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ORIGINAL PAPER

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# Efficient expression and secretion of *Aspergillus niger* RH5344 polygalacturonase in *Saccharomyces cerevisiae*

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**Abstract** An *Aspergillus niger* endopolygalacturonase (EC 3.2.1.15) cDNA was expressed in the yeast *Saccharomyces cerevisiae*. Secretion of the protein into the growth medium was efficiently directed by the fungal leader sequence, and processing occurred at the same site as in *Aspergillus*. The expression level was significantly enhanced by using a "short" version of the yeast *ADHI* promoter. An additional increase in the yield of heterologous protein was due to a higher plasmid stability and a rise in plasmid copy number. This was achieved by deleting most of the bacterial sequences from the expression vector. The yeast-derived enzyme showed the same enzymatic and biochemical properties as the fungal polygalacturonase, such as substrate specificity, pH and temperature optima and p*I* value. The yeast-derived enzyme, however, showed a higher degree of glycosylation and exhibited a more pronounced temperature stability than the fungal enzyme.

## Introduction

Preparations of pectinolytic enzymes are widely used in the food and beverage industry to clarify fruit juices and wine, to improve the cloud stability of fruit and vegetable nectars, and to increase the protein content of fruit juices (Rombouts and Pilnik 1978). Commercial products are mixtures of different pectic enzymes, such as endopolygalacturonase, pectate lyase and pectin esterase. There are, however, processes requiring only one kind of enzymatic activity, such as pectin esterase for clarifying cider and endopolygalacturonase (endo-PG) for preparing vegetable and orange and citrus juices. The filamentous fungus *Aspergillus niger* is one of the major industrial sources of pectinases and its pectic enzymes have been extensively studied (Harmsen et al. 1990; Kester and Visser 1990). Genomic or cDNA clones coding for endo-PG enzymes that degrade pectin by cleaving the polymer internally at random sites have been obtained from the *Aspergillus* species (Bussink et al. 1990; 1991a, b; Kitamoto et al. 1993; Ruttkowski et al. 1990).

The yeast *Saccharomyces cerevisiae* is well known both for its food-grade qualities and as a host for the production of heterologous proteins. *S. cerevisiae* does not synthesize any significant amounts of pectinases and is thus suited for producing a single type of pectic enzyme. In addition, this yeast secretes only a few of its own proteins in small amounts (Hitzeman et al. 1983), but can be efficiently manipulated to secrete foreign, especially fungal proteins (Innis et al. 1985).

We constructed a yeast expression vector in which the cDNA of the endo-PG of *A. niger* RH5344 (Ruttkowski et al. 1990), which is highly homologous to the *pgaII* of *Aspergillus tubigensis* (Bussink et al. 1991b), is under the control of the promoter of the *S. cerevisiae* alcohol dehydrogenase (*ADHI*) gene. We studied the expression of the heterologous protein using homologous and heterologous secretion signals and a deletion version of the *ADHI* promoter. We furthermore analysed the biochemical and molecular properties of the fungal enzyme in yeast and we optimized the protein yield by increasing the mitotic stability of the plasmid.

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## Materials and methods

### Strains

*Escherichia coli* K12 strains NM522 (*supE*, *thi*1, *Δ* (*lac-proAB*), Δ (*mcrB-hsdSM*) 5, ($r_k^-$, $m_k^-$), {*F′ proAB*, *lacI*$^q$ZΔ*M15*} or *SF8* (*recBC*, *lop11*, *tonA1*, *thr1*, *leuB6*, *thy1*, *lacY1*, *supE44*, *hsm*$^-$, *hsr*$^-$) were used as hosts in plasmid constructions.

Yeast host strains were *Saccharomyces cerevisiae* S3: a triploid wild-type baker's yeast, AH22 (*leu2–3*, *leu2–112*, *his4–519*, *can*1, *cir*$^+$, mating type a), X2180-1A (*SUC2*, *mal*, *mel*, *gal2*, *CUP1*, mating type a), X2180-1B (*SUC2*, *mal*, *mel*, *gal2*, *CUP1*, mating type α), GRF18 (*leu2–3*, *leu2–112*, *his*3–*11*, *his3–15*, *can1*, mating type α).

### Media and culture conditions

The bacterial cultures were grown at 37° C in Luria broth (Sambrook et al. 1989). Bacterial transformants were grown in Luria broth medium containing ampicillin at 50 µg/ml. Yeasts were cultured either in complex medium (YE: 0.5 % yeast extract, 2 % glucose, pH 6.3, supplemented with 150 µg/ml G418 for selecting plasmid-carrying cells or in minimal medium (WMVIII: sucrose 50 g/l, $NH_4H_2PO_4$ 0.25 g/l, $NH_4Cl$ 2.8 g/l, sodium glutamate 10 g/l, $MgCl_2 \cdot 6H_2O$ 0.25 g/l, $CaCl_2 \cdot 2H_2O$ 0.1 g/l, $KH_2PO_4$ 2 g/l, $MgSO_4 \cdot 7H_2O$ 0.55 g/l, *myo*-inositol 75 mg/l, $ZnSO_4 \cdot 7H_2O$ 1.75 mg/l, $FeSO_4 \cdot 7H_2O$ 0.5 mg/l, $CuSO_4 \cdot 5H_2O$ 0.1 mg/l, $MnCl_2 \cdot 4H_2O$ 0.1 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 0.1 mg/l, nicotinic acid 10 mg/l, pyridoxin-HCl 25 mg/l, thiamin-HCL 10 mg/l, biotin 2.5 mg/l, calcium pantothenate 50 mg/l). Amino acids were supplemented as required at 40 mg/l for shake-flask cultures and at 100 mg/l in fermentation media. Yeast transformants were selected on YE plates containing 250 µg/ml G418 (Gibco, Paisley, Scotland) or on minimal medium YNB (Difco Laboratories, Detroit, USA). Solid medium contained 20 g/l agar. Yeasts were cultured at 28° C, liquid cultures were grown at 120 rpm on a reciprocal shaker.

