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Heterogeneous glycosylation of the *EXG1* gene product accounts for the two extracellular exo-β-glucanases of *Saccharomyces cerevisiae*

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Two exo-β-glucanases of glycoprotein nature can be detected in culture supernatants of *Saccharomyces cerevisiae* cells. These exo-β-glucanases show different M_r values and kinetic properties, although they are immunologically related. Their carbohydrate content and the electrophoretic mobility of both endoglycosidase H-treated exo-β-glucanases suggest that they share the same protein fraction. Studies at genetic level relate the production of both extracellular exo-β-glucanases with the expression of a single-copy gene in *S. cerevisiae*. Expression of this gene in another yeast, *Schizosaccharomyces pombe*, demonstrates that it codes for a protein with exo-β-glucanase activity whose heterogeneous *N*-glycosylation accounts for both extracellular exo-β-glucanases of *S. cerevisiae*.

β-Glucanase; Extracellular enzyme; Glycosylation; Cell wall; (Yeast)

1. INTRODUCTION

β-Glucanases have been described and, in many cases, purified from almost all species of yeast. Usually, more than one enzymic form with β-glucanase activity can be detected in the same yeast. Owing to the composition of the yeast cell wall, it has been suggested that β-glucanases may play a role in yeast morphogenesis. These enzymes can be classified as endo- or exo-β-glucanases, according to their pattern of action [1–3].

Saccharomyces cerevisiae secretes into the culture medium three enzymic forms with β-glucanase activity; all three are glycoproteins. One is an endo-β-glucanase specific for β(1–3) linkages, while the other two are exo-β-glucanases with activity both on β(1–3) and β(1–6) glucans, as well as on synthetic β-glucosides such as MUG. These two exo-β-glucanases are immunologically related but show different M_r and K_m values and substrate specificity [4].

We have recently reported the cloning of a gene (named *EXG1*) which was proposed to be the structural gene for the major extracellular exo-β-glucanase activity in *S. cerevisiae* [5]. Here, we introduce results indicating that the *EXG1* gene codes for a protein whose heterogeneous glycosylation accounts for the two extracellular exo-β-glucanases detected in the culture medium of *S. cerevisiae*.

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Abbreviations: endo H, endo-β-*N*-acetylglucosaminidase H; MUG, 4-methylumbelliferyl-β-D-glucoside; PAGE, polyacrylamide gel electrophoresis

2. EXPERIMENTAL

S. cerevisiae strains X14, TD-28 and exo-β-glucanase-deficient (Exg⁻) mutant MAX18-9B,

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plasmids pRN4 and pRN5 carrying the *EXG1* gene and *EXG1*-transformed yeast clones have been described in [5].

Purification of exo-β-glucanases from culture supernatants of *S. cerevisiae* strain X14 was performed basically as in [6], except that the concanavalin A-Sepharose 4B chromatography was omitted.

Determination of total sugars in aliquots (20 μg) of the purified exo-β-glucanases, extensively dialysed against bidistilled water, was carried out by the phenol-sulfuric method [7].

Removal of carbohydrate from exo-β-glucanases was performed by endo H treatment. Exo-β-glucanases (1.5–3 U) were incubated for 48 h at 34°C with 0.005 U of endo H in 0.1 M acetate buffer (pH 5.6) containing 10 mM sodium azide, 2 mM phenylmethylsulfonyl fluoride and 50 μg/ml cycloheximide.

Extracellular enzyme preparations from regenerating *S. cerevisiae* protoplasts, gel-exclusion chromatography through Sephacryl S-200 and assay of β-glucanase activity on laminarin were performed as in [4]. Exo-β-glucanase preparations from supernatants of growing *Schizosaccharomyces pombe* cells, PAGE and detection of exo-β-glucanases in PAGE experiments using MUG as substrate were carried out as in [5].

