Isolation and expression of the gene which encodes a novel enzyme with polymethoxygalacturonate-degrading activity in *Trichosporon penicillatum*

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Abstract The novel gene named *PSX1*, encoding a new protopectinase with the polymethoxygalacturonase activity, was isolated from *Trichosporon penicillatum*. Nucleotide sequencing revealed that the *PSX1* gene is composed of 1080 bases (360 amino acids, 38747 Da). The *N*-terminal amino acid sequences of the open reading frame correspond to a signal peptide and propeptide processed by a Kex2-like proteinase. Mature PPase SX1 was composed of 334 amino acids (36121 Da). PPase SX1 produced by a *S. cerevisiae* transformant harboring the *PSX1* gene degraded methoxylated polygalacturonic acid as a substrate, but not degraded unmethoxylated polygalacturonic acid.

Key words: Gene cloning; Protopectinase; Polygalacturonase; Polymethoxygalacturonase; *Trichosporon penicillatum*; *Saccharomyces cerevisiae*

1. Introduction

Protopectin is the original water-insoluble parent pectic substance which occurs in plants and which yields pectin or pectic acids upon restricted hydrolysis [1]. Many microorganisms produce the enzyme, named protopectinase (PPase) by Sakai et al. [2], that releases a water-soluble pectin from protopectin. Two types of PPases ahve been reported: type A PPases, polygalacturonase (PGase), degrade the polygalacturonic acid (PGA) chain, which is composed of the unsubstituted region (smooth region) of the protopectin structure, and type B PPases degrade the neutral sugar chain, which connect the smooth region to the other cell wall constituents [2,3]. Type A PPases have been isolated from *Trichosporon penicillatum, Kluyveromyces wickerhamii, K. fragilis,* and *Aspergillus awamori* [4–6]. Three PPases, PPase SE1, SE2, and SE3 were isolated from *T. penicillatum* B2 strain, biochemically characterized, and found to have the different ratios of PPase activities to PGase activities [7]. We suggested that the three PPases have the different protein conformations which affect their enzymatic stabilities and immunological reactivities. However, it may be difficult to analyze the conformation of the two minor PPases, SE2 and SE3, since they are produced in only small amounts (0.15 and 0.007% of total PPases, respectively) by *T. penicillatum* B2 strain [7].

To determine the entire primary structure of the PPases and prepare a large amount of the minor PPases from *Saccharomyces cerevisiae* transformants, we have tried to isolate the PPase genes from the genomic library of the *T. penicillatum* B2 strain. In our another study, we isolate the *PSE3* gene, which encodes the PPase SE3, and analyzed its nucleotide and amino acid sequences [8]. A Southern hybridization analysis using the genomic DNA of *T. penicillatum* indicated that at least three genes are homologous to the *PSE3* gene. We tried to isolate these genes, which may be the genes of PPase SE1, PPase SE2, and a novel PPase-related enzyme. This paper deals with the isolation of the gene encoding a novel PPase and the analysis of its product.

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Abbreviations: PPase, protopectinase; PGA, polygalacturonic acid; PGase, polygalturonase; PMGase, polymethoxygalturonase; bp, base pairs; kb, kilobases; ORF, open reading frame

2. Materials and methods

2.1. Strains and culture condition

Strains used were *Trichosporon penicillatum* B2 [7], *Saccharomyces cerevisiae* DKD-5DH (*MATa trp1 leu2 his3*), and *Escherichia coli* DH5α (*supE*44 D*lac*U169(*f*80 *lac*ZDM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1). Culture media used were GYP (2% glucose, 0.5% yeast-extract and 0.5% peptone) and leucine-free SC (2% glucose, 0.7% yeast nitrogen base, and 50 μl/ml of each amino acids) for yeasts and LB for bacteria, added 50 μg/ml ampicillin when needed. Culture temperatures were 30°C for yeasts and 37°C for bacteria.

2.2. Manipulation of DNA

Plasmids used were pUC118, YEp13 (yeast-E. coli shuttle vector), and pMA91 (containing the promoter and terminator of the *PGK* gene). Restriction enzymes, T4 DNA ligase, and Exonuclease III (Takara Shuzo, Kyoto) were used as recommended by the manufacturer. DNA sequences were determined using a DNA Sequencer Core Kit (Toyobo, Osaka) and A.L.F. DNA sequencer (Pharmacia Biotech, Tokyo) and analyzed by GENETYX software (Software Development, Tokyo).

Plasmids were prepared from *E. coli* according as described by Sambrook et al. [9]. Genomic DNA of *T. penicillatum* B2 was prepared as described by Wach et al. [10] and Iguchi et al. [8].

