Occurrence of an Endo-1,3- β -glucanase in Culture Fluids of the Yeast *Candida utilis*

PURIFICATION AND CHARACTERIZATION OF THE ENZYME ACTIVITY

By TOMAS G. VILLA,* VICENTE NOTARIO† and JULIO R. VILLANUEVA
Department of Microbiology, Faculty of Sciences and Institute of Biochemical Microbiology,
C.S.I.C. University of Salamanca, Salamanca, Spain

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The endo-1,3- β -glucanase (EC 3.2.1.6) secreted into the culture medium by cells of *Candida utilis* was isolated and purified to homogeneity on polyacrylamide-gel electrophoresis and in ultracentrifugation studies ($S_{20,w} = 1.97S$). The purified enzyme represented only 0.001 % of the total 1,3- β -glucanase activity, the remainder being due to an exo-1,3- β -glucanase enzyme, and behaved as an acidic glycoprotein (pI 3.3) in isoelectric-focusing experiments. The mol.wt. was estimated to be 21 000 by gel filtration and polyacrylamide-gel electrophoresis. Studies on the hydrolysis of different substrates showed that the enzyme was only able to break down (1 $\rightarrow$ 3)- β -linkages, by an endo-splitting mechanism. Glucono- δ -lactone, D-glucoronolactone and heavy metal ions such as Hg^{2+} were inhibitors of the enzyme activity. The function of this endo- β -glucanase in *C. utilis* is discussed.

In yeasts, and particularly in those where 1,3- β -glucan is the component responsible for the rigidity and strength of the cell wall, it appears that 1,3- β -glucanases may produce architectural changes during morphogenetic processes. These enzymes may hydrolyse the substrates either by an endo-splitting system (Fleet & Phaff, 1974) or by an exo-splitting one (Bochkov *et al.*, 1972). Both kinds of enzyme would act synergistically to hydrolyse completely the cell-wall β -polymers, as suggested by Johnson (1968). Although both endo- and exo-glucanases have been found together in some yeasts (Villa *et al.*, 1975), the presence of exo- β -glucanases only in many other cases makes it more difficult to understand their mode of action.

Initial evidence showed that exo-splitting 1,3- β -glucanases were the exclusive β -glucanases present in culture fluids (Villa *et al.*, 1976b) as well as in cell-free extracts (Notario *et al.*, 1976b) of *Candida utilis*. However, experiments carried out in our laboratory showed that other yeast species thought to synthesize only exo- β -glucanases in fact produced and secreted into the culture media low amounts of endo- β -glucanases (Villa *et al.*, 1975). The present paper describes the isolation, purification and characterization of this type of enzyme from culture fluids of *C. utilis*.

* To whom reprint requests should be addressed.

† Present address: Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

Experimental

Materials

Micro-organism and culture conditions. *Candida utilis* (C.E.C.T. 1061) was obtained from the Spanish Type Culture Collection, Salamanca, Spain. Stock cultures were maintained in slant tubes on solid medium containing 20 g of agar, 5 g of Difco yeast extract and 20 g of D-glucose/litre. *C. utilis* cells were transferred to a liquid medium (Winge, 1935) containing 20 g of glucose and 3 g of Difco yeast extract per litre as the only ingredients. The cells were then incubated aerobically on an orbital shaker (240 rev./min) at 27 °C. Exponential-phase cells were harvested by centrifugation (5000g, 10 min) and discarded, whereas the supernatant fluid was dialysed overnight against water and used as source of enzymes.

Chemicals. Laminarin and nigeran were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Pustulan was prepared by the method of Lindberg & McPherson (1954) from *Umbilicaria pustulata*, and oat glucan was prepared by the method of Clarke & Stone (1966). Oxidized laminarin was obtained by the method of Goldstein *et al.* (1965). Yeast cell wall glucan and mannan were prepared from cell walls of *C. utilis* and *Saccharomyces cerevisiae* respectively, as recommended by Manners *et al.* (1973). DEAE-Sephadex A-50, Sephadex G-50, G-100 and G-200 and concanavalin A-Sepharose 4B were from Pharmacia, Uppsala, Sweden, and Bio-Gel P-10 was from Bio-Rad Laboratories, Richmond, CA,

U.S.A. Gentiobiose, cellobiose, glucono- δ -lactone, D-glucuronolactone, *p*-nitrophenol derivatives, yeast alcohol dehydrogenase (EC 1.1.1.1), L-amino acid oxidase (EC 1.4.3.2), NAD⁺ and Dowex 50W resin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Salicin, cellulose and dextran were purchased from BDH Chemicals, Poole, Dorset, U.K.

