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Cloning, Characterization, and Functional Analysis of the *EPG1-2* Gene: A New Allele Coding for an Endopolygalacturonase in *Kluyveromyces marxianus*

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A new allele, the *EPG1-2* gene, which codes for an endopolygalacturonase in *Kluyveromyces marxianus* CECT1043, has been cloned. The gene has 1086 bp and the protein 362 amino acids, one more than the previously described Epg1p. Epg1-2p shows a high degree of similarity with the polygalacturonases of fungi and yeasts. The sequences common to all of the polygalacturonases of prokaryotes, fungi, and higher plants are also conserved in Epg1-2p. The *EPG1-2* gene has been expressed in *Pichia pastoris*, and, when fused with the signal peptide of the α-factor of *Saccharomyces cerevisiae*, the protein is properly secreted into the media. The recombinant enzyme does not appear to be fully glycosylated by *P. pastoris* or not glycosylated in the same manner as in *K. marxianus*, but it maintains the same optimum temperature (55 °C) and pH (4.5) and the same stability at different temperatures and pH values as the native enzyme, also showing the same hydrolytic behavior. The recombinant strain produces 200-fold more enzyme than the wild-type strain of *K. marxianus*, making it a yeast of potential industrial interest for the production of endopolygalacturonase for the food industry.

KEYWORDS: ***Kluyveromyces marxianus*; polygalacturonase; *EPG1-2***

INTRODUCTION

Pectic substances are the main polysaccharides in the primary cell walls and middle lamella of higher plant cells, carrying out an essential role in the coherence and integrity of plant tissues (*1*). Pectin is a complex heteropolysaccharide, mainly composed of D-galacturonic acid units linked by α-1,4 bonds and partially esterified with methyl groups.

The enzymatic degradation of pectin is carried out through the concerted action of pectin methylesterases (PE), which remove methoxyl groups, and two types of depolymerases: polygalacturonases (PG) (endo- and exo-) or pectin lyases (PL). EndoPGs catalyze the random hydrolysis of α-1,4 glycosidic linkages between two nonmethylated acid residues, and PLs work by a mechanism of β elimination (*2*).

Pectolytic enzymes have been reported in plants (*3*) and, in the microbial world, in bacteria, fungi, and yeasts (*4*, *5*). In yeasts, two genes encoding endoPGs have been cloned and characterized in *Saccharomyces cerevisiae* (*6*) and *Kluyveromyces marxianus* (*7*).

Pectic enzymes have many applications in industry, particularly in fruit and vegetable processing. These applications include the extraction and clarification of fruit juices (i.e., grape and apple juice) and possible uses in textile production, papermaking, the fermentation of coffee and tea, and pectic wastewater treatments. Additionally, in complex mixtures with other hydrolases pectolytic enzymes can be used for the formulation of animal feeds (*8*).

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To date, most pectinase preparations consist of enzymatic mixtures of fungal origin with different pectolytic enzymes in variable proportions and also other enzymes, some of them with no desirable properties, such as methylesterases, arabinofuranosidases, or amylases. Bearing this in mind, together with the fact that the many industrial applications of pectinases may require the concerted action of several enzymes in the correct proportions (*9*), it would be useful if the different pectic enzymes could be produced separately and mixed as required.

Here we describe the cloning and characterization of the *EPG1-2* gene, a new allele coding for an endoPG in *K. marxianus* CECT1043, and its expression in *Pichia pastoris* to obtain an overproducer strain of industrial interest.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions. *Escherichia coli* TOP10 (Invitrogen) was used as a host for cloning and plasmid propagation. This strain was grown in LB (1% tryptone, 0.5% yeast extract, and 1% NaCl) or LB low salt (1% tryptone, 0.5% yeast extract, and 0.5% Na Cl) media supplemented with kanamycin (50 μg/mL) or zeocin (25 μg/mL) for plasmid selection. The yeast strains and plasmids used in this work and their relevant characteristics and sources are listed in **Table 1**. YEPD (1% yeast extract, 2% peptone, and 2% dextrose), supplemented with zeocin (100 μg/mL) when necessary, was used for growing yeast strains. PG production was detected on plates with 0.5% polygalacturonic acid (PGA) as reported previously (*10*).