2.3. Transformation

E. coli competent cells were prepared as described by Calvin et al. [11]. *S. cerevisiae* recipient cells were prepared by Becker and Guarente [12]. Transformations of *E. coli* and *S. cerevisiae* were done as described by Iguchi et al. [8].

2.4. Cloning strategy

Three gene libraries were prepared as follows. The *Hin*dIII-digests of the total DNA of *T. penicillatum* B2 were analyzed by Southern hybridization using the *PSE3* gene (encoding PPase SE3) [8] as a probe. Three DNA fractions (about 3.0, 5.5, and 6.2 kb) without the *PSE3* gene, hybridized with the probe, were prepared as described by Sambrook et al. [9]. Three gene libraries, GL1, GL2, and GL3, were constructed by insertion of DNA fragments of about 3.0, 5.5, and 6.2 kb, respectively, into pUC118. Probes were labeled with α-^{32}P-dCTP (Amersham Japan, Tokyo) with a Random Primer DNA Labeling KIT. Southern hybridization and colony hybridization were done as described by Sambrook et al. [9].

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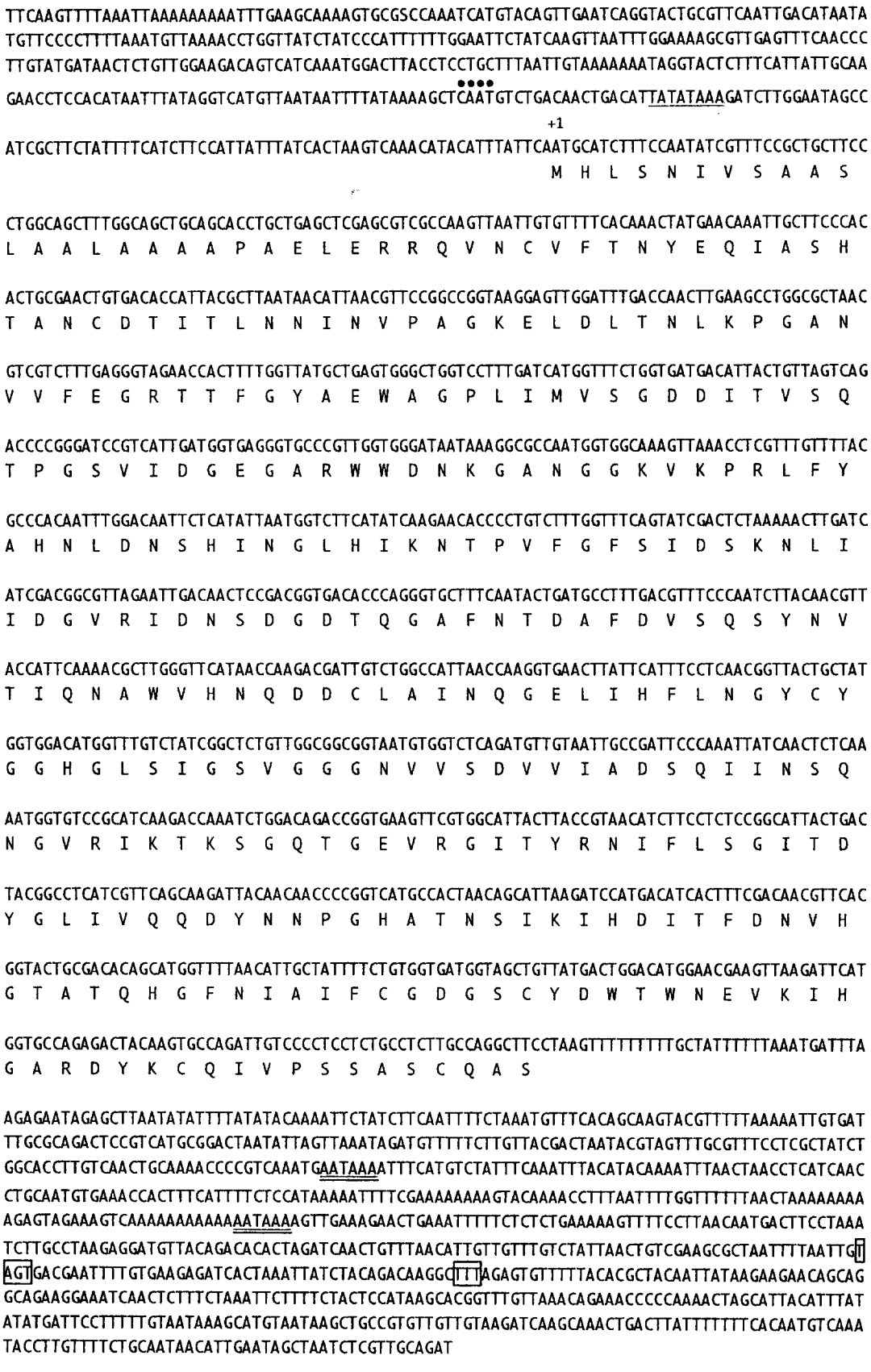