3. RESULTS AND DISCUSSION

3.1. *Structural characterization of the extracellular exo-β-glucanases*

Gel-exclusion chromatography through Sephacryl S-200 and SDS-PAGE showed an M_r of 56000 ± 2000 for the major exo-β-glucanase and an M_r of 83000 ± 6000 for the minor form (β-glucanases III and II, respectively, according to [4]). The carbohydrate content of both exo-β-glucanases was estimated by the amount of total sugars detected in aliquots of the purified enzymes: for β-glucanase III, carbohydrate represented 11% and protein 89% of the total weight of the molecule; however in β-glucanase II, 40% was carbohydrate and the remaining 60% was protein. When endo H was used to eliminate the oligosaccharides *N*-glycosidically linked to asparagine, we observed that both underglycosylated exo-β-glucanases displayed the same electrophoretic mobility (fig.1, lanes 1–4). In both cases, the endo H-treated exo-β-glucanases appeared as two bands of very similar size. Using SDS-PAGE, we estimated an M_r of 47000 for this underglycosylated form, which is in good agreement with the size calculated theoretically by considering the M_r of the glycosylated forms and their carbohydrate contents.

3.2. *The* EXG1 *gene codes for both extracellular exo-β-glucanases*

The possibility that both exo-β-glucanases might arise from the same protein by heterogeneous glycosylation was also supported at the genetic level. *S. cerevisiae* mutants affected in the *EXG1* gene (Exg^- mutants), obtained either by mutagenesis with ethylmethane sulfonate or by 'in vivo' disruption with an internal fragment of the cloned *EXG1* gene [5], were defective in both extracellular exo-β-glucanases. Conversely, Exg^- mutants transformed with *EXG1* gene-containing

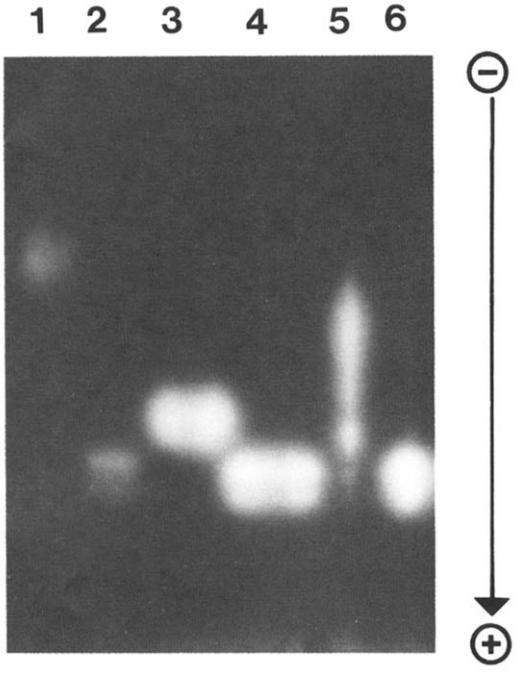

Fig.1. Gradient (5–15% polyacrylamide) gel electrophoresis of the exo-β-glucanases II (lanes 1 and 2) and III (lanes 3 and 4) purified from *S. cerevisiae* and exo-β-glucanase activity secreted by pRN4(*EXG1*)-transformed *Sc. pombe* cells (lanes 5 and 6): enzymic activity on MUG. Samples 2, 4 and 6 were incubated with endo H.

plasmids showed the two exo-β-glucanases in their culture supernatants according to Sephacryl S-200 chromatography (fig.2). In a similar way, transformation of an Exg^+ wild-type strain with yeast multicopy plasmids containing the *EXG1* gene increased the activity levels of both extracellular exo-β-glucanases. No significant changes in the extracellular endo-β-glucanase activity were observed in any of these cases. These results related the expression of the *EXG1* gene with the production of both extracellular exo-β-glucanases.

