SACCHAROMYCES CEREVISIAE SECRETES 2 EXO-β-GLUCANASES

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1. Introduction

An enzyme system capable of hydrolyzing β(1–3) glycosidic linkages has been shown in many species of yeast [1,2]. β-Glucanases may be classified into 2 groups according to their action pattern: endo- and exo-β-D-glucanases [3]. Some of the latter hydrolyze both β(1–3) and β(1–6) glycosidic linkages [4].

Fractionation of β-glucanases secreted into the culture medium by intact cells and protoplasts has shown the presence of >1 enzymic form with exo-β-glucanase activity in several yeasts of both ascomycetous and basidiomycetous origin [5–7].

This work shows 2 different enzymic forms in *Saccharomyces cerevisiae* with exo-β-glucanase activity. Evidence for different M_r-, K_m-values and substrate specificity has been obtained.

2. Materials and methods

Tunicamycin was kindly supplied by Dr R. L. Hamill (Lilly Res. Lab., Indianapolis IN).

Saccharomyces cerevisiae strains X_{14}, ts^{-136} Me10 and ts^{-136} were used [8].

Protoplasts, enzymic preparations, gel exclusion chromatography over Sephacryl S-200 and precipitation reactions with con A were performed as in [8], except that the Sephacryl S-200 column had larger dimensions (150 × 2 cm).

Concanavalin A–Sepharose 4B chromatography was done as in [9].

2.1. *Exo-β-glucanase activity determinations*

β-Glucanase activity was assayed on laminarin, periodate-oxidized laminarin, pustulan and *p*-nitrophenyl-β-D-glucopyranoside (PNPG). Reducing sugars, D-glucose and *p*-nitrophenol liberated were measured as in [5]. One unit of activity was defined as the amount of enzyme which released 1 μmol *p*-nitrophenol, glucose or equivalent reducing power per hour at 30°C.

When 4′-methylumbellipheryl-β-D-glucoside was employed as the substrate in bidimensional immunoelectrophoresis experiments, the exo-β-glucanase activity was detected by illumination under UV light (254 nm).

2.2. *Removal of carbohydrate from exo-β-glucanase by endo H treatment*

Endo-β-*N*-acetylglucosaminidase H (endo H) (0.01 units) was added to 15 units of exo-β-glucanase in 0.1 M acetate buffer (pH 5.2) which contained 0.05% sodium azide, and incubated for 30 h at 33°C.

2.3. *Immunological methods*

Anti-exo-β-D-glucanase III serum was obtained by immunizing rabbits for 1 month with purified enzyme III. Antibodies were prepared by ammonium sulphate precipitation and immunoprecipitation experiments were performed at 4°C for 20 h. Bidimensional immunoelectrophoresis were done as in [11].

3. Results

3.1. *Exo-β-glucanases in supernatants of growing cells or protoplasts*

Gel exclusion chromatography of supernatants of either growing cells or protoplasts through Sephacryl S-200 revealed the existence of ⩾3 active fractions against laminarin. β-Glucanase I (fig.1) eluted with a V_e/V_o ratio of 1.04 and was characterized as belonging to the endo-splitting type of β-glucanases by the following results:

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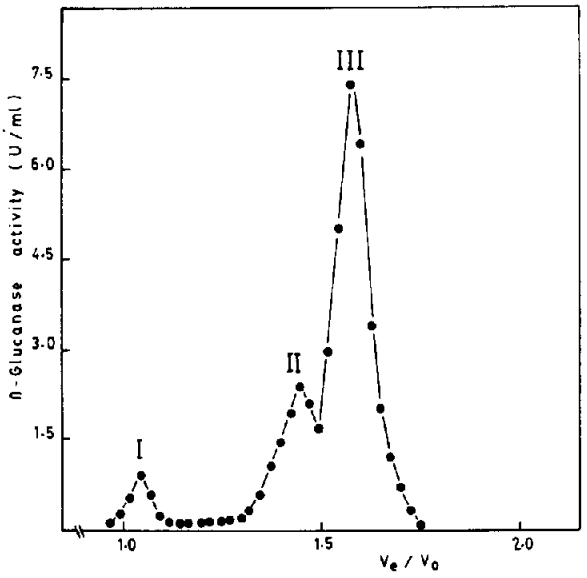

Fig.1. Sephacryl S-200 chromatography of β-glucanases I, II and III secreted by *S. cerevisiae* cells: enzymic activity on laminarin.

(i) The enzyme showed the same activity when evaluated against laminarin or oxidized laminarin;
(ii) No free glucose was detected after short incubation times;
(iii) No activity against both PNPG or pustulan was observed.

