

Impact of Glycosylation on *Saccharomyces cerevisiae* Endopolygalacturonase PGU1 Activity and Stability

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Abstract: Endopolygalacturonases are among the best selling enzymes for a number of commercial applications such as food processing. For such enzymes, a potentially important component of production cost is glycosylation. This important modification of endopolygalacturonase has been detected for a number of species, but its real importance has not been thoroughly studied. Here we investigated endopolygalacturonase PGU1 from *Saccharomyces cerevisiae* CECT 1389 produced in *S. cerevisiae* INVSc 1. Combinatorial mutagenesis of recombinant *S. cerevisiae* PGU1 putative glycosylation sites was performed, where asparagines 318 and 330 were replaced with either aspartic acid or glutamine. Electrophoretic analysis of the different recombinant enzymes studied here demonstrates that the putative sites 318 and 330 are indeed glycosylated when produced in *S. cerevisiae* INVSc 1. The optimal activity of these enzymes was detected at pH 4.5 and 55-60 °C. As for stability, all enzymes studied were less than 50% active after an incubation of two hours at 50 °C and at pH between 4.5 and 6.0. Glycosylation did not provide any significant stabilisation of PGU1, but the replacement of Asn 330 with Gln had a deleterious effect on stability. The secondary structure spectra are characteristics of proteins mostly composed of beta sheets. The T_m values measured for PGU1, PGU1 deglycosylated with endo H, and three mutants ranged from 53 to 55.4°C, indicating that glycosylation had no impact on PGU1 conformation.

Key Words: Endopolygalacturonase, glycosylation, directed mutagenesis, *Saccharomyces cerevisiae*, activity, stability.

1. INTRODUCTION

Pectic substances, a major constituent of plant cell walls, are degraded by a wide range of enzymes called polygalacturonases [1, 2]. Among them, endopolygalacturonases (endoPG) (E.C. 3.2.1.15) catalyze the random hydrolysis of α -1,4-D-glucosidic bonds between two non-methylated galacturonic acid residues [3, 4]. EndoPG production has been widely reported in plants and microorganisms. Plant endoPGs are important in fruit ripening and senescence [5], whereas the microbial enzymes are deployed during infection of plants [6-8]. The presence of polygalacturonases enzymes has been reported in different yeasts. The genes coding for endoPG from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* have been cloned and characterised [7-9]. Amino acids sequence derived from these genes show high similarity with each other and with endoPG from filamentous fungi. Recombinant expression systems have allowed for major advances in characterising endoPG, namely the identification of active site critical residues in *Aspergillus niger* [1], *S. cerevisiae* [10] and *Stereum purpureum* [11]. It was also useful for the elucidation of structural features associated with processivity in crystallised *A. niger* endoPGs [12].

Generally speaking, protein glycosylation has been related to solubility, stability and proteolytic resistance [13-

15]. Although most endoPGs characterised to date have been found to have one glycosylation site or more, the role of this modification in defining endoPG properties is not clear yet. First, as indicated earlier by [16] the number and sequence location of putative glycosylation sites vary across endoPG from different organisms. Then, divergent results have been reported regarding the impact of removing the oligosaccharides from (or avoiding their addition to) mature endoPGs. Enzymatic removal of oligosaccharides from native *A. niger* endoPG and from recombinant *Phytophthora parasitica* endoPG completely inhibited enzymatic activity of both enzymes. At variance, it was reported that glycosylation had no impact on activity for recombinant *S. purpureum* endoPG expressed in *Escherichia coli* [17]. For this particular case, enzyme thermostability decreased as the extent of glycosylation increased. Glycosylation of *S. cerevisiae* endoPG also appears to be accessory, as mutagenesis of the two putative glycosylation sites lead to partial enzymatic inhibition, or had no effect at all depending on the combination of sites mutated when produced in two strains of *Pichia pastoris* [10]. Stratilova *et al.* [16] indicated that while several endoPG were glycosylated near the active site region, PGU1 from *S. cerevisiae* was not. This observation may provide a rationale for the different behaviours reported for different endoPGs. A possible interaction between endoPG putative oligosaccharides and its substrate is not completely out of the question. The recent observation that *S. cerevisiae* endoPG properties are modulated by the different glycosylation pattern afforded by *S. pombe* suggests such interaction [18].