Polymerase Chain Reaction (PCR) Amplification. PCR was used to amplify the gene coding for the endoPG in different strains of

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Table 1. Yeast Strains and Plasmids Used in This Study

strain/plasmid	relevant characteristics	source/reference[a]
strain		
K. marxianus 1043	wild-type PG^{+b}	CECT
K. marxianus D5582	PG^{+}	Angel Domínguez
K. marxianus MW1031A	*Mata ade 2 ura3* K^{+} $PKD1^{+}$	Angel Domínguez
K. marxianus USC40	wild-type PG^{+}	our laboratory
K. marxianus USC41	wild-type PG^{+}	our laboratory
P. pastoris X33	wild-type PG^{-}	Invitrogen
plasmid		
PCRII-Blunt-TOPO	Kan^{R} Zeo^{R}	Invitrogen
PGAPZαA	Zeo^{R} PGAP	Invitrogen
PSIVI30	Kan^{R} Zeo^{R} *EPG1-2*	this study
pSIVI32	Zeo^{R} PGAP *EPG1-2*	this study

[a] CECT, Spanish Type Culture Collection; Domínguez, Department of Microbiology, University of Salamanca, Salamanca, Spain. [b] PG^{+} and PG^{-} indicate the ability to hydrolyze or not PGA on plate.

K. marxianus, using 10−50 ng of genomic DNA as template. Two primers were designed, consisting of *Eco*RI/*Xba*I restriction sites linked to sequences flanking the gene *EPG1* of *K. marxianus* (*7*): KM1 (5′-GAATTCATGTTATTCAGCAACACCTTATTGAT-3′) and KMREV (5′-TCTAGATTAACAGAAGGCTCCGCTACCAG-3′). Amplification was accomplished using a thermal cycler Gene Cycler (Bio-Rad) with *pfu* polymerase (Stratagene). Amplification conditions were (a) 94 °C for 2 min, (b) 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and (c) 72 °C for 5 min. Amplified products were purified after agarose gel electrophoresis using the Prep-A-Gene DNA purification system (Bio-Rad).

To express the cloned gene in *P. pastoris*, the mature protein obtained by removal of the first 25 amino acids of the precursor was fused in-frame with the signal peptide of the α-factor of *S. cerevisiae*. The mature protein was amplified using 10 ng of DNA containing the full length as template and the following primers flanking the mature protein and including the *Eco*RI/*Xba*I restriction sites, respectively: KK2 (5′-GAATTCGACAGTTGTACCTTGAGTGGGA-3′) and KKREV (5′-TCTAGAGCACAGAAGGCTCCGCTACCAGA-3′). Amplification conditions were the same as above.

DNA Manipulation and Cloning. Standard methods were used for the isolation of plasmid DNA and for restriction and ligation reactions (*11*). Alternatively, plasmid DNA was purified from recombinant clones employing the WizardPlus Midipreps-DNA Purification System (Promega). Genomic DNA from *K. marxianus* was isolated using the method described by Struhl et al. (*12*). Endonucleases and T_4 DNA ligase were purchased from Promega. *E. coli* transformation was carried out according to the $CaCl_2$ method (*11*), and transformation of *P. pastoris* was accomplished by electroporation, as previously described (*13*).

DNA Sequencing. Double-stranded DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (*14*) using the [^{35}S]dATP (Amersham) and the USB Sequenase kit, following the manufacturer's instructions.

Protein Sequence Analysis. Protein sequences were compared with those obtained from GenBank, using both the Blast and DNAStar packages. The alignment of amino acid sequences was performed using the ClustalW 1.74 program (*15*). The scoring matrix Blosum 62, GOP of 10, and GEP of 0.05 were defined for alignment. Prediction of the signal sequence of the protein was carried out with the SignalP version 2 signal peptide prediction software (*16*). The functional domain of the protein was analyzed using the PROSITE program (*17*) on the ExPASy server of the Swiss Institute of Bioinformatics.