Fig. 1. Nucleotide and amino acid sequences of the *PSX1* gene. A 2390-bp region that included an ORF was sequenced. Single and double underlined sequences are the putative TATA-like sequence and polyadenylation signals, respectively. Dotted and boxed sequences are the CAAT sequence and transcription termination signals, respectively.

2.5. Protein analysis

PPase SE1, SE2, and SE3 were provided by Iguchi et al. [7]. PPase SX1 was prepared from the culture filtrate of the *S. cerevisiae* transformant cultured with shaking on leucine-free SC for 3 days and then on GYP for 1 day, and partially purified by CM-Toyopearl 650M (Tosoh, Tokyo) column chromatography according to Iguchi et al. [7]. PPase and PGase activities were assayed as described by Iguchi et al. [7]. Pectinase and PMGase activities were assayed by the method used for PGase activity using pectin and methoxylated PGA as substrates, respectively. Methoxylation of PGA was done by the method described by Jansen and Jeng [13], and methoxylation frequency was determined by the method of Wood and Sidduqui [14].

3. Results

3.1. Isolation and sequencing of the PPase-related gene

To clone a new gene which encodes a new PPase, a colony hybridization analysis was done on three gene libraries, GL1, GL2, and GL3, using the *PSE3* gene as a probe. As the result of colony hybridization, one positive clone in the colonies harboring the gene library GL3 was hybridized with the *PSE3* gene. The plasmid recovered from this clone contained a 6.2-kb *Hin*dIII fragment.

The nucleotide sequence (2,390 bp) of the probe-hybridizing region in the cloned DNA was determined and was found to contain a 1080-bp ORF that encoded 360 amino acid residues with a calculated mass of 38737 Da (Fig. 1). The G+C ratio of the ORF was 46.6%. The frequencies of A, T, G, and C of the ORF were 24.2, 29.3, 22.1, and 24.4%, respectively. The third codon position in the ORF was relatively biased to T. These results are as same as those of PPase SE3. Putative promoter regions (TATA- and CAAT-box like sequence) were found 80 and 100 bases upstream from the ORF, respectively, and the transcriptional terminator sequences and the polyadenylation signal (AATAAA) were found 248 (and 415) and 570 bases downstream, respectively (Fig. 1).

3.2. Analysis of the deduced protein

The homologies of the deduced protein from the ORF to PPase SE3, PPase SE2 were 52.5% and 52.0%. The deduced protein contained a highly conserved domain (Asp200 to Thr258) of PGases (Fig. 2). These results suggests that the cloned gene encodes an enzyme related to the PGase of *T. penicillatum*. The cloned gene and the coding protein were named *PSX1* and PPase SX1, respectively.

The dipeptide residue, Arg25 and Arg26, of PPase SX1 was identical to the processing site of the Kex2 proteinase in *S. cerevisiae* [15]. This result suggests that the first 25 amino acid residues are probably processed by the signal peptidase and the KEX2-like proteinase in *T. penicillatum*. Mature PPase SX1 is probably composed of 334 amino acids with a calculated mass of 36,121 Da.