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Clearly, the importance of glycosylation in defining specific endoPG properties has to be addressed: this post-translational modification increases the metabolic cost of producing the enzyme. Further, the functional importance of such post-translational modifications may decrease the validity of prokaryote-based strategies for protein engineering. Both aspects may have a deleterious impact on the costs associated with the development and the commercialisation of endoPG for various industries.

Here we describe the use of a commercial *S. cerevisiae* strain for the recombinant expression of PGU1 isolated from *S. cerevisiae* strain CECT 1389. This expression system will be used to investigate the impact of glycosylation on *S. cerevisiae* PGU1 activity and stability.

2. MATERIALS AND METHODS

2.1. Strains and Plasmids

The gene coding for endoPG I (PGU1) and its native sequence signal were isolated from the strain *S. cerevisiae* CECT 1389 (ATCC). For polymerase chain reaction [19] gene amplification, the following primers were used 5' CCG CAA AGC TTG ATG ATT TCT GCT AAT TCA TTG C 3' and 5' CGC GCG GCC GCC TTA ACA GCT TGC ACC AGA TCC 3'. This strategy provided amplified gene flanked by *Hind*III and *Not*I restriction sites at their 5' and 3' ends, respectively. After PCR, the gene was cloned in pYES2/CT yeast vector (Invitrogen). The recombinant vector was transferred by electroporation in *Escherichia coli* *DH5a* for cloning, and in *S. cerevisiae* INVSc 1 (Invitrogen) for production of the enzyme.

2.2. Media Composition and Growth Conditions

Bacterial cells were grown overnight at 37 °C on Lowry Broth medium containing ampicillin (100 µg/mL) after transformation. INVSc 1 cells were grown on YPD medium (1% yeast extract, 2% peptone and 2% glucose) for two days at 30 °C. After transformation, yeast clones were grown at 30 °C for three days on Sc minimal medium as per manufacturer's instructions (pYES2/CT manual, Invitrogen, Burlington, Ontario). For enzyme production, yeast clones were grown in liquid Sc minimal medium for 24 h, with agitation (300 rpm) at 30 °C. The induction was performed in Sc minimal medium with galactose (equivalent to Sc minimal medium except for glucose being replaced by galactose) for 20 h, 300 rpm. Secretion of the PGU1 by the new production host was verified on Sc minimal media gelose [20] equilibrated at pH 5.0 with sodium acetate buffer and which contained polygalacturonic acid (PGA) 1%.

2.3. Purification and Quantification

After induction the supernatant was collected by centrifugation for 10 minutes at 2057 g and filtered on a 0.2 µm filter, then concentrated 10-fold using an ultrafiltration device (Amicon membrane 30 kDa, YM30). The proteins were then resuspended and conserved in sodium acetate buffer 50 mM at pH 5.0. Quantification of total proteins was carried out in a 96 multi-well plate (400 µL well) format using a bicinchoninic acid test using BSA as the standard for the calibration. Absorbance was measured at 560 nm on a Mul-

tiskan Ascent spectrophotometer (from Thermo Electron Corporation).

2.4. DNA Manipulation, Cloning and Mutagenesis

The substitution of the Asn by either Asp or Gln in Sc HN gene (recombinant PGU1, hereafter named Sc HN) was performed using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) as per manufacturer's instructions. DNA extracts were sequenced and analysed with an ABI Prism 3100.

2.5. Electrophoresis and Proteins Detection

Protein electrophoresis was performed using tricine 10% ready gel, a tricine buffer system and the Silver stain SDS-PAGE Standard, low range calibration mix from Biorad. Silver nitrate staining was used for detection, using the Pro-teosilver Silver Stain Kit from Sigma.