Enzymatic Assays. Samples from supernatants were filtered through 0.22 μm membranes and dialyzed for 48 h in 50 mM sodium acetate buffer (pH 4.5). Polygalacturonase activity was measured by evaluating the reducing power according to the method of Somogyi (*18*), as modified by Nelson (*19*). Typical reaction mixtures containing 0.5 mL of sample and 0.5 mL of 0.5% polygalacturonic acid in 50 mM acetate buffer (pH 4.5) were incubated at 37 °C for 1−10 min. One unit of activity was defined as the amount of enzyme that releases 1 μmol of galacturonic acid or the equivalent in reducing power per minute at 37 °C.

Expression, Purification, and Biochemical Characterization of the Enzyme. Four colonies of the transformants were picked up randomly from the plates and initially inoculated into 10 mL of YEPD medium in 250 mL flasks and cultured at 30 °C overnight in a shaking incubator at 200 rpm. The culture was then inoculated into 100 mL of YEPD in a 1 L flask and grown at 30 °C with shaking at 200 rpm for 72 h. Samples were taken at different times and used for enzymatic assays. The culture supernatant was collected, concentrated (40×) by ultrafiltration in Amicon devices (PM 10 membranes), and used for protein purification.

Gel filtration chromatography was used for enzyme purification. Concentrated supernatants (5 mL) were applied to a Sephacryl S-200 column (LKB 80 × 3.5 cm), equilibrated with 0.05 M sodium acetate buffer (pH 4.5). Chromatography was carried out with the same buffer, and 3.5 mL fractions were collected at a flow rate of 0.5 mL/min. Bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and lysozyme (14 kDa) were used as molecular weight standards. The optimum temperature was tested in the 20−65 °C range and the optimum pH in the 3−6 range, using 50 mM citrate buffer (pH 3−3.5) and 50 mM sodium acetate buffer (pH 4−6). All assays were performed in triplicate.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was used to obtain the molecular weight of polygalacturonase. Slab gels were prepared with 10% (resolving gel) and 5% (stacking gel) polyacrylamide according to the method of Ausubel et al. (*20*). After electrophoresis, gels were stained with Coomassie Blue G-250. Protein molecular weight markers were from Bio-Rad.

Viscosimetry Assays. Viscosimetry assays were performed in triplicate using an Ostwald 5354/2 viscosimeter submerged in a bath at 37 °C. The following control solutions were used to calibrate the viscosimeter: (i) 4 mL of 50 mM sodium acetate buffer (pH 4.5); (ii) 4 mL of 0.5% polygalacturonic acid in 50 mM sodium acetate buffer (pH 4.5), incubated for 15 min at 37 °C. A reaction mixture (containing 4.9 mL of polygalacturonic acid solution and 0.1 mL of enzyme) was prepared and incubated in the same conditions as above. Four milliliters of the reaction mixture was introduced into the viscosimeter, and viscosity was measured at different times. The percentage of viscosity reduction (PVR) was calculated as follows: $PVR = (V_i - V_r \times (V_s - V_r)^{-1} \times 100$, where V_i = viscosity of the reaction mix, V_r = viscosity of the buffer, and V_s = viscosity of the control (polygalacturonic acid).

Nucleotide Sequence Accession Number. The nucleotide sequence of the *EPG1-2* gene from *K. marxianus* CECT1043 was entered into Genbank under accession no. AY426825.

RESULTS

Cloning of the Gene *EPG1-2* of *K. marxianus*. The ability to produce enzymes that degrade polygalacturonic acid was tested in five strains of *K. marxianus*. All of the strains displayed a clear halo (around the colonies) on plates of polygalacturonic acid developed with HCl (**Figure 1A**). Two primers, KM1 and KMREV, based on the sequence of the *EPG1* gene, were used to amplify by PCR the gene that encodes the endoPG in the five strains of *K. marxianus* analyzed. A single PCR product of approximately 1 kb was obtained in all cases (**Figure 1B**). The DNA fragment corresponding to the strain of *K. marxianus* CECT1043 was cloned into the pCRII-Blunt-TOPO vector, thus generating plasmid pSIVI30. Sequencing of the cloned DNA revealed the presence of an ORF of 1086 bp that codes for a polypeptide of 362 amino acids, one more (Val^{122}) than the protein encoded by the *EPG1* gene (*7*), so that it can be considered to be a new allele of the *EPG1* gene and has been called *EPG1-2*.

