Accumulation of exoglucanase activity in yeast secretory mutants blocked at the endoplasmic reticulum level

Luis M. Hernández, Isabel Olivero, Manuel Ramírez and Germán Larriba

Departamento de Microbiología, Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain

Received 21 November 1985

Saccharomyces cerevisiae HMSF-176 (sec 18), a thermosensitive secretory mutant blocked at the endoplasmic reticulum (ER) level, drastically increased its osmotic sensitivity when grown at the restrictive temperature of 37°C in high glucose concentration. This fact led to the erroneous interpretation that glucanases were inactive when localized in the ER. The development of a suitable osmotic stabilizer now indicates that sec 18 accumulates exoglucanase activity. Another ER-blocked mutant behaved in a similar way. All the accumulated exoglucanase was found in a soluble form. By contrast, a significant portion of the accumulated invertase remained in a membrane-bound form.

Exoglucanase (Saccharomyces cerevisiae) *Secretory mutant* *Endoplasmic reticulum*

1. INTRODUCTION

A recent paper from this laboratory has focused on the secretory pathway of β-glucanases in yeast [1]. In that work we used a thermosensitive secretory mutant (sec 18-1) blocked at the endoplasmic reticulum (ER) level. Our experiments indicated that, in contrast with the accumulation pattern exhibited by invertase, glucanases did not accumulate when mutant cells were incubated at the restrictive temperature. In addition, a significant rise in internal glucanase activity followed the shift of the cells to the permissive temperature in the absence of protein synthesis. This was interpreted assuming that glucanases were synthesized as inactive precursors and then matured into active enzymes on their way through the secretory pathway.

To extend our observations to another ER-blocked thermosensitive mutant, we studied the behaviour of sec 12-4. Surprisingly, this mutant accumulated exoglucanase activity at the restrictive temperature (37°C). In this study we have carefully analyzed the reasons for these discrepancies and we have found that sec 18 protoplasts derived from cells maintained at the restrictive temperature in high glucose concentrations, significantly increase their osmotic sensitivity and lyse under our standard conditions of osmotic support (0.8 M KCl). Accordingly, a suitable osmotic stabilizer for sec 18 was developed. The results indicate now that our previous interpretation was wrong and that exoglucanases, at least, accumulate in an active form in both ER-blocked mutants (sec 12 and sec 18) when incubated at the restrictive temperature. We also show that, in contrast with secretory invertase that accumulated in both soluble and membrane-bound forms, exoglucanases only accumulated in a soluble form.

2. MATERIALS AND METHODS

Concanavalin A (Con A) was from Sigma. The rest of the chemicals were as in [1].

2.1. *Microorganism and culture medium*

S. cerevisiae haploid strain X2180-1A and mutants HMSF-176 (sec 18-1) and HMSF 162 (sec 12-4) were supplied by Dr Schekman (University of California, Berkeley). Both HMSF strains are

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

thermosensitive secretory mutants, derived from X2180-1A, blocked at the ER level [2].

Microorganisms were grown as described in [1].

2.2. *Preparation of protoplasts*

Protoplasts were obtained essentially as in [1] except that the osmotic support (0.8 M KCl) was substituted by 1.5 M glycerol, 1.4 M sorbitol or 1 M $MgCl_2$ as indicated. Protoplasts were washed once with 25 mM acetate buffer (pH 5.2) supplemented with 1.4 M sorbitol and 10 mM sodium azide. They were then lysed by resuspending in 25 mM acetate buffer (pH 5.2) supplemented or not with 1% Triton X-100 and homogenized in a glass homogenizer.

2.3. *Measurement of enzymatic activities*

Total internal (protoplast associated) activity was determined in protoplast lysates containing 1% Triton X-100. For determination of soluble vs membrane-bound forms of invertase or glucanase, protoplasts were lysed in the absence of detergent and the lysate spun down at 36 000 rpm in an SW 55Ti Beckman rotor (126 000 × *g*). Soluble activities were determined in the supernatants and membrane-bound activity in the pellets resuspended in the initial volume of acetate buffer (pH 5.2) and homogenized as before.

Periplasmic space (external cell-associated) activities were measured in cells resuspended in 25 mM acetate buffer (pH 5.2) supplemented with 10 mM sodium azide.