2.6. Deglycosylation with Endoglycosidase H

1.4 mg of proteins was deglycosylated with 0.125 U of endoglycosidase H (endo H) at 37 °C over night.

2.7. Detection of endoPG Activity

INVSc 1 clones, obtained on Sc minimal media gelose, were isolated and grown on Sc minimal media gelose with galactose and supplemented with 1% PGA. The yeast *Kluyveromyces marxianus* Y-49 (ATCC) which produces an active endoPG, was used as a positive control, while *S. cerevisiae* INVSc 1 which does not produce endoPG, provided a negative control. The gelose was left at 30 °C for 3 days and endoPG activity was detected by reacting with ruthenium red 0.075% using the method described by [21].

2.8. Enzymatic and Stability Assay

Quantitative measurements of enzymatic reaction initial velocity were performed using a reducing sugar detection method adapted from [22]. The enzymatic assay was carried out in an acetate/bis-tris (Bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane) buffer 180 mM adjusted from pH 4.0 to 6.5 as specified. The temperature was maintained at 50, 55 or 60 °C using a Digital heat block modified to transfer heat to a multi-well plate (1.2 mL well). Note that these conditions mimic the ones found in a typical TMP pulp and paper plant where pectate has to be controlled. The PGA 0.67% solution and all the acetate/bis-tris buffers were equilibrated at the specified temperature before addition of the enzyme. The reaction mixture was sampled every 35 s for 3 min and mixed with 1 mL of PABAH (4-hydroxybenzoyl hydrazine) 0.5% (previously dissolved in 0.5 M NaOH) in multi-well plates (2 mL well). The reactions were then heated at 110 °C for 10 min., cooled on ice for 10 min. and transferred to multi-well plates for reading at 405 nm. One unit was defined as the production of 1 µmole of galacturonic acid per minute. For thermostability measurements, endoPGs were equilibrated in the acetate/bis-tris buffers at the specified temperature and sampled every 30 min. Residual activity was then measured as indicated above, at a fixed temperature of 50 °C and pH 5.0. For sake of reproducibility, the reactions and incubations described in this section were auto-

mated using a Biomek 2000 workstation (Beckman Coulter) and all measures were performed in triplicates.

2.9. Circular Dichroism Measurements

For circular dichroism (CD) measurements enzymes were resuspended in sodium acetate buffer 50 mM (pH 5.0) at a concentration of 0.5 mg mL⁻¹. The samples were then degassed and equilibrated 20 minutes at 20°C before measurements. Spectra were measured with a Jasco J-720 spectropolarimeter, which was routinely calibrated with a 0.06% (W/V) ammonium (+)-10-camphorsulfonate solution. For measurements in the far-UV region a quartz cell with a path length of 0.01 cm was used. Ten scans were accumulated at a scan speed of 20 nm per minutes, with data being collected at every nm from 185 to 240 nm. Sample temperature was maintained at 20°C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvette. Spectra were corrected for buffer signal and conversion to $\Delta\epsilon_{MRW}$ (mean residue weight) was performed with the Jasco Standard Analysis software. In order to measure thermostability, temperature was increased from 45 to 70 °C at a rate of 20 °C per hour using a Neslab RTE-11 controlled by the Jasco spectropolarimeter software. For these experiments, data were collected at 215 nm using a 0.1 cm path quartz cell.

Thermal stability was calculated assuming a unimolecular, two-state process as previously described [23]. The $\Delta\epsilon_{MRW}$ calculated at various temperatures was used as the property (y) indicative of the extent of unfolding. In the folded state, the parameter $y = y_f$ and the fraction of folded protein f_f is equal to 1. When the protein is unfolded, the parameter $y = y_u$, and the fraction of unfolded protein f_u is equal to 1. For intermediate states, y is given by $y_f f_f + y_u f_u$. Thus, by measuring y , we can calculate the fraction of protein unfolded: $f_u = (y_f - y)/(y_f - y_u)$. The equilibrium constant for the unfolding process is $K_u = f_u/(1-f_u)$ and melting temperatures (T_m) are obtained at $K_u = 1$ [23].