Sequence Analysis of Epg1-2p. The BLAST analysis program was used to compare Epg1-2p with the proteins in databases. The results of this search indicated that Epg1-2p had a high degree of homology with polygalacturonases of different origins: *K. marxianus* BKM Y-719 (90% identity), *K. wickerhamii* (70% identity), *S. cerevisiae* (59% identity), *Penicillium olsoni* (53% identity), and *Aspergillus parasiticus* (52% identity). The alignment of Epg1-2p with these five amino acid sequences, using ClustalW 1.74, is shown in **Figure 2**.

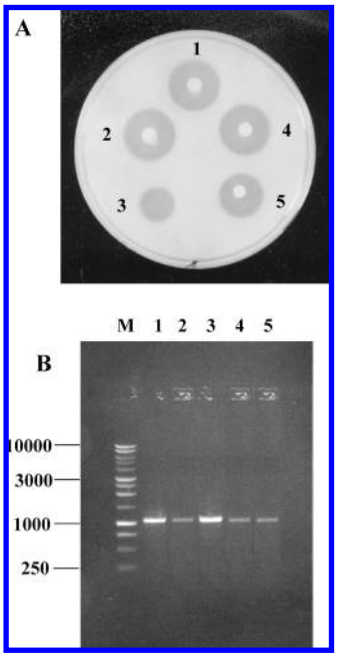

Figure 1. (**A**) Hydrolysis halos on plates with polygalacturonic acid for different strains of *K. marxianus*: 1, CECT1043; 2, D5582; 3, MW1031A; 4, USC40; 5, USC41. (**B**) Amplification of the *EPG* gene in different strains of *K. marxianus*: lane M, molecular weight marker (bp); lane 1, CECT1043; lane 2, D5582; lane 3, MW1031A; lane 4, USC40; lane 5, USC41.

Using SignalP version 2 signal peptide prediction software (*16*), it was observed that the first 25 amino acids in the extreme N-terminus show typical attributes of a signal peptide: a positively charged region, a hydrophobic region, and two cleavage sites typical of signal sequences, AEA^{19} (*21*) and KR^{25} (*22*). Assuming that the sequence corresponding to the signal peptide is cut as predicted, the resulting polypeptide would have 337 amino acids, with a molecular mass of 35 kDa and a theoretical isoelectric point (IEP) of 5.18.

The amino acid valine^{122} (present in Epg1-2p and absent in Epg1p) is the one that differentiates the two alleles. Besides Val^{122}, Epg1p and Epg1-2p show differences in another 12 amino acids (**Figure 2**).

Using the PROSITE database (*17*), the presence of a conserved motif, $C^{219}XGGHGXSIGSVG^{230}$, was detected in Epg1-2p, which would be the active site of endoPGs. Other motifs conserved in bacteria, fungi, and plants ($N^{178}TDG$, $D^{201}DC$, $R^{256}IK$) are also present in Epg1-2p. The polypeptide also has two hypothetical points of glycosylation situated at the amino acids N^{189} and N^{293}.

Functional Expression of *EPG1-2* in *P. pastoris*. To verify the functionality of the *EPG1-2* gene and to construct an overproducer strain of the endoPG enzyme of industrial interest, the *EPG1-2* gene was expressed in *P. pastoris*. The region of the gene corresponding to the mature protein was amplified by PCR and cloned in the expression vector pGAPZαA, fused with the signal peptide of the α-factor of *S. cerevisiae*, under the control of the constitutive promoter pGAP. Using the recombinant plasmid pSIVI32, strain X33 of *P. pastoris* was transformed and subsequently used for the expression of the protein.

The ability to produce the endoPG enzyme was tested, on plates and in liquid medium, in four colonies of *P. pastoris* transformed with plasmid pSIVI32 and in an untransformed colony. The four transformants hydrolyzed polygalacturonic acid on plates (see inset in **Figure 3**), whereas the untransformed colony lacked activity. When enzymatic activity was tested in liquid medium, differences were observed in the activity of the four transformants (**Figure 3**). Colony SIVI33 exhibited the highest activity (26 U/mL) after 48 h, such activity being 200-fold higher than that shown by the wild-type strain of *K. marxianus* CECT1043 (0.13 U/mL). After 72 h, the activity decreased by approximately 40% for all transformants tested.

