GLYCOSYLATION IS NOT NECESSARY FOR THE SECRETION OF EXO-1,3-β-D-GLUCANASE BY *SACCHAROMYCES CEREVISIAE* PROTOPLASTS

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1. Introduction

1,3-β-D-Glucanases are enzymes widely distributed among microorganisms [1]. According to their action pattern upon hydrolysis of the substrates they may be classified as exo-(EC 3.2.1.58) and endo-1,3-β-D-glucanases (EC 3.2.1.6). The first give only D-glucose as the end product whereas the latter produces a mixture of laminaridextrins, mainly laminaritriose and laminaribiose D-glucose being a minor end product. Both types of enzymes are present and easily detectable in yeast of ascomycetous origin.

The glycoprotein nature of *Candida utilis* exo- as well as endo-β-glucanases has been directly shown by carbohydrate analysis of the purified enzymes [2–4]. In contrast, glucanases from *S. cerevisiae*, have been studied to a lesser extent in this regard. Partially purified endo-β-glucanase from the latter microorganism was precipitated by concanavalin A (con A) to the same extent as invertase and acid phosphatase. However, the interaction of the exo-splitting enzyme with the lectin did not lead to unambiguous results [5]. To solve this problem we have used tunicamycin, an antibiotic shown to prevent the synthesis of dolichol pyrophosphate *N*-acetylglucosamine. This intermediate represents the first step in the pathway of biosynthesis of glycoproteins containing the *N*-acetylglucosaminyl type of bond [6]. It will be shown that the normal synthesis and secretion of the major exo-1,3-β-glucanase secreted by *S. cerevisiae* protoplasts was affected by the presence of the drug and a new, presumably non-glycosylated form, is exported to the culture medium. A similar form was also secreted in the presence of 2-deoxy-D-glucose.

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2. Materials and methods

Tunicamycin was kindly supplied by Dr Hamill (Lilly, Indianapolis).

2.1. *Strain*

Saccharomyces cerevisiae ts^{-136} Me_{10} strain was used. It can grow in galactose as carbon source and RNA synthesis is stopped at the restrictive temperature (37°C).

2.2. *Protoplast and enzyme preparations*

Exponentially growing cells in modified Winge medium (1% galactose, 1% yeast extract) were resuspended (10 mg cells/ml) in 50 mM Tris–HCl buffer (pH 7.3) osmotically stabilized with 1 M sorbitol and 0.8 mg/ml of zymolyase 5000 were added. After 30 min at 23°C in gentle shaking almost 100% of the cells were converted into protoplasts.

Protoplasts were washed 3 times by centrifugation and regenerated in the stabilized modified Winge medium for 4 h. Then they were pelleted, washed and resuspended in fresh, stabilized media supplemented or not with the corresponding drug.

Samples taken at the indicated times were centrifuged and the supernatants dialyzed in the cold and concentrated by ultrafiltration in Amicon cells (diaflo PM 10) to a small volume (2.5–3 ml). Pelleted protoplasts were lysed by osmotic shock in 0.1 M acetate buffer (pH 5.2). They were then centrifuged at 18 000 × *g* for 60 min. The resultant supernatant was considered as the cytosol fraction.

2.3. *Gel exclusion chromatography*

A column of Sephacryl S-200 (2.5 × 92 cm) was equilibrated with 0.1 M acetate buffer (pH 5.2). The

enzyme solutions (2.5–3 ml) were applied to the column and eluted with the same buffer at a flow rate of 30 ml/h and 5 ml fractions were collected. Standard calibration curves were constructed with bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), zymolyase (M_r 22 000) and lysozyme (M_r 14 000) and M_r-values of problems were calculated according to [7].

2.4. *Precipitation reaction with Con A*

Exo-glucanase (6.98 units) in 0.1 M acetate buffer (pH 5.2) (0.5 ml) were mixed with 2 mg Con A in 0.5 ml of the same buffer supplemented with 1 mM $MgCl_2$, $MnCl_2$, $CaCl_2$ and 1 M NaCl. Samples were incubated for 1 h at 30°C with gentle shaking, filtered through Whatman GF/C glass filter discs and the activity not retained by the filters estimated. Controls without Con A were done in each experiment.

2.5. *Assay of β-glucanases*

Glucanase activity was assayed on laminarin, periodate-oxidized laminarin and *p*-nitrophenyl-β-D-glucopyranoside (PNPG). Reducing sugars and *p*-nitrophenyl liberated was measured as in [2]. One unit of activity was defined as the amount of enzyme which released 1 nmol of the measured reactive per hour at 30°C.