2.10. 3D Structure Modelling

A hypothetical 3D structure of *S. cerevisiae* PGU1 was generated using Swiss-Model and Swiss-PDB Viewer [24, 25]. The crystallographic structures used for homology modelling were those of *Aspergillus niger* endoPG II [1], *A. aculeatus* PG [26] and *A. niger* endoPG I [27]. The RMS (Root mean square) calculated for the hypothetical 3D structure is 2.96 Å. The RMS calculated is an average of the backbone atoms positions of each crystallography structures, taken separately, and compared to the hypothetical 3D structure backbone position of PGU1 of *S. cerevisiae*.

3. RESULTS

3.1. Analysis on SDS-PAGE

PGU1 produced by *S. cerevisiae* has two putative glycosylation sites at positions 318 and 330. To verify that these two sites were indeed glycosylated in *S. cerevisiae*, those sites were mutated individually or in a combinatorial mode and the resulting enzymes were produced and purified. Electrophoretic investigation of PGU1 extracts (95% Sc HN purity) reveals that in *S. cerevisiae*, three bands separated by about 4 kDa are associated with endoPG activity (Fig. 1).

Mutating either of the two putative glycosylation sites resulted in the loss of the 45 kDa band, which is the heaviest species, while mutating both sites lead to disappearance of both bands at 45 and 41 kDa. For double mutated enzymes analysed, only one band at 37 kDa was detected on the gel, this could correspond to the molecular mass of deglycosylated PGU1 [28]. These results suggest that in *S. cerevisiae*, PGU1 exist as three populations: one non-glycosylated, one with one glycosylation and a third group with both sites glycosylated.

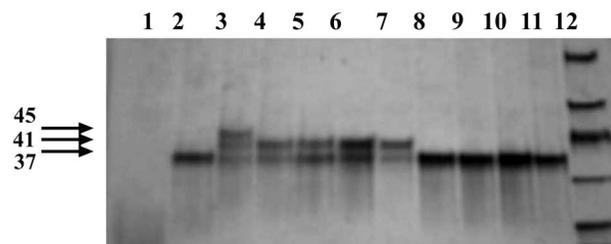


Fig. (1). SDS-PAGE analysis of Sc HN and all mutants. Lane 1: INVSc 1 (host without expression vector); lane 2: Sc HN deglycosylated with endo H; lane 3: Sc HN; lane 4: Sc N318D; lane 5: Sc N318Q; lane 6: Sc N330D; lane 7: Sc N330Q; lane 8: Sc N318D-N330D; lane 9: Sc N318D-N330Q; lane 10: Sc N318Q-N330D; lane 11: Sc N318Q-N330Q and lane 12: standards. Approximate size of proteins detected is indicated by arrows on the left.

3.2. Analysis of the Enzymatic Activity

The activity of Sc HN, Sc HN deglycosylated with endo H and all mutated enzymes was measured at various temperatures and pH. As shown in Fig. (2), all curves were characterised by a peak at pH 4.5-4.75 and a shoulder at pH 5.5. None of the enzymes were active at pH 6.5. These curves were similar for all enzymes studied at 50, 55 and 60 °C (not shown). Similar activity curves were obtained for another native endoPG from the laboratory strain *S. cerevisiae* IM1-8b. Removing one or both glycosylation sites resulted in no significant difference in enzymatic activity at all pH and at