### Plasmids

Plasmid pPG1 contained the *Aspergillus niger* RH5344 PG gene. It is inserted as a cDNA copy at the *Pst*I site plasmid pUC9 (Ruttkowski et al. 1990). The yeast expression vector was pPT2b or pPTK-NP (Lang-Hinrichs et al. 1989; Looman et al. 1991). The plasmids pUC8 (Vieira and Messing 1982), pMFα8 (Miyajima et al. 1985) and YEp13 (Parent et al. 1985) have been described previously.

### Recombinant DNA procedures

Standard methods were used for DNA isolation, Southern analysis, ligation and subcloning (Sambrook et al. 1989). Restriction endonucleases, T4 DNA ligase and Klenow fragment DNA polymerase I were purchased from Pharmacia (Freiburg, Germany) and used as recommended by the supplier. Sequencing of the cDNA clone pPG1 and further constructions was performed by enzymatic double-strand DNA sequencing (Chen and Seeburg 1985) with T7 DNA polymerase (Pharmacia, Freiburg, Germany) and oligonucleotide primers hybridizing either 5′ or 3′ from the insert in pUC9 or on the *ADH1* promoter sequence upstream of the insert in pPG2, pPG3α6, pPG5 and pPG6. To reduce band compression when sequencing "tail" sequences, deazadideoxynucleotides were used. Nucleotide and amino acid sequences were analysed using the Mac Molly program (Soft Gene GmbH, Berlin, Germany).

### Plasmid DNA isolation from yeast

Routine recombinant plasmid isolation from yeast was done by the method of Hoffman and Winston (1987). The isolated plasmids were retransformed to *E. coli* SF8 and analysed after isolation (Sambrook et al. 1989). Plasmid pPG6 was isolated from yeast transformants grown in 500 ml YE medium. Whole-cell DNA was prepared after zymolyase (Zymolyase 20T, ICN Biochemicals USA) treatment and sodium dodecyl sulphate (SDS) lysis. Proteins were precipitated with NaCl, and nucleic acids were precipitated from the supernatant with 10 % polyethyleneglycol (PEG) 6000. Plasmid DNA was separated from chromosomal and mitochondrial DNA by caesium chloride density fractionation in the presence of ethidium bromide. Plasmid DNA (pPG6 plus endogenous 2 µm DNA) was isolated as a separate fraction of higher density distinct from chromosomal and mitochondrial DNA.

### Transformation of *E. coli* and *S. cerevisiae*

Transformation of *E. coli* and *S. cerevisiae* was performed as previously described (Lang-Hinrichs et al. 1989).

### Assay for polygalacturonase activity

PG activities were measured by a reducing-sugar assay (Miller 1959) with polygalacturonate (Sigma P-7276) as a substrate. A typical assay consisted of 0.1 ml enzyme solution, 0.65 ml buffer (2 mM citric acid, 1 mM $CaCl_2$, pH 4.5) and 0.25 ml polygalacturonate (10 g/l) . An enzyme unit is the amount that releases 1 µmol galacturonic acid/min at 25° C with galacturonic acid (Sigma G-2125, St. Louis, USA) as a standard.

### Purification of PG from yeast culture medium and protein analysis

The recombinant PG was purified from 500 ml culture medium. Yeast cells were separated by centrifugation and the medium supernatant was applied to a carboxymethylcellulose cation-exchange column (Sigma, Deisenhofen, Germany) in 10 mM sodium acetate, pH 4.0. Protein was eluted with a linear gradient from 0 to 1.5 M NaCl in 10 mM sodium acetate, pH 4.0. Fractions containing pure PG were eluted between 0.6 M and 0.75 M NaCl. Pooled fractions were dialysed against distilled water and freeze-dried. The molecular mass of the yeast-derived PG in comparison to the *Aspergillus* PG was analysed by SDS/polyacrylamide gel electrophoresis (PAGE) before and after deglycosylation with endoglycosidase H in 30 mM potassium phosphate, pH 6.0, for 18 h at 37 °C (Boehringer, Mannheim, Germany). Stained gels were analysed by an imaging densitometer (BioRad, Munich, Germany). The isoelectric points of both proteins were determined by electrophoresis in a gradient gel from pH 3 to pH 7. The N-terminal amino acids of the secreted recombinant PG were determined by the dimethylaminoazobenzene-4′-isothiocyanate microsequencing method as described by Wittmann-Liebold and Kimura (1986).

### Fermentation studies

Fermentation studies were performed in a 5-l bioreactor (Biostat E, Braun Diessel Biotech, Melsungen, Germany) with temperature and pH controllers. Batch and fed-batch processes were performed at 28° C and pH 5.0 maintained by the automatic addition of $H_2SO_4$ or KOH. Dissolved oxygen was measured using a $pO_2$ electrode (Ingold, Steinbach, Germany) and was maintained above 20 % saturation. Foam was suppressed, when necessary, by manual addition of the anti-foam polypropylene glycol 2000.

