Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b

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**Abstract**

A structural polygalacturonase-encoding gene (*PGU1*) from *Saccharomyces cerevisiae* IM1-8b was cloned and sequenced. The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18 and two potential glycosylation points in residues 318 and 330. The putative active site is a conserved histidine in position 222. This polygalacturonase showed 54% homology with the fungal ones and only 24% homology with their plant and bacterial counterparts. The gene is present in a single gene copy per haploid genome and it is detected in all strains, regardless of their phenotype. The expression of *PGU1* gene in several strains of *S. cerevisiae* revealed that the polygalacturonase activity depended on the plasmid used and also on the genetic background of each strain but in all cases the enzymatic activity increased. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** *Saccharomyces cerevisiae*; Polygalacturonase; Polygalacturonase-encoding gene; Protein sequence; Over-expression

**1. Introduction**

Polygalacturonases (PGs) are pectolytic enzymes which degrade pectic substances by splitting 1,4-α-glycosidic bonds between two galacturonic acid residues [1]. They are classified by activity as endo-PG and exo-PG; the former hydrolyzes polygalacturonic acid in a random fashion, thus releasing oligosaccharidic chains of variable length, whereas the latter catalyzes the release of single galacturonic acid residues starting from the non-reducing end. The production and characterization of PGs has been reported in plants, filamentous fungi, yeasts and bacteria.

These enzymes are involved as virulence determinants in some bacterial plant diseases, especially by soft-rot *Erwinia* species and genes encoding PGs have been isolated and characterized from several strains of *Erwinia carotovora* and *Erwinia chrysanthemi* [2]. A role of PGs and other cell-wall-degrading enzymes has also been invoked for phytopathogenic filamentous fungi. In the case of *Aspergillus niger*, because of its relevance as a producer of extracellular enzymes for the food industry, its molecular biology has been more developed than in other filamentous fungi [3,4].

Polygalacturonase production in yeasts is not so
well known and documented as in bacteria and fungi. The presence of this kind of enzymes has been reported in some yeast species including *Saccharomyces fragilis* [5], *Kluyveromyces marxianus* [6], and *Cryptococcus albidus* [7]. Although *Saccharomyces cerevisiae* is an important yeast in the food industry, PG production has only recently been described and characterized in a few strains [8,9].

Until now, the reports on yeast pectinases have focused mainly on the biochemical characterization of the enzymes, but nothing has been published about their genetic determination, except the report by Blanco et al. [10] who described several genes involved in PG production and the presence of several PGs in *S. cerevisiae*. Their work also reported the presence of an open reading frame in the chromosome X of *S. cerevisiae* exhibiting high homology with PG genes of *A. niger*. In the present work we report on the cloning and molecular characterization of an endo-polygalacturonase-encoding gene from *S. cerevisiae* IM1-8b, as well as the over-expression of this gene in several *S. cerevisiae* strains.

2. Materials and methods

2.1. Strains, culture media and plasmids

*Escherichia coli* DH5α (F−, end A1, hsd R17 (rK−, mK+), sup E44, thi-1, lac, rec A1, gyr A96, Δ lac U169 (Δ 80 lacZAM15)) was used as host for cloning. This strain was grown in LB medium [11], supplemented with ampicillin (100 µg ml−1) for plasmid selection. Relevant characteristics and sources of *S. cerevisiae* strains and plasmids employed in the present study are listed in Table 1. YEPD and minimal medium (SD) supplemented with the required amino acids in each case [12] were used for growing yeasts strains. PG production was detected on plates with polygalacturonic acid (PGA) as previously reported by Blanco et al. [8].

2.2. Enzymatic assays

Crude enzyme sample preparation was performed as described elsewhere [8]. PG activity was measured by evaluating the reducing power according to the method of Somogyi [13] as modified by Nelson [14].

2.3. PCR amplification

Polymerase chain reaction (PCR) was carried out to amplify the coding region of the *PGU1* gene using genomic DNA (10–50 ng) from *S. cerevisiae* as the template. Two primers were designed consisting of a *Bam*HI restriction site linked to sequences flanking the ORF YJR153w from *S. cerevisiae* Genome Database: PG-1 (5′-CGCGGATCCATGATTTCTGCT AATTCATTACTTATTT-3′) and PG-1r (5′-CGCGGATCCACAGCTTGCACCAGATCCAG-3′). These oligonucleotides were previously phosphorylated with T4 Polynucleotide Kinase (Promega). Amplification was performed employing a thermal cycler Gene Cycler® (Bio-Rad) and using a mix of Taq DNA Polymerase (Promega) and *Pfu* Polymerase (Stratagene). Amplification conditions were: (a) 94°C for 2 min, (b) 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, (c) 72°C for 5 min. Amplified products were purified after agarose gel electrophoresis using the Prep-A-Gene® DNA Purification System (Bio-Rad).

