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Mutagenesis of Endopolygalacturonase from *Fusarium moniliforme*: Histidine Residue 234 Is Critical for Enzymatic and Macerating Activities and Not for Binding to Polygalacturonase-Inhibiting Protein (PGIP)

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The sequence encoding the endopolygalacturonase (PG) of *Fusarium moniliforme* was cloned into the *E. coli*/yeast shuttle vector Yepsec1 for secretion in yeast. The recombinant plasmid (pCC6) was used to transform *Saccharomyces cerevisiae* strain S150-2B; transformed yeast cells were able to secrete PG activity into the culture medium. The enzyme (wtY-PG) was purified, characterized, and shown to possess biochemical properties similar to those of the PG purified from *F. moniliforme*. The wtY-PG was able to macerate potato medullary tissue disks and was inhibited by the polygalacturonase-inhibiting protein (PGIP) purified from *Phaseolus vulgaris*. The sequence encoding PG in pCC6 was subjected to site-directed mutagenesis. Three residues in a region highly conserved in all the sequences known to encode PGs were separately mutated: His 234 was mutated into Lys (H 234→K), and Ser 237 and Ser 240 into Gly (S 237→G and S 240→G). Each of the mutated sequences was used to transform *S. cerevisiae* and the mutated enzymes were purified and characterized. Replacement of His 234 with Lys abolished the enzymatic activity, confirming the biochemical evidence that a His residue is critical for enzyme activity. Replacement of either Ser 237 or Ser 240 with Gly reduced the enzymatic activity to 48% and 6%, respectively, of the wtY-PG. When applied to potato medullary tissue, *F. moniliforme* PG and wtY-PG caused comparable maceration, while the variant PGs exhibited a limited (S 234→G and S 240→G) or null (H 234→K) macerating activity. The interaction between the variant enzymes and the *P. vulgaris* PGIP was investigated using a biosensor based on surface plasmon resonance (BIAlite). The three variant enzymes were still able to interact and bind to PGIP with association constants comparable to that of the wild type enzyme.

Additional keywords: surface plasmon resonance, yeast.

Fungal endopolygalacturonases (PGs, E.C. 3.2.1.15) catalyze the fragmentation and the solubilization of the homogalacturonan in the plant cell wall. These enzymes might have important functions during plant colonization by a fungus. For example, they are responsible for tissue maceration (cell separation) (Hahn et al. 1989; Walton 1994). PGs have also been shown to activate plant defense responses, likely because they release oligogalacturonides with elicitor activity from the plant cell wall (Bruce and West 1982; Cervone et al. 1989a; Darvill et al. 1992). A polygalacturonase-inhibiting protein (PGIP), found in the cell wall of many dicotyledonous plants, forms a specific complex with fungal PGs (Cervone et al. 1987) and in vitro favors the accumulation of elicitor-active oligogalacturonides (Cervone et al. 1986; Cervone et al. 1989b; De Lorenzo et al. 1996).

We have previously reported the isolation, characterization, and cloning of the gene encoding PG of the phytopathogenic fungus *Fusarium moniliforme* (Caprari et al. 1993b). We have also shown that the four forms of PG secreted by *F. moniliforme* into the medium derive from differential glycosylation of the polypeptide encoded by this gene (Caprari et al. 1993a). As a starting point for a systematic study of the structure and the different, often antithetic, functions of the enzyme, we have now developed a yeast-based expression system for the *F. moniliforme* PG gene and undertaken site-directed mutagenesis studies. Here we describe the expression of the *Fusarium* enzyme in *Saccharomyces cerevisiae*, the site-directed mutagenesis of three amino acid residues located at the putative active site, and the characterization of the variant enzymes in terms of enzymatic and macerating activities and ability to interact with the PGIP of *Phaseolus vulgaris*. Our results indicate that a single mutation in the His 234 is critical for the enzymatic and macerating activities but not for the PGIP-binding capacity of the enzyme.

RESULTS

Expression and analysis of the *Fusarium moniliforme* PG into *Saccharomyces cerevisiae*.

The plasmid Yepsec1, a *E. coli*/yeast shuttle vector constructed for secretion of homologous and heterologous proteins in *S. cerevisiae* under the control of a galactose-inducible