3. Results

3.1. *Glucanases in* S. cerevisiae *protoplasts*

Fig.1a,b shows the elution pattern in Sephacryl S-200 of β-glucanases associated to the supernatant and cytosol fractions of 3 h incubated protoplasts. The supernatant fraction apparently contained only one major form of β-glucanase. This activity was characterized as belonging to the exo-splitting type of β-glucanase by its ability to hydrolyze PNPG, production of glucose as the only end product of laminarin hydrolysis, and by its inability to hydrolyze periodate-oxidized laminarin. By contrast, at least 2 β-glucanases were found in the cytosol. The peak eluting first (V_e/V_o, 1.05) showed endo 1,3-β-glucanase activity (i.e., it hydrolyzed oxidazed laminarin, and the reducing power liberated from laminarin extensively exceeded that produced from PNPG). The second peak, which eluted at the same V_e/V_o (i.e., 1.58) as the supernatant-associated enzyme exhibited exo-β-glucanase activity.

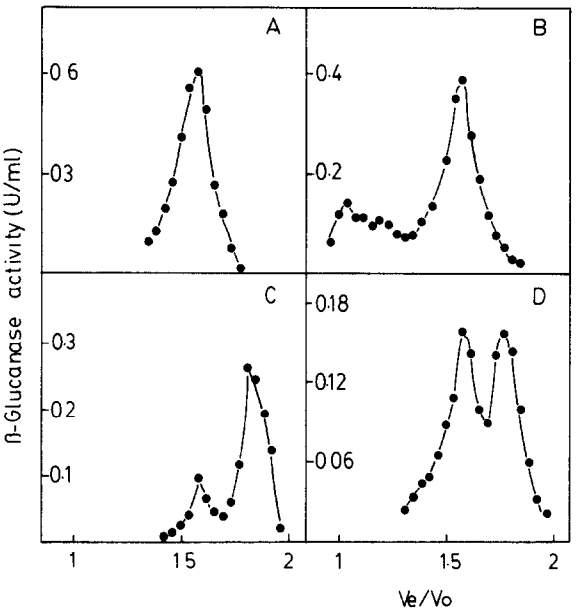

Fig.1. Fractionation of 1,3-β-glucanases produced by *S. cerevisiae* protoplasts. Regenerating protoplasts were incubated in the absence (a,b) or presence (c,d) of tunicamycin. After 3 h supernatant (a,c) and cytosol (b,d) fractions were obtained and fractionated as in section 2.

3.2. *Effect of tunicamycin*

Addition of tunicamycin (30 μg/ml) to the incubation media lowered the total β-glucanase activity in supernatant and cytosol fractions to 60% of the controls. Under these conditions, the incorporation of L-[U-^{14}C]threonine in total acid-precipitable material was unaffected during the period studied (table 1).

Table 1
Incorporation of L-threonine into trichloroacetic acid-insoluble fraction by regenerating *S. cerevisiae* protoplasts

Incubation time (min)	Control (cpm)	Tunicamycin (30 μg/ml) (cpm)
30	3423	3470
60	6319	5830
90	8106	8206
120	10 529	10 175
180	15 825	15 654

Protoplasts were incubated in the usual medium plus and minus tunicamycin and 2.5 μCi L-[U-^{14}C]threonine (spec. act. 589 μCi/mmol) were added. Aliquots were taken at the indicated times, made 5% trichloroacetic acid, filtered in GF/C Whatman discs and counted in a Packard scintillation spectrometer

Fractionation of supernatant and cytosol fractions from protoplasts incubated with tunicamycin is shown in fig.1c,d. Most of the supernatant associated activity now eluted with a subtantially smaller size (V_e/V_o, 1.8) and only a minor fraction remained at the original position. By calibration of the column, the M_r for the native and the new form of glucanases was estimated to be 54 000 and 42 000, respectively.

Cytosol fraction again showed two peaks of activity. The first peak eluted at the same elution volume as the native exoenzyme but, interestingly, it appeared to exhibit both endo- and exo-β-glucanase activities. The second peak, whose activity belonged to the exo-splitting type, eluted at the same position as the new form secreted into the supernatant. The low activity found in the cytosol precluded at present further characterization of the enzymes associated to this fraction. The following studies were thus carried out with exo-β-glucanases from supernatant.

