Post-secretional modification of $\text{exo-1,3-}\beta\text{-D-glucanases}$ from *Saccharomyces cerevisiae*

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Exo-1,3- $\beta\text{-D-glucanase}$ secreted by *Saccharomyces cerevisiae* undergoes extracellular modifications in its carbohydrate moiety that change the affinity towards the lectin concanavalin A. The transition of negatively reacting enzyme form into positively reacting one depends on temperature. Results from experiments with glucono- δ -lactone and from treatments *in vitro* with hydrolases suggest a glycosidase-mediated mechanism.

1,3- $\beta\text{-D-Glucanases}$ have been described in many yeasts of both ascomycetous and basidiomycetous origin (Brock, 1965; Notario *et al.*, 1976a; Villa *et al.*, 1978a). They may be classified as *exo-* (EC 3.2.1.56) or *endo-1,3-}\beta\text{-D-glucanases} (EC 3.2.1.6). The former has been purified from different yeast species such as *Candida utilis* (Villa *et al.*, 1976b), *Kluyveromyces aestuarii* (Lachance *et al.*, 1977), *Pichia polymorpha* (Villa *et al.*, 1978b) and *Saccharomyces cerevisiae* (Sánchez *et al.*, 1982), and in all cases they were found to be glycoproteins. The carbohydrate moiety is formed mainly of D-mannose, variable quantities of D-glucose and small amounts of D-glucosamine. The relative percentage of each sugar varies according to the micro-organism used as the enzyme source. The linkage established between the polypeptide and carbohydrate moieties has been characterized as belonging to the *N*-acetylglucosaminoyl-asparagine type (Sánchez *et al.*, 1981). Other glycoproteins of yeast origin, such as α -galactosidase from *Saccharomyces carlsbergensis*, also contain D-glucose in the mature enzymic form (Lazo *et al.*, 1978).*

It has been known for several years now that, despite the similar overall composition among yeast *exo-}\beta\text{-glucanases}*, some do interact with concanavalin A whereas others do not (i.e. *exo-}\beta\text{-glucanases}* from *S. cerevisiae* and from *C. utilis* respectively). Chu & Maley (1980) have reported that the effect of glucose on invertase synthesis occurred at the level of glycosylation and that the non-glycosylated polypeptide was rapidly degraded by endogenous proteinases. Basing our criteria on these results, we studied the effect of high glucose concentrations on

exo-}\beta\text{-glucanase} synthesis and secretion by strains of *S. cerevisiae*. Contrariwise to what has been described for catabolite-regulated external enzymes in yeasts, the synthesis and secretion of *exo-}\beta\text{-glucanases}* did not halt, but, rather, showed a different pattern of glycosylation, since they did not exhibit affinity for concanavalin A. These enzymic forms were not stable, however, and were rapidly converted into ones with a positive affinity for the lectin, and this was taken as an indication of post-secretional modification.

Materials and methods

Chemicals

α -Glucosidase (from brewer's yeast), α -mannosidase (from jack bean), *p*-nitrophenyl α -D-glucoside, *p*-nitrophenyl β -D-glucoside, concanavalin A, salicin and laminarin were purchased from Sigma Chemical Co. Sephacryl S-200 and concanavalin A–Sephacryl 4B were from Pharmacia. Radioactive precursors were from Amersham International. All other reagents were of analytical grade from commercial sources.

Methods

Saccharomyces cerevisiae strains ts⁻¹³⁶Me₁₀, ts⁻¹³⁶ and X₁₄ were grown aerobically in liquid media containing 10 g of yeast extract and either 5 g of glucose, 5 g of fructose, 5 g of galactose or 100 g of glucose per litre, at 25°C in a rotary shaker at 240 rev./min.

Concanavalin A–Sephacryl 4B chromatography and precipitation with the lectin were carried out as reported by Notario *et al.* (1976b), and Sephacryl S-200 filtration was as described by Villa *et al.* (1978a).

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Exo-1,3- β -glucanase activity was assayed on laminarin and *p*-nitrophenyl β -D-glucoside. α -Glucosidase and β -glucosidase were tested by the hydrolysis of *p*-nitrophenyl α -D-glucoside and salicin respectively. The reducing sugars, D-glucose and *p*-nitrophenol liberated were measured as indicated by Villa *et al.* (1978a). One unit of activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol, glucose or equivalent reducing power per h at 30°C.

Treatments with α -glucosidase and α -mannosidase were carried out with 1 unit of the respective enzyme and assayed for 12 h at 30°C, in accordance with the manufacturer's recommendations.

