Detection of inactive precursors of β-glucanases in *Saccharomyces cerevisiae*

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Received 10 May 1983; revised version received 21 June 1983

Accumulation and secretion of β-glucanases have been studied in vivo by using a thermosensitive secretory mutant of *Saccharomyces cerevisiae* blocked at the endoplasmic reticulum level (sec 18-1). When incubated at the restrictive temperature no accumulation of active glucanases was observed. Following a shift to permissive conditions in the presence of cycloheximide a rise in the internal activity took place. The increase in total glucanase activity was partially due to the activation of an exo-glucanase that hydrolyzes PNPG. It is concluded that glucanases are synthesized in inactive precursor forms and are converted to the active forms in their secretory pathway.

β-Glucanases Saccharomyces cerevisiae *Secretory mutant* *Endoplasmic reticulum* *Precursor* *Activation*

1. INTRODUCTION

The knowledge of the secretory process in yeast has suffered a delay compared to that in plant and animal cells. A determinant factor has been the lack of concentration and intracellular storage of secretory products which in turn has precluded the unambiguous recognition of the organelles involved in the secretory process. Secretory mutants that show a conditional block in the secretory pathway have been isolated [1,2]. The combination of genetic and biochemical approaches to mutants blocked at different stages has allowed them to order events in the yeast secretory pathway [3,4]. Initially they used repressible enzymes (invertase, acid phosphatase) as markers for secretion but recently they also showed accumulation of carboxypeptidase Y (CPY), a vacuolar enzyme, in mutants blocked in the early stages of the secretory process [5].

β-glucanases are constitutive enzymes in *Saccharomyces cerevisiae* [6] and they appear to have a role in the morphogenetic events which in turn involve modifications of the yeast cell wall. We assumed that their action should be controlled in a spatial and temporal manner in order to produce at very determined places the correct number of nicks in the glucan molecules. One possibility is that some control is exerted at the secretion rate level. Accordingly we initiated a systematic study to determine the secretory pathway of β-glucanases in *S. cerevisiae*, by using thermosensitive secretory mutants.

We introduce here results which indicate that β-glucanases are synthesized as inactive precursors and that they accumulate in that form in an endoplasmic reticulum (ER) blocked secretory mutant (sec 18-1) when incubated at the restrictive temperature.

2. MATERIALS AND METHODS

Zymoliase 5000 was obtained from Seikagaku Kogyo (Tokyo). Cycloheximide, glucose oxidase, peroxidase, O-dianisidine, *p*-nitrophenyl-β-D-glucopyranoside (PNPG) and *p*-nitrophenol were from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

2.1. *Microorganisms and culture medium*

S. cerevisiae haploid strain X2180-1A and HMSF-176 (sec 18-1) [2] were supplied by Dr

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001457593/82/0000—0000/$2.75

Schekman (Univ. California, Berkeley). The latter is a thermosensitive secretory mutant blocked at the ER level; at the restrictive temperature cell division stops but protein synthesis continues. It was derived from X2180-1A.

Microorganisms were grown in YPD medium containing 1% Bacto yeast extract, 2% Bacto peptone and either 0.1% or 5% glucose. Cell number was determined with a hemocytometer.

2.2. *Preparation of enzymatic samples*

Intracellular activities were measured in protoplast lysate. Pelleted cells were resuspended at a final concentration of 5 mg (dry wt/ml) in 50 mM Tris–HCl buffer (pH 7.3) supplemented with 0.8 M KCl, 10 mM SO_4Mg, 15 mM mercaptoethanol, 10 mM sodium azide and 1 mg/ml of zymoliase 5000.

After 45 min at 37°C almost 100% of the cells were converted into protoplast. Protoplasts were collected by centrifugation and washed once with 0.1 M acetate buffer (pH 5.2) osmotically stabilized with 1 M sorbitol and supplemented with 10 mM sodium azide. They were then resuspended in 0.1 M acetate buffer (pH 5.2) with 1% Triton X-100. The protoplast lysates were homogenized in a glass homogenizer.