This enzyme is not taken into account here.

β-Glucanases II and III eluted from the column with V_e/V_o ratios of 1.46 and 1.58, respectively. The former represented ~15% of the total exo-β-glucanase activity.

3.2. *Characterization of β-glucanases II and III*

β-Glucanases II and III were characterized as belonging to the exo-splitting type by their ability to hydrolyze PNPG, production of glucose as the only end-product of laminarin hydrolysis and by its inability to hydrolyze periodate-oxidized laminarin, cellobiose or salicin. Studies using different substrates showed that enzymes II and III were unspecific exo-β-glucanases able to break down both β(1–3) and β(1–6) glycosidic linkages. Table 1 summarizes the percentage of activity exhibited by the enzymes against different substrates. The Michaelis-Menten constants (K_m) of exo-β-glucanases II and III with PNPG, laminarin and pustulan are summarized in table 2.

Table 1
Substrate specificity of the exo-β-glucanases II and III

Substrate	Exo-β-glucanase	
	II	III
Laminarin	100	100
Pustulan	47	46
PNPG	15	38
Periodate-oxidized laminarin	0	0
Salicin	0	0
Cellobiose	0	0

The values are percentages of activity relative to that obtained with laminarin as the substrate

Determinations of M_r were made in a pre-calibrated column packed with Sephacryl S-200. The approximate values obtained were 66 000 M_r for exo-β-glucanase II and 58 800 M_r for exo-β-glucanase III. These 2 enzymes showed affinity towards con A and ≥95% of the activity was bound to the lectin.

Once learning that both enzymes were precipitated by antibodies raised against purified exo-β-glucanase III, bidimensional immunoelectrophoresis proved to be the best method for visualizing these exo-β-glucanases. Two peaks active against 4′-methylumbellipheryl-β-D-glucoside were detected (fig.2). β-Glucanase II migrated faster than β-glucanase III and corresponds to the peak with a higher R_F-value.

3.3. *Effect of tunicamycin and endo H*

The antibiotic tunicamycin prevents the synthesis of dolichol pyrophosphate *N*-acetylglucosamine, which in turn represents the first step in the biosynthetic pathway of the oligosaccharides *N*-glucosidically-linked to asparagine [12]. Addition of tunicamycin (30 μg/ml) to the incubation media of either cells or protoplasts provoked the synthesis and secretion of an enzymic form of exo-β-glucanase III with lower

Table 2
K_m-Values for exo-β-glucanases II and III based on Lineweaver-Burk plots [14]

Substrate	Exo-β-glucanase	
	II	III
Laminarin (mg/ml)	3.8	48
Pustulan (mg/ml)	3.2	10
PNPG (mM)	3.1	4

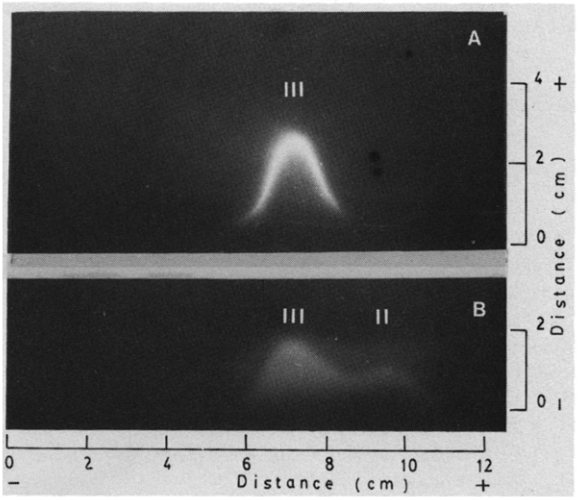

Fig.2. Bidimensional immunoelectrophoresis of purified β-glucanase III (A) and exo-β-glucanases from supernatant of growing *S. cerevisiae* protoplasts (B): enzymic activity on 4'-methylumbellipheryl-β-D-glucoside.

M_r. This enzyme proved to be underglycosylated by its inability to bind to con A.

To determine whether or not the different physicochemical properties observed for both β-glucanases were due to the carbohydrate content, the effect of tunicamycin upon exo-β-glucanase II production and secretion was investigated. Different supernatant fractions obtained from protoplasts growing in the presence of tunicamycin (30 μg/ml), once concentrated and dialyzed, were filtered through con A–Sepharose 4B and the resulting adsorbed and unadsorbed material was then fractionated over Sephacryl S-200. Two underglycosylated enzymes were detected which corresponded to the underglycosylated forms of exo-β-glucanase II and III (fig.3). These glucanases belong to the exo-splitting type of est. M_r 58 800 and 43 000, respectively.

