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Two ionic forms of exoglucanase in yeast secretory mutants

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Analysis of exoglucanase activity accumulated by sec mutants from *Saccharomyces cerevisiae* revealed the presence of two ionic forms of the major exoglucanase (exo II) secreted into the culture medium. From the accumulation pattern of representative sec mutants and the carbohydrate composition it appears that the less acidic form is converted into the more acidic one by addition of one phosphate to one of the oligosaccharide cores as the enzyme progresses through the secretory pathway. Exoglucanase I, the heavier isoenzyme, was not accumulated by the mutants. Accordingly, it should arise from exoglucanase II after the execution point of sec1 mutation.

Exoglucanase; Secretory pathway; Ionic form; (Yeast)

1. INTRODUCTION

Saccharomyces cerevisiae secretes two exoglucanases into culture media [1-3]. Recent results from this laboratory have indicated that both isoenzymes share a very similar, if not identical, protein portion [3] and accordingly there exists the possibility that they are products of the same or related genes. The final forms would arise from differential modifications (glycosylation?) of the primary gene product during its transport to the cell surface. This hypothesis is further supported by the fact that the carbohydrate content of the major exoglucanase (exoglucanase II) is 11-14%, whereas that of the minor isoenzyme (exoglucanase I) exceeded 40% [4].

We have also shown [5] that exoglucanase activity follows the secretory pathway defined by sec mutants isolated by Novick et al. [6]. This feature makes it possible to investigate the step of the pathway at which exoglucanase I, the heavier enzyme, is first detected, by analysis of the molecules accumulated in representative sec mutants blocked at specific stages of the secretory pathway.

Here, we show that independently of the mutant used, only variants of exoglucanase II, the lighter enzyme, are accumulated inside the cells. In addition, it was found that two ionic forms of this exoglucanase account for the intracellular pool of secretory exoglucanase.

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Abbreviations: endo H, endo-β-*N*-acetylglucosaminidase H; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline; ER, endoplasmic reticulum

2. MATERIALS AND METHODS

Goa anti-rabbit IgG antibodies coupled to peroxidase were from Bio-Rad. Other chemicals were obtained as in [3].

2.1. *Microorganisms and culture conditions*

S. cerevisiae haploid strain X2180-1A and sec mutants blocked in transport of secretory and plasma membrane proteins from the ER (sec18), Golgi body (sec7) and in the transport of secretory vesicles (sec1) were supplied by Dr Schekman (University of California, Berkeley). They were grown as in [5]. These mutants accumulate secretory products at the indicated stages of the secretory pathway when incubated at the restrictive temperature of 37°C.

2.2. *Ion-exchange chromatography*

Dialyzed samples were applied to a column (6×1.2 cm) of DEAE-Bio-Gel A equilibrated at 4°C with 25 mM acetate buffer, pH 5.2, and eluted with 400 ml of a linear gradient of NaCl (0-0.5 M in acetate buffer) at a flow rate of 25 ml/h. Fractions of 4 ml were collected.

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2.3. *Immunoblots*

Proteins subjected to electrophoresis were electrophoretically transferred to nitrocellulose filters [7]. The filters were then incubated in blocking solution (3% BSA in TBS) for 1 h at 37°C. Antibody binding was carried out with 100 μl of the purified IgG fraction [4] in TBS containing 0.05% Tween 20 (TBS-T) and 1% BSA for 2 h at 37°C under gentle shaking. Nitrocellulose filters were washed once with TBS-T, twice with TBS-T containing 1 M NaCl and once with TBS-T, each time for 10 min. Paper-bound antibodies were detected by incubation of commercial goat anti-rabbit IgG antibodies coupled to peroxidase in TBS containing 1% BSA for 1 h at 37°C. After washing with TBS-T buffer, filters were incubated until color development with a solution containing TBS (100 ml), methanol (20 ml), 4-chloro-1-naphthol (60 mg) and hydrogen peroxide (60 μl from 30 volume commercial solution). The reaction was stopped by dipping the filter in distilled water.

2.4. *Other methods*

Transformation of cells in protoplasts, separation of soluble and membrane-bound forms of exoglucanase and enzymatic assays were carried out as in [8]. Purification of exoglucanases I and II, endo H treatment, PAGE, SDS-PAGE and preparation and purification of antibodies against the protein portion of exoglucanase II have been described [3,4].

3. RESULTS

All the exoglucanase activity accumulated by sec mutants at the restrictive temperature is found in the high-speed supernatant from homogenized protoplast lysates [5]. Aliquots of these supernatants (0.1 U exoglucanase) derived from sec18, sec7 and sec1 cells, which had accumulated secretory products for 2 h at the restrictive temperature, were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose filters and exoglucanase detected with specific antibodies. In all cases only one protein band, migrating at the level of exoglucanase II, was detected, whereas no traces of exoglucanase I could be visualized (fig.1). Pellets from the high-speed centrifugation were also analyzed similarly. In this case, immunodetection revealed the presence of a series of bands of low molecular mass whose relationship to secreted exoglucanases is at present uncertain. Since the detection of these bands required the use of large amounts of membranes and long incubation times with peroxidase, we suspect that they represent non-specific material. However, even under these conditions no traces of exoglucanase I were detected either (fig.1).