Periplasmic space activities were measured in resuspended cells in 0.1 M acetate buffer (pH 5.2) containing 10 mM sodium azide.

External activity (periplasmic space plus culture medium) was measured in culture samples supplemented with 10 mM sodium azide.

2.3. *Enzymatic assays*

Invertase was tested for 10 min at 30°C as in [7]; units of activity are μmol of glucose released per min. β-Glucanase activity was tested on laminarin and PNPG. Reducing sugars were measured by the Somogyi method as modified in [8], D-glucose by the glucose-oxidase method [9] and *p*-nitrophenol as in [10]. One unit of β-glucanase activity was defined as the amount of enzyme which released 1 μmol *p*-nitrophenol, glucose or an equivalent reducing power per hour at 30°C.

Protein synthesis was followed by determining the incorporation of [^{14}C]threonine (0.025 μCi/ml, spec. act. 0.25 μCi/μmol) in 5% trichloroacetic acid-insoluble material. After 30 min at 0°C samples were filtered on Whatman GF/C filters; the filters were washed and dried and the radioactivity was measured in a Beckman LS-100 liquid scintillation counter.

3. RESULTS

3.1. *Comparison of the secretion pattern of invertase and glucanase*

As described in [2] sec 18-1 accumulated invertase inside the protoplast at the restrictive temperature. Fig. 1 shows the result of an experiment similar to that described for sec 1-1 in [1]. The intracellular pool of invertase increased in the mutant at 37°C (but not at 24°C) following a shift to derepressive conditions and remained almost undetectable outside the protoplast. When shifted

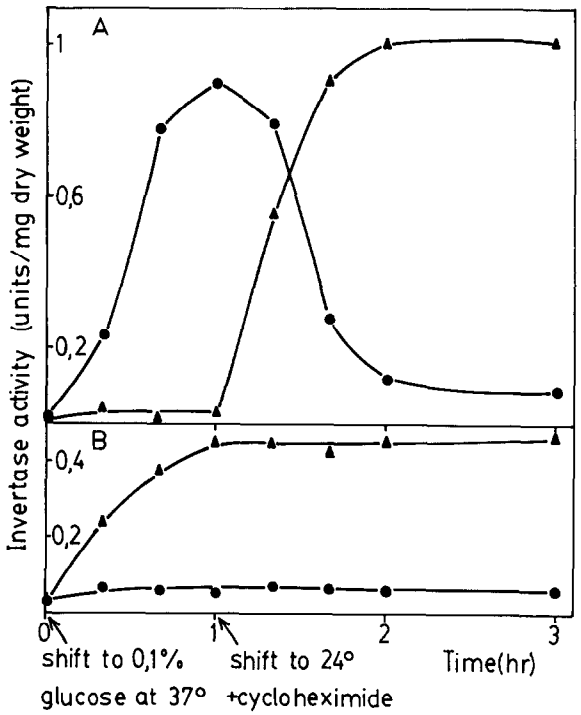

Fig.1. Invertase secretion and accumulation in HMSF-176 (sec 18-1) (A) and X2180-1A (B) cells. Cells were grown overnight in YPD medium containing 5% glucose at 24°C and shifted to a 37°C prewarmed medium with 0.1% glucose. After 1 h cycloheximide was added and cells were transfered to 24°C. Samples were withdrawn chilled at 0°C, centrifuged and resuspended in cold 10 mM sodium azide [1]. Invertase activity was determined in cells (▲—▲) and in protoplasts (●—●) as indicated in section 2. Results are means of 2 experiments.

to permissive temperature in the presence of cycloheximide the intracellular pool invertase dropped and a concomitant increase of the activity in the periplasmic space was observed (fig. 1A).

In contrast, wild type did not accumulate invertase. Invertase synthesis and secretion were observed following a shift to derepressive conditions and no intracellular accumulation occurred during incubation at either 37 or 24°C (fig. 1B). Cycloheximide stoped synthesis and secretion of invertase as well as total protein synthesis (not shown).