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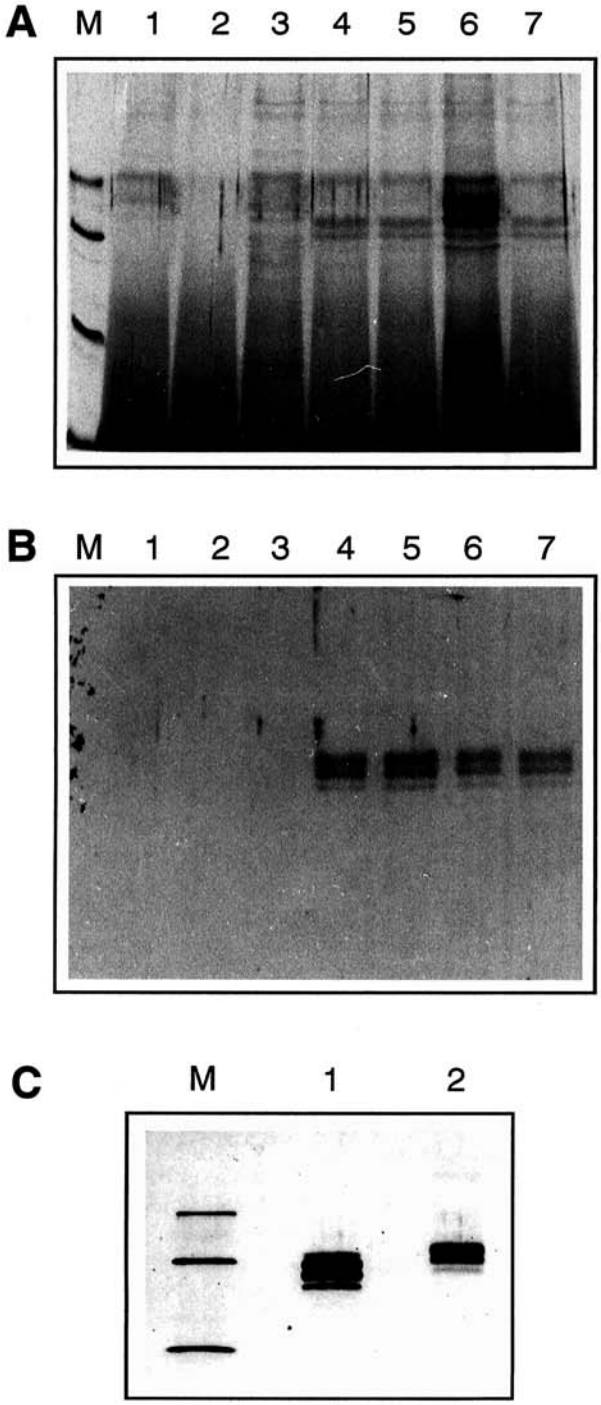

Fig 1. Analysis of PGs produced by transformed *Saccharomyces cerevisiae*. **A,** SDS-PAGE analysis of the culture filtrate of untransformed S150-2B and transformed yeast pCC6-PG. Lane M, molecular mass standards (66, 48.5, 29, 18.4 kDa); lane 1, untransformed yeast cells grown in glucose-containing medium; lane 2, untransformed yeast cells grown in galactose-containing medium; lane 3, pCC6-PG yeast cells grown in glucose-containing medium; lane 4, pCC6-PG yeast cells grown in galactose-containing medium; lane 5, H→K 234 yeast cells grown in galactose-containing medium; lane 6, S→G 237 yeast cells grown in galactose-containing medium; lane 7, S→G 240 yeast cells grown in galactose-containing medium. **B,** Western blot analysis of the gel shown in A. Proteins were separated by SDS-PAGE and subjected to Western blot analysis using polyclonal rabbit antibodies prepared against *Fusarium moniliforme* PG (De Lorenzo et al. 1987). **C,** SDS-PAGE analysis of purified *F. moniliforme* PG (lane 1) and purified wtY-PG (lane 2). M, molecular mass standards (66, 48.5, 29 kDa).

promoter (Baldari et al. 1987), was used in this study. A polymerase chain reaction (PCR)-generated *Bam*HI/*Pst*I 1,063-bp fragment corresponding to the coding region, including the translation termination codon, of the mature PG of *F. moniliforme* was inserted into the *Bam*HI and *Pst*I sites of the Yepsec1 plasmid, downstream of the secretion signal peptide sequence of the *Kluyveromyces lactis* killer toxin gene (k1 signal peptide sequence) (Baldari et al. 1987). The recombinant plasmid, pCC6, was introduced into *S. cerevisiae*. Putatively transformed yeast colonies were tested by PCR to confirm the presence of pCC6; one colony (yeast pCC6-PG) was selected. The presence of PG mRNA in the transformant yeast pCC6-PG was analyzed by hybridization of total RNA extracted from cells grown in rich medium containing either galactose or glucose as a carbon source. A transcript of 1.4 kb was detected only in the RNA prepared from cells grown on galactose medium and not in those grown on glucose. The transcript was absent in untransformed S150-2B yeast cells (data not shown).

The culture filtrates of pCC6-PG yeast cells grown in the presence of galactose or glucose as a carbon source were assayed for PG activity. Activity was present only in the filtrates of the cells grown in the presence of galactose but not in those grown in the presence of glucose, and was absent in the culture filtrates of untransformed yeast cells. The same culture filtrates were also subjected to SDS-PAGE. Two major bands with molecular masses of 46.75 kDa and 50.25 kDa, and a minor band with a molecular mass of 43.25 kDa were present in the filtrate of the yeast pCC6-PG grown in the presence of galactose and not in that of the same cells grown in the presence of glucose, or of untransformed cells (Fig. 1A). Immunoblotting showed that the three bands present in the culture filtrate of the transformant yeast pCC6-PG reacted with a polyclonal antibody prepared against *F. moniliforme* PG (Fig. 1B).