Purification and Characterization of the Recombinant Enzyme. The recombinant enzyme was purified from the supernatant of clone SIVI33 by gel filtration chromatography and characterized biochemically. The enzymatic preparation obtained had a homogeneous aspect and migrated along a single band in SDS-PAGE (**Figure 4**). The molecular mass of the mature protein, calculated by the sequence of amino acids (35 kDa), approximately matched that estimated by SDS-PAGE (37 kDa). The recombinant enzyme displayed activity equal to that of the native enzyme in the 20 and 60 °C temperature ranges, the optimum being 55 °C (**Figure 5A**) and in a pH range between 3 and 6, the optimum being 4.5 (**Figure 5B**). The hydrolytic behavior of the recombinant enzyme was monitored by viscosimetry and compared with that of the native enzyme. The two enzymes (2 U) showed the same pattern of reduction in viscosity, reducing the viscosity of a 0.5% polygalacturonic acid solution by 60% in 5 min (data not shown).

DISCUSSION

The *EPG1-2* gene coding for an endoPG enzyme in the *K. marxianus* CECT1043 strain has been cloned. Analysis of the enzyme reveals a high homology with the protein encoded by the previously described *EPG1* gene, as well as with the polygalacturonase of *K. wickerhamii* (*23*) and other endoPGs of yeasts and fungi.

Analysis of the N-terminal sequence of the protein suggests that the first 25 amino acids may constitute a signal peptide that would be cut in two consecutive steps: the AEA^{19} sequence would be cut by the STE13-type protease (*21*), freeing a signal peptide of 19 amino acids, and the KR^{25} sequence would be cut by the KEX2 endoprotease (*22*). This same cleavage pattern has been described for Epg1p, where the second event has been confirmed (*7*), and probably occurs in the same way for the PG of *K. wickerhamii*.

Besides the $C^{219}XGGHGXSIGSVG^{230}$ region, considered to be the active site according to mutagenesis and chemical modification studies (*24*), other motifs conserved in bacteria, fungi, and plants ($N^{178}TD$, $D^{201}D$), which have been directly involved in the catalysis of the PGII of *A. niger* (*25*), are also present in Epg1-2p and in the PG of the *K. wickerhamii* species. Asp^{180} and Asp^{202} may activate the hydrolytic water molecule, as in the catalytic mechanism proposed for *A. niger*, whereas Asp^{201} would act as a proton donor. Similarly, the $R^{256}IK$ domain, present in all PGs described so far, is also conserved in Epg1-2p and in the PG of *K. wickerhamii*. Arg^{256} and Lys^{258} would be directly involved in the interaction with the substrate (*25*).

In contrast to the previously described *EPG1* gene (*7*), *EPG1-2* has an extra triplet that codes for the amino acid Val^{122} and can therefore be considered as a new allele of *EPG1*. In this sense, it can be concluded that in *K. marxianus* the polygalacturonase-positive phenotype can be due to two different alleles. This particular amino acid is not present in the polygalacturonases of the yeasts *K. wickerhamii* and *S. cerevisiae*, whereas in fungi it normally appears to be substituted by a threonine and, in some cases, by an isoleucine or glutamine. Epg1p and Epg1-2p also have differences in 12 other amino acids, although none of them affects the above-mentioned conserved regions. It is interesting to note that the amino acids Ser^{186} and Leu^{328} (present in Epg1-2p) are identical in all of the polygalacturonases analyzed except Epg1p. The amino acids Ser^{118}, Lys^{130}, and Cys^{195} differentiate

