Purification of an Exo- β -D-glucanase from Cell-Free Extracts of *Candida utilis*

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β -Glucanase present in cell-free extracts from *Candida utilis* was isolated and purified 562-fold by procedures that include adsorption on DEAE-Sephadex A-50 and filtration through columns of Sephadex G-50, G-100 and G-200, Bio-Gel P-10, and Concanavalin A-Sepharose 4B. The purified enzyme appeared homogeneous on polyacrylamide-gel electrophoresis and in ultracentrifugation studies ($s_{20,w} = 1.74S$). The enzyme behaved as an acidic glycoprotein (pI 4.1) with 68% carbohydrate and a high content of acidic amino acids. The mol.wt. was estimated to be 20000 from gel filtration and polyacrylamide-gel electrophoresis and 36000 from sedimentation experiments. Studies on the hydrolysis of different substrates showed that the enzyme is an unspecific β -glucanase able to break down both (1 $\rightarrow$ 3)- β - and (1 $\rightarrow$ 6)- β -linkages by an exo-splitting mechanism. Glucono- δ -lactone, Zn^{2+} and Hg^{2+} inhibited the enzyme activity.

β -Glucanases may be classified into two groups by their mode of action: endo- and exo- β -D-glucanases. The presence of the exo- β -D-glucanases in different micro-organisms has been widely reported. Huotari *et al.* (1968) purified an exo- β -D-glucanase from culture fluids of the basidiomycete QM 806. Abd-El-Al & Phaff (1968) studied exo- β -D-glucanases from several yeasts, and Notario *et al.* (1976) reported the existence of exo- β -D-glucanase in cell-free extracts of the yeast *Cryptococcus albidus* (var. *aerius*). In all these cases exo- β -glucanases were the only type of glucanase present in these micro-organisms. Moreover Fleet & Phaff (1974) and Villa *et al.* (1975a) described the coexistence of both endo- and exo- β -D-glucanases in different yeasts.

It appears that β -glucanase enzymes may produce architectural changes in the cell wall during yeast and fungal morphogenesis. With respect to this, the mechanism of β -glucanase activity is easier to understand when there are both endo- and exo- β -D-glucanases in the same micro-organism than when only one of them is present. Johnson (1968) proposed a co-operative action between both types of glucanases to explain processes such as budding and cell-wall elongation. This kind of mechanism has been described by Jones *et al.* (1974) to account for the degradation of *Sclerotinia sclerotiorum* cell-wall glucans. This mechanism cannot be considered when only exo- β -D-glucanase activity is present in the cells. Nelson *et al.* (1969) and Bochkov *et al.* (1972) studied the activity pattern of some exo- β -D-glucanases. However, little is known of their role *in vivo*. We now describe the purification of one exo- β -D-glucanase, which is the only type of β -glucanase in cell-free

extracts of *Candida utilis*. The present paper also deals with the analysis of the composition and with the biochemical characterization of the purified enzyme.

Experimental

Micro-organism and culture conditions

Candida utilis (C.E.C.T. 1061) was from the Spanish Type Culture Collection, Salamanca, Spain. Stock cultures were grown in slant tubes with a solid medium containing 20g of agar, 20g of glucose and 5g of Difco yeast extract per litre. *C. utilis* cells were transferred to a liquid Winge (1935) medium containing 20g of glucose and 3g of Difco yeast extract per litre. The cells were then incubated aerobically in a rotary shaker (240 rev./min) at 27°C.

Enzyme preparation

Cells grown for 24 h in the liquid Winge medium as above were harvested by centrifugation and washed with 50 mM-sodium acetate buffer (pH 5.5). After washing, the cells were resuspended in the same buffer and broken by treatment for 2 min in an MSK Braun homogenizer with refrigeration. The unbroken cells and the cell-wall residues were removed by low-speed centrifugation. The supernatant was again centrifuged at 40000g for 30 min. The soluble fraction was adjusted to pH 7.5 by the addition of Sigma 7-9 biochemical buffer (Sigma Chemical Co., St. Louis, MO, U.S.A.) and its conductivity to 2 p Ω . DEAE-Sephadex A-50 (5g) was added and it was then shaken

for 30 min. The mixture was filtered and the gel resuspended in 2 M-NaCl and filtered again. The filtrate was first dialysed against water and then against 50 mM-sodium acetate buffer (pH 5.5). Polyethylene glycol was used to concentrate the non-diffusible material in the dialysis bags, that was then used in the following steps of the purification process.