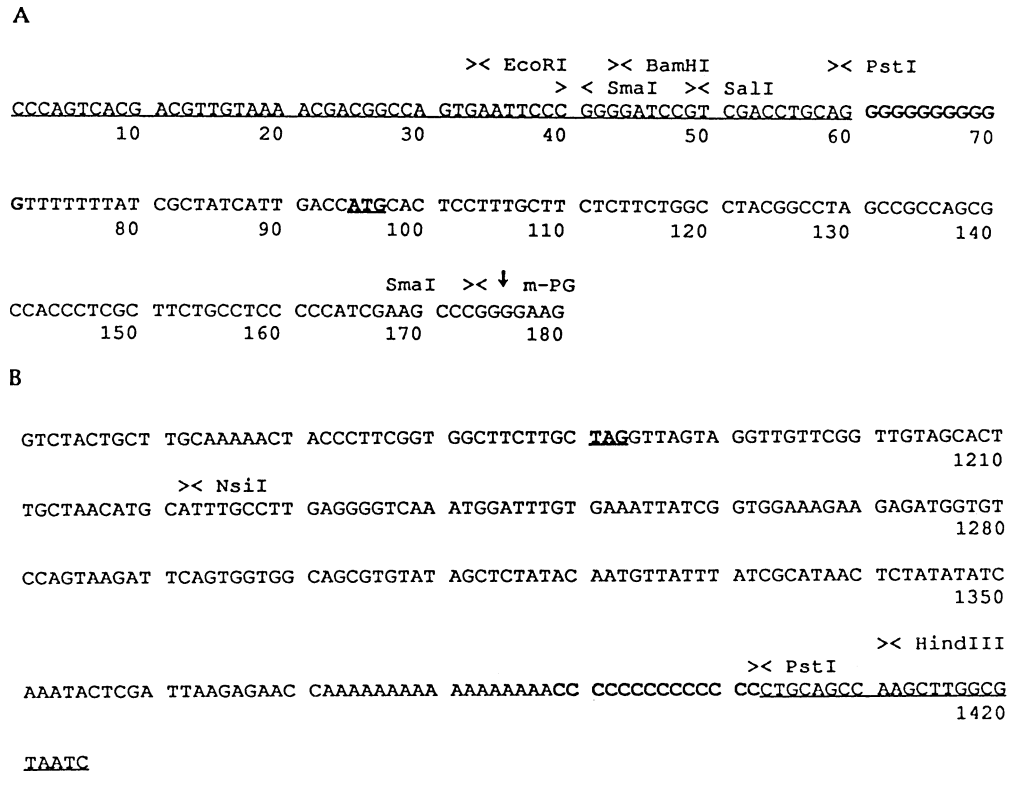


**Fig. 1A, B** 5′ and 3′ DNA sequence of the pPG1 cDNA in pUC9. **A** 5′ sequence of the *A. niger* polygalacturonase (PG) cDNA in pPG1. The sequence of the oligo (G) tail as well as the initiation codon are given in bold characters. *Arrow* the site on the DNA corresponding to the processing site in the PG protein. Restriction sites for several enzymes are indicated. *m-PG* the coding part of the mature PG. Vector sequences are underlined. **B** Sequence of the 3′ part of the PG cDNA in pPG1. The stop codon of the PG gene is underlined. The oligo (C) tail is given in bold characters. Nucleotides 1371–1388 originate from the reverse-transcribed poly (A) tail

## Results

### Adaptation of the *Aspergillus* cDNA for expression in yeast

Sequence analysis of the pPG1 cDNA clone confirmed the presence of an 11-base oligo(G) tail 5′ from the coding sequence and a 17-base oligo(A) and 14-base oligo(C) tail 3′ from the coding sequence, which were introduced when the *Aspergillus* cDNA was cloned (Ruttkowski et al. 1990; Fig. 1). Yeast transformants with a plasmid carrying the *Aspergillus* gene in its original form, with 5′ and 3′ tails, did not notably express the PG gene (pPTPG6L10 and 12, data not shown). Since this could be due to interference with replication and translation by the oligo(G) tail or general instability of the insert due to recombination on the 5′ and 3′ tails, we chose to remove both sequences. In order to remove the 5′ tail, plasmid pPG1 was digested with *Bam*HI and filled up with thionucleotides and Klenow fragment DNA polymerase I, using the nested deletion kit from Pharmacia (Freiburg, Germany). After this blocking step, the plasmid was digested with *Sal*I and treated with exonuclease III and S1 nuclease. The deletion plasmids were self-ligated, transformed to *E. coli* and analysed by restriction and sequencing. A plasmid that lacked the 5′ tail and the following 7 T nucleotides was chosen for further construction. The 3′ tail was removed starting from this plasmid. The plasmid was opened at the *Nsi*I restriction site, which is located 38 bases downstream from the TAG (see Fig. 1) and treated with Klenow DNA polymerase I and low amounts of nucleotides to remove the overhanging 3′ end (Sambrook et al. 1989). At this point a *Bam*HI linker (GGGATCCC) was introduced. The *Eco*RI-*Bam*HI fragment, now containing the optimized PG cDNA, was introduced into plasmid pPTK-NP (Looman et al. 1991) to produce plasmid pPG2 (Fig. 2). Transcription of the PG gene is under the control of the yeast *ADH1* promoter sequence and terminated on the yeast *TRP*1 termination sequence in this plasmid. As a selection marker, the plasmid contains the Tn903 kanamycin-resistance gene, which confers G418 resistance in *S. cerevisiae*. The expression of the PG gene from this plasmid was tested in several yeast strains, among which were the wild-type baker's yeast strain S3, and the laboratory strains AH22 and GRF18. All of the strains exhibited enzymatic activity in the culture supernatant (see Table 1 for data on AH22 transformants). Although the dominant G418-resistance marker enabled us to transform wild-type production strains, the antibiotic itself, when used to keep selection pressure on the plasmid during cultivation, influences growth and would not be practical for the production of a food-grade enzyme. The use of G418 in synthetic or molasses-based media is hampered by the fact that its effectiveness is decreased in

salt-containing media. For this reason the expression plasmid was reconstructed to optimize expression further and to delete bacterial sequences successively.