Radioactive labelling of exo- β -glucanases was carried out as reported by Sánchez *et al.* (1982) except that larger quantities of [14 C]mannose and [14 C]glucose were used (i.e. 20 μ Ci of D-[U- 14 C]-mannose, sp. radioactivity 11.1 GBq/mmol; 20 μ Ci of D-[U- 14 C]glucose, sp. radioactivity 12 GBq/mmol).

Anti-exo-1,3- β -glucanase serum was obtained as specified by Sánchez *et al.* (1982). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

Saccharomyces cerevisiae ts⁻¹³⁶Me₁₀ synthesizes and secretes one endo- and two exo-1,3- β -D-glucanases into the culture medium. The physico-chemical properties of the two exo-enzymes have been published elsewhere (Sánchez *et al.*, 1981). These are constitutive enzymes, are produced in nearly the same proportion independently of the carbon source employed for growth, and are glycoproteins exhibiting affinity towards concanavalin A. However, growth with high glucose concentrations (10%) is characterized by the appearance of exo-1,3- β -D-glucanase activity that is not adsorbed on the lectin. This property was also found for other strains of the same species (i.e. X₁₄, ts⁻¹³⁶ and X-2180-1A), suggesting that this may be a generalized property for *S. cerevisiae* exo- β -glucanases.

To rule out the possibility that non-adsorption on concanavalin A was due to interference with the still unused glucose present in the culture broth, the enzymic solution was divided into two samples. One of them was rapidly dialysed at 2°C until all the unused glucose was removed, by using an Amicon ultrafiltration system, and then both were subjected to affinity chromatography with concanavalin A-Sephrose 4B. In all cases activity remained as non-adsorbed.

An unexpected result was obtained when we tried to study the percentage of non-concanavalin A-adsorbable and concanavalin A-adsorbable exo-

β -glucanase activities during yeast growth at 10% glucose. Here, the former represented 65% of the total activity in the exponential phase, whereas the value was only 1% if the culture was allowed to enter the stationary phase. This transformation was apparent when a culture broth containing high non-precipitable activity was incubated at different temperatures. Invariably at the end of the experiments all the enzyme activity showed affinity for the lectin (Table 1). The mechanism by which such a transformation occurred was accelerated by temperature, and this, in turn, suggested an enzyme-mediated change.

Both concanavalin A-precipitable and non-concanavalin A-precipitable exo- β -glucanases were then subjected to filtration through Sephacryl S-200, showing nearly the same molecular mass. These results suggest that the differences in the carbohydrate moiety of both enzymic forms that are presumably responsible for their distinct behaviour in terms of affinity for the lectin must not be very important quantitatively, but, rather, qualitatively.

It is now known that *S. cerevisiae* exo- β -glucanases contain in their mature form a carbohydrate moiety formed by D-mannose, small quantities of D-glucose and two residues of *N*-acetyl-D-glucosamine that establish a linkage with the asparagine of the polypeptide chain (Sánchez *et al.*, 1982). The use of antibodies prepared against exo- β -glucanase (Sánchez *et al.*, 1981) has allowed us to study the extracellular modifications in the carbohydrate portion of the total exo- β -glucanase activity secreted by cells growing in glucose-rich media. The experiments were carried out by growing *S. cerevisiae* in 10%-glucose-containing media supplemented with the radioactive precursors indicated in the Materials and methods section. After 24 h at 25°C, the cells were spun down and the

Table 1. Transition, at different temperatures, of exo- β -glucanase activity (40 units) from a form non-precipitable by concanavalin A into the precipitable form

The enzymic sample was obtained from broths of cells growing exponentially in 10% glucose. For full experimental details see the text.

Temp. (°C)	Time	Non-precipitable activity (%)
1	0	65
1	14 days	20
1	35 days	0
4	0	65
4	2 days	45
4	4 days	27
4	8 days	0
30	0	65
30	3 h	40
30	6 h	10
30	12 h	0

supernatant fluids subjected to precipitation with concanavalin A. The non-precipitated exo- β -glucanase was then precipitated with antibodies. The radioactive pellets were resuspended in 0.05 M-sodium-acetate buffer, pH 5.2, and samples prepared as indicated in Table 2.