Chemicals

Gentiobiose, cellobiose, glucono- δ -lactone, *p*-nitrophenyl β -D-glucopyranoside, *p*-nitrophenyl phosphate, yeast alcohol dehydrogenase, L-amino acid oxidase (from *Crotalus adamanteus* venom), Dowex-50W and β -diphosphopyridine nucleotide were from Sigma. Laminarin and nigeran were from Koch-Light Laboratories, Slough, Bucks., U.K. Dried pyridine, chloromethylsilane and 1,1,1,3,3,3-hexamethyldisilazane were from Merck, Darmstadt, Germany. DEAE-Sephadex A-50 and Sephadex G-50, G-100 and G-200 were from Pharmacia, Uppsala, Sweden, and Bio-Gel P-10 and P-2 from Bio-Rad Laboratories, Richmond, CA, U.S.A. Pustulan was prepared from *Umbilicaria pustulata* as indicated by Lindberg & McPherson (1954). Oxidized laminarin was obtained by the method of Goldstein *et al.* (1965). Yeast glucan and mannan were prepared from cell walls of *C. utilis* and *Saccharomyces cerevisiae* respectively, as recommended by Manners *et al.* (1973). Oat glucan was prepared by the method of Clarke & Stone (1966).

Methods

Gel filtration. Filtrations were performed on columns (2 cm $\times$ 37 cm, 2.5 cm $\times$ 40 cm and 3 cm $\times$ 70 cm respectively) of Sephadex G-50, G-100 and G-200 previously equilibrated with 50 mM-sodium acetate buffer (pH 5.5). Columns (2 cm $\times$ 40 cm and 0.9 cm $\times$ 2 cm respectively) of Bio-Gel P-2 and P-10 prepared as above were also used. Samples were eluted with the same buffer. Concanavalin A-Sepharose 4B filtration was performed on a column (1 cm $\times$ 10 cm) previously equilibrated with 0.1 M-Tris/maleate buffer (pH 6.8). Samples were first eluted with the same buffer and then with 7% (w/v) methyl α -D-mannoside prepared in the same buffer. In all cases, samples were eluted at a flow rate of 23 ml/h and 5 ml fractions were collected. Protein was measured with a u.v. monitoring system.

Analytical gel electrophoresis. At the end of each purification step polyacrylamide-gel electrophoresis was performed at pH 8.0 (200 V, 2.5 mA/gel, 3 h) on 7.5% (w/v) gel as described by Weber & Osborn (1969). In every case the electrophoretic behaviour of the β -glucanase was followed by measuring enzymic activity in the gels parallel to the protein determination. Therefore sodium dodecyl sulphate

was omitted and protein denaturation was not performed when enzymic activity was checked in this way. After electrophoresis the gels were cut into 1 mm slices, which were transferred to tubes containing 0.5 ml of the buffered substrate. After 5 h incubation at 37°C samples were assayed for reducing sugars. Periodically the gels were tested for total carbohydrate content.

Isoelectric focusing. Isoelectric focusing of the purified enzyme was performed by using a focusing system obtained from LKB-Produkter AB, Stockholm, Sweden. The density gradient was formed with glycerol and ampholyte (pH 3–6) and used at a concentration of 1% (v/v) in a total volume of 110 ml. The voltage was adjusted to 500 V. After 48 h at 6°C, focusing was finished and 2 ml fractions were collected. The protein profile was recorded by using a u.v. monitoring system. The pI value was checked by a Radiometer Copenhagen Titrator 11 pH-meter no. 28.

Sedimentation behaviour. Sedimentation coefficient of the purified enzyme was determined in a Spinco L2 65B ultracentrifuge by comparing with proteins of known molecular weight and $s_{20,w}$, as proposed by Martin & Ames (1961). Yeast alcohol dehydrogenase and L-amino acid oxidase from *Crot. adamanteus* venom were used as standards and assayed as indicated by Martin & Ames (1961) and Ratner (1955) respectively.

Enzyme hydrolysis and amino acid analysis. The purified enzyme was hydrolysed by treatment for 12 h with 6 M-HCl at 100°C. On occasions 3 M-HCl was used. In all cases the excess of HCl was removed by evaporating the hydrolysate under vacuum. Then the hydrolysate was filtered through a column of Dowex-50W (2 cm $\times$ 15 cm). Neutral sugars were obtained by eluting the column with water and the amino acids by eluting with 0.5 M-NH₃. The amino acid composition was determined in a Jeol-JLC-5AH amino acid autoanalyser.