## Shortening of the yeast *ADHI* promoter

The yeast *ADHI* promoter is regulated by glucose in that glucose is essential for efficient transcriptional induction. The upstream region of the promoter can be removed to avoid this regulation (Ruohonen et al. 1991). This was done by partial *Sph*I digestion of plasmid pPG2. The resulting fragment containing the "short" *ADHI* promoter and the PG cDNA was integrated into plasmid YEp13 (Parent et al. 1985) restricted with *Sph*I. The resulting plasmid was called pPG4 (Fig. 2). In order to see whether the selection marker (LEU2 or G418 resistance) influences PG production, plasmid pPG5 containing the Tn903 *kan*$^r$ gene at the unique *Pvu*II site of pPG4 was constructed.

## Deletion of bacterial sequences from the expression plasmid

In order to reduce the size of the expression plasmid and to remove the bacterial sequences that have frequently been found to cause plasmid instabilities (Ludwig and Bruschi 1991), the plasmid pPG4 was digested with *Xho*I and partially with *Sal*I and self-ligated. The ligation mixture was transformed in the yeast strain AH22, and the identity of the resulting plasmid pPG6, which is not able to replicate in *E. coli*, was confirmed by Southern analysis of yeast transformants and by restriction analysis of the plasmid purified from yeast as described in Materials and methods. We did not find any evidence for recombination between the expression plasmid and the endogenous 2μm DNA.

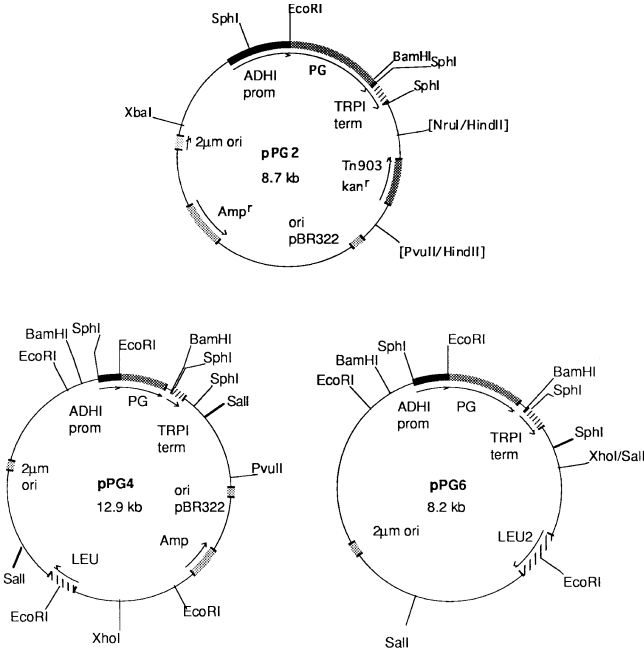


**Fig. 2** Plasmids pPG2, pPG4 and pPG6. Relevant restriction sites and gene locations are indicated. See text for details on the construction of plasmids. *ADHI prom*, promoter of the *S. cerevisiae ADHI* gene; *TRPI term*, teminator of the *S. cerevisiae TRPI* gene; 2 μ*m ori*; origin of replication of the 2 μm plasmid; *ori pBR322*, origin of replication of the *E.coli* pBR322 plasmid; *Tn903 kan*$^r$, kanamycin-resistance gene of Tn903 conferring G418 resistance in yeast; *Amp*$^r$, ampicillin-resistance gene; *LEU2*, *LEU2* gene of *S. cerevisiae*

## Exchange of the *Aspergillus* PG leader sequence for the yeast α-factor leader

Although the fungal leader sequence apparently directs the enzyme to the yeast growth medium, we also tested the yeast-homologous α-factor leader sequence as a secretion signal. For this purpose we first had to remove the fungal leader sequence. The first 27 amino acid codons of the *Aspergillus* PG cDNA code for the leader sequence of the immature protein (Fig. 1; Ruttkowski et al. 1990). Digestion of plasmid pPG2 with *Sma*I almost completely deletes this leader sequence. The single *Sma*I site thus generated was used to insert the homologous yeast α-factor leader sequence. The α-factor leader sequence comprising the *Hin*fI site at nucleotide-67 (relative to the A of the initiation codon; Singh et al. 1983) to the *Stu*I site in pMFα-8 (Miyajima et al. 1985) was subcloned in the *Sma*I site of pUC8 (Vieira and Messing 1982). It was then inserted in pPG2 as a Klenow-filled-in 334-bp *Eco*RI- *Bam*HI fragment giving plasmid pPG3α-6. Transcription initiates from the yeast *ADHI* promoter in plasmid pPG3α-6, translation initiation and secretion are directed by the homologous α-factor leader sequence. The sequence contains the *KEX2* processing site (Kurjan and Herskowitz 1982) and the putative PG processing sequence Arg-Gly (Ruttkowski et al. 1990) separated by two additional amino acids, Gly-Asp, which were introduced in the effort to maintain the reading frame of the α-factor–PG fusion (see Fig. 3). This construct, when transformed into different yeast strains (S3 as well as in X2180-1A and X2180-1B or AH22), induced PG expression and secretion into the culture medium. The amount of secreted protein and enzyme activity was, however, much lower than from the original *Aspergillus* PG leader constructs (Table 1), and plasmid pPG3α-6 was not used for further optimization.

