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EXTRACELLULAR β-GLUCANASES OF THE YEAST, *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Fractionation of proteins secreted by protoplasts and intact cells of *Saccharomyces cerevisiae* by DEAE-cellulose chromatography in 0.05 M phosphate buffer (pH 7) using a linear gradient of NaCl revealed the presence of at least three different fractions hydrolyzing laminarin (β-1,3-glucan). The first fraction, not retained by the column, appeared to consist of two enzymes exhibiting activity for laminarin, *p*-nitrophenyl-β-D-glucopyranoside, pustulan (β-1,6-glucan) and a low but detectable activity for laminarin treated with $NaIO_4$. The second fraction, eluted from the DEAE-cellulose column at 0.23 M NaCl possessed the substrate specificity of an endo-β-1,3-glucanase. It exhibited the maximum activity at pH 5.5 in 0.1 M acetate buffer. The third fraction, eluted from the column at 0.35 M NaCl, was an unspecific exo-β-glucanase hydrolyzing terminally both laminarin and pustulan. The pH optimum of the latter enzyme was in the range 4.5–5.0 with laminarin, *p*-nitrophenyl-β-D-glucopyranoside or pustulan as substrates. The β-glucanases found extracellularly were compared with those occurring in protoplast lysates and cell-free extracts of intact cells.

INTRODUCTION

Studies carried out by several authors[1–3] indicate that a portion of yeast β-glucanases (β-1,3-glucan glucanohydrolases, is closely associated with the cell wall. It is evident that such an association would facilitate the direct participation of these enzymes in degrading processes taking place in the cell wall during cell-wall growth, lysis and cell conjugation[4–8]. Our recent finding[9] that the protoplasts of *Saccharomyces cerevisiae* secrete into the growth medium, under suitable conditions, enzymes capable of hydrolyzing laminarin and *p*-nitrophenyl-β-D-glucopyranoside (PNPG), confirms the extracellular location of these enzymes.

The present study was undertaken to separate the glucan-hydrolyzing enzymes secreted by protoplasts and by intact cells of *S. cerevisiae* and to determine their

Abbreviation: PNPG, *p*-nitrophenyl-β-D-glucopyranoside.

substrate specificity in order to reveal their possible role in the cell-wall degrading processes. A comparative study was also done with the intracellularly located enzymes.

MATERIALS AND METHODS

Yeast and preparation of protoplasts

S. cerevisiae, diploid strain CCY 21-4-13, was grown on a reciprocal shaker at 27 °C in a synthetic medium containing glucose, 20 g; Yeast Nitrogen Base (Difco), 6,7 g; asparagin, 2 g; KH_2PO_4, 5 g per l. The protoplasts were prepared from log-phase cells by the method described in our previous study[9]. For the preparation of one batch of protoplasts usually 10–15 g (wet weight) of cells were used.

Preparation of crude extracellular enzymes

The protoplasts (after being washed 6 times with 0.02 M phosphate buffer, pH 5.5, containing 0.8 M mannitol) were suspended in 300–400 ml of the synthetic growth medium which was osmotically stabilized with 0.8 M mannitol. The suspension was kept at 27 °C with occassional gentle stirring. After 3 h cultivation the suspension was centrifuged at 1500 × *g* for 10 min and the clear supernatant was dialyzed at 4 °C for 3 days against six 3-l changes of distilled water containing 1 mM 2-mercaptoethanol and 10 μg/ml of cycloheximide. The contents of the dialysis bag were then lyophilized, reconstituted in a small volume of 0.05 M phosphate buffer (pH 7) and dialyzed at 4 °C for 24 h against two 1-l changes of the same buffer. This preparation was then fractionated by means of DEAE-cellulose chromatography as described below.

The culture fluid from the cultivation of yeast cells was used as a source of crude extracellular enzymes secreted by intact growing cells. After removing the cells by centrifugation the supernatant was treated in the same manner as the supernatant from growing protoplasts.