Culture medium-released exoglucanase activity was determined in supernatants of culture (obtained by removal of cells by centrifugation) supplemented with an equal volume of 25 mM acetate buffer.

Enzymatic assays of exoglucanase against *p*-nitrophenyl-*β*-D-glucopyranoside (p-NPG) and invertase were carried out as in [1]. Protein was determined according to [3].

2.4. *Concanavalin A precipitation of invertase*

Enzymatic samples were mixed with an equal volume of acetate buffer containing 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 2 mg/ml Con A. After incubation at 37°C for 30 min, samples were filtered through glass fiber filters and non-precipitated invertase activity was determined in the filtrate.

3. RESULTS

3.1. *Exoglucanase accumulation by sec 12 at non-permissive temperature*

Fig.1A shows that sec 12, an ER-blocked thermosensitive secretory mutant, accumulated internal p-NPG-hydrolyzing activity when incubated at 37°C. During the accumulation period the external cell-associated activity dropped as a consequence of its diffusion out into the culture medium without a supply of new enzyme from inside. After its transfer to 24°C in the presence of cycloheximide the internal activity slowly decreased. The observed drop was concomitant with a rise in the external cell-associated and, to a lesser extent, culture medium-released activities. It may be concluded that accumulated exoglucanase activity was steadily secreted at the permissive temperature. Wild type, when incubated at the restrictive temperature, did not accumulate internal activity,

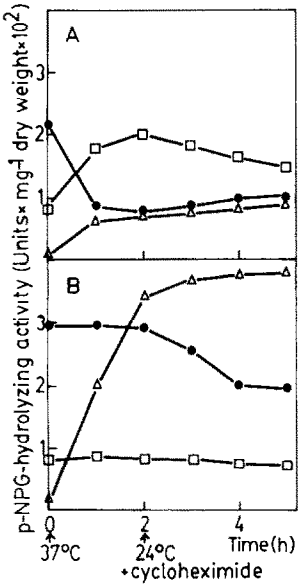

Fig.1. Evolution of exoglucanase activity against p-NPG in sec 12 (A) and wild type (B). Cells were grown overnight in 5% glucose at 24°C and shifted at 37°C prewarmed fresh medium. After 2 h cycloheximide was added and cultures were transferred to 24°C. Samples were withdrawn, chilled at 0°C and processed as indicated in section 2.3 to determine culture medium (△), external cell-associated (●) and protoplast-associated (□) exoglucanase activity. Each experiment was performed at least twice with almost identical results.

maintained constant levels of external cell-associated activity and all the newly synthesized exoglucanase appeared in the culture medium. Following the blockage in synthesis caused by cycloheximide part of the external cell-associated activity diffused into the culture medium (fig.1B). sec 12, when incubated at 24°C during the whole experiment, behaved like wild type (not shown).

3.2. *Secretion patterns of sec 18*

The accumulation of active exoglucanase by sec 12 at 37°C was, at first, surprising since we had reported that another ER-blocked mutant (sec 18) accumulated exoglucanase in an inactive form under the same conditions. Accordingly, the behaviour of sec 18 was more thoroughly investigated. Fig.2 shows the evolution of the absorbance of the cells and resulting protoplasts as well as total protein in protoplast lysates of sec 18 throughout accumulation-secretion experiments in which different osmotic stabilizers were used in the protoplast treatment step. Under standard conditions used to prepare protoplasts from wild type cells (0.8 M KCl), the increase in cell absorbance at 37°C was concomitant with a drop in both protoplast absorbance and total protein in protoplast lysates (fig.2A). The substitution of 0.8 M KCl by 1.5 M glycerol did not improve the yield of protoplasts (fig.2B). This was, however, accomplished by using 1 M $MgCl_2$ as osmotic stabilizer (fig.2D). 1.4 M sorbitol gave intermediate results (fig.2C). Fig.2 also shows the evolution of the internal activity during the experiments. When expressed per mg dry wt of initial cells, the patterns obtained under conditions of protoplast lysis (fig.2A,B) suggest accumulation of an inactive precursor which is transformed into the active form at the permissive temperature. However, the greater the yield of protoplasts, the greater was the amount of accumulated activity (fig.2C,D). When exoglucanase activity was expressed per mg protein in protoplast lysates, an accumulation pattern instead, was observed under all the conditions tested (fig.2).