## Copy number and mitotic plasmid stability

The mitotic stability of the various expression plasmids constructed was determined. The data revealed dramatic differences between the yeast/*E. coli* shuttle vectors (pPG2, pPG4) and the yeast-only plasmid (pPG6),

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        KEX2↓              m-PG
Asp Lys Arg Gly Asp Arg Gly Ser Cys Thr Phe Lys
GAT AAA AGG GGG GAT CGG GGA AGC TGC ACC TTC AAA
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**Fig. 3** Site of processing in the construct pPG3α-6. The sequence shows the part of the construct where the yeast pre-pro α-factor sequence from plasmid pMFα-8 (Miyajima et al. 1985) (underlined) was fused at the *Stu*I/ *Sma*I site to the truncated part of the *Aspergillus* cDNA. *Arrow* the yeast *KEX2* processing site; *m-PG* the beginning of the mature PG in *Aspergillus*. The Gly-Asp-Arg amino acid spacer between the *KEX2* processing site and the beginning of the mature PG is given in bold face. Italics show the DNA sequence originating from pUC8

as the latter exhibits a considerably higher mitotic stability (Table 1). Plasmid copy number was also determined in yeast strains transformed with the various plasmids. The copy numbers calculated and corrected for the mitotic stability of the corresponding plasmid were similar for the different 2μm-based plasmids (about 20 per plasmid-carrying cell, Table 1). As a plasmid with low copy number, a yeast centromere vector was constructed by inserting a $1.5 \times 10^3$-base (1.5-kb) *Xho*I fragment of plasmid YCp19 (Stinchcomb et al. 1982), which carries the CEN4 sequences, into *Xho*I-digested pPG4 yielding plasmid pPG7.

The data show that there is no marked difference in copy number between the different 2μm-based vectors, whether or not they carry the expression cassette (pPG4/YEp13) or possess *E. coli* pBR322 sequences (pPG4/pPG6). The centromere plasmid is a low-copy-number vector as expected. Differences in plasmid behaviour are, however, significant with respect to mitotic stability in that the deletion of bacterial sequences (pPG6) greatly increases plasmid stability compared to the conventional 2μm-based vectors (pPG4 and YEp13). Under selective conditions the centromeric plasmid pPG7 behaves as expected; it exhibits, however, extremely low mitotic stability under non-selective conditions, which might be caused by incompatibility between the 2μm origin and the CEN sequence (Apostol and Greer 1988).

## Polygalacturonase synthesis by yeast transformants

To compare the synthesis level of yeast transformants carrying the different expression vectors, cells were grown to stationary phase in minimal medium (WMVIII), and PG activity in the supernatant was determined (Table 1). In contrast to the first-generation expression vectors (pPTPG6L10 and 12), which carried the unmodified cDNA (data not shown), the deletion of the cDNA tail sequences in plasmid pPG2 results in a readily detectable level of PG activity in the medium. The partial deletion of the *ADH1* promoter sequences in plasmid pPG4 gives a 2.5-fold increase in productivity. This increase in enzymatic activity is parallelled by higher amounts of PG-specific mRNA in the transformants as seen by Northern blotting (data not shown). The choice of the selection marker (LEU2 or G418 resistance) has no effect, as transformants carrying pPG5 and grown in the presence of G418 yield similar PG activities to strain AH22/pPG4. Another 2.3-fold increase to 7000 U PG/ml was achieved by using plasmid pPG6. This rise can be largely attributed to the enhanced mitotic stability of the expression plasmid, which is devoid of bacterial DNA sequences (see Table 1). The centromere plasmid (pPG7), which carries the partially deleted and thus more active *ADH1* promoter, and has a copy number of only two per cell, yields 1500 U/ml. The recombinant protein is also detectable in Coomassie-stained gels as a dominant band (Fig. 5).

**Table 1** Mitotic stability and copy number of expression plasmids in AH22 and comparison of polygalacturonase (PG) activities of transformants as an effect of the different plasmids (*ND* not determined). Mitotic stability of markers transformed in yeast was measured after cells had been grown in non-selective or selective medium to stationary phase. Diluted samples were plated on selective and non-selective medium. The percentage of cells on the selective medium versus that on the non-selective medium was taken as a measure of plasmid stability. To determine the copy number, total yeast DNA was prepared and digested to completion with *Pst*I. The fragments were separated by agarose gel electrophoresis and blotted onto a nitrocellulose membrane (0.45μm pore size, Sartorius, Göttingen, Germany). This was hybridized with a *Sal*I/*Xho*I fragment of YEp 13 that contains the *LEU2* gene. DNA probes were labelled using the multi-prime DNA labelling kit of Amersham and [α-$^{32}$P] dATP (Amersham, Braunschweig, Germany). After hybridization, the amount of radioactivity bound to the chromosomal and to the plasmid-derived band was quantified by counting the corresponding filter spots in a scintillation counter (Beckman LS1701). Copy number was calculated in relation to the chromosomal band. For assay of PG activity, strains were grown in WMVIII medium, i.e. selective medium, in shake-flask culture. All the strains tested yielded comparable dry weights. Aliquots of the medium supernatant were taken as the culture reached the stationary growth phase (approx. 72 h); appropriate dilutions were used directly for determining PG activity