Preparation of crude intracellular enzymes

Thoroughly washed protoplasts (see above) were lysed in 10–15 ml of 0.05 M phosphate buffer, pH 5.5 and sonicated (100 W, 9 kcycles/s) at 0 °C for 3 min. All subsequent operations were carried out at 0–4 °C. The suspension was then diluted with the buffer to 30 ml and centrifuged at 10 000 × *g* for 15 min. The microsomes were eliminated by a 1-h centrifugation at 100 000 × *g*. The supernatant was dialyzed against 5 mM phosphate buffer containing 1 mM 2-mercaptoethanol and 10 μg/ml of cycloheximide (six 1-l changes, 3 days). The protein precipitated during dialysis was removed by centrifugation and the supernatant was lyophillized. After reconstitution in a small volume of 0.05 M phosphate buffer (pH 7) the intracellular proteins were dialysed against two 1-l changes of the same buffer for 24 h.

In order to obtain crude extracts from intact cells the yeast was grown to the late logarithmic phase. The cells were collected by centrifugation, washed twice with ice-cold water and disintegrated in a cooled homogenizer with glass beads in the presence of 0.05 M phosphate buffer (pH 5.5). After removing the ballotini beads, the cell-wall debris was removed by low speed centrifugation; the supernatant was processed in the same manner as was the sonication of protoplasts.

DEAE-cellulose chromatography

A sample containing 1–30 mg of protein was applied to a DEAE-cellulose column (1.5 cm × 15 cm) previously equilibrated with 0.05 M phosphate buffer (pH 7) and eluted first with 50 ml of the buffer and then with a linear concentration gradient (0–1.0 M) of NaCl in the same buffer. The volume of the solutions in the reservoir as well as in the mixing chamber of the gradient mixer was 130 ml. 2.5–3.0-ml fractions were collected and assayed for the hydrolytic activities against different substrates.

Substrates

Laminarin and PNPG were purchased from Koch-Light, England. β-Gentiobiose was from Sigma Chem. Co., U.S.A. Laminarin oxidized at both ends of its molecule by $NaIO_4$ was prepared by a modified method of Goldstein *et al.*[10]. 0.3 g of laminarin was dissolved in 100 ml of water at 70 °C and 100 ml of 0.08 M $NaIO_4$ was added. The mixture was stirred at 4 °C for 10 days. 0.4 g of ethyleneglycol was then added and after standing at room temperature for 30 min the mixture was dialyzed for 5 days against several changes of distilled water. The content of the dialysis bag was then lyophilized and used as a substrate in the assays of endo-β-1,3-glucanases.

Alkali-soluble cell-wall glucan was prepared from the cell walls of used strain of *S. cerevisiae*. After fractionation of the cell walls by ethylenediamine according to Korn and Northcote[11] the insoluble Fraction C (enriched in glucan content) was extracted with 1 M NaOH at 4 °C for 16 h. The supernatant was then diluted by 2 vol. of 96% (v/v) ethanol and the precipitate was isolated by centrifugation (10 min at 10 000 × *g*). The pellet was successively washed with 0.05 M acetate buffer (pH 5) and with water.

Pustulan (β-1,6-glucan) from *Umbilicaria pustulata* was a gift from Dr E. T. Reese and carboxymethylpachyman (CM-pachyman) was obtained by the courtesy of Professor B. A. Stone. The degree of substitution of CM-pachyman was 0.29.

Enzyme assays

Assay mixtures (0.5 ml) contained 0.1 M phosphate or acetate buffer of desired pH, enzyme and the substrate. The concentrations of the respective substrates in the assay mixtures were 1 mg/ml. The incubations were carried out at 30 °C for different time intervals. The measurements of viscosity changes of CM-pachyman were carried out at 30 °C as described by Cortat *et al.*[1]. The concentration of CM-pachyman in the assay mixtures was 0.38% (w/v).

Analytical methods

Reducing sugar was determined by the method of Somogyi and Nelson (see ref. 12) using glucose as a reference standard. Glucose was determined by glucose oxidase coupled to peroxidase (blood sugar test, Boehringer, Mannheim, Germany). Hydrolysis of PNPG was followed by measuring the liberated *p*-nitrophenol[7]. Proteins were determined by the method of Lowry *et al.*[13] or by measuring the absorbance of solutions at 280 nm.