2.4. DNA manipulation and molecular cloning

Standard methods for plasmid DNA isolation, restriction and ligation reactions were used [11]. Alternatively plasmid DNA was purified from recombinant clones employing the Wizard® Plus Midipreps-DNA Purification System (Promega). Genomic DNA from *S. cerevisiae* was isolated using the method described by Struhl et al. [15]. Endonucleases and T4 DNA ligase were purchased from Promega. *E. coli* and *S. cerevisiae* transformations were carried out according to the CaCl2 method [11] and the LiAc procedure [16], respectively. The PCR product (*PGU1*) from strain IM1-8b was cloned into the *Eco*RV site of pBluescript SK+ generating pBSK-PGU1 plasmid which was amplified and used as a source for sequencing. The *PGU1* gene from *S. cerevisiae* IM1-8b was cloned into the yeast expression vectors pBEJ16 and pYES2 (Table 1). pBEJ-PGU1 recombinant plasmid was created by cloning the 1.1 kb *Bam*HI fragment from pBSK-PGU1, containing the *PGU1* gene, into the pBEJ16 previously linearized with *Bg*II. pYES-PGU1 construct was obtained by ligating the *Xba*I-*Hind*III insert recovered from pBSK-PGU1 into the pYES2 vector cleaved with the same enzymes.
2.5. DNA sequencing and sequence analysis

The pBSK-PGU1 was used as a template for sequencing PGU1 gene. The DNA sequence was determined by the dideoxy chain termination method of Sanger et al. [17], using \([35S]dATP\) (Amersham) and the USB Sequenase\textsuperscript{TM} (Version 2.0) Kit following the manufacturer’s instructions. Analysis of DNA sequences was performed using the GCG package (Wisconsin University Biotechnology Center). Search for homologies was carried out using the BLAST [18] or FASTA [19] programmes. The protein sequence prediction and multiple sequence alignments were obtained using Swiss-Prot databases.

2.6. Southern blot analysis

Total genomic DNA digested with HindIII and separated by electrophoresis (5 \(\mu\)g per lane) was denatured and then transferred to positively charged nylon membranes (Boehringer Mannheim) employing the Pharmacia 2016 VacuGene Vacuum Blotting System. Probes were labelled with digoxigenin using the DIG High DNA Labeling and Detection Kit II (Boehringer Mannheim). Hybridizations were carried out under standard high stringency conditions. The detection of sequences was performed as recommended by the manufacturer.

3. Results and discussion

3.1. Cloning and sequencing of a polygalacturonase gene from \(S.\) ceriseiae

The biochemical and genetic characterization of PGs produced by \(S.\) ceriseiae strains 1389 and IM1-8b have been previously described [8–10]. We have reported the existence of different genes involved in PG production in the above strains, although no molecular characterization of the genes was presented. According to the data from the \(S.\) ceriseiae Genome Database there is an ORF (YJR153w) located on chromosome X that shows high homology with a polygalacturonase-encoding gene from \(A.\) niger [3]. In order to investigate if that ORF codes for a PG in \(S.\) ceriseiae, the genomic DNA of the three polygalacturonase positives (PG\textsuperscript{+}) strains (IM1-8b, 1389, and C6) and one polygalacturonase negative (PG\textsuperscript{−}) strain (MI-2B) was used as template for PCR amplification of such gene. The results demonstrated that a single band of ca. 1 kb was amplified from all strains used, re-