Paper chromatography. Descending paper chromatography was used to characterize the hydrolysis products of the enzymic reactions and the carbohydrate composition of the purified enzyme. Glucose was used as the only standard. Chromatography was carried out for 36 h on Whatman no. 1 filter paper with ethyl acetate/pyridine/water (8:2:1, by vol.) as solvent. Spots were developed by the method of Trevelyan *et al.* (1950).

Gas-liquid chromatography. G.l.c. was used to characterize the neutral sugars obtained from the hydrolysis of the purified enzyme. The solution containing neutral sugars was prepared for g.l.c. by the method of Sweely *et al.* (1963). G.l.c. was performed on a column (1.83 m $\times$ 3 mm) of 10% UCC-W982 Chromosorb W HP 80–100. Column temperature was maintained at 190°C for 16 min and then programmed to increase from 190° to 250°C, at 4°C per

min, and maintained at 250°C for 2 min. A Hewlett-Packard 5710-A gas chromatograph with a flame-ionization detector was used. Detector temperatures were maintained at 300°C and the carrier gas was N₂ at a flow rate of 40 ml/min.

Enzyme assays. Reaction mixtures (1 ml) contained enzyme plus buffered substrate (50 mM-sodium acetate buffer, pH 5.5). The final concentration of substrates was 2.5 mM. Enzymic activity is expressed in arbitrary units as nmol of *p*-nitrophenol, glucose or equivalent reducing power released from each substrate/min per mg of protein, at 37°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 using glucose oxidase coupled to peroxidase (Keston, 1956). Enzymic activity on *p*-nitrophenyl derivatives was measured spectrophotometrically. Protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard, or by E_{280} measurements. The amount of protein was determined as E_{254} when a u.v. monitoring system was used.

Results

Enzyme purification

The breakage solution from *C. utilis* cells was used as source of enzyme for purifying β -glucanase activity.

Fig. 1. Concanavalin A-Sepharose 4B filtration of an enzymic fraction containing *exo*- β -D-glucanase activity

The elution pattern was obtained by using 0.1 M-Tris/maleate buffer, pH 6.8, as effluent. ○, Enzymic activity; ●, total carbohydrate content (determined by the phenol/H₂SO₄ method and expressed as E_{490}); △, E_{254} .

The purification process included adsorption on DEAE-Sephadex A-50 and filtration through columns of Sephadex G-50, G-100 and G-200, Bio-Gel P-10 and concanavalin A-Sepharose 4B (Fig. 1). After these experiments a final purification of 562-fold was obtained. Table 1 summarizes the results obtained in the purification of the enzyme. In every case the purity of the enzyme was checked on polyacrylamide-gel electrophoresis at the end of each purification step and also by ultracentrifugation at the end of the complete purification process.

When the electrophoresis were performed samples were placed on three polyacrylamide gels and run as described. The first was stained for protein and the other ones were cut into 1 mm slices; the second was tested for β -glucanase activity and the third one for total carbohydrate. In all cases only one band was detected after the last purification step. Protein (Fig. 2b), β -glucanase activity (Fig. 2a) and total carbohydrate (Fig. 2c) were detected at the same distance from the origin of the gels.

The purified enzyme sedimented as a single protein when ultracentrifugation was used to check the enzymic purity (Fig. 3). The estimated sedimentation coefficient ($s_{20,w}$) was 1.74S.

Enzyme composition

The purified enzyme was tested for total carbohydrate and protein. A value of 68% was found for the carbohydrate content and 32% for protein concentration. Samples of purified enzyme were hydrolysed as described. Descending paper chromatography of the neutral sugar solution obtained after hydrolysis showed that the main spot corresponded to glucose, but another spot with an R_F value different from glucose was also detected (Fig. 4a). The hydrolysate was checked for free glucose and the results showed that glucose formed 53.4% of the total carbohydrate.