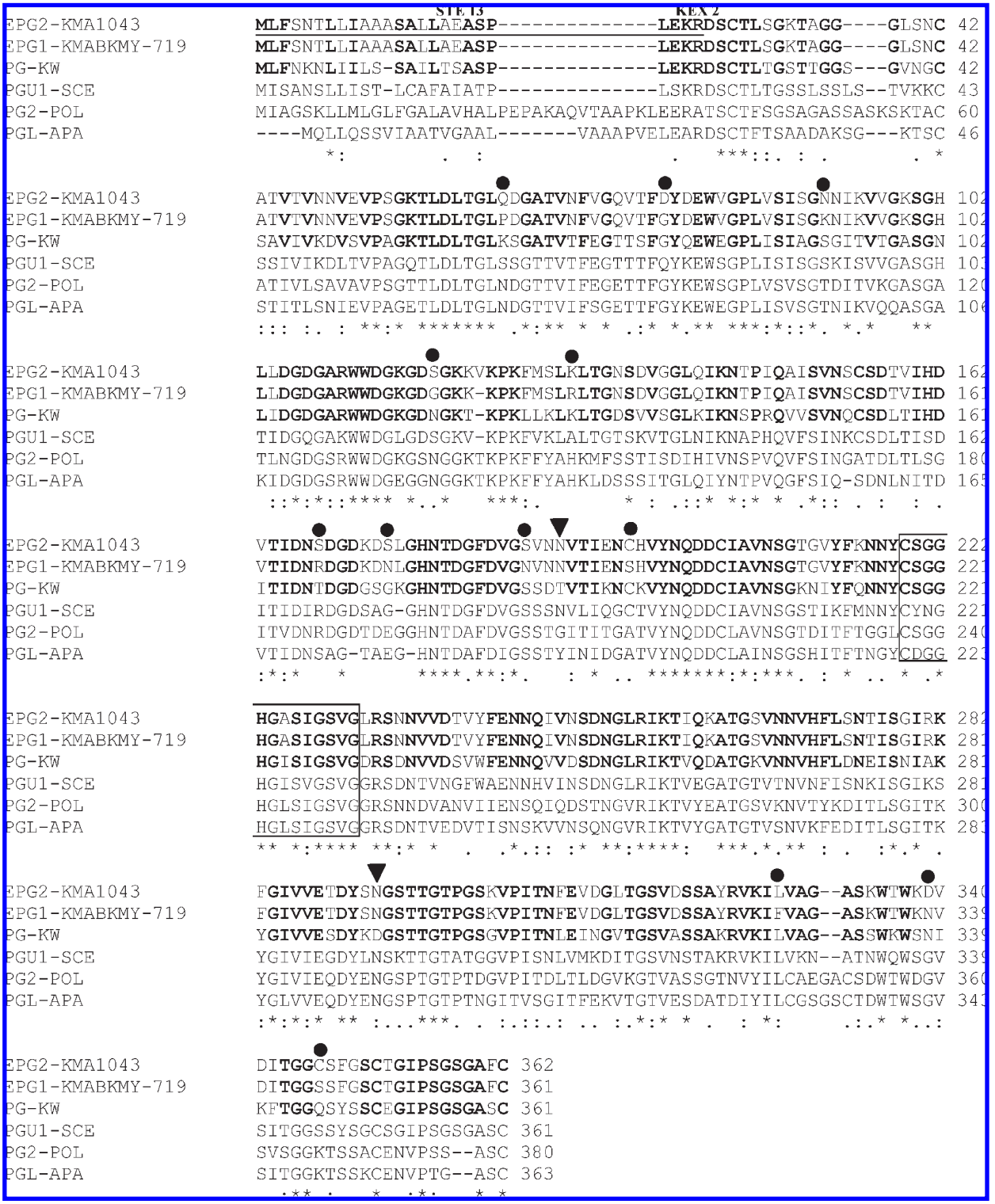

Figure 2. Amino acid alignment of *K. marxianus* Epg1-2p (EPG2-KMA1043) and high-matching sequences: EPG1-KMABKMY-719, *K. marxianus* KMAJ76 (AJ000076); PGKW, *K. wickerhamii* (AB059425); PGU1-SCE, *S. cerevisiae* (NP-012687); PG2-POL, *P. olsoni* (Q9Y833); PGL-APA, *A. parasiticus* (P49575). (∗) Identical residues in all sequences aligned; (:), conserved substitutions; (.) semiconserved substitutions. Amino acids that are identical in the genus *Kluyveromyces* are indicated by boldface letters. Amino acids that differ between Epg1p and Epg1-2p are indicated by •, and hypothetical glycosylation sites are indicated by ▼. The box indicates the catalytic domain. The putative signal peptide is underlined, and the cleavage sites for the STE 13 and KEX2 endoproteases are marked.