The failure to detect exoglucanase I inside the mutant cells was at first attributed to its low level,

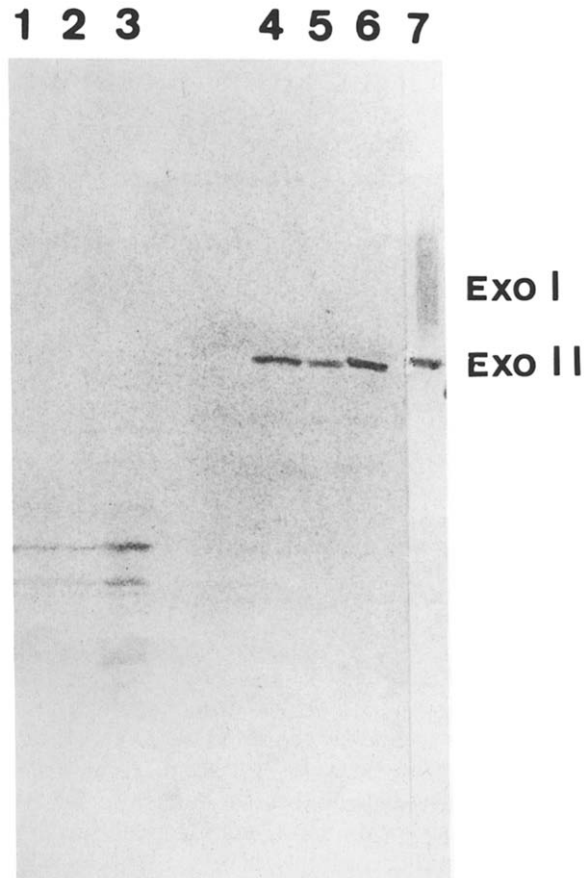

Fig.1. Immunoblot analysis of exoglucanase accumulated by sec mutants. Sec1 (lanes 1,4), sec7 (lanes 2,5) and sec18 (lanes 3,6) cells were incubated at 37°C for 2 h and transformed in protoplasts. These were lysed, homogenized and centrifuged at high speed to obtain supernatant (lanes 4–6) and pellet (lanes 1–3) fractions. Aliquots of these fractions were electrophoresed on an SDS-8% polyacrylamide gel, transferred to nitrocellulose and immunodetected. Lane 7: immunodetection of purified exoglucanase I and II.

since this isoenzyme accounts for only about 10% of the total activity present in the culture medium [3,4] (see also fig.2d). Accordingly, we prepared greater amounts of exoglucanase (3 U) from mutant cells incubated for 2 h at the restrictive temperature. Since both isoenzymes are easily separated by ion-exchange chromatography, high-speed supernatants from protoplast lysates were fractionated in DEAE-Bio-Gel A. Regardless of the mutant used two peaks of activity were always apparent (fig.2a–c). The second peak (B) eluted at the same position as the exoglucanase II present in the

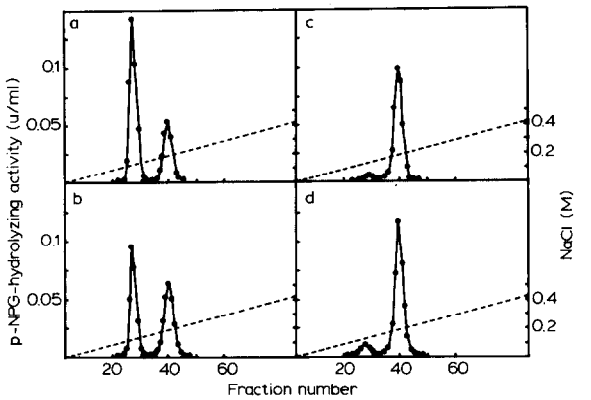

Fig.2. Separation of two ionic forms of the exoglucanase accumulated by sec mutants at 37°C. Soluble fractions of protoplasts lysates from sec18 (a), sec7 (b) and sec1 (c) obtained as described in fig.1 (1–3 units of activity) were applied to the DEAE-Bio-Gel column and eluted as indicated in section 2. The profile of the exoglucanase activity secreted by wild-type cells into the culture medium is represented in d.