RESULTS

As in our previous study[9], it should be also stressed here that the enzymes

found in the supernatants of growing protoplasts were not contaminated by the glucan-hydrolysing enzymes originating from the snail gut juice used for the preparation of protoplasts. Control cultivations of protoplasts grown in the presence of cycloheximide or with the lysed and washed protoplasts showed that all the enzymes found in the supernatants of growing protoplasts were synthesised *de novo* and secreted into the medium. The protoplasts were well stabilised by the used cultivation medium and no lysis was observed during the experiments.

The DEAE-cellulose chromatography in 0.05 M phosphate buffer (pH 7) using the linaer gradient of NaCl for the elution of the proteins proved to be the most efficient method for the separation and purification of yeast β-glucanases in our experiments. Attempts to obtain partial purification of the enzymes by $(NH_4)_2SO_4$ precipitation, adsorbtion on the cold-water-insoluble laminarin, gel filtration on the Sephadex G-100 or G-200 or by DEAE-cullulose chromatography at pH 5.5 did not give satisfactory or sufficiently reproducible results.

Separation and characterization of extracellular β-glucanases secreted by yeast protoplasts

Fig. 1 shows the pattern of extracellular β-glucanases after their resolution on the DEAE-cellulose column. At least three peaks of activity against laminarin were recorded. The minor Peak 1 was not retained by the column and exhibited activity against laminarin, PNPG and pustulan and low but detectable activity against oxidized laminarin. The bulk of non-diffusible carbohydrate and a considerable portion of protein were eluted together with the Peak I from the column. Occasionally, a

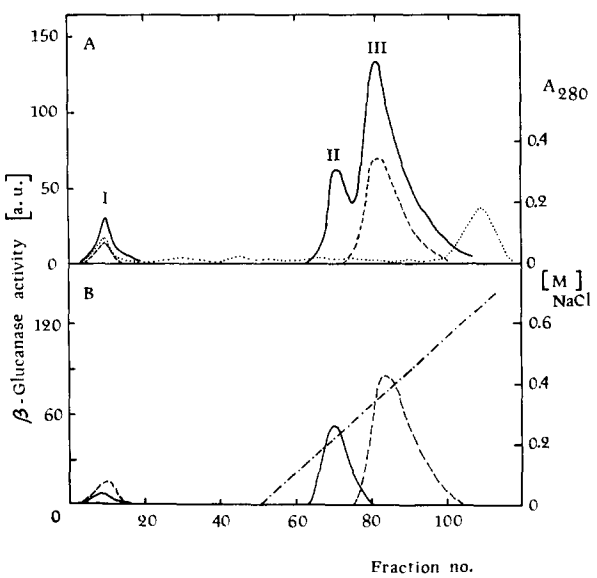

Fig. 1. DEAE-cellulose chromatography of proteins secreted into the growth medium by yeast protoplasts. 3 mg of protein were applied to a column (1.5 cm × 15 cm) and eluted as described in the Materials and Methods section. 2.6-ml fractions were collected. (A) Absorbance at 280 nm (. .); activity against laminarin (———) and PNPG (– –). (B) NaCl concentration (. — . —); activity against oxidized laminarin (———) and pustulan (– –). β-Glucanase activity is expressed in arbitrary units as nmoles of *p*-nitrophenol, glucose or equivalent reducing power liberated from the respective substrate per fraction per h at 30 °C. a.u., arbitrary units.

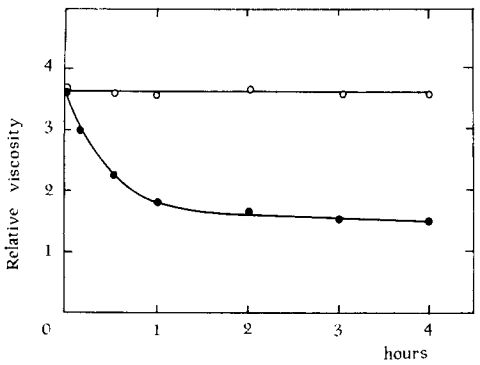

Fig. 2. Viscosity changes of CM-pachyman solutions on incubation with the enzymes from Peak II (●) and from Peak III (○) (see Fig. 1.) The total amounts of the respective enzymes in the incubation mixtures were approximately equal when assayed against laminarin. The viscosities of the complete incubation mixtures at different time intervals were compared with the viscosity of the mixture without CM-pachyman.

little shoulder in Peak I was observable as it came off from the column indicating its possible heterogeneity. Attempts to fractionate Peak I on the DEAE-cellulose column, starting with 0.01 M phosphate buffer (pH 7) did not lead to a better resolution.