Table 1

<table>
<thead>
<tr>
<th>Strains of (S.) ceriseiae and plasmids used in this study</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S.) ceriseiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1389</td>
<td>Wild-type diploid PG\textsuperscript{+}</td>
<td>CECT</td>
</tr>
<tr>
<td>IM1-8b</td>
<td>(MATa) leu2-3 leu2-112 his4 PG\textsuperscript{+}</td>
<td>A. Jimenez\textsuperscript{a}</td>
</tr>
<tr>
<td>MI-2B</td>
<td>(MATa) ura3-52 trp1 PG\textsuperscript{+}</td>
<td>T. Benítez\textsuperscript{b}</td>
</tr>
<tr>
<td>C6</td>
<td>(MATa) trp1 PG\textsuperscript{−}</td>
<td>This laboratory</td>
</tr>
<tr>
<td>AH22</td>
<td>(MATa) leu2-3,112 his4-519 CAN1 PG\textsuperscript{−}</td>
<td>T. Benítez\textsuperscript{b}</td>
</tr>
<tr>
<td>STX347-1D</td>
<td>(MATa) his2 ura3 gal1 PG\textsuperscript{+}</td>
<td>YGSC</td>
</tr>
<tr>
<td>1389-8b</td>
<td>(MATa) ade2 ura3 his7 PG\textsuperscript{−}</td>
<td>This laboratory</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK+</td>
<td>(Ap\textsuperscript{R}) (\beta)-GAL</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pYES2</td>
<td>(Ap\textsuperscript{R}) URA3 GALp</td>
<td>Invitrogen Corporation</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>(Ap\textsuperscript{R}) LEU2 PGKp PGKt</td>
<td>[21]</td>
</tr>
<tr>
<td>PBSK-PGU1</td>
<td>(Ap\textsuperscript{R}) ( \beta)-GAL PGU1</td>
<td>This study</td>
</tr>
<tr>
<td>PYES-PGU1</td>
<td>(Ap\textsuperscript{R}) URA3 GALp PGU1</td>
<td>This study</td>
</tr>
<tr>
<td>PBEJ-PGU1</td>
<td>(Ap\textsuperscript{R}) LEU2 PGKp PGU1</td>
<td>This study</td>
</tr>
</tbody>
</table>

CT, Colección Española de Cultivos Tipo; YGSC, Yeast Genetic Stock Center, Berkeley, CA, USA.
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\textsuperscript{b}Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Spain.
PG\textsuperscript{+} and PG\textsuperscript{−} indicate the ability to hydrolyze or not polygalacturonic acid on plate, respectively.
Regardless of their PG phenotype. This result strongly suggested that ORF YJR153w was probably present and conserved in both positive and negative strains. The PCR product size was in close agreement with those observed for other known fungal PGs [3,4].

The PCR product cloned into pBluescript SK+ was amplified for sequencing. The gene was named as PGU1 and comprises an ORF of 1086 bp. The nucleotide sequence shows complete homology with the ORF YJR153w, thus indicating that this is a
highly conserved region in *S. cerevisiae*. The sequence encodes a 361-amino acid polypeptide with a predicted molecular mass of 37,287 Da (Fig. 2) which is in close agreement with the value 36,000 Da previously obtained by gel exclusion chromatography [10]. The first 18 amino acids of the deduced protein represent a potential signal peptide with an alanine residue as the cleaving site. The putative active site is a histidine residue in position 222, which is common for all PGs so far described. The protein has two possible N-glycosylation sites at the residues 318 and 330, which correspond with the typical sequence N\(\alpha\)T.

Comparison of the amino acid sequence for yeast polygalacturonase and other PGs from different origins revealed that the protein encoded by *PGU1* gene shares similarity with PGs from bacteria and plants (24%) but more specifically with fungal PGs (54%). The predicted domain structures for these enzymes showed a common C-terminal region where the putative active site (a histidine residue) is located (Fig. 1). This region also includes positively charged residues, which could facilitate protein interaction with the substrate which carries a strong negative charge. An alignment among PGs from different origins including yeast, fungi, plants and bacteria showed the existence of similarity between PGs from yeast and filamentous fungi in all domains. However, with bacteria and plants the identity was only found within the C-terminal region. The similarity between *S. cerevisiae* PG and fungal PGs in Domain 1 was 13%, whereas the similarity for Domain 2 was found to be 64%. The Domains 3 and 4 are common to all sequences compared and contain a highly conserved region (Fig. 1B). The similarity among PGs from all origins was 25% in Domain 3 and 50% in Domain 4. Evolutionary conservation of these domains suggests that they must be essential for the structure and formation of the active site of PGs and may indicate the existence of a common ancestral gene for prokaryotic and eukaryotic PGs.

### 3.2. Distribution of the cloned gene in *S. cerevisiae* strains

Southern blot analysis was used to investigate the distribution of the *PGU1* gene in several strains of *S. cerevisiae*. When the *PGU1* gene was used as the probe, a hybridization signal composed of a single band was detected in all strains tested regardless of their PG phenotype (in perfect agreement with PCR results), thus indicating that *PGU1* gene is in only
one copy per haploid genome. No restriction fragment length polymorphism was observed when the genomic DNA was digested with *HindIII*.