3.3. Analysis of PPase SX1 produced by Saccharomyces cerevisiae

The cloned *PSX1* gene was inserted in a YEp13 vector and

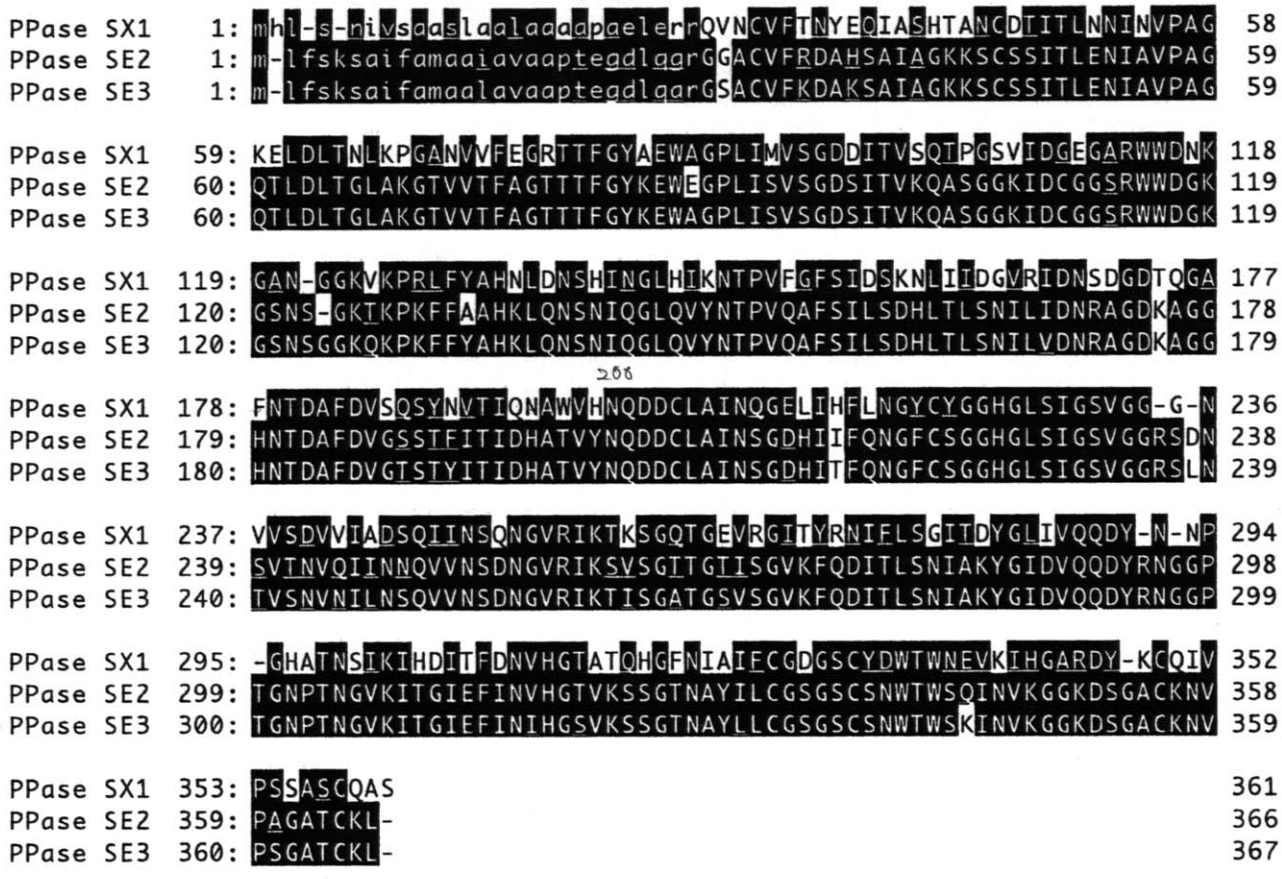

Fig. 2. Alignments of homologous PGase sequences with the *T. penicillatum* PPase SX1 sequence. Sequences of PPase SX1, PPase SE2 and PPase SE3 are predicted from our cloning genes, *PSX1, PSE2* (our unpublished data), and *PSE3* [8], respectively. Black-boxed letters are identical amino acids and black-boxed and underlined letters are similar amino acids. Small capitals are the processed peptide residues.

Table 1
Enzyme activities on various substrates

Substrate	Enzyme activity (U/mg protein)			
	PPase SX1	PPase SE1	PPase SE2	PPase SE3
PGA[a]	< 1	160000	26000	130000
Protopectin[b]	230000	190000	25000	110000

[a]PGase activity was assayed.
[b]PPase activity was assayed using protopectin prepared from lemon peel as a substrate.

the new plasmid (pYEPSX1) was used to transform the *S. cerevisiae* DKD-5DH strain. However, PPase activity was not detected in the culture supernatant of the transformant. Next, the plasmid pMKPSX1 was constructed by the inserting the ORF-region of *PSX1* into pMA91. PPase activity was detected in the culture supernatant of the transformant harboring pMKPSX1. PGase activity was assayed using the culture supernatant of the pMKPPSX1 transformant as a crude enzyme preparation. However, PPase SX1 showed less activity than PPase SE1, SE2, and SE3 using pure PGA as a substrate (Table 1). When the enzyme activities on protopectin prepared from a lemon peel were assayed, the PPase activity of PPase SX1 was the same as that of PPase SE1, SE2, and SE3 (Table 1). These results suggest that PPase SX1 more strongly act on PGA-related compounds (probably modified PGA) than on pure PGA in the pectin and protopectin.