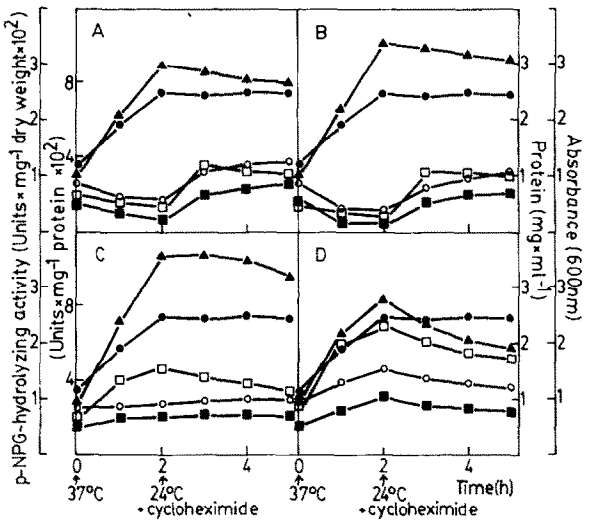

Fig.2. Accumulation-secretion patterns of sec 18 as a function of the osmotic stabilizer used in the protoplast treatment step. (A) 0.8 M KCl, (B) 1.5 M glycerol, (C) 1.4 M sorbitol and (D) 1 M $MgCl_2$. Other conditions were as in fig.1. (A–D) A_{600} evolution of culture (●) and resulting protoplasts (○), protein (mg/ml) in protoplast lysates (■) and protoplast-associated p-NPG hydrolyzing activity expressed by mg dry wt in the corresponding culture sample (□) or by mg protein present in the protoplast lysate (▲). Each experiment was performed at least twice with almost identical results.

3.3. *Soluble vs membrane-bound forms of accumulated exoglucanase and invertase*

The breakage of protoplasts during the protoplasting step did not appear to be the only reason for the patterns observed in fig.2A,B and [1] since more than 90% of the protoplast-associated exoglucanase activity was membrane-bound (table 1) and we recovered about 60% of this activity in the low-speed (1800×g) pellet from a wild type protoplast lysate (not shown). Table 1 shows that in contrast with wild type, sec 12 and sec 18 mutants accumulated all the activity in a soluble form and did not modify the total amount of the membrane-bound pool.

Secretory invertase showed a somewhat different behaviour. While traveling inside the cell this invertase is found as an intermediate membrane-bound form [4,5] that, in contrast with the cytoplasmic soluble invertase, has immature oligosaccharides of variable length [6]. A significant part (20–50%) of the secretory invertase accumulated in a membrane-bound pool in sec 12 and sec 18 cells; the rest accumulated in a soluble form (table 1). The levels of the cytoplasmic (small) invertase did not vary throughout the experiment. Finally, the thermoreversibility of sec 12 and sec 18 with regard to invertase (80–95%) was far higher than for exoglucanase (30–60% of the accumulated activity).

Table 1

Intracellular location of exoglucanase and secretory invertase in wild type and sec 12 and sec 18 mutants

	Units/mg dry wt						% release[a]	
	$t=0$		2 h 37°C		2 h 37°C → 3 h 24°C		S	M
	S	M	S	M	S	M		
Exoglucanase[b]								
Wild type	0.10	1.00	0.10	1.00	0.10	1.00	0	0
sec 12	0.10	0.95	1.40	0.92	0.90	0.85	30–50[c]	0
sec 18	0.09	0.92	1.42	0.90	0.70	0.89	35–65[c]	0
Invertase								
Wild type	0.00	0.002	0.00	0.01	0.00	0.01	0	0
sec 12	0.00	0.002	1.79	0.51	0.33	0.07	>80	>85
sec 18	0.00	0.002	1.32	0.64	0.13	0.03	>90	>95

[a] $(\text{internal}_{37}-\text{internal}_{37\rightarrow 24})/(\text{internal}_{37}-\text{internal}_{t=0})$