The enzyme endo H hydrolyzes the linkage between the 2 *N*-acetylglucosamine residues of the asparagine-linked oligosaccharides core [13] producing an effect similar to that described for tunicamycin. Treatment of exo-β-glucanase II with this enzyme resulted in the formation of an underglycosylated form (fig.4) which exhibited M_r 58 800 and lacked affinity towards con A.

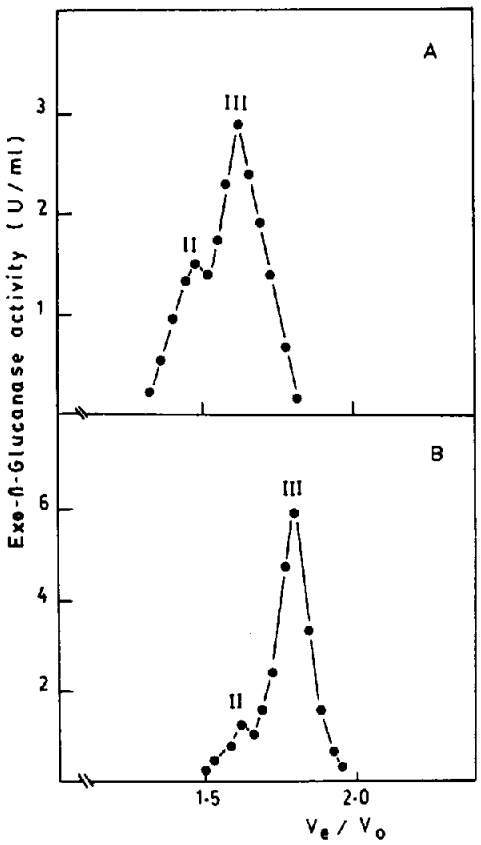

Fig.3. Sephacryl S-200 chromatography of exo-β-glucanases secreted by *S. cerevisiae* protoplasts growing in the presence of tunicamycin (30 μg/ml). Supernatants were first filtered through con A–Sepharose 4B and the resulting adsorbed (A) and unadsorbed (B) fractions were then applied on a Sephacryl S-200 column.

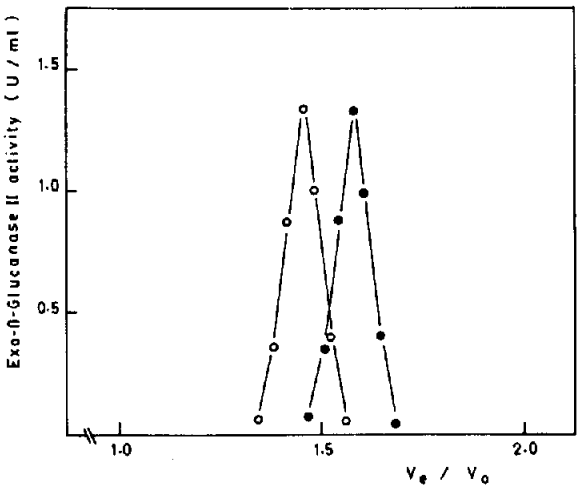

Fig.4. Sephacryl S-200 chromatography of β-glucanase II treated with endo H (•); elution pattern of native β-glucanase II (○).

4. Discussion

The existence of more than one exo-β-glucanase has been described in ascomycetous and basidiomycetous yeasts [5–7]. Two fractions with exo-β-glucanase activity were reported in the supernatants of *S. cerevisiae* growing cells and protoplasts [15], although the results were inconclusive. Recent work deals with exo-β-glucanase III only, which is the predominant form in crude enzymic preparations.

This work shows 2 exo-β-glucanases in the culture medium of both *S. cerevisiae* growing cells and protoplasts. The enzymes must be closely related because antibodies raised against exo-β-glucanase III readily precipitated exo-β-glucanase II. This property made it possible to visualize both β-glucanases in bidimensional immunoelectrophoretic experiments (fig.2B). However, an est. M_r of 58 800 and 66 000, respectively, allowed us to separate them also by filtration through a Sephacryl S-200 column (fig.1). The fact that the heavier enzymic form corresponds to the one which migrated faster in immunoelectrophoretic experiments (fig.2B) may be due to additional effects of agarose on some glycoproteins.

Evidence for differences between the 2 exo-β-glucanases is provided by the K_m-values determined against substrates such as laminarin and pustulan (table 2) and also by the substrate specificity shown by both enzymes (table 1). Glucanases II and III must be glycosylated as suggested by their affinity for con A and sensitivity towards tunicamycin and endo H. The carbohydrate content *N*-glycosidically linked to asparagine was similar for both exo-β-glucanases (~24%).

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