Methods

Enzyme preparation. After dialysis overnight at 4°C against water, the culture medium was adjusted to pH 7.0 by addition of Sigma 7-9 biochemical buffer and its conductivity to 2 pS. DEAE-Sephadex A-50 (3 g) was then added and, after 30 min shaking, the gel was collected by filtration, resuspended in 1 M-NaCl, shaken for 10 min and filtered again. The filtrate (53 ml) was first dialysed 20 h against water and then overnight against 50 mM-sodium acetate buffer (pH 5.0), the dialysis fluid being renewed at 4 h intervals. Poly(ethylene glycol) was used to concentrate the non-diffusible material in the dialysis bags to a final volume of 2.0 ml (protein content 57.5 mg/ml), which was then used in the following purification steps.

Gel filtration. Filtrations were performed on columns (2.5 cm $\times$ 40 cm) of Sephadex G-50, G-100, G-200 and Bio-Gel P-10 previously equilibrated with 50 mM-sodium acetate buffer (pH 5.0). Samples were eluted with the same buffer. Concanavalin A-Sepharose 4B filtrations were carried out on columns (1 cm $\times$ 8 cm) previously equilibrated with 0.1 M-Tris/maleate buffer (pH 6.8). Samples were first eluted with the same buffer and then with 0.36 M-methyl α -D-mannoside in the same buffer. In all case samples were eluted at 4°C at a flow rate of 32 ml/h and 5 ml fractions were collected. The protein content in the fractions was measured with a u.v.-monitoring system.

Paper chromatography. Descending paper chromatography was used to characterize the hydrolysis products of the enzymic reactions. The products were chromatographed on Whatman no. 1 filter paper with ethyl acetate/pyridine/water (8:2:1, by vol.) as solvent for 24 h. Glucose, gentiobiose and laminari-dextrins were used as references. Spots were developed by the method of Trevelyan *et al.* (1950).

Sedimentation pattern. The sedimentation velocity pattern of the purified enzyme was studied in a Spinco L2 65B ultracentrifuge. According to the method of Martin & Ames (1961) the sedimentation coefficient was determined by comparing with proteins of known $s_{20,w}$ values and molecular weights. Rotor speed was 183000 g (r_{av} , 6.4 cm), and the protein concentration of the sample 10 μ g/ml in 50 mM-sodium acetate buffer (pH 5.0). Yeast alcohol dehydrogenase and L-amino acid oxidase from *Crotalus adamanteus* venom were used as standards, being assayed as indicated by Martin & Ames (1961) and Ratner (1955) respectively.

Analytical disc electrophoresis. After each purification step, disc electrophoresis was performed on polyacrylamide gel (75 g/l) at pH values of 6.5, 7.0 and 8.0 (200 V, 2.5 mA/gel, 3 h) as described by Weber & Osborn (1969). The enzyme activity was measured directly on the gels in parallel with the protein content and total carbohydrate determinations, as reported by Notario *et al.* (1976b).

Isoelectric focusing. Isoelectric focusing was carried out in an LKB column (110 ml) at 6°C in the pH range 3-6. The gradient was formed with glycerol and the samples were run for 48 h at 500 V. Then the column was emptied into 1.5 ml fractions. The protein content was recorded by using a u.v.-monitoring system. The pH of each fraction was checked with a Radiometer (Copenhagen) Titrator 11 pH-meter no. 28.

Enzyme assays. The standard reaction mixtures (0.5 ml) contained enzyme (0.1 ml) and buffered substrate (0.4 ml) at a final concentration of 2.5 mg/ml in 50 mM-sodium acetate buffer, pH 5.0. The enzyme activity is expressed in units as nmol of glucose or equivalent reducing power released from each substrate/min per mg of protein at 37°C.

The enzyme assays to elucidate the optimum pH for the purified enzyme were carried out by using different buffers (0.1 M solutions in all cases) within the pH range 2.5-10.5. Each assay contained 0.1 ml of enzyme, 0.2 ml of laminarin in water solution and 0.2 ml of the suitable buffer.

The influence of the temperature on the reaction rate was evaluated from enzyme assays at temperatures from 20 to 80°C, under the same conditions as above.

The effect of pH and temperature on the denaturation of the enzyme was determined from enzyme assays developed under usual conditions, but with enzyme samples treated for 10 min at different pH and temperature values. The optimum pH and temperature values were established from the results of both types of experiments.