| Strain/plasmid | Characteristics | Mitotic stability (%): non-selective/selective | Copy number/ plasmid-crrying cell | Polygalacturonase activity (U/ml) |
|---|---|---|---|---|
| AH22/YEp13 | Negative control | 45.6/59.4 | 20 | 0 |
| AH22/pPG2 | "Long" *ADHI* | 47.0/54.6 | ND | 1200 |
| AH22/pPG4 | "Short" *ADHI* promoter | 56.7/61.6 | 20 | 3000 |
| AH22/pPG6 | Yeast-only plasmid | 87.7/98.0 | 18 | 7000 |
| AH22/pPG7 | Centromere plasmid | 36.5/98.7 | 2 | 1500 |
| AH22/pPG3α-6 | α-factor leader | ND | ND | 800 |

Secretion of the fungal enzyme is directed by the fungal signal sequence in the expression plasmids pPG2, pPG4, pPG6 and pPG7. Secretion efficiency is very high as no activity or considerable amounts of protein can be found in the periplasmic or the cellular fraction of enzyme-producing cells (data not shown). Exchange of the fungal leader sequence with the yeast α-factor secretion signal in plasmid pPG3α-6 also leads to secretion of the PG enzyme; the amount of secreted protein is, however, much lower (800 U/ml).

## Functional analysis of the yeast-derived PG

The yeast-derived, recombinant PG was compared with the *Aspergillus*-derived enzyme for its pH optimum, temperature optimum and temperature stability. The recombinant PG secreted into the culture supernatant by an AH22 transformant (AH22/pPG4) was used without further purification. Its enzymatic properties were compared to PG present in an enzyme mixture produced by *A. niger* that also contained pectinesterases and pectate lyases (Rohament P, Röhm GmbH, Darmstadt, Germany) and to PG purified from this mixture (kindly provided by Dr. Schuster, Röhm GmbH). The results of these comparisons show that the recombinant enzyme has the same enzymatic properties as the native *Aspergillus* enzyme with respect to pH optimum (which is between 5 and 6) and temperature optimum (which is at 50° C). Thermal stability is, however, increased for the yeast-derived enzyme in that the recombinant protein retains 95 % of its activity compared to 65 % for the *Aspergillus* PG after incubation for 1 h at 40° C , and still exhibits 15 % of the initial activity after 1 h at 50° C while the *Aspergillus* PG has lost all of its activity under these conditions.

## Biochemical characterization of recombinant PG from *S. cerevisiae*

Recombinant PG was purified from *S. cerevisiae* medium supernatant using an ion-exchange column (see Materials and methods). The eluted protein was analysed by SDS-PAGE for molecular mass determination. The recombinant enzyme from yeast has an apparent size of 38.4 kDa on SDS gels and is thus somewhat larger than the enzyme synthesized by *Aspergillus* (37.3 kDa). Both the recombinant yeast PG and the *Aspergillus* PG were treated with endoglycosidase H to determine the molecular mass after removal of the asparagine-linked *N*-glycosylation (Fig. 4). There is one potential glycosylation site in the PG sequence at asparagine residue 213 (Ruttkowski et al. 1990). The recombinant protein as well as the *Aspergillus* protein have an apparent molecular mass of 35.5 kDa after deglycosylation. Figure 4 shows that deglycosylation results in a protein band of identical size for both

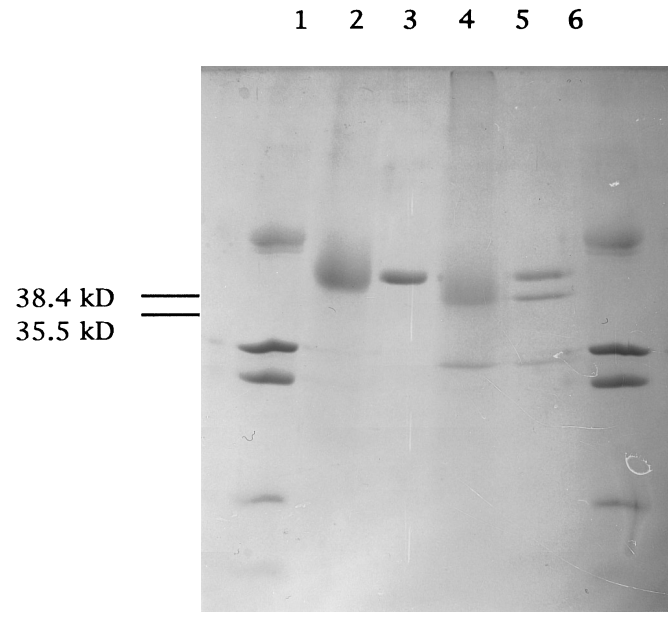


**Fig. 4** Endoglycosidase H treatment of purified *Aspergillus* and yeast-derived enzyme. Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using a 15% resolving gel. After electrophoresis the gels were stained with Coomassie brilliant blue R250. Endoglycosidase H (Boehringer, Mannheim, Germany) treatment was done with 0.01 U in 30 mM potassium phosphate buffer, pH 6.0 for 18 h at 37° C. Lanes: *1*, *6* marker proteins of 45 kDa, 29 kDa, 24 kDa, 14.2 kDa; *2* untreated *Aspergillus* PG; *3* untreated yeast-derived PG; *4* endoglycosidase-H-treated *Aspergillus* PG; *5* mixture of untreated and endoglycosidase-H treated yeast-derived PG in equal quantities. The PG enzymes were purified as described in Materials and methods. The lower band in both endoglycosidase H-treated enzymes at 26 kDa results from non-specific breakdown of PG and is sometimes observed in fermentation