[b] Units/mg dry wt $\times 10^2$

[c] Highest and lowest values of 3 experiments

Cells were grown overnight at 24°C and shifted to a 37°C prewarmed medium containing 5% (exoglucanase) or 0.1% (invertase) glucose. After 2 h cycloheximide was added and cultures were transferred to 24°C. At the indicated times samples were taken and processed as described in section 2.3. to determine internal soluble (S) and membrane-bound (M) activities. When sec 18 cells were incubated in 5% glucose the standard osmotic stabilizer in the protoplasting medium (0.8 M KCl) was substituted by 1 M $MgCl_2$. Separation of secretory (glycosylated) and constitutive invertases in soluble fractions was achieved by Con A precipitation of the former; the difference in non-precipitable activity between the sample treated with the lectin and a control without it was considered as secretory invertase. Results are means of at least 2 experiments

4. DISCUSSION

Our data confirm those reported by Ramirez et al. [7] showing that sec mutants increase their osmotic sensitivity when incubated at 37°C. This is particularly evident in the case of sec 18 protoplasts derived from cells maintained at the restrictive temperature in high glucose. Most of these protoplasts lysed when obtained in 0.8 M KCl as osmotic stabilizer which preserved most protoplasts derived from wild type and some other mutant cells such as sec 1 and sec 7 (unpublished). The osmotic stabilizer used in [7] (1.4 M glycerol) did not improve the yield of intact sec 18 protoplasts. Since the accumulated exoglucanase was soluble most of it was lost during the protoplasting step in 0.8 M KCl. If one does not take into account these facts [1] it appears that sec 18 does not accumulate active exoglucanase. Moreover, once returned to the permissive temperature the osmotic sensitivity decreased as a consequence of the secretion of part of the accumulated products. Concomitantly an increase in the activity associated with protoplast lysates was observed giving the impression of endogenous activation of an inactive precursor. These problems have now been solved by using an appropriate osmotic stabilizer. Under these conditions exoglucanase, a constitutive activity in yeast [8], showed an accumulation-secretion pattern similar to repressible enzymes such as invertase.

It should be mentioned that the increase in osmotic sensitivity was hardly noticeable under stringent conditions of the carbon source, as that used for depression of invertase (i.e. 0.1% glucose). Under these conditions 0.8 M KCl preserved the integrity of most protoplasts and, accordingly, the pattern shown in [1] for invertase remains valid.

Total glucanase activity appeared to evolve in a similar way to that shown for exoglucanase. However, we have also observed that part of the

endoglucanase activity remains bound to residual glucan molecules on the surface of protoplasts in such a way that it makes it difficult to discern internal from secreted activity.

Why is all the exoglucanase activity accumulated by sec 12 and sec 18 soluble whereas a large part of secretory invertase remains membrane-bound? It is unclear but it may be related to the number of specific receptors postulated to be involved in the transport of secretory molecules from ER to Golgi [9]. This would explain, in turn, the differences in thermoreversibility of these mutants with regard to invertase (more than 85%) and exoglucanase (less than 60% of the accumulated activity). A search in this matter in other sec mutants might provide new insights in the field.

ACKNOWLEDGEMENT

This work was supported by grant no. 1315/82 from CAICYT to G.L.

REFERENCES

[1] Hernández, L.M., Olivero, I. and Larriba, G. (1983) FEBS Lett. 161, 190–194.
[2] Novick, P., Field, C. and Schekman, R. (1980) Cell 21, 205–215.
[3] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 19, 265–275.
[4] Babczinski, P. and Tanner, W. (1978) Biochim. Biophys. Acta 538, 426–434.
[5] Babczinsky, P. (1980) Biochim. Biophys. Acta 614, 121–133.
[6] Esmon, B., Novick, P. and Schekman, R. (1981) Cell 25, 451–460.
[7] Ramirez, R.M., Ishida-Schick, T., Krilowicz, B.L., Leish, B. and Atkinson, K. (1983) J. Bacteriol. 154, 1276–1283.
[8] Olivero, I., Hernández, L.M. and Larriba, G. (1985) Arch. Microbiol., in press.
[9] Schauer, I., Emr, S., Gross, C. and Schekman, R. (1985) J. Cell Biol. 100, 1664–1675.