Bio-Gel P-2 filtration was performed to separate these compounds. The elution was as indicated. The fractions were tested for reducing sugars by the method of Park & Johnson (1949). The results (Fig. 4b) showed that there were three components which eluted with different K_d values. The first was eluted with a K_d of 0.23, the second with one of 0.32 and the third with one of 0.37. G.l.c. of the hydrolysate showed that glucose and mannose are two of the components of the enzyme. The ratio glucose/mannose was 7.3. The component eluted in the last peak formed 42% of the carbohydrate and has not yet been identified (Fig. 5).

The results obtained from the amino acid analysis are indicated in Table 2. This enzyme has a high content of acidic amino acids. Other amino acids such as serine, glycine and alanine are also present in high percentages.

Table 1. Summary of steps involved in the purification of the *exo*- β -D-glucanase from cell-free extracts of *Candida utilis*

A unit of activity is defined as the amount of enzyme needed to liberate 1 nmol of glucose from the substrate under the conditions indicated in the text.

Purification step	Volume (ml)	$10^3 \times \beta$ -Glucanase activity (units/ml)	Total protein (mg/ml)	$10^5 \times$ Specific activity (units/ml)	Purification (fold)	Yield (%)
Crude extract	254.0	2.4	19.7	0.1	—	100
Adsorption on DEAE-Sephadex A-50	7.2	24.9	69.3	3.6	27	28.4
Sephadex G-100 filtration	6.0	13.6	13.0	10.4	79	12.9
Sephadex G-200 filtration	3.2	18.9	5.5	34.6	262	9.5
Sephadex G-50 filtration	4.0	12.8	2.2	57.8	436	8.1
Bio-Gel P-10 filtration	2.6	17.2	2.2	71.7	540	7.1
Concanavalin A-Sepharose 4B filtration	1.2	6.3	0.8	74.7	562	1.2

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

Enzymic activity (a), protein (b) and total carbohydrate content (c) (expressed as nmol of the glucose equivalent/ml) were determined as indicated in the text.

Enzyme properties

Isoelectric focusing. From amino acid analysis, isoelectric-focusing experiments showed that the purified enzyme behaved as an acidic protein focusing in a single peak (Fig. 6). The isoelectric point (pI) corresponded to a value of 4.1.

Molecular weight. Ultracentrifugation, gel filtration and polyacrylamide-gel electrophoresis were used to determine the molecular weight of the enzyme. Bovine serum albumin (mol.wt. 67000),

Fig. 3. Sedimentation behaviour of the purified *exo*- β -D-glucanase (I)

The standards were L-amino acid oxidase of *Crotalus adamanteus* venom (II) (expressed as μ l of O_2 /h per mg of protein) and yeast alcohol dehydrogenase (III) (expressed as the increase in $E_{340}/20$ s per 0.1 ml). Experimental conditions were those described by Martin & Ames (1961).

Fig. 4. Carbohydrate composition of the purified *exo*- β -D-glucanase

Results of paper chromatography (a) and Bio-Gel P-2 filtration (b) of an enzyme hydrolysate are shown. Experimental conditions are given in the text. The fractions obtained from Bio-Gel P-2 columns were tested for total carbohydrate.

Fig. 5. Gas-liquid chromatography of an enzyme hydrolysate

Glucose and mannose were detected forming part of the enzyme molecule. Conditions are indicated in the text. (1) α -Mannose; (2) α -glucose; (3) β -mannose; (4) β -glucose; (5) unknown component.

Table 2. Amino acid composition of the purified *exo*- β -D-glucanase from *Candida utilis*

The sample analysed contained 0.85 mg of protein/ml. The results refer to 1 mg of enzymic protein.

Amino acid	Composition	
	(nmol/mg of protein)	(% of each residue)
Alanine	96.8	12.0
Arginine	10.2	1.2
Aspartic acid	75.0	9.3
Cysteine	3.0	0.3
Glutamic acid	82.0	10.2
Glycine	110.5	13.7
Histidine	6.2	0.7
Isoleucine	29.2	3.6
Leucine	47.8	5.9
Lysine	71.6	8.9
Methionine	9.2	1.1
Phenylalanine	7.4	0.9
Proline	41.6	5.1
Serine	112.8	14.0
Threonine	44.7	5.5
Tyrosine	1.9	0.2
Valine	53.1	6.6

ovalbumin (mol.wt. 45000), trypsin (mol.wt. 23800) and cytochrome *c* (mol.wt. 12300) were used as standards in the last two cases. Results obtained from Sephadex G-100 filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis suggest a mol.wt. of 20000. However, its sedimentation behaviour indicated a higher value (36000). The cause of this difference remains unknown. Abeles *et al.* (1970) obtained similar results and indicated that one reason may be the formation of protein aggregates during sedimentation.