Epg1-2p from Epg1p, but they are present (S^{118} and C^{195}) in *S. cerevisiae* and (K^{130} and C^{195}) in *K. wickerhamii*.

The functionality of the *EPG1-2* gene was verified by its expression in the yeast *P. pastoris*. The expression system, based on the yeast *P. pastoris*, has been demonstrated to be very efficient for expressing large quantities of several heterologous proteins (*26*). Furthermore, the *P. pastoris* system is well developed for undertaking fermentations on an industrial scale, because the yeast grows rapidly, reaching high cellular densities in economically viable culture media. When the *EPG1-2* gene is fused with the

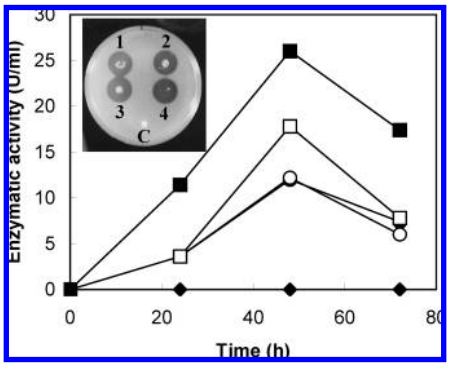

Figure 3. Endopolygalacturonase activity over a time course of four transformants of *P. pastoris*: ●, SIVI30; ○, SIVI31; □, SIVI32; ■, SIVI33; ◆, negative control (untransformed host strain). Activity is expressed in μmol/mL/min. (Inset) Hydrolysis halos on plates with polygalacturonic acid originated by four transformants of *P. pastoris* that express the *EPG1-2* gene: 1, SIVI30; 2, SIVI31; 3, SIVI32; 4, SIVI33; C, negative control (untransformed host strain).

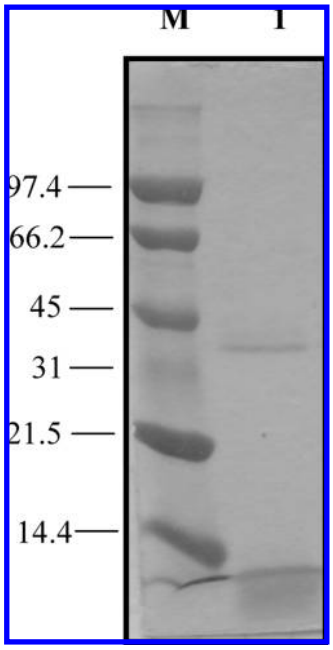

Figure 4. SDS-PAGE of recombinant endopolygalacturonase purified by gel filtration chromatography. Lanes: M, molecular size standards (kDa); 1, Epg1-2p.

signal peptide of the α-factor of *S. cerevisiae*, *P. pastoris* expresses the endoPG encoded by that gene in a constitutive manner and secretes it adequately into the culture medium. Three of the four recombinant colonies of *P. pastoris* analyzed showed differences in the quantity of the enzyme produced, probably due to the different numbers of copies of the *EPG1-2* gene integrated into the genome of the yeast. This explanation is consistent with the fact that the activity of the SIVI33 transformant (200-fold greater than that observed for the wild-type strain of *K. marxianus*) is practically double that shown by transformants SIVI30 and SIVI31. The decrease in enzymatic activity after 48 h of culture (in all four transformants analyzed) could be explained by proteolysis of the recombinant protein. It was suggested earlier that the extracellular proteolysis of recombinant proteins is partly due to release of proteases from dead cells in high cell density cultures (*27*).