Inhibition experiments. Inhibition studies with glucono- δ -lactone and D-glucuronolactone were carried out by adding 0.3 ml of buffered laminarin and 0.1 ml of freshly prepared inhibitor solutions (both at final concentrations up to 10 mM) to 0.1 ml of enzyme preparation. This same procedure was used for experiments involving metal ions (Hg²⁺, Ag⁺). The sources of these ions were HgCl₂ and AgNO₃ respectively, and the final concentration of each ion in the tests was up to 3 mM. In all the inhibition experiments, controls involved the same amount of enzyme previously inactivated by 5 min heating at 100°C.

Analytical measurements. Total carbohydrate content was determined by the method of Dubois *et al.* (1956) or by that of Park & Johnson (1949). Reducing sugars were measured by the method of Somogyi (1952) as modified by Nelson (1957), and the results

referred to glucose as the standard. Free glucose determination was performed by the enzymic method of Keston (1956). The activity on *p*-nitrophenol derivatives was measured spectrophotometrically. Protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, or by A_{280} measurements. The amount of protein was determined as A_{254} when a u.v.-monitoring system was used.

Results

Enzyme purification

The same purification process as described by Villa *et al.* (1976b) was used. Table 1 summarizes the results obtained in the purification of the enzyme. The enzyme studied in the present paper behaved exactly as the exo-1,3- β -glucanase reported by Villa *et al.* (1976b), and it could be only separated at the last purification step by its different behaviour in concanavalin A-Sepharose 4B filtration, where this enzyme was strongly adsorbed on the gel, being eluted only after intensive washing of the column with 0.36M-methyl α -D-mannoside in 0.1M-Tris/maleate buffer, pH6.8.

After dialysis against water and 50mM-sodium acetate buffer (pH5.0), the purity of the enzyme was checked on polyacrylamide-gel electrophoresis and by ultracentrifugation experiments. The purified enzyme sedimented as a single protein after ultracentrifugation (Fig. 1), and the estimated sedimentation coefficient ($s_{20,w}$) was 1.97S. When electrophoresis were performed samples were applied to three gels and run as described. At the end the first gel was stained for protein and the other two were cut into 1 mm slices; the second was tested for β -glucanase and the third one for total carbohydrate. In all cases a single band only was recovered at the same distance from the origin of the gels (Fig. 2).

Fig. 1. Sedimentation pattern of the purified endo-1,3- β -D-glucanase (●)

L-Amino acid oxidase from snake venom (○) and yeast alcohol dehydrogenase (Δ) were used as standards. Experimental conditions were those indicated by Martin & Ames (1961).

Fig. 2. Polyacrylamide-disc electrophoresis of the enzyme after the last purification step

The electrophoretic behaviour of protein (a) and enzymic activity (b) was determined directly on the gels as indicated in the text. Protein, enzyme activity (□) and total carbohydrate (■) moved as single bands always at the same distance from the origin of the gels. M, marker of the front.

Table 1. Summary of steps involved in the purification of the endo-1,3- β -D-glucanase from culture fluids of the yeast *Candida utilis*

To avoid interference by the exo-1,3- β -D-glucanase, periodate-oxidized laminarin was used as substrate for the endo-enzyme throughout the purification process.

Purification step	Volume (ml)	$10^{-3} \times \beta$ -Glucanase activity (units/ml)	Total protein (mg/ml)	$10^{-2} \times$ Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Adsorption on DEAE-Sephadex A-50	2	78.7	57.5	13.7	—	100
Sephadex G-50 filtration	2.5	62.9	34.3	18.3	1.33	100
Sephadex G-200 filtration	2.5	44.0	10.7	41.1	2.99	70.1
Bio-gel P-10 filtration	2.5	26.6	5.7	46.2	3.36	42.3
Concanavalin A-Sepharose 4B filtration	1	0.15	0.03	48.2	3.51	0.1

Enzyme composition

No clearly conclusive results were obtained on the glycoprotein nature of the purified enzyme either from gel filtration or from total carbohydrate staining of the polyacrylamide gels. Samples of purified enzyme were hydrolysed by treatment with 6M-HCl at 100°C for 12 h. After removal of the excess of HCl by evaporating under vacuum, the hydrolysate was filtered through a Dowex 50 W column (2 cm × 15 cm). The neutral sugar fraction was collected by elution of the column with water and, after concentration under vacuum, was tested for total carbohydrate. This experiment showed that the enzyme had a low carbohydrate content which would correspond to about 3% of the molecule. The monosaccharides in the hydrolysate were tentatively identified by paper chromatography. These analyses indicated that, although very little glucose and mannose could be detected, mannose was rather predominant. The ratio glucose/mannose (determined from samples eluted with water from the chromatographic paper) was about 0.8.