A similar experiment was carried out to check the behaviour of the 1,3-β-glucanase activity. Exponentially growing cells were transferred to YPD medium containing either 5 or 0.1% glucose. Results were similar in 4 different experiments carried out at both glucose concentrations. Sec 18-1 cells maintained at 37°C for 2 h did not show any increase in the intracellular activity. After transfer to 24°C in the presence of cycloheximide a significant rise in the internal activity occurred in the next 2 h (4–5-fold). Then it started dropping (fig. 2A). Estimation of glucanase activity by determination of reducing sugars gave values slightly higher than by valuation of free glucose. It is due to the presence of endo, as well as exo, 1,3-β-D-glucanases in our extracts.

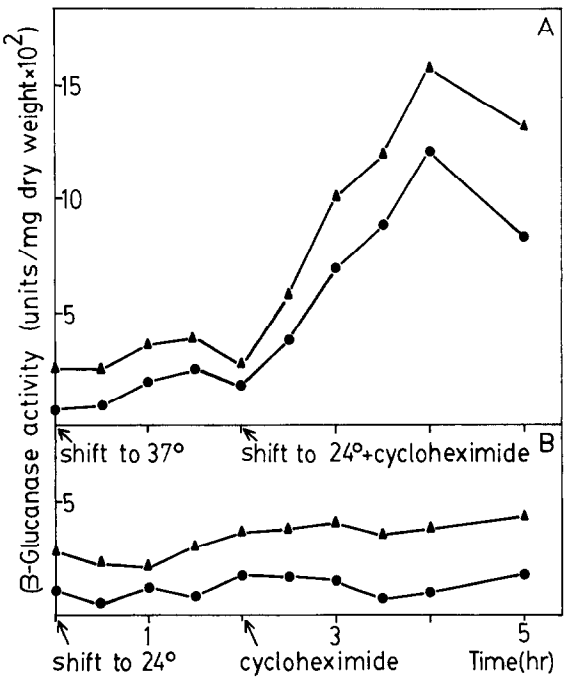

Fig.2. Intracellular glucanase activity evolution in HMSF-176 (sec 18-1) cells preincubated at 37°C (A) and 24°C (B). Cells were grown overnight in YPD medium containing 5% glucose at 24°C and shifted to new medium prewarmed at 37°C or 24°C. After 2 h cells were supplemented with cycloheximide and incubated at 24°C for an additional 3 h. Samples were withdrawn chilled at 0°C, centrifuged and protoplasts were obtained. Laminarinase activity was determined (incubation time 6 h) in protoplast lysate by measuring reducing groups (▲—▲) or D-glucose (●—●) as indicated in section 2. Results are means of 4 experiments. Standard deviation was never greater than 20% of the mean.

A different pattern was observed when the mutant was incubated at 24°C from the beginning of the experiment (fig. 2B). In this case, the intracellular glucanase activity did not show significant changes during the whole period. The same pattern was exhibited by wild type when incubated at either 24 or 37°C (not shown).

3.2. *Secretion pattern of the PNG-hydrolyzing activity*

The presence of both exo- and endo-glucanases in the protoplast lysate, plus the long incubation times required to measure the glucanase activity, raised the question whether the increase in activity observed was due to activation of the exo- and/or the endo-splitting enzyme(s). In order to solve this we took advantage of the PNPG-hydrolyzing capacity of the yeast exo-glucanase [11]. Since reducing sugars of the culture medium did not interfere in this case with the valuation of the activity, the secreted enzyme could also be determined. Protoplast lysates, whole cells and culture medium were devoid of β-glucosidase activity when assayed against salicine.

As shown in fig. 3A the intracellular pool of PNPG-hydrolyzing activity did not vary significantly during preincubation of sec 18-1 cells at the restrictive temperature. During this period cells secreted only negligible amounts of enzyme. Following the shift to 24°C in the presence of cycloheximide the intracellular activity rose 2-fold and maintained this level for 2 h. At the same time the secreted activity showed a significant and continuous increase.