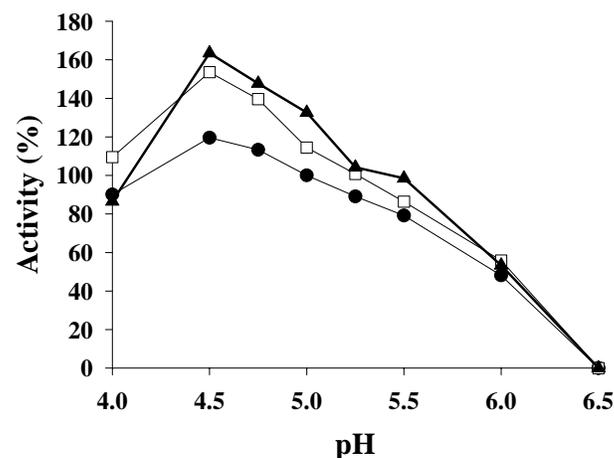


Fig. (2). Relative activity of Sc HN measured at various pHs and at 50 (●), 55 (□) and 60 (▲) °C. Enzymatic activities were measured on a period of 3 min and expressed as percentage of enzymatic activity measured for Sc HN at 50 °C, pH 5.0 (defined as 100%). Only Sc HN results are shown for clarity. Average error margin: +/- 5%.

all temperatures studied except for the double mutated enzyme. At 60°C the activity was decreased by 10 to 20% depending on pH, for the double mutated enzymes. Removal of both oligosaccharides from Sc HN by treatment with endo H lead to a similar decrease in activity at 60°C (see also Table 1).

Table 1 summarises the results obtained for enzymatic activity at pH 4.5, 5.0, 5.5 and 6.0, measured at 50, 55, and 60 °C. Most enzymes had their maximal activity at 60 °C and pH 4.5. Enzymes Sc N318Q-N330D and Sc N318Q-N330Q were more active at 50-55 °C for all pH, with no significant difference in the enzymatic activity measured for both temperatures. For all proteins studied, it was found that as the pH increased (from 4.5 to 6.5), the activity decreased, with no activity left at pH 6.5. The impact of temperature was found to vary depending on pH and on the nature of mutations. The six first enzymes in Table 1 (i.e. with a single mutation or none) showed an increase of activity with the increase of temperature at all pH. Double mutants behaved differently. Mutants with D in position 318 (Sc N318D-N330D/Q) had similar activities at 55 and 60 °C, for most of the pH range studied. For the other double mutants (Sc N318Q-N330D/Q) the activity peaked at 55°C at all pH studied. Despite the moderate variations observed in Table 1, it remains that PGU1 activity is not dependant on glycosylation. Only a moderate impact on activity was detected when PGU1 was deglycosylated with endo H or when glycosylation sites were mutated individually, regardless of the residues chosen for the positions 318 and 330. The differences observed for the double mutated enzymes appear to be controlled by the residues chosen to replace the asparagines 318 and 330.

Sc HN enzymatic activity was compared with Sc HN deglycosylated with endo H at temperatures between 30 and

70 °C at pH 4.5, 5.0 and 6.0 (Fig. 3). The enzymatic activity of both enzymes was similar with a maximum at 60 °C for all pH studied. Activity was maximal at pH 4.5 and decreased by 60% at pH 6.0. These results corroborate those in Table 1.

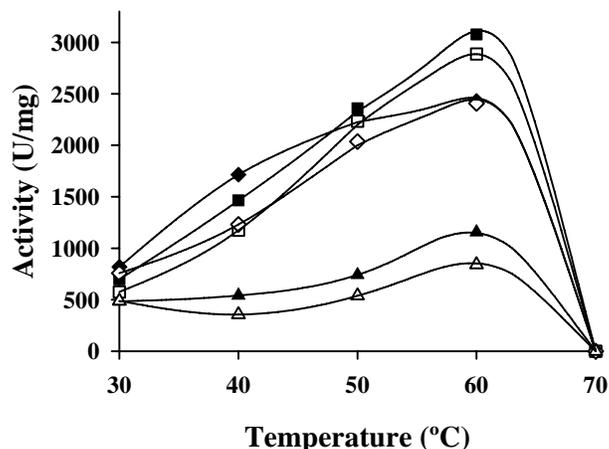


Fig. (3). Activity measured at different temperatures and pH 4.5 (■, □) 5.0 (◆, ◇) and 6.0 (▲, △) for Sc HN (filled symbol) and Sc HN deglycosylated with endo H (open symbol). Average error margin: +/- 5%