The amino acid-containing fraction was obtained by eluting the Dowex 50 W column with 0.5M-NH₃. The amino acid analysis proved that the enzyme had a high content of acidic amino acids, although other amino acids such as glycine and serine were also present in high percentages. The amino acid composition was determined in a JEOL JLC-5AH amino acid autoanalyser. The composition was very similar to those described for exo- β -glucanases from *C. utilis* (Villa *et al.*, 1976b; Notario *et al.*, 1976b).

Enzyme properties

Molecular weight. Molecular weight determination was carried out by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) and by gel filtration by the method of Whitaker (1963). Bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), trypsin (mol.wt. 23800) and cytochrome *c* (mol.wt. 12300) were used as standards for both techniques. The average mol.wt. from these experiments was estimated to be 21000.

Effect of pH and temperature. The highest enzyme activity was obtained at pH 5.0 and at 50°C. Whereas no degradation of the enzyme could be detected at pH 5.0, 7% loss of activity was observed after 10 min at 50°C. From the results shown in Fig. 3, the optimum values were pH 5.0 and 37°C, so the enzymic assays were usually performed under these conditions.

Isoelectric focusing. The purified enzyme behaved as an acidic protein in isoelectric-focusing experiments with pI 3.2 (Fig. 4).

Substrate specificity. The activity of the purified β -glucanase was tested against several substrates showing different glycosidic linkage types (Table 2). No

Fig. 3. Effect of pH (a) and temperature (b) on the purified endo-1,3- β -D-glucanase

Assays were carried out under different pH or temperature conditions (○), or under the usual conditions described in the text, but with enzyme samples maintained for 10 min at the pH or temperature values (●).

Fig. 4. Isoelectric-point determination for the purified β -glucanase

The pH range (Δ) of the carrier Ampholines was 3–6. Both β -glucanase activity (○) and protein (●) focused at pH 3.2, were detected as single bands.

activity could be detected when the substrates contained (1 $\rightarrow$ 4)- β -, (1 $\rightarrow$ 6)- β - or α -linkages as the main linkage in the molecule, and only very low activity was found on substrates containing mixed (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)- β -linkages such as cereal glucans. Therefore the purified enzyme was a specific 1,3- β -glucanase.

Pattern of hydrolysis. Chromatographic analysis of the hydrolysis products of the enzyme reactions showed that the first products liberated when the activity was tested against laminarin, oxidized laminarin or yeast cell-wall 1,3- β -glucan were laminaridextrins, whereas no glucose could be detected. However, as indicated in Table 3, glucose began to appear after long incubation periods.

The hydrolysis products formed by the enzyme after 2 h incubation against oxidized laminarin were identified by paper chromatography as trace amounts of glucose, laminaribiose and laminaritriose, and high concentration of laminaritetraose. However, higher laminaridextrins could not be detected. These results, in addition to the fact that the enzyme did not show activity against *p*-nitrophenyl β -D-glucopyrano-

Table 2. *Substrate specificity of the purified endo-1,3- β -D-glucanase from culture fluid of Candida utilis*
The assays were performed for 1 h at 37°C. The assay mixture contained 0.2 ml of enzyme plus 0.8 ml of substrate (2.5 mg/ml) in 50 mM-sodium acetate buffer (pH 5.0).

Substrate	Main linkage type	Hydrolysis
Laminarin	(1 $\rightarrow$ 3)- β	+
Periodate-oxidized laminarin	(1 $\rightarrow$ 3)- β	+
Pustulan	(1 $\rightarrow$ 6)- β	—
Nigeran	(1 $\rightarrow$ 3)- α ; (1 $\rightarrow$ 4)- α	—
Oat glucan	(1 $\rightarrow$ 3)- β ; (1 $\rightarrow$ 4)- β	+
Yeast glucan	(1 $\rightarrow$ 3)- β	+
Yeast mannan	(1 $\rightarrow$ 6)- α ; (1 $\rightarrow$ 2)- α	—
	(1 $\rightarrow$ 3)- α ; (mannose)	—
Gentiobiose	(1 $\rightarrow$ 6)- β	—
Cellobiose	(1 $\rightarrow$ 4)- β	—
Cellulose	(1 $\rightarrow$ 4)- β	—
Salicin	β -Glycosidic	—
Dextran	(1 $\rightarrow$ 3)- α	—
<i>p</i> -Nitrophenyl β -D-glucopyranoside		—
<i>p</i> -Nitrophenyl β -D-xylopyranoside		—
<i>p</i> -Nitrophenyl phosphate		—

Table 3. *Mechanism of hydrolysis of the endo-1,3- β -D-glucanase purified from culture fluids of Candida utilis*
The purified enzyme was incubated under the usual conditions with oxidized laminarin. At different times samples were taken, tested for reducing sugars and free glucose, and hydrolysis products characterized by paper chromatography, as indicated in the text. Enzymic activity is given as units/ml of assay.