### 3.3. Expression of PGU1 gene in *S. cerevisiae*

In order to confirm that the cloned ORF encodes an active protein, a chimeric plasmid in which the coding region was controlled by either inducible or constitutive promoters was constructed. Thus the *PGU1* gene was cloned into pBEJ16 (containing the constitutive *PGK* promoter) and also into pYES2 as indicated in Section 2. In the latter construct the *PGU1* gene was under the control of the inducible *GAL* promoter. Several strains of *S. cerevisiae* were transformed with these plasmids and tested for the PG phenotype on plates. All transformed strains were able to hydrolyze PGA on plates indicating that *PGU1* gene was a structural gene for PG production.

When a PG− strain (AH22) was transformed with the plasmid pBEJ-PGU1 all the recombinants obtained displayed a positive phenotype, whereas when pYES-PGU1 was introduced in PG− strains (MI-2B and STX347-1D), the phenotype of the transformants was dependent on the carbon source. They showed a PG+ phenotype when they were grown in a synthetic medium containing galactose and PG− when glucose was used as the carbon source (Fig. 2). These results therefore confirm that the *PGU1* gene is the responsible one for the PG phenotype. All the transformants of the PG+ strain IM1-8b with both recombinant plasmids (pYES-PGU1 and pBEJ-PGU1) exhibited a positive phenotype after 4 days incubation, while the parental strain required at least 6 days.

The enzymatic activity in some strains transformed with both recombinant plasmids was quantified in liquid medium (Table 2). The increase in PG activity was variable (between 2–200 times), depending on the plasmid and strain used. This fact indicated that the genetic background of the receptor strain was relevant for PG production. The highest enzyme yield was obtained with the plasmid pYES-PGU1 (inducible promoter), except in the case of strain IM1-8b which showed higher activity when transformed with plasmid pBEJ-PGU1 than with the inducible one. The low increase in the enzymatic activity in strains MI-2B and IM1-8b could be due to the poor growth exhibited by these strains on galactose when transformed with the pYES-PGU1 construct.

It is important to note that with all the strains and plasmids used the PG activity was enhanced, suggesting that in the negative strains the phenotype may be the result of having a promoter-less *PGU1* gene or else a non-functional one, since we have demonstrated that all strains so far tested contain this structural gene and that they are able to express it under plasmidic promoter control. In addition, the fact that the positive strain 1389-8b showed a very low activity in liquid medium could be due to the effect of shaking, which is an important factor on polygalacturonase activity in yeasts. This aspect

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Enzymatic activity U (µg protein)−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1389-8b</td>
<td>0.5% galactose</td>
<td>0.025</td>
</tr>
<tr>
<td>1389-8b+pYES-PGU1</td>
<td>0.5% galactose</td>
<td>5.026</td>
</tr>
<tr>
<td>MI-2B</td>
<td>0.5% galactose, 0.5% glucose</td>
<td>0.314</td>
</tr>
<tr>
<td>MI-2B+pYES-PGU1</td>
<td>0.5% galactose, 0.5% glucose</td>
<td>1.853</td>
</tr>
<tr>
<td>AH22</td>
<td>1% glucose</td>
<td>0.239</td>
</tr>
<tr>
<td>AH22+pBEJ-PGU1</td>
<td>1% glucose</td>
<td>1.492</td>
</tr>
<tr>
<td>IM1-8b</td>
<td>1% glucose</td>
<td>0.816</td>
</tr>
<tr>
<td>IM1-8b+pBEJ-PGU1</td>
<td>1% glucose</td>
<td>9.425</td>
</tr>
<tr>
<td>IM1-8b</td>
<td>0.5% galactose, 0.5% glucose</td>
<td>1.382</td>
</tr>
<tr>
<td>IM1-8b+pYES-PGU1</td>
<td>0.5% galactose, 0.5% glucose</td>
<td>2.680</td>
</tr>
</tbody>
</table>

*a* The strains transformed with the plasmid pBEJ-PGU1 were grown with glucose as the carbon source whereas the strains transformed with pYES-PGU1 were grown with galactose; strains MI-2B and IM1-8b were cultivated with galactose and glucose since galactose did not support abundant growth. All strains were incubated with shaking (120 rpm) except strain IM1-8b, that was grown under static conditions.
and the promoter regions of PG will be investigated in the future. Finally, we would like to comment that this enzymatic activity increase (up to 200 times) can be regarded as a significant first stage for over-production PGs for industrial purposes in \textit{S. cerevisiae}. This type of enzymes could possibly be good substitutes for pectinases from fungal origin reported in some cases to contain undesirable enzymes \cite{5,20}.

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\begin{enumerate}
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\end{enumerate}