Peak II was eluted from the column at 0.23 M NaCl and exhibited activity against laminarin and oxidized laminarin but not against PNPG and pustulan. It readily decreased the viscosity of CM-pachyman solutions (Fig. 2). The paper chromatographic resolution of the products of its action on laminarin showed the presence of oligosaccharides of the laminaribiose series in addition to the free glucose (Fig. 3). It may be seen that higher oligosaccharides predominate in the mixture of

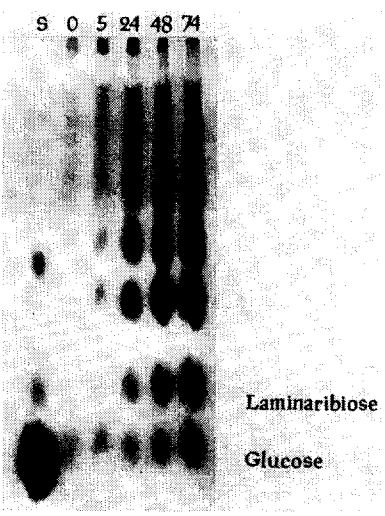

Fig. 3. Paper chromatographic resolution of the products of an endo-β-1,3-glucanase (Peak II, Fig. 1) action on laminarin. Descending chromatography was carried out in the solvent system ethyl acetate–pyridine–water (5:3:2, v/v) for 20 h. The components were detected with alkaline $AgNO_3$; 0, 5, 24, 48, 74, incubation time in h; S, glucose *plus* β-1,3 markers.

hydrolytic products. Free glucose, biose, triose and traces of tetrose were liberated from the alkali-soluble cell wall glucan, although at much slower rate. The described substrate specificity of the enzyme eluted in Peak II behaves as an endo-β-1,3-glucanase. It exhibited maximum activity at pH 5.5 with laminarin as substrate. Approx. for 80% lower activity values were recorded when 0.1 M phosphate buffer was used instead of acetate in the assays. The enzyme activity was not appreciably inhibited by δ-gluconolactone, and of the various divalent cations tested Hg^{2+} was most inhibitory.

Peak III was eluted from the column at 0.35 M NaCl and exhibited activity against laminarin, PNPG, gentiobiose and pustulan but no activity against oxidized laminarin. It practically did not reduce the viscosity of CM-pachyman solutions (Fig. 2) and free glucose was identified as the sole product of its action on laminarin and pustulan. On the basis of available evidence the enzyme eluted in the Peak III may be considered as a non-specific exo-β-glucanase splitting both β-1,3 and β-1,6 linkages. An enzyme possessing similar substrate specificity as that eluted in the Peak III has been described in baker's yeast by Brock[4] and Abd-El-Al and Phaff[5]. Evidence that in our case a single enzyme is also responsible for splitting both β-1,3 linkages in laminarin and PNPG and β-1,6 linkages in pustulan is obtained from the observation that the rate of heat inactivation was identical for both of the described activities (Fig. 4).

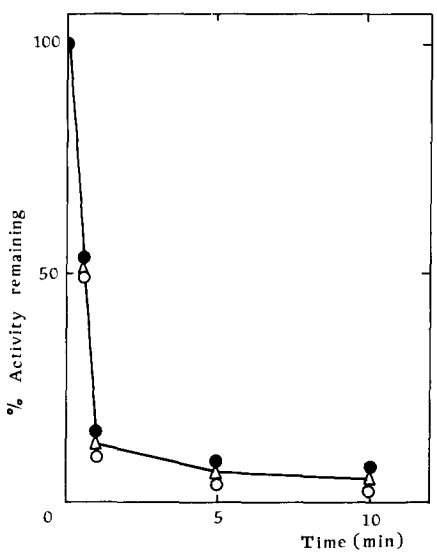

Fig. 4. Rate of heat inactivation of exo-β-1,3-glucanase (see Fig. 1, Peak III). The dialysed enzyme was heated at 50 °C for the time intervals indicated and then assayed with laminarin (○), *p*-nitrophenyl-β-D-glucoside (●), and pustulan (△) in 0.05 M acetate buffer (pH 5.5).