Since a part of the PGA molecule is methoxylated in the pectin and protopectin, the effect of methoxylation of PGA on the activity of PPase SX1 was assayed. Fig. 3 shows the enzyme activities of PPase SX1, SE1, SE2, and SE3 on the substrates which contain a various frequency of the methoxylated PGA. The activity of PPase SX1 was larger but the activities of PPase SE1, SE2, and SE3 were smaller using the more methoxylated PGA as the substrate. These results suggest that PPase SX1 is a PMGase which degrades only the methoxylated PGA. Since a PMGase has not yet been reported, PPase SX1 is a novel PPase.

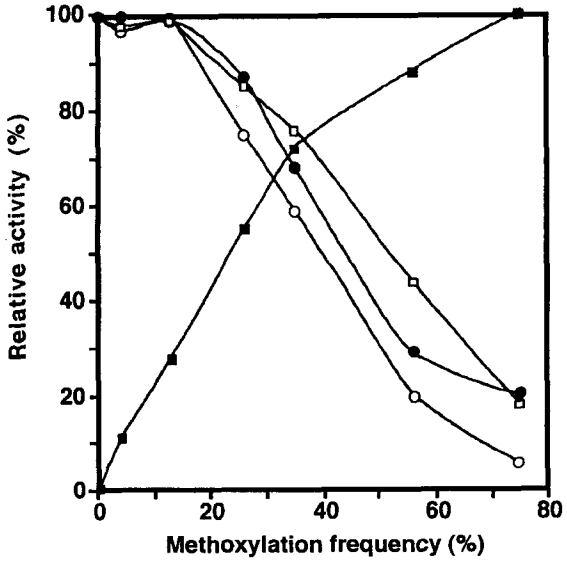

Fig. 3. Effect of methoxylation of PGA on enzyme activity. Enzymes were reacted with 10 mg substrate at 40°C for 30 min. Maximum activities of each enzymes are 100%. PPase SE1, ○; PPase SE2, ●; PPase SE3, □; PPase SX1, ■.

4. Discussion

We have succeeded in isolating an interesting gene that encodes a novel PPase that is more active on methoxylated PGA than on pure PGA. The DNA sequence indicates that the *PSX1* gene contains a TATA-sequence and other units necessary for the initiation of transcription. However, *PSX1* was not expressed in transformed *S. cerevisiae*. These results are different from those of *PSE3*, which encodes PPase SE3, since *PSE3* was expressed in *S. cerevisiae* [8]. As the promoter region of *PSE3* functions in *S. cerevisiae* and *T. penicillatum*, the inability of the promoter region of PSX1 to function in *S. cerevisiae* suggests that *PSX1* is a gene that is not expressed in *T. penicillatum*. These results suggest that the third position of the codons of the *PSX1* ORF is biased to T. As the codons of highly expressed genes in fungi are biased to C at third position [16], *PSX1* is probably expressed at a low level in *T. penicillatum*. The reason why *PSE3* can express and *PSX1* can not express while the both genes have the same units of the transcriptional initiation has not yet been elucidated. But the results that the 5′-upstream sequence of *PSX1* is different from *PSE3* except for the transcriptional initiation units may suggest that the different cis-elements which regulate the expression of *PSX1* and *PSE3* is present in the 5′-upstream sequences of the both genes.

The PPase SX1 produced by the *S. cerevisiae* transformant harboring the *PSX1* gene contains PPase activity. However, PPase SX1 is more active against methoxylated PGA than pure PGA, while the other PPases (SE1, SE2, and SE3) of *T. penicillatum* are more active against pure PGA than methoxylated PGA. The primary structure PPase SX1 has a high similarity to that of PPase SE2 and SE3 (Fig. 2), and especially the conserved domains of the PGases are almost identical. To our knowledge, there are no other reports of fungal PGases with PMGase activity. The results of this study indicate that the substrate specificity of PPase SX1 is much different from those of the other PGases, although the primary structures of these enzymes are very similar. This suggests that the specificity of PPase SX1 for methoxylated PGA is due to a few differences in the amino acid sequence. In fact, the structure of the enzyme surface of PPase SX1 appears to be different from those of the other PPases (SE1, SE2, and SE3) of *T. penicillatum*, since PPase SX1 does not react with the anti-PPase S antibody that interacts with PPase SE1, SE2, and SE3 (our unpublished data). The study of PPase SX1 will provide important data on the mechanism of protopectin solubilization by the PPases, since PPase SX1 is a novel PPase that has a substrate specificity for methoxylated PGA.

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