proteins and that the recombinant enzyme isolated from yeast thus has a higher degree of glycosylation than the *Aspergillus* enzyme. The isoelectric point of both proteins was determined to be at pH 5.7. The N-terminal amino acids of the recombinant PG were analysed to determine whether the processing of the fungal leader sequence in yeast occurred at the same site as in *Aspergillus*. The amino acid sequence was confirmed to be Gly-Ser as in the mature *Aspergillus* protein (Ruttkowski et al. 1990).

## Fed-batch fermentation

Strain AH22/pPG6 was used for fermentation cultures. The strain was inoculated from 400 ml preculture grown in selective medium (WMVIII) to stationary phase. Fed-batch culture was started with salts and minerals in the vessel and sucrose was continuously added with a constant feeding rate of 5 g/h for 120 h. Samples were removed at regular intervals and were analysed for dry matter content, ethanol concentration, PG activity and protein concentration in the supernatant. PG activity increased in correlation with yeast

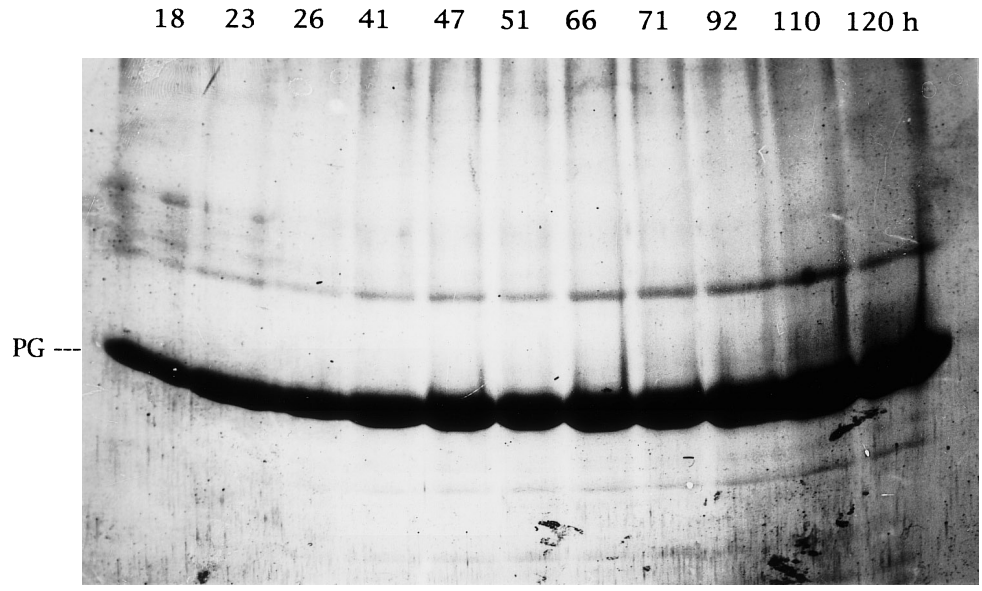


**Fig. 5** Coomassie-stained gel of the culture supernatant of yeast fermentation. Aliquots of medium supernatant, taken at different times during fed-batch fermentation of strain AH22/pPG6, were run on a 12% sodium dodecyl sulphate polyacrylamide gel and stained with Coomassie blue. The lanes were loaded with 300 µl culture supernatant; times after the start of fermentation are indicated. The major protein band in culture supernatant (*arrow*) was identified as PG by functional and biochemical analysis

dry matter and activities up to 45 000 U/ml were measured. The amount of PG produced during shake-flask and fed-batch fermentations was estimated from Coomassie-stained gels (Fig. 5) and from protein concentration measurement of the supernatant of control and transformant cells. Secreted protein concentrations were found to be 22 mg/l in shake-flask cultures and 200 mg/l in fed-batch cultures, where PG constitutes up to 89 % of the total protein secreted. *S. cerevisiae* synthesizes recombinant *Aspergillus* PG at approximately 1 %–2 % of the total cell protein.

## Discussion

The *A. niger* PG was expressed in *S. cerevisiae* as a secreted protein and its expression level was increased in several successive steps. The aim was to yield a high level of active enzyme in the culture medium without reducing the viability of the yeast cell. The expression was not to be limited by the selection pressure, the culture medium or the production strain. To improve plasmid stability and its chances of being used for producing a food-grade recombinant enzyme, an "all-yeast" plasmid was constructed, in which most of the bacterial sequences were removed.

The first improvement involved the 5′/3′ ends of the cDNA. The original cDNA clone (pPG1) contains, apart from the PG coding sequence, several short single-base sequences most of which were introduced in the process of cDNA cloning. They include the 12-base G tract at the 5′ end followed by 7 T bases coded by the PG gene and the 15-base C tract at the 3′ end preceded by the reverse-transcribed 17-base poly(A) tail. By cloning the original cDNA sequence of the PG gene (*Eco*RI-*Hin*dIII) in the yeast expression vector pPT2b we were not able to detect enzymatic activity in the yeast transformants. We attribute this fact to either an interference of the oligonucleotide sequences with translation or transcription or to the instability of the plasmid construct. It was shown that short repeats of bases are the cause of extreme instability either by slippage of the DNA polymerase or by induction of recombination (Henderson and Petes 1992). Removal of interfering 5′- and 3′- homopolymer tails improved the expression of mouse α-amylase cDNA in yeast (Olsen 1987). By removing the sequences containing the single-base repeats as in pPG2 we could raise the expression to a measurable level.