Characterization of the PG secreted by transformed *S. cerevisiae*.

The PG secreted by the yeast pCC6-PG (wtY-PG) was purified to homogeneity by preparative isoelectric focusing and gel permeation fast-performance liquid chromatography (FPLC). Enzyme activity focused in one distinct peak at a pH of 6.6 ± 0.1, a value which is very close to the pI of the enzyme secreted by *F. moniliforme* (De Lorenzo et al. 1987). The purified enzyme, analyzed by SDS-PAGE, consisted of the same protein bands with molecular masses of 43.25, 46.75, and 50.25 kDa described above. The electrophoretic mobility of these protein bands was lower than that of the four PG glycoforms secreted by *F. moniliforme* (Fig. 1C). The specific activity of the wtY-PG was 500 RGU/mg, slightly lower the that of the *F. moniliforme* enzyme (660 RGU/mg).

The apparent K_m values of the two enzymes were determined by measuring the initial reaction rates at different concentrations (from 1 up to 25 mg/ml) of polygalacturonic acid (PGA) as a substrate. Both enzymes displayed Michaelis-Menten kinetics at the substrate concentrations tested; *F. moniliforme* PG, with a K_m of about 8 mg/ml, exhibited a slightly higher affinity for PGA than the wtY-PG (K_m of about 10.7 mg/ml).

The stabilities of the two PGs were determined by following the loss of enzyme activity at 37°C, 45°C, and 50°C as a

function of time. Both enzymes exhibited comparable curves of inactivation at 37°C, while at 45°C and 50°C the wtY-PG was slightly more stable than the *F. moniliforme* PG.

Activities of the two enzymes were measured in a range of pH values from 3 to 8. Both PGs displayed similar pH-dependence curves with maximal activity at pH 4.0.

Site-directed mutagenesis and properties of the mutated PGs.

Three oligonucleotides were designed to separately introduce a point mutation in the amino acid sequence of the *F. moniliforme* PG. Thus, target sequence of these mutations was a 15-amino acid region which is highly conserved in all PGs so far characterized (Scott-Craig et al. 1990; Caprari et al. 1993b): His 234 was mutated into Lys, Ser 237, and Ser 240, respectively into Gly. Each mutated sequence was used to transform *S. cerevisiae*. A colony of each transformant yeast was isolated. The three yeast transformants expressing the variant PGs were named yeast H 234→K, S 237→G, and S 240→G, respectively.

The culture media from yeast cells containing the mutated genes and grown in the presence of galactose were assayed for PG activity. Activity was present only in the supernatant of S 237→G and S 240→G yeast cells, and was absent in the supernatant obtained from H 234→K yeast cells.

SDS-PAGE analysis of the culture medium of each of the three transformed yeasts exhibited protein patterns similar to that of of pCC6-PG yeast (Fig. 1A). The three bands of 43.25, 46.75, and 50.25 kDa were observed in all three yeast transformants carrying the mutated PG genes; these bands reacted with polyclonal antibody prepared against *F. moniliforme* PG (Fig. 1B).

The variant PGs were purified to homogeneity and characterized. Their specific activity and macerating activities are reported in Table 1. The *F. moniliforme* and the wtY- PGs, when applied to potato medullary tissue, caused a comparable extent of maceration, while the variant PGs exhibited a limited (S 237→G and S 240→G) or null (H 234→K) macerating activity.

Interaction with PGIP.

The wtY-PG was inhibited by the purified PGIP of *P. vulgaris* to an extent comparable to that of the *F. moniliforme* enzyme (Table 2).

The interaction between PGIP of *P. vulgaris* and the different PGs was studied by using a biosensor based on surface plasmon resonance (SPR) (Granzow and Reed 1992; Schuster et al. 1993). PGIP was immobilized as a ligand on the sensor surface, while PG was passed in solution as an analyte over the surface. The results of the analyses of the interaction of PGIP with increasing amounts of the different PGs are reported in Figure 2. The increase in RU (resonance units) from the initial baseline represents the binding of the polygalacturonase to the surface-bound PGIP. The plateau line represents the steady-state phase of the polygalacturonase-PGIP interaction while the decrease in RU from the plateau represents the dissociation phase.

The different interactions were analyzed kinetically and the kinetic parameters of each interaction are reported in Table 3. These data show that the equilibrium association constant of the wtY-PG with PGIP was five times lower than that of the *F. moniliforme* enzyme, and that the mutations introduced did not alter the capacity of the molecules to interact with PGIP.

DISCUSSION

The study of the structure and function of fungal PGs and their plant-derived interacting proteins PGIPs is becoming of great importance in understanding some of the recognition phenomena occurring between plants and microorganisms. The recent finding that several resistance gene products in plants exhibit structural similarity to PGIP (Staskawicz et al. 1995) prompted us to investigate the structural basis of the interaction between polygalacturonases and PGIPs as a model for understanding how plants recognize "antigenic" molecules from pathogenic microorganisms (De Lorenzo et al. 1994).