The enzyme eluted in Peak III exhibited maximum activity in the range pH 5.0–5.5 when assayed with laminarin, PNPG and/or pustulan as substrates. While the phosphate did not affect the reaction rate, δ-gluconolactone acted as a competitive inhibitor. The inhibitor constant, K_i, for δ-gluconolactone was $3.0 \cdot 10^{-3}$ M with laminarin and $2.9 \cdot 10^{-3}$ M with PNPG as the respective substrates (Fig. 5).

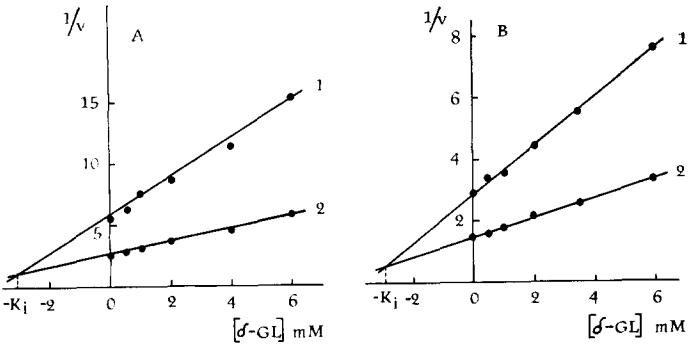

Fig. 5. Effect of δ-gluconolactone (δ-GL) on the activity of exo-β-1,3-glucanase (*cf.* Fig. 1, Peak III). (A) Curve 1, 1.2 mg/ml; Curve 2, 1.0 mg/ml of laminarin as substrate, respectively. (B) Curve 1, 0.25 mg/ml; Curve 2, 1.0 mg/ml of *p*-nitrophenyl-β-D-glucoside as substrate, respectively. The data were plotted according to method of Dixon[20].

Separation and identification of β-glucanases secreted by intact growing yeast cells

The pattern of enzymes secreted into the growth medium by intact growing yeast cells (Fig. 6) closely resembled that obtained after resolution of enzymes released by growing protoplasts (*cf.* Fig. 1). The obtained three β-glucanase Fractions 1, 2 and 3, had in all examined aspects identical properties with those described for the corresponding Fractions I, II and III from Fig. 1. The quantitative proportions of the individual enzymes were, however, somewhat different. The proteins released into the growth medium by intact cells were relatively more abundant in the endo-β-1,3-glucanase activity.

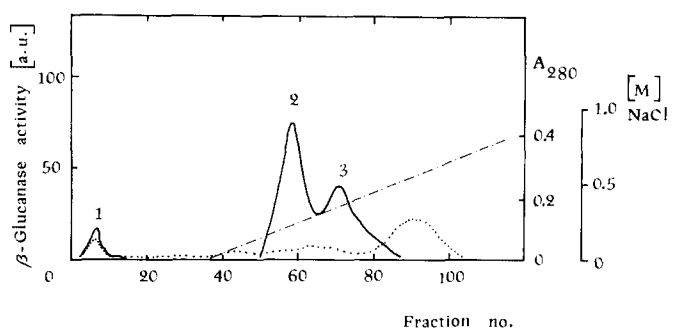

Fig. 6. DEAE-cellulose chromatography of proteins secreted into the growth medium by intact yeast cells. The conditions were the same as in Fig. 1. 1.2 mg of protein were applied to the column. 2.5-ml fractions were collected and $A_{280\ nm}$ (. .), NaCl concentration (— . — .) and the activity against laminarin (———) were measured.

Separation and identification of the intracellular β-glucanases

Fig. 7 shows the pattern of intracellular enzymes obtained by lysis of fresh yeast protoplasts after their resolution on the DEAE-cellulose column. In addition to the three enzyme fractions appearing extracellularly, a minor enzyme fraction (Peak B) which eluted from the column at 0.03 M NaCl was observed. The enzyme was active when assayed with laminarin and PNPG but not with oxidized laminarin. Due to the

variability of its occurence in different batches of protoplasts we did not investigate its properties any further. Since this fraction was not found to appear extracellularly its occurence seems to be limited to the cell interior.

