{g} \)) were put on the 10\% polyacylamide gel. lanes 1, protein standard; 2,3, PGase-X2; 4, PGase-X2 treated with Endoglucanase H; 5, Endoglucanase H; lanes 1,2,4 and 5, stained with CBB; 3, stained with Schiff reagent.

![SDS-PAGE image]

**Table 1.** Physicochemical and Enzymatic Properties of Purified PGase-X2

<table>
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<th>Properties</th>
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<th>recombinant</th>
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<tr>
<td>for protocatepin</td>
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<td>Temperature optimum</td>
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<td>Specific activity (U/mg)</td>
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Protopectinase activity was assayed using lemon protopectin. Thermal stability was assayed by incubating the enzyme at pH 5.0 for 30 min and pH stability was by incubating at 30\°C for 12 h. Stable means the remaining activity up to 80\%.

**Fig. 6.** Putative Cleavage Positions of Signal Peptide.
N-terminal amino acid sequences of recombinant PGase-X2 and similar enzymes are shown. Signal sequences deduced from the nucleotide sequence are also shown at the bottom. Two cleavage sites are dashed lined and shown by arrows; A, cleavage site of signal peptidase; B, cleavage site of KEX-2 like protease. Hydrophobic amino acids of PGase-X2 were underlined.

![Signal Peptide image]

**Fig. 7.** Effects of pH on the Production of PGase-X2.
Total RNA (10 \( \mu \text{g} \)) from mycelia grown in media at the various pHs was extracted, electrophoresed, and analyzed by Northern hybridization.

![Northern Blot image]

**General properties of recombinant PGase-X2**
We studied a PGase corresponding to native PGase-X2 in the culture filtrate of the strain used in this research, then we isolated and characterized it.9) Some physicochemical and enzymatic properties of recombinant and native PGase-X2 are listed in Table 1. These two enzymes have quite similar physicochemical and enzymatic properties, and both are assumed to be \textit{pgx2} products. The N-terminal amino acid sequence of native enzyme was determined to be DSDXFTTTT. This fact supports the hypothesis that PGase-X2 has a pre-pro-structure. The both PGase-X2 showed optimum at pH 5.0 and 40\°C. The pH stability of the enzyme was also studied, by incubating the enzyme in a 100 mm acetate-HCl buffer, various pHs, at 30\°C for 12 h. The enzyme is stable at pH 4.0-6.0 under these conditions.

**pH dependent regulation of expression of \textit{pgx2} gene**
As mentioned in our previous paper, \textit{A. awamori} IFO 4033 produces acid-stable PGase, named PPase-AS.10) The production of PPase-AS is affected by ambient pH, and the productivity is higher at pH 2.0 than at pH 5.0, and that of PGase-X2 is opposite with that of PPase-AS, in submerged culture.11) Considering our knowledge obtained up to now, together with the fact that \textit{pgx2} contains a PacC binding site, we hypothesized that PGase productions of \textit{A. awamori} IFO 4033 are regulated by ambient pH. To test this hypothesis, the transcription levels of \textit{pgx2} were analyzed. The transcription of \textit{pgx2} was higher at high pHs than at lower pHs (Fig. 7). Regarding the pH-mediated regulation of PGase production, the research has been done, and details will be published elsewhere.
References