We have undertaken a systematic study of the structure and function of PGIP from *P. vulgaris* and of PG from *F. moniliforme*. In this paper we have reported on the site-directed mutagenesis of the PG. The gene encoding this enzyme was introduced into *S. cerevisiae* and an active PG was secreted into the medium by the transformed yeast cells. Like the enzyme produced by *F. moniliforme* (Caprari et al. 1993a), the yeast-secreted PG also consists of multiple forms differing in their electrophoretic mobility and likely corresponding to differentially glycosylated polypeptides. Enzymological and biological properties of the yeast-secreted enzyme are very similar to those of the enzyme produced by *F. moniliforme*. Small differences in the biochemical properties of the yeast- and the *Fusarium*-secreted enzymes, as for example the higher thermal stability of the yeast enzyme, can probably be ascribed to their different glycosylation. In fact, the yeast-secreted forms have an electrophoretic mobility lower than that of the *Fusarium* glycoforms, possibly due to the well-

Table 1. Specific activities and macerating activities of the wild-type and variant PGs produced by *S. cerevisiae*

PG	Specific activity (RGU/mg)	Maceration index [a]
native *Fusarium*	660	5 ± 0
wtY- (yeast)	500 (100%)	5 ± 0
H→K 234 (yeast)	0 (0%)	0
S→G 237 (yeast)	240 (48%)	1 ± 0.1
S→G 240 (yeast)	30 (6%)	0.5 ± 0.1

[a] A maceration index with a 0 to 5 scale was used. A rating of 0 indicates that cellular cohesion is comparable to that of freshly cut tissue and a rating of 5 indicates a completely loss in cohesion of tissue disks. Each value is the average of six determinations ± SE.

Table 2. Inhibition of *Fusarium* and wtY-PGs by *Phaselous vulgaris* PGIP

PGIP (ng)	Inhibition (%)[a]	
	Fusarium	wtY-
0	0	0
10	1	1.9
60	65	67
100	75	87
200	96	98

[a] Measurements were performed with the standard PAHBAH assay. The 300 µl reaction mixture in 50 mM sodium acetate (pH 5.0) contained 0.3% polygalacturonic acid, 12.7 mRGU of PG and the indicated amounts of PGIP.

known characteristic of *S. cerevisiae* to hyperglycosylate heterologous secreted proteins (Buckholz 1993).

As a first step, we decided to target the active site of the PG. Previous observations had implicated a histidine residue as essential for the activity of PGs purified from *Aspergillus niger*. Inhibition of enzyme activity at pH 6.0 by diethyl pyrocarbonate closely followed the rate of increase in protein absorbance at 250 nm, and was reversed by neutral hydroxylamine, suggesting the involvement of a histidine residue (Cooke et al. 1976). Photooxidative inactivation paralleled the decomposition of imidazole groups. On the basis of pH vs. activity profiles an imidazole group was again regarded to be part of the active center, together with the carboxyl groups of glutamic or aspartic acid. Moreover, the behavior of the kinetic parameters as a function of pH suggested the participation of a protonated imidazole group of histidine and a carboxylate group in the reaction catalyzed by PG (Rexová-Benková and Mracková 1978, and references therein). On the basis of these observations His 234 was a good candidate for being the His residue critical for catalysis, as in all the PG sequences so far determined this amino acid is highly conserved (Caprari et al. 1993b). We also introduced mutations into other two residues, the Ser 237 and the Ser 240, located in the same conserved region. The Ser residues were selected because it is known that for other enzymes in which His is involved in the active site, Ser residues contribute together with His to form a hydrogen bonding pocket for the substrate at the active site (Poulos 1993).

The replacement of His 234 with Lys abolished enzymatic activity, confirming the previous biochemical evidence (Cooke et al. 1976; Rexová-Benková and Mracková 1978). Replacement of either Ser 237 or Ser 240 with Gly reduced