3.3. Analysis of Stability

The stability of Sc HN, Sc N318D, Sc N330D and Sc N318D-N330D exposed at 37 °C, pH 5.0 for one hour was measured (data not shown). After one hour treatment, all enzymes retained 100% of their initial activity. The stability of Sc HN, Sc HN deglycosylated with endo H and all mutated enzymes was then analysed after different incubation

Table 1. Enzymatic Activity of Sc HN, Sc HN Deglycosylated with Endo H and all Mutated Enzymes Measured at pH 4.5, 5.0 and 6.0 and at 50, 55 and 60 °C

Enzymes	Activity U/mg ^a											
	pH 4.5			pH 5.0			pH 5.5			pH 6.0		
	50°C	55°C	60°C	50°C	55°C	60°C	50°C	55°C	60°C	50°C	55°C	60°C
Sc HN	2300	3000	3200	2000	2200	2600	1500	1700	1900	940	1100	1000
Sc HN deglycosylated	2100	2100	2500	1900	1900	2200	1200	1600	1400	470	640	890
Sc N318D	1800	3000	3100	1500	2400	2700	1300	1900	2300	560	1100	1200
Sc N318Q	2500	2500	2700	1900	2000	2300	1500	1600	1800	640	700	800
Sc N330D	2600	2800	3000	2100	2200	2400	1600	1700	1800	900	920	630
Sc N330Q	2300	2500	3200	1900	2000	2600	1400	1400	2000	830	880	1200
Sc N318D-N330D	2000	2500	2400	1700	2200	2200	1400	1600	1700	660	640	630
Sc N318D-N330Q	2300	2300	2500	1900	2000	2100	1600	1700	1700	630	700	420
Sc N318Q-N330D	2600	2600	2200	2200	2200	1600	1600	1700	1200	870	860	570
Sc N318Q-N330Q	2400	2500	2200	2200	2200	1900	1800	1900	1500	760	800	370

^aOne unit is the concentration of GA produced in μmol per mL per minute ($U = \mu\text{mol mL}^{-1} \text{min}^{-1}$). The activity is expressed as $U \text{ mg}^{-1}$, with an error margin of 10%.

time during a two hours treatment at 50 °C from pH 4.5 to 6.0. The half-life of these enzymes differed depending on the mutation (Table 2). Sc HN, Sc HN deglycosylated and both mutants Sc N318D/Q lost 50% of their initial activity in 30 to 60 min. When Asn 330 was replaced by an Asp, the half-life increased between 60 and 90min at pH 4.5, 5.0 and 5.5. As for the enzymes with the mutation N330Q, the half-life was reached in less than 30 min for all pH. The stability was not dependant on glycosylation and like the activity, the differences observed were rather due to the amino acid replacing Asn 318 and 330. All of these enzymes are still active after the two hours treatment and their residual activity ranged from 5 to 50% (data not shown).

Table 2. Approximate Half-Life of Residual Enzymatic Activity of Sc HN, Sc HN Deglycosylated with endo H and all Mutants Measured at 50 °C at pH 4.5, 5.0, 5.5 and 6.0. *: 0 to 30 min; **: 30-60 min; * 60-90 min**

Enzymes	Half-life (min)			
	pH 4.5	pH 5.0	pH 5.5	pH 6.0
Sc HN	**	**	**	**
Sc HN deglycosylated	**	**	**	**
Sc N318D	**	**	**	**
Sc N318Q	**	**	**	**
Sc N330D	***	***	***	**
Sc N330Q	*	*	*	*
Sc N318D-N330D	***	***	***	**
Sc N318D-N330Q	*	*	*	*
Sc N318Q-N330D	***	***	***	**
Sc N318Q-N330Q	*	*	*	*