Fig. 6. Isoelectric focusing of the purified *exo*- β -D-glucanase

The pH range ($\blacktriangle$) of the carrier ampholines was from 3 to 6. Δ , E_{254} ; $\circ$, activity on laminarin.

Table 3. Substrate specificity of the purified *exo*- β -D-glucanase from *Candida utilis*

The assays were performed for 1 h at 37°C. The assay mixture (1 ml) contained enzyme plus buffered substrate. In every case the substrate concentration was 2.5 mM in 50 mM-sodium acetate buffer, pH 5.5.

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	—
Gentiobiose	(1 $\rightarrow$ 3)- α ; (mannose)	—
Cellobiose	(1 $\rightarrow$ 6)- β	—
<i>p</i> -Nitrophenyl β -D-glucopyranoside	(1 $\rightarrow$ 4)- β	+
<i>p</i> -Nitrophenyl phosphate		—

Substrate specificity. Several substrates have been used to test the action of β -glucanase enzyme on different linkage types (Table 3). The purified enzyme showed no activity when the enzymic reactions were performed with substrates with α -linkages as the main linkage type. Although highest activity was obtained when the substrate was laminarin, it seems clear that this enzyme is an unspecific β -glucanase, able to split both (1 $\rightarrow$ 3)- β - and (1 $\rightarrow$ 6)- β -linkages.

Michaelis constant determination. K_m values were calculated with purified enzyme acting on laminarin,

Fig. 7. Michaelis constants determination

K_m values were determined by the system of Lineweaver & Burk (1934) when the enzyme activity was tested on laminarin (a), *p*-nitrophenyl β -D-glucopyranoside (b) and pustulan (c).

p-nitrophenyl β -D-glucopyranoside and pustulan. The K_m for the enzyme when incubated against laminarin was 0.31 mM, 3.1 mM when tested on *p*-nitrophenyl β -D-glucopyranoside and 0.682 mg/ml when incubated with pustulan. For laminarin a mol.wt. of 4000 was assumed, equivalent to a degree of polymerization of 25 glucose residues (Reese, 1963). An inhibitory effect on the action of β -glucanase was observed at high substrate concentration when the rate of hydrolysis was measured as a function of laminarin (Fig. 7a) and pustulan (Fig. 7c) concentrations. The extent of inhibition was higher when pustulan was used as substrate than in the case of laminarin. Such inhibitory effect was not found when *p*-nitrophenyl β -D-glucopyranoside was used as substrate (Fig. 7b). This inhibitory effect has also been described for some yeast β -glucanases (Abd-El-Al & Phaff, 1969; Notario *et al.*, 1976).

Pattern of activity. Chromatographic analysis of the hydrolysis products of the enzymic reactions

Fig. 8. Inhibition of the exo- β -D-glucanase activity

Inhibition constant determination for glucono- δ -lactone (a), Zn^{2+} (b) and Hg^{2+} (c). K_i values were determined by the graphic method of Dixon (1953) by plotting the enzyme activity with laminarin when tested in the presence of increasing concentrations of each inhibitor.

showed that glucose was the main product when either laminarin, (1 $\rightarrow$ 3)- β -glucan from yeast cell wall or pustulan was used as substrate. In addition, the evaluation of free glucose indicated that glucose was produced from the beginning of the enzymic reaction. The fact that almost all the reducing power was due to glucose was established by using simultaneously the methods of Somogyi (1952) and Keston (1956) to evaluate the hydrolysis products of the same enzymic assay. In addition to the fact that the purified enzyme showed high activity with *p*-nitrophenyl β -D-glucopyranoside and had no activity against oxidized laminarin, these results support the idea of considering this enzyme as an exo- β -D-glucanase.