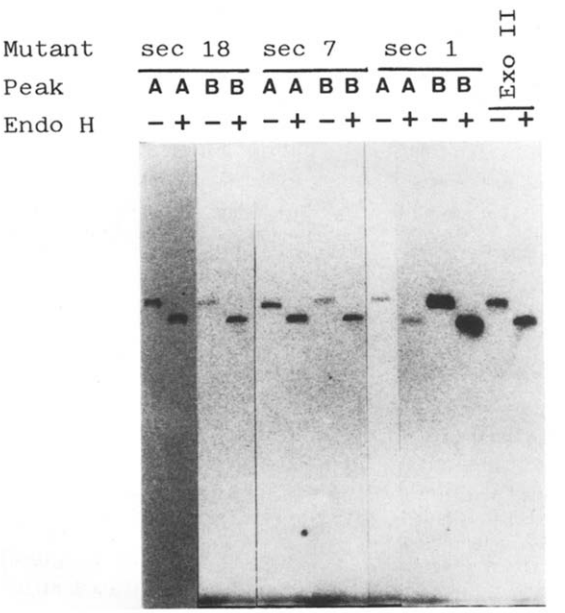

Fig.3. Immunoblot analysis of the ionic forms of exoglucanase accumulated by sec mutant cells. Aliquots of the material included under peaks A and B from fig.2 were subjected to SDS-PAGE and immunodetected as in fig.1. Purified exoglucanase II was used as a control. In each case half of the sample was treated with endo H.

culture medium (0.21 M NaCl), whereas the first (A) eluted at a salt concentration (0.14 M) very close, but not identical, to that calculated for exoglucanase I (0.13 M NaCl). In addition, exoglucanase I always eluted as a wide peak (fig.2d) which contrasted with peak A which was very sharp.

The ratio between peaks A and B in terms of enzyme activity decreased as the block in the secretory pathway was more advanced. Thus, whereas in sec18, and ER-blocked mutant, peak A accounted for most of the total activity, nearly 90% of the activity accumulated by sec1, which is blocked at the level of secretory vesicles, corresponded to peak A. Sec7, which is blocked at an intermediate step of the secretory pathway (Golgi body), accumulated similar proportions of both peaks.

Fractions corresponding to peaks A and B of each mutant were pooled separately and an aliquot treated with endo H. Thereafter, samples were prepared for SDS-PAGE and the corresponding immunoblot was obtained. As shown in fig.3 the different exoglucanase forms from the DEAE-Bio-Gel column exhibited the same electrophoretic mobility, which coincided with that of mature exoglucanase II purified from the culture medium. Moreover, all the glycoproteins were equally susceptible to endo H, generating a product which has been characterized as the deglycosylated form of both exoglucanases [3]. Surprisingly, no exoglucanase I was accumulated inside any of the mutant cells.

Since both forms A and B appear to differ in charge we tried to resolve them in non-denaturing PAGE. This technique also failed to provide a useful tool for a rapid analysis of the molecules accumulated by mutant cells.

4. DISCUSSION

The major exoglucanase secreted by *S. cerevisiae* (exoglucanase II) containst two *N*-glycosidically linked oligosaccharide cores [9]. One is neutral whereas the other is acidic due to the presence of a phosphate. The higher carbohydrate content of exoglucanase I compared with exoglucanase II raises the suspicion that the former arises from the latter by addition of an outer carbohydrate chain to one or both oligosaccharide cores. Since in other glycoproteins this addition seems to occur at the level of the Golgi body [10,11], it would then be possible to detect exoglucanase I in mutants

blocked at this step or in a further stage of the secretory pathway. Our results indicate, however, that even sec1, a mutant blocked at the level of secretory vesicles, does not accumulate this isoenzyme. All the activity included under peak A of this mutant, i.e. the best candidate for representing exoglucanase I, was identified as exoglucanase II.

In an effort to detect exoglucanase I inside the cells we investigated whether it was present in membrane fractions of mutants and wild-type cells, but again the results were negative. Accordingly, it should arise from exoglucanase II. This conclusion is in agreement with our previous suggestion that both exoglucanases share the same protein portion and thus are products of a single gene [4]. In addition, whatever the modification implied in the conversion of exoglucanase II into exoglucanase I, it takes place after the execution point of sec1 mutation. In order to ascertain the nature of such a modification the carbohydrate moiety of exoglucanase I is currently being characterized.

Nevertheless, our efforts proved to be fruitful in revealing the presence of two ionic forms of exoglucanase II inside the cells. The accumulation pattern exhibited by representative sec mutants strongly suggests that form A is transformed into form B as exoglucanase molecules progress through the secretory pathway. This second form (B) corresponds to the mature secreted exoglucanase II. On the basis of the carbohydrate composition of exoglucanase II we suggest that the conversion of A into B occurs by addition of one phosphate to one of the oligosaccharide cores present in the molecule.

Such conversion would be in agreement with the fact that both ionic forms of exoglucanase II are not separated in SDS-PAGE. Purified carboxypeptidase Y was also resolved on DEAE-Sephadex into two peaks of enzyme activity whose differences were attributed to a different phosphate content [12]. On the other hand, the failure to resolve both exoglucanase forms in non-denaturing PAGE indicates that the presence of one phosphate does not significantly modify the net charge of the protein. However, the projection of the carbohydrate chains from the enzyme surface would allow the acidic form to interact efficiently with the ion-exchange resin.

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