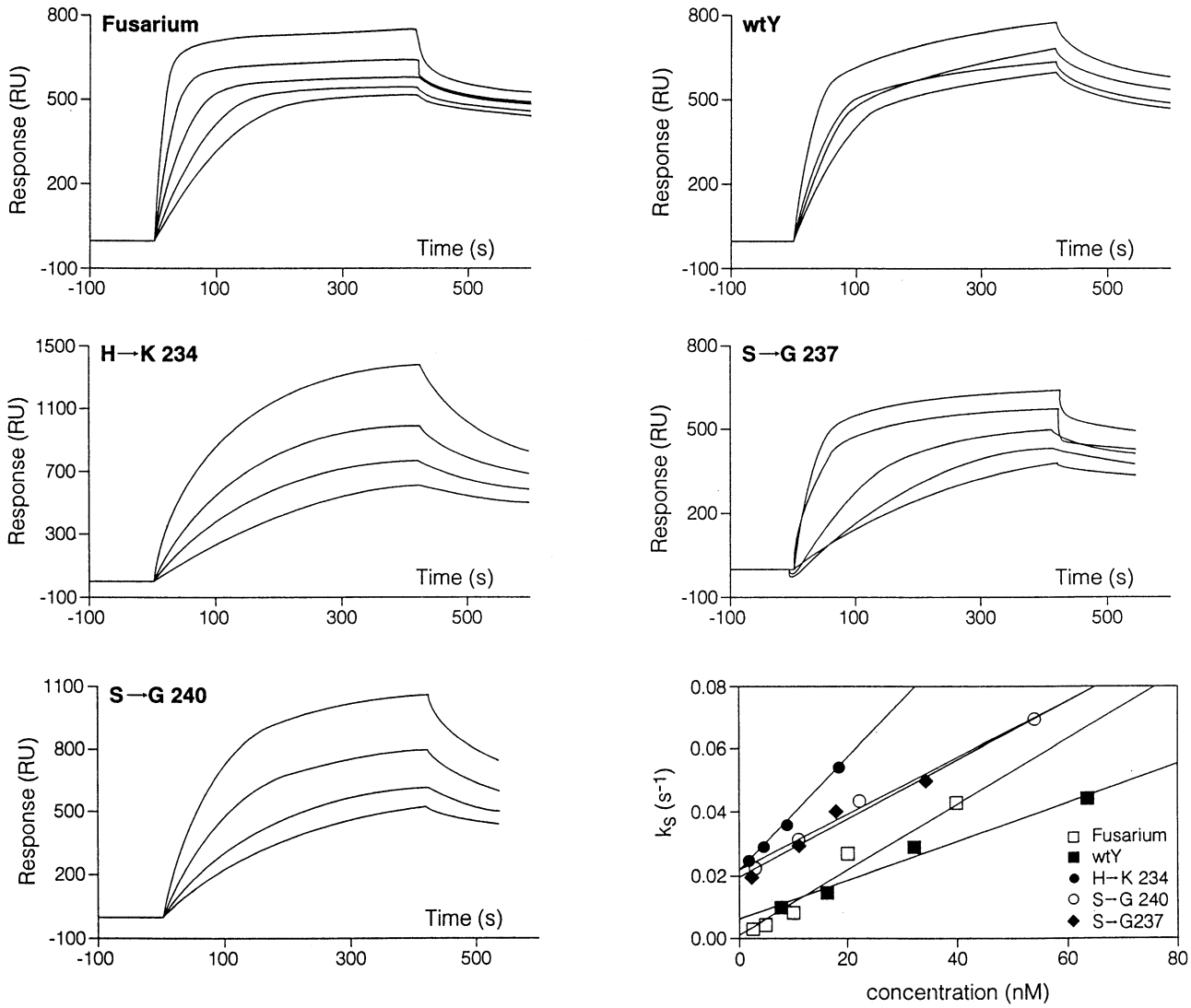

Fig. 2. Interaction between *Phaseolus vulgaris* PGIP and PGs. The different panels report the surface plasmon resonance sensorgrams obtained with the *Fusarium moniliforme* enzyme (Fusarium), the wild-type enzyme expressed in yeast (wtY), and the enzymes mutated at the His 234 (H→K 234), at the Ser 237 (S→G 237) and at the Ser 240 (S→G 240), respectively. Concentration of each enzyme used in this study were (from bottom to top curve of each panel) : Fusarium, 4.2, 6.2, 12.5, 25.0, 50.0 nM; wtY, 7.6, 9.5, 12.5, 25 nM; H→K 234, 4.2, 8.0, 12.5, 25.2 nM; S→G 237, 3.8, 6.2, 12.5, 25.0, 88.0 nM; S→G 240, 4.4, 6.6, 12.5, 25.3 nM. Bottom right panel reports the plots of k_s (= $k_{on}c + k_{off}$) versus the concentration of PG. From the kinetic analysis of the slopes, the association parameters of the different interactions reported in Table 3 were derived.

the enzyme activity to 48% and 6%, respectively, indicating that these Ser residues are also important for enzyme activity.

PGs are thought to be important pathogenicity factors of fungi since they are able to "macerate" the plant tissue, allowing a rapid and extensive colonization by the invading hyphae. Our results show that, as expected, the macerating ability of the enzyme fully depends on the enzymatic activity since the inactive enzyme mutated in the residue His 234 was completely unable to affect the texture and the coherence of potato medullary tissue.