Further proof that the *EXG1* gene codes directly for a protein with exo-β-glucanase activity was obtained by the expression of this gene in *Sc. pombe*. Culture supernatants of *Sc. pombe* cells transformed by the *EXG1* gene showed exo-β-glucanase activity, which was not detected in the untransformed *Sc. pombe* cells, and immunologically related to that of *S. cerevisiae* [5]. The exo-β-glucanase activity secreted by *EXG1*-transformed *Sc. pombe* appeared, according to PAGE analysis, as a diffuse band between the two forms of *S. cerevisiae* with exo-β-glucanase activity. However, after incubation with endo H, this exo-β-glucanase produced in *Sc. pombe* showed the same electrophoretic mobility as those of the endo H-treated exo-β-glucanases of *S. cerevisiae* (fig.1, lanes 5 and 6).

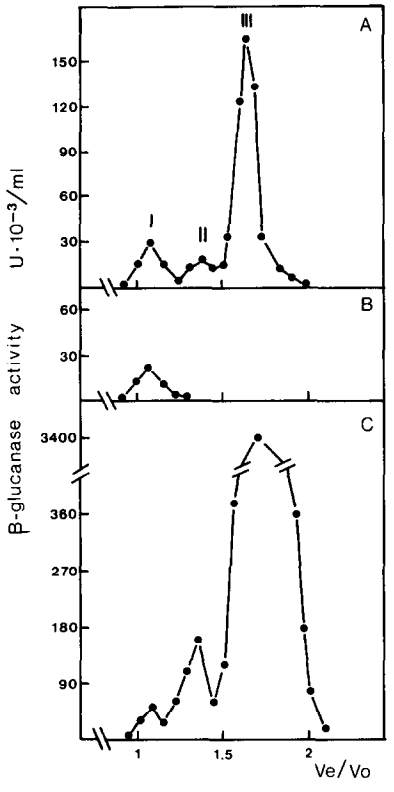

Fig.2. Sephacryl S-200 chromatography of β-glucanase activity secreted by *S. cerevisiae* protoplasts: enzymic activity on laminarin. Panels: A, exo-β-glucanase-producing strain TD-28; B, exo-β-glucanase-deficient strain MAX18-9B; C, pRN5(*EXG1*)-transformed strain MAX18-9B. Aliquots of 400 μg of total extracellular protein were applied to the columns. Endo-β-glucanase I and exo-β-glucanases II and III are indicated.

Accordingly, the *EXG1* gene codes for a protein whose glycosylation pattern in *S. cerevisiae* mainly determines the appearance of the β-glucanase III (major exo-β-glucanase in the culture supernatant of *S. cerevisiae*). A small part of the *EXG1*-encoded protein is glycosylated in a different way, thus accounting for the other minor exo-β-glucanase (β-glucanase II) with a larger ratio of carbohydrate. Another example has been reported in *S. cerevisiae* – the expression of an endoglucanase from *Trichoderma reesei* – showing that a single gene product may give rise to extracellular enzymes glycosylated at different levels [8]. Similar results have also been described for viral glycoproteins that are translated from a single mRNA, even though they exhibit different forms of glycosylation [9]. Such heterogeneity is presumably due to differences in protein folding which in turn would modify the number of potential glycosylation sites utilized and/or the level of glycosylation at each site. A similar explanation would be feasible for the results obtained in *Sc. pombe*. In this case, the *EXG1*-encoded protein was not glycosylated according to a defined major pattern, as observed in *S. cerevisiae*, leading to a wider heterogeneity in the amount of carbohydrate attached to the protein. The data reported in *Sc. pombe* demonstrate that heterogeneity, at least quantitatively, in the *N*-glycosylation level of the *EXG1*-encoded protein is possible, thus changing the electrophoretic properties of the secreted and glycosylated exo-β-glucanases.

Despite the differences observed in the process of glycosylation between both yeasts, the endo H-treated exo-β-glucanases, from either *S. cerevisiae* or *Sc. pombe*, appeared as bands that were identical on PAGE. This fact, also described for other

endo H-treated glycoproteins [8,10,11], indicates that both bands arise from the *EXG1* gene and regardless of the process determining them, it must be produced in exactly the same way in both yeasts.

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