Inhibition of β -glucanase activity. Glucono- δ -lactone as well as different metal ions have been described as inhibitors of β -glucanase enzymes (Bull, 1967). Glucono- δ -lactone, Zn^{2+} and Hg^{2+} were used for inhibition experiments. The inhibition tests were

performed by measuring β -glucanase activity on laminarin in the presence of increasing concentrations of the inhibitors. In these assays the substrate concentration was kept constant. All three agents had an inhibitory effect on the activity of the purified enzyme. The strongest inhibition corresponded to Hg^{2+} . When the inhibitor concentration was 0.15 mM a 100% loss of activity could be detected. Glucono- δ -lactone inhibited the exo- β -D-glucanase activity more strongly than Zn^{2+} did. A notable fact was that at low inhibitor concentrations an increase of activity could be observed when the effect of glucono- δ -lactone was assayed. The inhibitor constants (K_i) were determined by the graphic method of Dixon (1953). The K_i values were 0.44 mM for glucono- δ -lactone (Fig. 8a), 0.20 mM for Zn^{2+} (Fig. 8b) and 0.025 mM for Hg^{2+} (Fig. 8c).

Discussion

Low degrees of purification have been reported for exo- β -D-glucanases from culture fluids of different organisms (Huotari *et al.*, 1968; Abeles *et al.*, 1970). Such results would agree with the fact that a high proportion of the protein secreted into the medium by the organisms is β -glucanase. Higher amounts can be obtained in purification processes of β -glucanases located inside the cell wall. Fleet & Phaff (1974) obtained a 299-fold purified cell-wall-associated exo-(1 $\rightarrow$ 3)- β -D-glucanase from the yeast *Schizosaccharomyces versatilis*. Our results (562-fold purification) agree with the low percentage of β -glucanase in cell-free extracts.

Brown (1972) and Notario *et al.* (1976) found that β -glucanases behaved as acidic proteins on isoelectric-focusing experiments. The purified enzyme from *C. utilis* also appears as an acidic protein. This fact is suggested, not only by the results obtained from isoelectric-focusing experiments, but also from those obtained by adsorption on DEAE-Sephadex A-50, polyacrylamide-gel electrophoresis and amino acid analysis. In all these cases, the enzyme seems to have a net negative charge. The amino acid composition is very similar to those reported by other workers for different β -glucanases (Huotari *et al.*, 1968; Abeles *et al.*, 1970). This enzyme presents a high percentage of acidic amino acids, and other amino acids such as glycine and alanine have been found in a high proportion.

It is thought that β -glucanases, as typical extracellular enzymes, might be considered as glycoproteins. However, Winterburn & Phelps (1972) indicated that not all extracellular proteins should be necessarily glycosylated. Results obtained by Huotari *et al.* (1968) and Farkas *et al.* (1973) suggest that β -glucanases possess a small proportion of carbohydrate as part of their molecule. Moreover Villa *et al.* (1975b) reported that 2-deoxy-D-glucose did not

affect β -glucanase synthesis, whereas the production of other typical glycosylated enzymes such as invertase and acid phosphatase was clearly inhibited. In our case data from gel electrophoresis, paper chromatography and g.l.c. indicate that the purified enzyme is a glycosylated protein with a high carbohydrate content. A notable fact was that amino acids such as serine and threonine were in a high proportion. Sentandreu & Northcote (1968) indicated that the carbohydrate is attached to the protein portion by means of serine and threonine bridges in typical yeast glycoproteins.

In general, enzyme properties such as molecular weight and sedimentation coefficient are in the range reported by other authors (Bull, 1967; Kitamura *et al.*, 1974). It seems clear that the purified enzyme is an unspecific one, which may hydrolyse both (1 $\rightarrow$ 3)- β - and (1 $\rightarrow$ 6)- β -linkages. It is noteworthy that this enzyme shows higher activity against laminarin than on (1 $\rightarrow$ 3)- β -D-glucan from yeast cell wall. The different degree of polymerization, the difference in solubility and the different proportions of non-reducing ends between both laminarin and (1 $\rightarrow$ 3)- β -D-glucan from yeast cell wall may be some of the arguments to explain the distinct affinity for these substrates. Our results show that the mode of action of the enzyme corresponds to an exo-splitting mechanism when it acts on laminarin, (1 $\rightarrow$ 3)- β -D-glucan from yeast cell wall or pustulan.

Glucono- δ -lactone, Zn^{2+} and Hg^{2+} inhibit β -glucanase activity. The inhibitory effect of Hg^{2+} would indicate that thiol groups are implicated in the active centre of the enzyme or that these groups are important in maintaining the β -glucanase structure. A notable fact was the enzyme activation found when glucono- δ -lactone was used at concentrations lower than 0.15 mM. Experiments to explain the reason for this activation have not been developed in the present work.

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