The ability of the mutated forms of the yeast-expressed PG to interact with a PGIP purified from bean was investigated using a biosensor based on SPR. This new powerful technique has shown considerable potential for the characterization of high-affinity interactions such as those between antigens and antibodies (Zeder-Lutz et al. 1993; O'Shannessy et al. 1993), biologically active ligands and receptors (Ward et al. 1995) and (oligo)saccharides and lectins (Shinohara et al. 1995). Our measurements show that the K_A for the interaction of PGIP with the yeast-expressed wild-type PG is five times lower when compared to that of the *F. moniliforme* enzyme. This is probably due to the steric hindrance caused by hyperglycosylation of the yeast enzyme. The K_A of the three variant enzymes was comparable to that of the wtY-PG. Since the modification that causes loss of activity in the enzyme H→K 234 does not interfere with the formation of the PG-PGIP complex, the site responsible for PGIP recognition must reside in a domain different from the active site. Studies are now under way to establish which site(s) and amino acid residues of the PG are crucial for interaction with PGIP.

MATERIALS AND METHODS

Strains and media.

Saccharomyces cerevisiae strain S150-2B (*leu2-3 leu2-112 ura3-52 trp1-289 his3-Δl cir*$^+$) was grown at 28°C either in minimal medium (0.67% yeast nitrogen base [Difco], 2% carbon source [glucose or galactose]) supplemented with the required amino acids (40 μg/ml each), or in rich medium (1% yeast extract, 2% bactotryptone, 2% carbon source). The lithium chloride method described by Eble (1992) was used for transformation.

Escherichia coli strain DH5α was used as a recipient cell for plasmid amplifications. The bacterial cells were grown in rich medium Terrific Broth (2.4% yeast extract, 1.2% bactotryptone, 0.04% glycerol, 70 mM KH_2PO_4, 170 mM K_2HPO_4) (Lee and Rasheed 1990). Preparation of competent cells and transformation of *E. coli* cells were carried out as described by Hahanan et al. (1989).

Plasmid construction.

All recombinant DNA manipulations (restriction endonuclease digestion, DNA modification, ligation, and agarose gel electrophoresis) were performed according to Sambrook et al. (1989).

A *Bam*HI/*Pst*I 1,063-bp fragment, corresponding to the coding region of the mature PG of *Fusarium moniliforme*, was amplified by standard polymerase chain reaction (PCR) from the PG cDNA (plasmid pCC2 previously described by Caprari et al. [1993b]). The oligonucleotides OCC2 (5′-CAT AGT CTG CAG CTA GCT GGG GCA AGT GTT-3′; reverse) and OCC3 (5′-CTC CAG GGA TCC GAT CCC TGC TCC GTG ACT-3′; forward) were used as primers, at a final concentration of 1 μM. These two oligonucleotides introduced unique 5′ *Bam*HI and 3′ *Pst*I sites onto the ends of the amplified fragment. The pCC2 plasmid DNA template was used at a final concentration of 125 ng/ml. PCR was performed as follows: 30 cycles (1 cycle = 1.5 min at 94°C [denaturation], 1.5 min at 55°C [annealing] and 3 min at 72°C [extension]) followed by 7 min of final extension at 72°C. For a 50-μl reaction mixture 0.5 units of Amplitaq (Perkin-Elmer) were used. PCR was done in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT). The PCR-amplified fragment was ligated into the *Bam*HI and *Pst*I sites of the vector Yepsec1 (Baldari et al. 1987) to obtain the recombinant plasmid pCC6. Yepsec1 is a *E. coli*/yeast shuttle vector, constructed for secretion of homologous and heterologous proteins in yeast, and comprises a galactose-inducible hybrid promoter GAL-CYC, the secretion signal peptide sequence of the *Kluyveromyces lactis* killer toxin gene, a polylinker for cloning, and sequences of the 3′ end of the FLP gene of the 2-μm plasmid for transcription termination and polyadenylation signals. The pCC6 plasmid DNA was used to transform *Saccharomyces cerevisiae* strain S150-2B.

Transformant yeast and bacterial cells were analyzed by colony PCR as described by Sandhu et al. (1989) using the specific oligonucleotides OCC3 and OCC2 as primers (see above).

Site-directed mutagenesis.

Mutations were introduced into the pCC6 plasmid using the U.S.E. Mutagenesis Kit (Pharmacia Biotech) according to the manufacturers' instructions. Mutagenic primers were the following oligonucleotides (mismatched bases are underlined): FmPG8 (5′-TGC TCC GGC GGC AAG GGT CTT AGT ATC-3′) to substitute a Lys for the Hys 234; FmPG10 (5′-GGC CAT GGT CTT GGT ATC GGA TCT GTT-3′) and FmPG12 (5′-CTT AGT ATC GGA GGT GTT GGT GGA AAG-3′) to substitute Gly for the Ser 237 and Ser 240, respectively. Selection primer was the oligonucleotide OYep1 (5′-CAAGGTACCCGGAAATCCGATCCCTGCTCC 3′) that eliminated a unique *Bam*HI restriction site in pCC6. Oligonucleotides were 5′ phosphorylated using T4 polynucleotide kinase.