By contrast, mutant cells incubated at the permissive temperature, maintained their intracellular

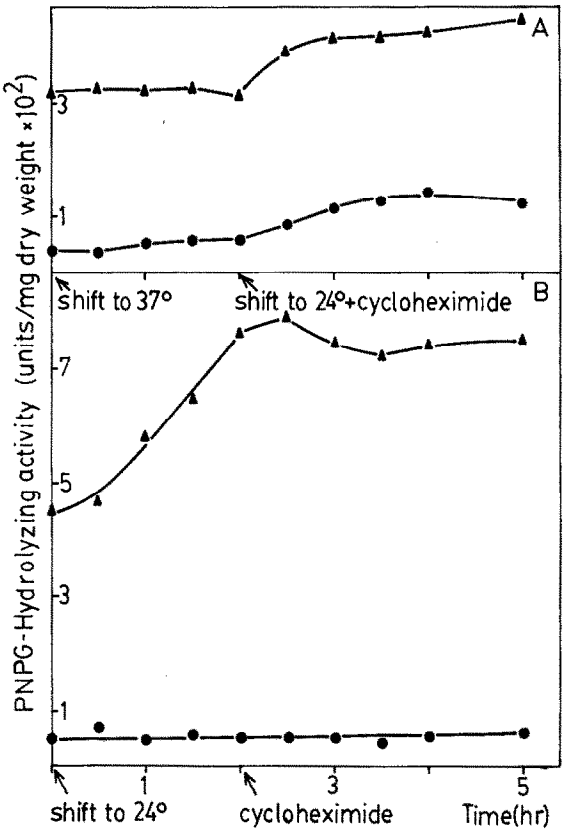

Fig.3.PNPG-hydrolyzing activity accumulation and secretion in HMSF-176 (sec 18-1) cells preincubated at 37°C (A) and 24°C (B). The experiment was performed as in fig.2. For determination of external activity (periplasmic space plus culture medium) samples were withdrawn, made up to 10 mM in sodium azide and incubated with the substrate for 1 h (▲—▲). Determination of internal activity was carried out as in fig.2 (incubation time 8 h, (●—●). Results are means of 4 experiments. Standard deviation was never greater than 10% of the mean.

levels of activity constant and the enzyme was actively secreted until the addition of cycloheximide (fig. 3B). The same profile was obtained for wild type when preincubated at either 24 or 37°C (not shown).

4. DISCUSSION

The results shown here provide preliminary evidence for the presence of precursors of glucanases at the ER level in *S. cerevisiae*.

No accumulation of active internal glucanases was observed at the restrictive temperature in sec 18-1 in spite of the fact that the strain behaved as a secretory mutant with regard to the secretion of invertase. However, after the shift to permissive temperature in the presence of cycloheximide, a rise in the amount of total internal glucanase took place indicating the accumulation of inactive precursors at the restrictive temperature and their further maturation to active enzymes when shifted to permissive conditions.

On the basis of our experiments, part of the surge may be attributed to the maturation of the exo-glucanase capable to hydrolyze PNPG [11]. This activity was secreted when cells were incubated at the permissive temperature (fig. 3). The rise in total units of PNPG-hydrolyzing activity does not account however per se for the whole increase in total laminarinase activity, indicating the additional activation of endo-glucanases.

With regard to the secretory process, glucanases behaved in a similar way to carboxypeptidase Y (CPY). This lysosomal hydrolytic enzyme has been shown to be synthesized as a proenzyme, (pro-CPY) which is converted in vivo to the mature form by proteolytic cleavage [12]. Maturation of pro-CPY is prevented in mutants that, like sec 18-1, are blocked in the movement of secretory molecules at the ER level [2] and precursors accumulate at this level [5].

The maturation of glucanases may involve proteolytic cleavage, glycosyl-trimming and glycosyl-transfer reactions or both since exo- as well as endo-glucanases appear to be glyco-proteins [13]. In particular, it has been reported that the exo-glucanase described here has the carbohydrate moiety *N*-glycosydically bound to protein [14]. Secretory studies in the presence of tunicamycin as well as in vitro treatment of the accumulated precursors with proteases may help to decide the nature of the activation.

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