The yeast *ADHI* promoter is a constitutively expressed promoter only in the presence of glucose (Denis et al. 1983). Since the regulatory sequences upstream of the promoter sequence are responsible for catabolite repression (Ruohonen et al. 1991), we chose to remove these sequences from the construct. The resulting plasmid pPG4, which carries the short version of the *ADHI* promoter and is no longer subject to catabolite repression, gives high-yield transcription initiation. We assume that an upstream activating sequence present in pBR322 sequences (Sidhu and Bollon 1990) is responsible for the improved transcription rate.

Enzyme synthesis yields were further improved by altering the composition of the expression plasmid. Interestingly, the effect of removing the pBR322 part of the vector resulted mainly in a dramatic increase in mitotic stability under selective and non-selective conditions. There was no measurable effect on plasmid copy number. In contrast to the 2- µm expression plasmids described by Ludwig and coworkers (1993),

recombination with the endogenous 2 μm plasmid was not required for its stable maintenance.

The use of *S.cerevisiae* for the expression and secretion of heterologous proteins has been described in numerous cases (Hitzeman et al. 1990; Romanos et al. 1992). Foreign proteins can be directed to the secretion pathway by fusing them to yeast secretion signals such as the pre-pro region of the α-factor (Miyayima et al. 1985), or the invertase leader sequence (Chang et al. 1986). Numerous cases have also been reported where heterologous signal sequences, e.g. from bacteria (Ruohonen et al. 1987), fungi (Hiramatsu et al. 1991), plants (Nagahora et al. 1992) up to rat and man (Kanda et al. 1992), direct secretion. Several *Aspergillus* leader sequences, such as the one described in this paper, have also been successful in directing secretion from yeast. These include the leader sequences of *A. niger* glucose oxidase (Frederick et al. 1990), *Aspergillus oryzae* α-amylase (Randez–Gil and Sanz 1993), *A. oryzae* alkaline and neutral proteases (Tatsumi et al. 1989, 1991), and *Hormoconis resinae* glucoamylase (Vainio et al. 1993). The fact that even random protein sequences function as export signal sequences in yeast with a frequency of 20% (Kaiser et al. 1987) points to the considerable degeneracy of these signals (Ferenci and Silhavy 1987). Together with the observation that the molecular machinery for secretion is conserved throughout a large variety of species (Bennett and Scheller 1993), it is not surprising that the PG signal sequence from *Aspergillus* functions in *S. cerevisiae*. The efficiency of the process, however, and the fact that the enzyme is correctly processed, is an unexpected result. It was shown for the yeast α-factor precursor that the first 19 amino acids of the presequence provide sufficient information for the efficient secretion into the medium (Ernst 1988). When the 27 N-terminal amino acids of the leader sequence of the immature *Aspergillus* PG are compared with the yeast α-factor presequence, there is no large homology on an amino-acid basis, but a striking structural homology, as the hydropathy plot of the first 27 amino acids of both leader sequences (after Kyte and Doolittle 1982, data not shown) is identical. The *Aspergillus* leader sequence is composed of a short, charged N-terminal sequence, a hydrophobic core followed by a polar region of 5 amino acids with a helix-bending proline at position − 5 (von Heijne 1985). This fact, as well as the presence of the *N*-glycosylation sequence (Caplan et al. 1991), could explain the efficient secretion of the *Aspergillus* PG in yeast. In our case the exchange of the PG leader sequence of the yeast α-factor sequence reduced the secretion of PG. This indicates that the *Aspergillus* leader sequence provides a better fit between leader and mature peptide as does the α-factor leader and the mature PG. That different leader sequences influence the quantity of secreted protein in yeast has previously been observed for secreted human serum albumin (Sleep et al. 1990).

N-terminal protein sequencing showed that the recombinant PG is correctly processed in yeast although the cleavage site does not follow the "( − 3, − 1) " rule as defined by von Heijne (1983). The last amino acids of the PG leader peptide are Glu-Ala-Arg, which does not constitute a *KEX*2-like endoproteolytic cleavage site (Lys-Arg or Arg-Arg). The cleavage site only partly fits the consensus sequence for monobasic processing sites (Devi 1991). The nature of the peptidase performing the correct processing of the recombinant PG in yeast thus remains unclear.

The PG coding sequence contains one potential *N*-glycosylation site at position Arg-213. Endoglycosidase H treatment of both *Aspergillus* and yeast-derived PG showed that both enzymes are *N*-glycosylated. Endoglycosidase H treatment of the yeast-derived enzyme resulted in the reduction in molecular mass of about 2.9 kDa, which points to one core oligosaccharide moiety per molecule (Caplan et al. 1991). The degree of glycosylation is higher in the yeast enzyme than in the *Aspergillus* PG. A hyperglycosylation of a fungal enzyme in yeast has also been reported for *Aspergillus* glucose oxidase (Frederick et al. 1990). The different degree of glycosylation could account for the differences in temperature stability of both enzymes. An influence of glycosylation on thermostability of recombinant glucanases from *S. cerevisiae* has also been reported by Meldgaard and Svendsen (1994); these authors suggest that phosphorylation of yeast *N*-glycans might influence thermostability.

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