Starting from 0.025 pmol of pCC6 DNA, each mutant plasmid DNA was synthesized, digested with *Bam*HI for primary selection, introduced into competent *E. coli* BMH71-18 *mutS*, and purified again. After *Bam*HI digestion for second

Table 3. Kinetic constants of the interaction between PGIP and *endo*-polygalacturonases (PGs)[a]

PGs	$k_{on} \times 10^{-5}$ (M^{-1} s^{-1})	$k_{off} \times 10^{3}$ (s^{-1})	$k_A \times 10^{-7}$ (M^{-1})
Fusarium	1.2 ± 0.1	1.2 ± 0.2	10.0
wtY-	0.60 ± 0.1	3.0 ± 0.2	2.0
H→K 234	1.9 ± 0.1	3.5 ± 0.2	5.4
S→G 237	1.1 ± 0.4	3.0 ± 0.2	3.6
S→G 240	1.0 ± 0.1	3.3 ± 0.2	3.0

[a] The values were obtained from the analyses of the interactions between PGs and immobilized *P. vulgaris* PGIP performed with BIAlite and reported in Figure 2. Each value is expressed as the mean ± SD for three measurements.

round selection, DNA was introduced into competent *E. coli* DH5α. The mutant plasmid DNA was purified from transformed bacterial cells according to Lee and Rasheed (1990) and, before transformation of *S. cerevisiae*, analyzed by DNA sequencing using the dideoxynucleotide method (Sequenase II Kit, U.S. Biochemical, Cleveland, OH).

RNA extraction, blotting, and hybridization.

RNA was extracted from yeast cells using the procedure described by Sambrook et al. (1989). Total RNA was size-fractionated by electrophoresis on formaldehyde agarose gels (Lebrach et al. 1977) and blotted onto Hybond-N filters (Amersham, Arlington Heights, IL). Prehybridization was carried out in 6× SSPE, 0.1% SDS, 5× Denhardt (Denhardt 1966) and 50 μg/ml salmon sperm DNA as a carrier at 65°C for 3 h. The hybridization solution was the same except for the presence of the PG-specific DNA probe radioactively labeled by random priming. Blots were washed twice in 6× SSPE, 0.1% SDS at 65°C for 15 min, once in 1× SSPE, 0.1% SDS at 65°C for 30 min. The final wash was in 0.1× SSPE, 0.1% SDS at 65°C for 20 min.

Protein assay.

Protein was determined by the method of Bradford (Bradford 1976) using bovine serum albumin as a standard.

Purification and characterization of PGs.

F. moniliforme PG was prepared and purified as previously described (De Lorenzo et al. 1987).

Culture medium of transformed yeast cells grown for 4 days in galactose-containing medium were centrifuged at 10,000 × g for 20 min and the supernatant concentrated to 60 ml with a Minitan pump system (Millipore, Bedford, MA). The concentrated culture filtrate was dialyzed against 20 mM sodium acetate, pH 5.0, then loaded on a 2.0 × 20 cm DEAE column equilibrated with the same buffer. Enzyme activity eluted in the flow through. This was dialyzed against 0.5% glycine and subjected to isoelectric focusing at 4°C using an LKB 8100 column containing 3 ml of 40% ampholine in a linear sucrose gradient (0 to 40%, w/v). Focusing was complete in 36 h when the initial voltage was 0.2 kV, and 1 kV was applied for the last 24 h. The accuracy of the pI value is given as the difference between the highest and the lowest results from five experiments.

Fractions containing the highest PG activity were pooled, dialyzed against water, and lyophilized. Enzyme was redissolved in 100 μl of 100 mM sodium acetate, pH 5.0, and subjected to FPLC purification through a Superose 12 column (Pharmacia) equilibrated with the same buffer containing 100 mM NaCl. Flow rate was 0.5 ml/min; fractions of 1 ml were collected. One peak of activity was detected within fractions 11, 12, and 13. These fractions were pooled and analyzed by SDS-PAGE.

Mutant PGs were purified using the same procedure.

Enzyme assays.

PG activity was determined by reducing end-group analysis using the PAHBAH procedure (York et al. 1985). One activity unit (RGU) was defined as the amount of the enzyme producing one microequivalent of reducing groups per min at 30°C with 0.5% (w/v) polygalacturonic acid as substrate.

Thermal stability was assayed by the standard PG assay using 40 ng of wild-type yeast-secreted (wtY-) PG and 30 ng of *F. moniliforme* PG at three different temperatures (37°C, 45°C, and 50°C) and over different times (from 0 to 5 h).

Effect of pH was determined using the standard PG assay with 48 ng of *F. moniliforme* enzyme and 26 ng of wtY-enzyme. The different pHs were obtained using, in the reaction mixture, the following buffers: succinic acid (pH 3.0, 3.5, and 4.0); sodium acetate (pH 4.5, 5.0, 5.5, 6.0) and phosphate buffer (pH 6.5, 7.0, 7.5, 8.0).

The apparent K_m value of *F. moniliforme* and wtY-PGs were determined using the standard PG assay (York et al. 1985) by measuring the initial reaction rates at substrate concentrations ranging from 0.1 to 2.5% in the presence of Na acetate (50 mM, pH 5.0).