3.3. *Characterization of the secreted glucanases*

Preliminary experiments indicated that, in addition to displaying an exo-hydrolytic mode of action, the low M_r form had a K_m-value for PNPG identical to the native enzyme (4.048 mM). These similarities, plus

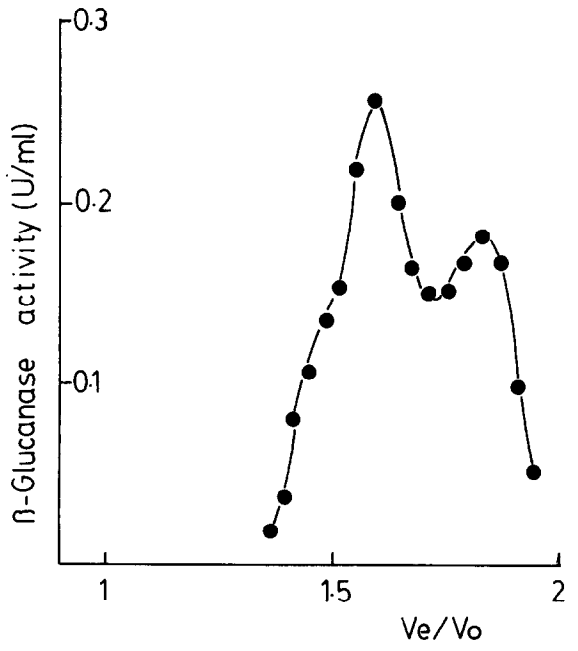

Fig.2. Fractionation of supernatant-associated 1,3-β-glucanases from 2-deoxyglucose treated protoplasts. Supernatant of 5 h treated protoplasts was processed as in fig.1.

Table 2
Absortion by Con A of native and tunicamycin-induced β-glucanases from supernatant of protoplasts

Exo-β-glucanase	Activity remaining soluble (%)
Native	0%
Tunicamycin-induced	84.4%

Native and tunicamycin-induced exo-β-glucanases from fig.1a and 1c, respectively, were used in this experiment. Activity was assayed against laminarin

the fact that the small form was secreted in the presence of tunicamycin, suggested that it represents the non-glycosylated form of the native exoglucanase.

Accordingly, native and tunicamycin-induced exoglucanases were tested by their ability to bind to Con A. As shown in table 2, all the native activity was precipitated by the lectin; in contrast, ~85% of the new form remained soluble under the same conditions. It may be concluded that the enzyme secreted in the presence of tunicamycin was devoid of carbohydrate.

3.4. *Effect of 2-deoxyglucose on the synthesis and secretion of exo-β-glucanase*

2-Deoxyglucose depressed the accumulation of exo-β-glucanase activity in the supernatant to a greater extent than tunicamycin (90%). This was probably due to its additional interference with the normal sugar metabolism. Fractionation of the supernatant revealed two broad peaks of β-glucanase activity with elution volumes corresponding to the native and tunicamycin-induced enzymes.

4. Discussion

These results provide strong evidence for the glycoprotein nature of the major exo-β-glucanase produced by *S. cerevisiae*. From the mode of action of tunicamycin it may also be inferred that the carbohydrate moiety is bound to the protein through *N*-glycosidic bonds.

The inhibition caused by tunicamycin in the amount of supernatant associated activity is very similar to that described for carboxypeptidase Y [8,9]. In addition, the differences in M_r between the glycosylated and non-glycosylated forms of our enzyme (12 000) are very close to that reported for the res-

pective forms of carboxypeptidase Y (10 000) [9]. The last enzyme appears to contain 4 oligosaccharide chains composed on average of 2 *N*-acetylglucosamine and 13 mannose residues. From the similarities between the effect of tunicamycin on both enzymes, a similar carbohydrate composition may be expected for exo-β-glucanases.

2-Deoxy-D-glucose, an inhibitor of the secretion of glycoproteins by yeast protoplast [10], strongly affected the production of exo-β-glucanase. Analysis of the residual activity secreted into the supernatant revealed a molecular form with a size very similar to the putative non-glycosylated enzyme. It is possible that this peak also contains intermediate forms with shortened oligosaccharides as described for carboxypeptidase Y [9].

With regard to the secretory process, our results show for the first time that carbohydrate addition is not a neccesary requirement, neither for the synthesis, nor for the transport of glycoproteins to their final destination in yeast. Synthesis of vacuolar glycoproteins also occurs in the absence of glycosylation [9,11]. However, the precise location of the non-glycosylated forms of these enzymes has not been definitively established.

Work is in progress to obtain specific antibodies against the pure enzyme. It will help us to decide whether the partial inhibition observed in the rate of secretion of the non-glycosylated exo-β-glucanase is a reflection of an impairment at the level of synthesis or whether transport is the step altered by the absence of carbohydrate.

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