Table 2. *Variation in radioactivity in immunoprecipitated exo- β -glucanase labelled with [14 C]glucose or [14 C]-mannose after different treatments*

Values express the percentage of radioactivity compared with that observed in sample A (640 d.p.m./ μ g of exo- β -glucanase when labelled with [14 C]-glucose, and 320 d.p.m./ μ g of exo- β -glucanase when labelled with [14 C]-mannose). The values obtained for the various samples indicate the following: A, radioactivity detected in immunoprecipitated exo- β -glucanase lacking affinity for concanavalin A; B, the same as A but before counting of radioactivity the sample was maintained at 30°C for 12 h in 0.05 M-sodium acetate buffer, pH 5.2; C, radioactivity detected in immunocomplexes after treatment for 12 h at 30°C with a culture broth from which exo- β -glucanase activity had been removed in a batchwise manner with 1,3- β -D-glucan from *S. cerevisiae* (Villa *et al.*, 1976a); D, the same as C except that the treatment was performed with purified exo- β -glucanase (10 units); E and F represent the radioactivity after treatment with commercial α -glucosidase and α -mannosidase respectively. For full experimental details see the text.

Sample	Labelled with...	Radioactivity (% of that in sample A)	
		[14 C]Glucose	[14 C]Mannose
A		100	100
B		96	98
C		87	92
D		77	92
E		50	92
F		50	10

From these data it may be inferred that, although exo- β -glucanases may be involved in a self-modification process (i.e. the molecules would contain small amounts of β -D-glucose), there must be other enzymes splitting some of the sugar residues and therefore transforming the non-concanavalin A-precipitable exo- β -glucanases into precipitable forms. High glucose concentration lowers the activity of glucosidases (Table 3), which could favour the appearance of exo- β -glucanase activities without affinity towards concanavalin A. It should be pointed out, however, that this is a transient characteristic, as evidenced in Table 1, and therefore it is difficult to obtain in a stabilized state the enzymic form with the aforementioned property.

We were, however, able to stabilize such an enzyme by using glucono- δ -lactone. This compound has been described as a competitive inhibitor of glycosidases (Villa *et al.*, 1979). The addition of glucono- δ -lactone (10 mM) into the growth media resulted in the secretion of higher concentrations (up to 80%) of exo- β -glucanases without affinity towards concanavalin A. The use of this compound is somewhat laborious, since, as it is rapidly hydrolysed to gluconic acid, the media had to be buffered (0.2 M-sodium acetate buffer, pH 5.2) and glucono- δ -lactone added every hour. We have taken these results as evidence that extracellular modification of exo- β -glucanase may be mediated by glycosidases. The nature of these enzymes is still unknown, but it is worth noting that *S. cerevisiae* has been reported (Saunier *et al.*, 1982) to produce glucosidases that specifically removed the glucose residues from oligosaccharides such as Glc₂-Man₉-GlcNAc₂ and Glc₁-Man₉-GlcNAc₂, but did not show activity against aryl glucosides. This may be taken as an indication that possibly similar enzymes occur in the supernatant fluid of growing cells (perhaps as a result of cellular leakage) and that they are not detected, or only partially, if total glucosidase is assayed with commercial aryl or alkyl glucosides.

Table 3. *α - and β -Glucosidase activities in cytosol and supernatant fractions of *S. cerevisiae* ts⁻¹³⁶Me₁₀ protoplasts growing on different carbon sources*

For full experimental details see the text.

Carbon source	Activity (munits/mg)			
	Cytosol		Culture fluid	
	α -Glucosidase	β -Glucosidase	α -Glucosidase	β -Glucosidase*
Glucose (0.5%)	69	215	Traces	10
Glucose (10%)	16	109	Traces	4
Galactose (0.5%)	33	209	Traces	10
Fructose (0.5%)	82	40	Traces	Traces

* These values have been corrected, since salicin is also slowly hydrolysed by exo-1,3- β -glucanases (Villa *et al.*, 1978a).

Candida utilis synthesizes and secretes an exo- β -glucanase that is a glycoprotein (Villa *et al.*, 1976b) and that does not exhibit affinity for concanavalin A independently of the carbon source employed for growth. Treatment *in vitro* of 2 units of exo- β -glucanase with 1 unit of commercial α -glucosidase results in the enzyme being precipitated by the lectin, which indicates that α -glucosidases do modify the affinity of these enzymes towards concanavalin A.

From the results given in the present paper we conclude that glycoproteins such as yeast exo- β -glucanases may undergo post-secretional modifications. To the best of our knowledge these involve the carbohydrate moiety and must consist mainly of the splitting of α -glucose residues. The end result is that, depending on the time of cell harvesting and the carbon source used, the carbohydrate composition of the mature enzymes may change. In this sense, the quantitative evaluation of monosaccharides in hydrolysates of pure exo- β -glucanases (Sánchez *et al.*, 1982) is probably an inadequate reflection of those present in the enzyme molecule before undergoing the extracellular modifications.

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