PGIP purification and assay.

PGIP was purified from hypocotyls of *Phaseolus vulgaris* cv. Pinto as previously described (Toubart et al. 1992). Inhibition was measured with the standard PG assay. One unit of inhibitor was defined as the amount of PGIP required to reduce the activity of one RGU of *F. moniliforme* PG by 50%.

SDS-PAGE and immuno-western blotting.

SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with silver as described by Blum et al. (1987).

For immunoblotting experiments, the proteins were electrophoretically transferred to nitrocellulose after SDS-PAGE according to Towbin et al. (1979). Goat antirabbit IgGs conjugated to horseradish peroxidase (HRP) were used as secondary antibody. For HRP color development the blot was soaked in a solution prepared by adding 30 mg 4-chloro-1-naphthol, dissolved in 10 ml of cold methanol, to 50 ml of 10 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.02% H_2O_2.

Macerating activity bioassay.

Macerating activity of the PGs was assessed on disks (8 mm diameter) of potato medullary tissue as described (Cervone et al. 1978). Eight disks were allowed to stand at 22°C in a tube containing 0.5 RGU/ml of PG in 20 mM MES buffer, pH 5.5. The solution was removed after 4 h of enzymatic treatment and the disks were washed with MES buffer. Macerating activity was estimated by testing the loss of coherence of tissue disks. The degree of cohesion of cells was estimated by determining the ease with which the disks could be pulled apart with a spatula.

Surface plasmon resonance determinations.

The biosensor BIAlite (Pharmacia, Uppsala, Sweden) based on surface plasmon resonance (SPR) was used to study the interaction between PGIP and PG. The specific interface of BIAlite is an exchangeable sensor chip consisting of a glass slide coated with a thin gold film to which a matrix of carboxylated dextran is bound. One molecule (the ligand) is covalently attached to the dextran matrix while the other molecule (the analyte) is introduced in a flow passing over the surface. As the two molecules bind, the refractive index in the vicinity of the surface is altered, and the angle at which plasmon resonance occurs is correspondingly modified. This

change, measured in resonance units (RU), directly correlates with the amount of protein interacting with the surface. One thousand RU correspond to about 1 ng ligand bound/mm^2. The resulting sensorgram, a plot of RU versus time, can be divided into three phases: association during sample injection, steady state where the rate of analyte binding is balanced by dissociation from the complex, and dissociation from the surface during buffer flow at the end of sample injection.

PGIP, purified from *P. vulgaris* hypocotyls, was immobilized to sensor chip CM5 (Pharmacia Biosensor, Uppsala, Sweden) via amine coupling. A continuous flow (5 µl/min) of HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% [v/v] surfactant P20 from Pharmacia Biosensor, in distilled water) was maintained over the sensor surface. The carboxylated dextran matrix of the sensor surface was first activated by a 6-min injection of a mixture of *N*-hydroxy-succinimide and *N*-ethyl-N′-(3-diethylaminopropyl)carbodiimide, followed by a 7-min injection of PGIP (10 ng/µl in 10 mM acetate, pH 5.0). The immobilization procedure was completed by a 7-min injection of 1 M ethanolamine hydrochloride to block remaining ester groups. The amount of PGIP immobilized on the surface was 700 to 1,500 RU for the surfaces used.

Solutions of PGs in acetate buffer, pH 5.0, were injected into the flow cell and passed over the PGIP surface at a flow rate of 10 µl/min, and their interaction was followed in real time at different analyte concentrations.

Sensorgrams were analyzed by nonlinear least squares curve fitting using the Pharmacia BIAevaluation 2.0 software (O'Shannessy et al. 1993). A single-site binding model (A + B $\rightleftharpoons$ AB) was used for the analysis of the interactions. The association rate and the dissociation rate can be expressed with the following equations, respectively:

$$dR/dt = -(k_{on}c + k_{off})R + k_{on}cR_{max} \quad (1)$$

$$dR/dt = - k_{off} R \quad (2)$$

where R_{max} is the maximum analyte binding capacity (in RU) of the PGIP surface and R is the SPR signal in RU at time t.

The association phase was analyzed fitting the integrated form of equation (1):

$$R_t = R_{eq} (1 - \exp(-k_s (t-t_0)) \quad (3)$$

where $R_{eq} = k_{on}\ c\ R_{max}/(k_{on}\ c + k_{off})$ in the amount of ligand bound in RU at equilibrium, t_0 is the time the injection started and $k_s = k_{on}c + k_{off}$, where c is the concentration of the protein injected over the sensor surface. The association rate constant, k_{on}, was determined from the slope of a plot of k_s versus c.

The dissociation rate was determined fitting the integrated form of equation (1)

$$R_t = R_0 \exp(-k_{off} (t-t_0)) \quad (4)$$

by nonlinear least square analysis: R_t is the amount of ligand (in RU) remaining bound at time t and t_0 is the beginning of the dissociation phase.

The equilibrium association constant K_A was then calculated from k_{on}/k_{off}.

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