Incubation period (min)	Reducing sugars	Glucose	Hydrolysis products
15	90	0	Laminaritetraose Laminaribiose
30	164	0	Laminaritetraose Laminaribiose
60	227	38	Laminaritetraose Glucose
90	284	62	Laminaritetraose Laminaribiose Glucose
120	338	89	Laminaritetraose Laminaritriose Laminaribiose Glucose
150	395	129	Laminaritetraose Laminaritriose Laminaribiose Glucose

side (a typical substrate for exo- β -glucanases), suggested that the purified enzyme was an endo-splitting one.

Determination of Michaelis constant. The K_m values were calculated by the method of Lineweaver & Burk (1934) with purified enzyme acting on laminarin and oxidized laminarin (Fig. 5), being estimated to be 0.21 and 0.19 mM respectively. For laminarin a mol.wt. of 4000 was assumed, equivalent to a degree of polymerization of 25 glucose residues (Reese & Mandels, 1963). An inhibitory effect on the enzyme activity was observed at high substrate concentrations when the rate of hydrolysis was measured as a function of the concentrations of both laminarin and periodate-oxidized laminarin. The extent of inhibition was similar in both cases, but clearly lower than that reported for the exo- β -glucanases purified from *C. utilis* (Villa *et al.*, 1976b; Notario *et al.*, 1976b).

Inhibition of β -glucanase activity. Glucono- δ -lactone, D-glucuronolactone and Hg^{2+} inhibited the enzyme activity, whereas Ag^+ had very little effect on the β -glucanase. The inhibitor constants (K_i) were determined by the graphic method of Dixon (1953). The estimated K_i values were 30.75 mM for glucono- δ -lactone, 17.5 mM for D-glucuronolactone and 0.45 mM for Hg^{2+} . The strongest inhibition corresponded to Hg^{2+} which promoted 60 % loss of activity at 0.1 mM concentration.

D-Glucuronolactone inhibited β -glucanase activity more strongly than glucono- δ -lactone did (Fig. 6), and the inhibition kinetics were somewhat different for both compounds. When the enzyme activity was tested by adding a series of increasing inhibitor concentrations to the usual assays, but keeping the sub-

Fig. 5. Influence of substrate concentration on the activity of the purified enzyme

The activity was tested against increasing concentrations of laminarin under the usual conditions (a), and the K_m value was estimated from these results by the graphic method of Lineweaver & Burk (1934) by plotting $1/v$ versus $1/[S]$ (b).

Fig. 6. Non-competitive inhibition of the purified enzyme by glucono- δ -lactone

The activity of the purified enzyme against several laminarin concentrations was tested in the presence of increasing inhibitor concentrations under conditions described in the text. The substrate concentrations were 0.5 mg/ml (1), 1 mg/ml (2), 2.5 mg/ml (3) and 5 mg/ml (4) prepared in each case in the usual buffer. The K_i value was 30.75 mM.

strate concentration constant, a straight line was obtained for glucono- δ -lactone on plotting $1/v$ versus the inhibitor concentration, as shown by Dixon (1953). However, this straight line was only obtained for low D-glucuronolactone concentrations. In all cases the inhibitory mechanism was non-competitive.

Discussion

As previously reported (Villa *et al.*, 1976b), preliminary evidence on the presence of two different types of β -glucanase enzymes in culture fluids of *C. utilis* was obtained after filtration through concanavalin A-Sepharose 4B columns. The same authors reported the purification of the major form, an exo- β -glucanase, which was not retained on concanavalin A-Sepharose 4B. The activity present in much lower proportion (only 0.001 % of the total) has now been isolated and purified to a degree that is in agreement with those reported for other endo- β -glucanases from yeast (Villa *et al.*, 1975) or bacterial (Fleet & Phaff, 1974) preparations. In general, enzyme properties such as molecular weight and sedimentation pattern are within the range reported by other workers. Thus the ratio molecular weight to $s_{20,w}$ for the purified enzyme was 1.07×10^4 , a value very close to those calculated from data described by Huotari *et al.* (1968), Villa *et al.* (1976b) and Notario *et al.* (1976b), which were respectively 1.3×10^4 , 1.2×10^4 and 1.15×10^4 .