DEAE-cellulose chromatography of the crude extracts from the log-phase yeast cells showed the presence of enzymes identical to those found in the extracts from protoplasts. The only exception was that the above-mentioned Peak B was scarcely detectable in the cell-free extracts from yeast cell.

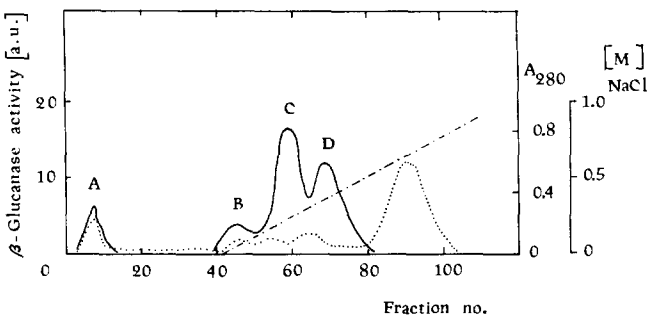

Fig. 7. DEAE-cellulose chromatography of proteins obtained by lysis of yeast protoplasts. 30 mg of protein were applied to the column. The conditions and the explanation of symbols are the same as in Fig. 6.

All enzyme fractions were stable when stored at −10 °C for several months or at 4 °C for at least a week. The repeated freezing and thawing did not influence the activity of the enzymes. However, they lost a considerable portion of their activity during concentration by freeze-drying. Due to this fact we were not able to express quantitatively the degree of purification achieved. The amount of protein in the enzyme fractions was very low in most cases and determination of specific activities was practically impossible. Our attention was therefore concentrated mainly on the qualitative aspects of the individual enzymes.

DISCUSSION

As was already shown in our previous study[9] the yeast protoplasts secrete *de novo* synthesized enzymes capable of hydrolyzing of laminarin and PNPG into the cultivation medium. The present study shows that the β-glucanase activity found in the supernatant of growing protoplasts is not due to a single enzyme but that the protoplasts, as well as the intact growing cells, secrete at least three β-glucanases differing both in their physicochemical properties and in their substrate specificity.

The use of yeast protoplasts allows a reliable differentiation of the extracellular enzymes from the intracellular ones. The finding that both protoplasts and intact cells secrete enzymes possessing endo-β-1,3-glucanase and exo-β-1,6-glucanase specificities into the growth medium seems to be of basic importance for the understanding of processes involved in the cell-wall extension and degradation. Even though the endo-β-1,3-glucanase, with relation to the chemical structure of the cell-wall glucan in *S. cerevisiae*[14,15], seems to play the crucial role in the degrading processes involved in the cell-wall growth, the enzyme exhibiting the hydrolytic action against β-1,6-linkages in

glucan may cooperate as well. Contrary to the findings of Arnold[8], we did not prove the presence of an endo-β-1,6-glucanase in our yeast strain.

As reported by Cortat *et al.*[3] about 87% of the total exo-β-1,3-glucanase activity in *S. cerevisiae* is released upon the conversion of intact cells into naked protoplasts. Since the washed intact cell walls do not exhibit measurable exo-glucanase activity[1], one may assume that this enzyme is localized in the periplasmic space and that a part of the enzyme is released into the growth medium during the budding process[3]. Nevertheless, slow but significant autohydrolysis proceeds in the isolated cell walls of *S. cerevisiae*[1]. It appears that this process is due to the action of endo-β-1,3-glucanases since in the course of autohydrolysis oligosaccharides of laminaribiose series are liberated from the cell walls[1].

The presence of endo-β-1,3-glucanases in the yeast cell walls brings about the question of how they are prevented from uncontrolled action on cell-wall glucans. Such an action would inevitably lead to lysis and death of the cells when they are deprived of substrate or when the anabolic processes in the cells are blocked. One explanation is that the bulk of extracellular endo-β-1,3-glucanases is not retained by the cell wall but that it is released into the medium as was demonstrated in the present study. Another possibility is that the enzymes trapped in the cell wall become largely converted into the latent form and that only the portion released into the medium remains fully active.