3.4. Analysis by Circular Dichroism

The secondary structures were analysed by circular dichroism. Spectra were indicative of significant beta sheet content, with a minimum around 217 nm and a maximum around 195 nm. This spectroscopic analysis fully supported the theoretical 3D structure obtained by modelling (Fig. 4). The secondary structures of selected mutants prepared here were measured under the same conditions. There was no significant difference in the spectra obtained for the enzymes Sc HN, Sc HN deglycosylated with endo H, Sc N318D, Sc N330Q and Sc N318D-N330D (not shown). This suggests that the oligosaccharides have little impact on the protein structure, providing a rationale for the absence of large differences in the activity and in the stability of these enzymes.

3.5. Analysis of the Melting Temperature

The melting temperatures (T_m) calculated for enzymes studied by CD were similar. The T_m of Sc HN deglycosylated with endo H was 55.3 °C, while the least stable enzyme studied by CD had a T_m of 52.6 °C (Sc N318D-N330D).

Other enzymes studied had intermediate melting temperature. Analysis of conformational stability appears to be closely related to the stability challenge performed at 37 and 50 °C. A direct comparison of T_m calculated from denaturation curves and results in Table 1 is not possible. One has to consider that for Table 1 activity data, enzymes were exposed to 60 °C (or any other temperature) only few minutes, while denaturation studied by CD were performed over 70 min.

DISCUSSION

The importance of glycosylation for protein stability is well established. Glycosylation may provide conformational stability by its steric impact on the denatured state entropy, and may protect otherwise exposed proteolytic targets from hydrolysis by proteases [14]. Glycosylation is also an important factor regarding protein engineering strategies as well as commercial application of enzymes. Examination of endoPGs sequences by Stratilova [16] revealed that, in comparison with several other endoPGs, putative glycosylation sites in *S. cerevisiae* PGU1 are remote from its active site, and according to homology-based 3D structure modelling, these sites point outward from the substrate binding cleft (Fig. 4). On the basis of this observation one would predict a limited impact of glycosylation in *S. cerevisiae* PGU1 activity. Obviously, an experimental verification of this postulate was mandatory before considering production of PGU1 in organism devoid of post-translational modification machinery.

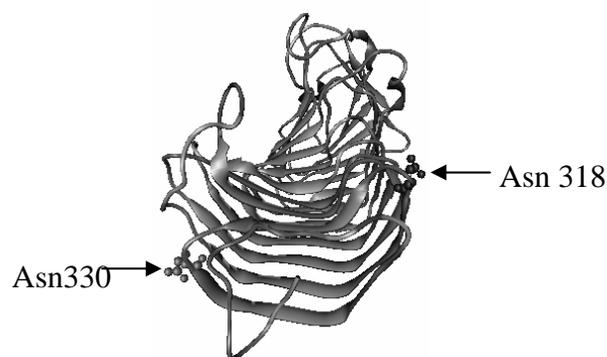


Fig. (4). Structural model of PGU1 from *S. cerevisiae*. Putative glycosylation sites Asn 318 and 330 are indicated by arrows.

Blanco *et al.* [10] have reported a partial characterisation of enzymes with their putative glycosylation sites (Asn 318, Asn 330) mutated for Asp in *S. cerevisiae* PGU1 from the strain IM1-8b produced in *P. pastoris* X-33 and KM71. Modification of pectinase activity was detected for N318D-N330D, with 50% activity left compared to the control, when produced in strain X-33. For the same enzyme produced in strain KM71, the activity detected was 12.5% compared to the control. Since nothing in this study proves that these sites were glycosylated in the native enzyme and not in the double mutated enzyme, interpretation is not straightforward: The possibility that this change in activity was due to deleterious mutations of Asn to Asp themselves was not addressed. In addition, since activity was calculated on the ba-

sis of volume used for the activity assay, one cannot rule out a possible difference in protein expression yield as an explanation for the apparent decrease in activity. Since glycosylation may offer protection against proteolysis, avoiding glycosylation by mutating both Asn may lead to increased proteolysis, reduced protein recovery and lower apparent endoPG activity. Also, depending on the *P. pastoris* strain used for the production, the activity detected was different. The organism chosen for the production seems to have a direct impact on the activity detected, that may explain the difference between their results and the ones we obtained.