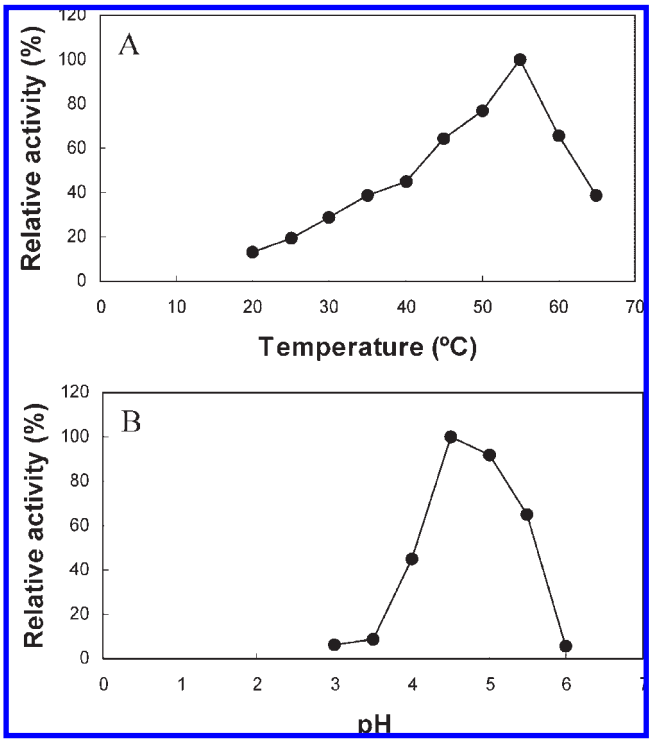

Figure 5. Optimum temperature and pH of the recombinant enzyme: effect of temperature (**A**) and pH (**B**) on the activity of the recombinant enzyme.

The biochemical parameters of the recombinant protein, its optimum pH and temperature, and its stability at different pH values and temperatures all coincide with those previously estimated for the native enzyme. The hydrolytic behavior of the enzyme, which confirms it as an endoPG, is also similar to that of the native enzyme. When the *EPG1* gene was cloned, its product was not biochemically characterized. With respect to Epg1-2p, the optimum temperature of 55 °C is significantly higher than the 40 °C for the PGs of *K. marxianus* and the 45 °C for those of *S. cerevisiae* reported by Schwan et al. (*28*) and Blanco et al. (*4*), respectively. However, it does coincide with that reported by Serrat et al. (*29*) for strain CCEBI 2011 of *K. marxianus*. Due to this high optimum temperature, the enzyme is attractive for a wide range of food industrial applications such as vegetable softness, juice extraction and clarification from different fruits (*8*), and especially pomace liquefaction (*30*): a new alternative technique for the production of apple juice. The use of enzymes in these industrial processes may require that reactions be conducted at high temperatures to improve productivity and also reduce microbial contamination. Clarification processes are mainly carried out at temperatures between 35 and 60 °C. As the temperature increases, the rate of clarification also increases as long as the temperature is below the denaturation temperature for the enzyme (*8*). The optimum pH of 4.5 coincides with that described for other yeast PGs (*4*) and makes the enzyme suitable for use in the fruit-processing industry, where acid conditions are the norm.

By comparison of the theoretical molecular mass for the deglycosylated protein (35 kDa) with the one estimated for the recombinant protein (37 kDa), it can be inferred that the expressed protein is glycosylated. However, it does not appear to be fully glycosylated by *P. pastoris* or not glycosylated in the same manner as in *K. marxianus*, because the native protein exhibited 41.7 kDa of molecular mass, like other polygalacturonases characterized in *K. marxianus* (*29*). This difference in size

affects neither its biochemical properties nor its stability, in contrast to what has been described for other deglycosylated proteins (*31*) or for those for which the pattern of glycosylation has been modified (*32*). The theoretical IEP of Epg1-2p is 5.18, that is, lower than that of Epg1p (6.05) or the PG of *S. cerevisiae*, Pgu1p (8.87).

The large variety of industrial applications of the pectinases may require the action of a single pectic enzyme or that of several enzymes in the correct proportions (*9*). Accordingly, it would be of considerable use to produce the different pectic enzymes individually, and in large quantities, so that they can be mixed as required. Although other yeasts that overproduce PGs have been described (*33*), to our knowledge strain SIVI33 of *P. pastoris* shows the highest values so far reported. It also has the advantage over other recombinant yeasts of producing the enzyme in a constitutive form, the maximum being reached in 48 h in a simple and economic medium (YEPD), in comparison with the media that are used for the expression of inducible strains. The significant increase in the production of enzyme observed for clone SIVI33 demonstrates that this yeast has a potential application as an alternative source of PG for the formulation of preparations for the food-processing industry.

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