It seems clear that this enzyme is a specific 1,3- β -glucanase only able to break down 1,3- β -linkages through an endo-splitting mechanism, as evidenced

by the analysis of its hydrolysis products (Table 3). The explanation of why this endo-enzyme produced glucose as a hydrolysis product is not clear. In fact some other purified endo- β -glucanases produced glucose together with laminaridextrins (Farkas *et al.*, 1973). The possibility of contamination with any exo- β -glucanase or β -glucosidase activity was taken into account, but all our attempts to obtain some endo-glucanase preparation not producing glucose were unsuccessful. According to this it may be postulated that the enzyme shows a mixed mode of activity, behaving firstly as an endo-enzyme, but being able to act as an exo-enzyme on substrates with lower degrees of polymerization. This would explain the fact that no laminaridextrin larger than laminaritetraose was detected among the hydrolysis products. However this problem requires more detailed studies.

The purified enzyme was found to be an acidic protein with pI 3.2. β -Glucanases with low pI values (about 4.0) have been reported by other authors (Brown, 1972; Notario *et al.*, 1976a), but always in agreement with their content of acidic amino acids. However, the enzyme now studied, although containing similar proportions of these acidic amino acids, showed a lower isoelectric point, which was rather in the range of that described by Villa *et al.* (1976b) for the exo- β -glucanase from culture fluids of *C. utilis*. On account of their amino acid compositions both exo- and endo- β -glucanases had a net negative charge and therefore moved towards the anode in polyacrylamide-gel electrophoresis (Fig. 2) and were easily adsorbed on DEAE-Sephadex A-50, but their pI values were too low to be explained only on the basis of their amino acid compositions. The use of several methods (Svennerholm, 1957; Miettinen & Takki-Luukkainen, 1959; Aminoff, 1961) to detect the possible presence of sialic acids as part of the molecule did not allow any significant explanation.

Different reports have been published during the last few years in which β -glucanase enzymes were shown as glycoproteins (Villa *et al.*, 1976b; Notario *et al.*, 1976b; Biely *et al.*, 1976; Lachance *et al.*, 1977). According to these authors, and as could be expected from its interaction with concanavalin A, the purified enzyme appeared as a glycoprotein, although its carbohydrate content was lower than those reported for exo-glucanases from the same yeast. Another important difference with respect to the exo-enzymes was that those enzymes contained glucose as the main component of their carbohydrate moieties, whereas this endo- β -glucanase seems to be mainly formed by mannose. This result is in agreement with those obtained by Biely *et al.* (1976), by indirect techniques, for only partially purified β -glucanases of *Sacch. cerevisiae*.

Although Reese *et al.* (1971) described glucono- δ -lactone and other similar compounds as 'poor inhibitors' for exo- and endo-glucanases, the purified

enzyme was clearly inhibited by both glucono- δ -lactone and D-glucuronolactone, according to Villa *et al.* (1976a). The strong inhibition promoted by small amounts of Hg^{2+} might indicate either that thiol groups of the cysteine residues are involved in the active catalytic site or that these groups are essential in maintaining the enzyme structure.

The activity of the purified enzyme either against purified yeast cell-wall 1,3- β -glucans (Table 2) or 1,3- β -glucans forming part of the whole structure of purified cell walls may be important for improving our understanding of the role that this enzyme must play in morphogenetic processes, probably through a co-operative system with exo-enzymes (Johnson, 1968). The fact that no endo-glucanase has been found in cell-free extracts of *C. utilis* may be explained as suggested by Farkas *et al.* (1973). The enzyme would be trapped in some way by the cell wall after its secretion to the periplasmic space and converted into a latent form to prevent uncontrolled actions on cell-wall glucans. This latent form would be fully active after being secreted into the culture medium.

However, the presence of the active form in the culture fluids makes it more difficult to understand why cell-wall lysis does not take place in the stationary phase of growth, when the budding process is absent and all the cellular structures are in progressive disorganization. Villa *et al.* (1976b) proposed that the protection of glucans in the absence of budding mechanisms would be determined by 'some architectural factor' affecting the cell walls. This problem and that of the activation of the latent β -glucanase form at the beginning of the budding process have not yet been elucidated.

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