Sieiro *et al.* [18] reported on the production of PGU1 from *S. cerevisiae* IM1-8b in *S. pombe*. Using this host, an endoPG with modified properties (optimal pH and temperature, stability) was generated. It was suggested that differences in glycosylation pattern between this host and *S. cerevisiae* was responsible for the modulation of optimal conditions for enzymatic activity. The authors suggested that glycosylation may affect *S. cerevisiae* PGU1 but such interpretation was not supported by experimental evidence. Further, actual glycosylation of Asn 318 and 330 was neither demonstrated nor characterised in this study.

We attempted here to shed some light on this important issue by reassessing the experimental strategy: First, a recombinant system based on *S. cerevisiae* as a host was chosen. The purpose was to use an organism which does not produce endoPG and which is close to the native organism Sc CECT 1389. Second, we compared all activities on the basis of endoPG concentration (mg L^{-1}), eliminating the impact of expression yield on measured endoPG activity. Third, we adapted an enzymatic assay for direct and continuous measurement of product accumulation, that can be carried out at the pH and temperature that one selects to investigate. Earlier works were based on the method of Somogyi [29] as modified by Nelson [30] where all measurements are “end point” and are carried out at 37 °C, after incubation under selected conditions. Obviously, as seen here, changing temperature greatly affect the outcome of activity measurements.

The successful expression of recombinant *S. cerevisiae* PGU1 in a commercial *S. cerevisiae* host was achieved. Expression yield in the range of 25-40 mg L^{-1} of broth was achieved, and recombinant PGU1 (Sc HN) was found to represent at least 95% of all secreted proteins. The electrophoretic analysis shown in Fig. (1) indicates that *S. cerevisiae* PGU1 was glycosylated at Asn 318 and 330 when produced in *S. cerevisiae* INVSc strain and that Sc HN secreted by *S. cerevisiae* were not homogeneously glycosylated. Depending of the presence of one, two or no putative glycosylation sites at 318 and 330, three forms of different masses were observed. The observation that most single and double mutated enzymes were found to be as active as Sc HN, is compatible with three isoforms having substantial endoPG activity. In a previous work, [9] characterized the native PGU1 produced by Sc CECT 1389. After partial purification of PGU1 by gel exclusion chromatography, only one active enzyme with a molecular mass of 39 kDa was isolated. This suggests that PGU1 from Sc CECT 1389 was not glycosylated when produced by this strain. Also, [9] measured the optimal pH and temperature of PGU1, which are pH 5.5 and 40 °C. At variance, we found here that when using INVSc 1 as production host, PGU1 has an optimal pH and temperature of 4.5 and

60°C. Comparison of our results and those of other researchers [10, 18] indicate that the production host has a significant impact on PGU1 glycosylation and enzymatic properties.

Eight enzymes were analysed, using a combinatorial approach for double mutations. All of them displayed substantial activity and all had a similar pH – activity curve. Glycosylation also had limited impact on the stability of the enzymes at 50 °C measured at four different pH. Only the replacement of Asn 330 with Gln had a negative effect on stability. These results are confirmed by the similarity of the secondary structure spectra measured for enzymes treated with endo H or with mutations. Also, the melting temperature was similar for all enzymes studied confirming that the oligosaccharides have no significant impact on enzyme conformation. This suggests that the structure is not affected by the presence or absence of glycosylation. Overall, this study clearly demonstrated that *S. cerevisiae* PGU1 does not need to be glycosylated for maintaining wild-type behaviour in the range of conditions studied here. Current work is under way with the goal of using simple bacterial expression system(s). Production of PGU1 in bacterial hosts is being investigated and new improvement strategies based on high-throughput protein engineering are being developed.

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