From our earlier studies it is known that 2-deoxy-D-glucose, present in the growth medium in the concentration not influencing the protein synthesis, effectively blocks the synthesis of cell-wall polysaccharides and the lysis of yeast cells ensues[16,17]. Blocking protein synthesis by cycloheximide or transfer of the lyzing cells from the medium with 2-deoxy-D-glucose to the buffer inhibits the lysis almost completely, (Farkaš, V., Biely, P. and Bauer, Š., unpublished). These facts indicate that *de novo* synthesis of the glucan-hydrolyzing enzymes, rather than their local activation is a necessary prerequisite for their function in the cell wall during various morphogenetic changes. This assumption is supported by the finding of Cortat *et al.*[3] that the synthesis of β-glucanases during the cell cycle slightly precede the initiation of budding in a synchronously growing culture of *S. cerevisiae*. Nevertheless, the possibility that the enzymes located in the cell wall may be repeatedly set into operation cannot be entirely ruled out when assuming that their activation is mediated through the action of some proteinaceous compound (enzyme). This very interesting feature of the budding process, as well as the mechanism of cell separation after finishing the septum between the mother and daughter cells deserves further investigation.

Except for one minor fraction (Peak B, Fig. 5) all β-glucanases present intracellularly were found also to occur extracellularly in the growth medium of protoplasts or intact cells. The extracellular enzymes had similar physicochemical properties to the corresponding intracellular ones, *i.e.* they were eluted from the DEAE-cellulose column at the identical concentrations of NaCl. It can be assumed that the intracellular enzymes represent a pool of the enzymes found extracellularly.

So far, all extracellular enzymes of yeast studies were found to be the glycoproteins[18]. In our experiments, only the enzyme fractions not retained by the DEAE-cellulose column contained non-diffusible carbohydrate. The heavy, glycoprotein form of invertase eluted from the column together with these enzymes. To answer the question whether all extracellular β-glucanases are glycoproteins would require fur-

ther study. In view of the recent critical survey of Winterburn and Phelps[19] not all extracellular proteins should be necessarily glycosylated.

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REFERENCES

1 Fleet, G. H. and Phaff, H. J. (1973) in *Yeast Mould and Plant Protoplasts (Proc. 3rd Int. Symp. Yeast Protoplasts, Salamanca, Spain, October 1972)*, Academic Press, London, in the press
2 Barras, D. R. (1972) *Antonie van Leeuwenhoek J. Microbiol. Serol.* 38, 65–80
3 Cortat, M., Matile, P. and Wiemken, A. (1972) *Arch. Mikrobiol.* 82, 189–205
4 Brock, T. D. (1965) *Biochem. Biophys. Res. Commun.* 19, 623–629
5 Abd-el-Al, A. and Phaff, H. J. (1968) *Biochem. J.* 109, 347–360
6 Shimoda, C. and Yanagishima, N. (1971) *Physiol. Plant.* 24, 46–50
7 Tingle, M. A. and Halvorson, H. O. (1971) *Biochim. Biophys. Acta* 250, 165–171
8 Arnold, W. N. (1972) *J. Biol. Chem.* 247, 1161–1169
9 Biely, P., Farkaš, V. and Bauer, Š. (1972) *FEBS Lett.* 23, 153–156
10 Goldstein, I. J., Hay, G., Lewis, B. A. and Smith, F. (1965) in *Methods Carbohydr. Chem.* (Whistler, R. L. and Wolfrom, M. L., eds), Vol. 5, pp. 361–370, Academic Press, New York
11 Korn, E. C. and Northcote, D. H. (1960) *Biochem. J.* 75, 12–17
12 Paleg ,L. G. (1959) *Anal. Chem.* 31, 1902–1904
13 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
14 Bacon, J. S. D., Farmer, V. C., Jones, D. and Taylor, I. F. (1969) *Biochem. J.* 114, 557–567
15 Manners, D. J. and Masson, A. J. (1969) *FEBS Lett.* 4, 122–124
16 Farkaš, V., Svoboda, A. and Bauer, Š. (1970) *Biochem. J.* 118, 755–758
17 Biely, P., Krátký, Z., Kovařík, J. and Bauer, Š. (1971) *J. Bacteriol.* 107, 121–129
18 Lampen, J. O. (1968) *Antonie van Leeuwenhoek J. Microbiol. Serol.* 34, 1–18
19 Winterburn, P. J. and Phelps, C. F. (1972) *Nature* 236, 147–151
20 Dixon, M. (1953) *Biochem. J.* 55, 170–171