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SUSCEPTIBILITY OF SCLEROGLUCAN TO THE (1→3)-β-D-GLUCANASE ZYMOLYASE

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ABSTRACT

Exposure of the (1→3,6)-β-D-glucan, scleroglucan, to 0.2M NaOH at room temperature rendered it susceptible to hydrolysis by the (1→3)-β-D-glucanase zymolyase. Fractionation of the products revealed that 42% of the polysaccharide remained intact and that the remainder was converted into glucose, laminaribiose, and gentiobiose. The implications of these findings for selectively solubilising glucans in yeast cell walls prior to their enzymic characterisation are discussed.

INTRODUCTION

The use of aqueous alkali at various concentrations, temperatures, and exposure times for selectively extracting populations of polysaccharides and glycoproteins from the cell walls of yeast and fungi is widely practised. Reasonably reproducible results are obtained and the method is useful not only as a preparative procedure for cell-wall components but also as a fingerprint for the characterisation of cells. However, as the complexities of wall architecture become apparent, it is now appreciated that certainly the harsh, and most probably the mild, conditions of extraction with alkali and acid modify the components of the wall during dissolution. In the presence of air, oxidation of the reducing-end residues occurs and this may be minimised by purging with nitrogen. For large polysaccharides with a small proportion of reducing units, this effect is of no concern. Of more concern are the bonds susceptible to alkali, *e.g.*, the glycosyl-seryl and -threonyl bonds in mannoproteins and *N*-acetyl groups.

Polysaccharides in helical and ribbon conformation tend to associate to give double or triple helices, as in amylose or curdlan, respectively, or as a complex of multi-ribbon structures containing 30–40 molecules, as in the microfibrils of cellulose[1]. In alkali, the hydrogen bonds that maintain these associations are broken to give random-coil structures. Unless the helical or microfibrillar structures can be re-formed accurately and completely on neutralisation, the susceptibility of

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bonds to enzymic hydrolysis, a process in part dependent on the flexibility and conformational state of the substrate, may change.

Scleroglucan and curdlan are useful model polymers for the study of yeast and fungal cell-wall assemblies, as their structures approximate to those of cell-wall glucans. Indeed, schizophylan, which has a structure identical to that of scleroglucan, is a cell-wall component of *Schizophylum commune*[2].

We now report that, after treatment with alkali, scleroglucan becomes susceptible to attack by a (1→3)-β-D-glucanase.

EXPERIMENTAL

Materials. — Scleroglucan, prepared from *Sclerotium rolfsii,* and curdlan were gifts from Dr. D. Lecacheux, Dr. G. Brigand, and Professor T. Harada. Zymolyase 20T, a (1→3)-β-D-glucanase from *Arthrobacter luteus,* was purchased from Miles Scientific, and novozyme 234, a mixed glucanase from *Trichoderma harzianum,* from Novo Biolabs (Denmark). The latter enzyme was partially purified by fractionation on ion-exchange cellulose and contained (1→3)- and (1→6)-β-D-glucanase activity in the ratio 3:1 as measured by hydrolysis of soluble laminarin and pustulan, respectively, and is referred to as mixed glucanase. The activity of this enzyme is expressed with respect to hydrolysis of laminarin.

Enzyme digestions. — Solutions of scleroglucan or curdlan (10 mg) in 2-(*N*-morpholino)ethanesulphonate (MES) buffer (pH 6.0; 800 μmol) were mixed with zymolyase or mixed glucanase (5 nkat) in a final volume of 5.0 mL and incubated at 37°. Hydrolysis was assayed by the copper reductometric method[3], using absorbance at 520 nm.

Exposure to alkali. — Scleroglucan (10 mg) was rapidly dissolved in 0.2M NaOH (4.0 mL) at 20° through which N_2 had been previously passed for 30 min. The flow of N_2 was continued for 20 min, and the appropriate amount of MES was added to rapidly (30 s) lower the pH to 6.0 ±0.1. The temperature was raised to 37°, enzyme (1 mL) added, and the digestion proceeded with as described above.

Gel-permeation chromatography. — Enzyme digest (3 mL) was applied to a column (1 × 30 cm) of Sephadex G-200 and eluted with water containing 5mM NaN_3 at 0.7 mL/h. Fractions were monitored for carbohydrate by the phenol–sulphuric acid procedure[4].

Fractions in the peak of low M_r (Fig. 2) produced by zymolyase were combined and concentrated to dryness at 70°, and a solution of the residue in a small amount of water was subjected to descending p.c. on Whatman 3MM paper, using ethyl acetate–pyridine–H_2O (10:4:3) and detection with alkaline silver nitrate, or h.p.l.c., using a dextropack column (Waters Associates) and elution with water at 1 mL/min and room temperature.

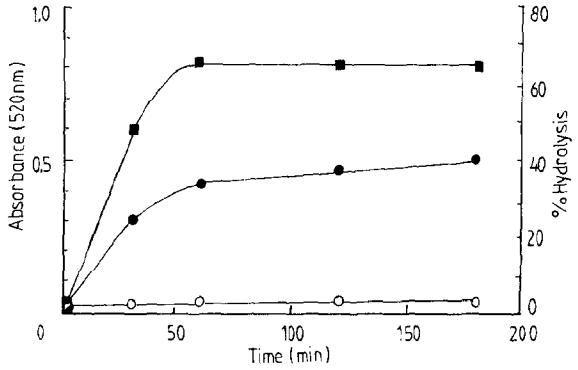

Fig. 1. Action of zymolyase on solutions of scleroglucan in 0.16M MES buffer (pH 6.0) (—○—), 0.2M NaOH for 20 min at 20° before adjusting to pH 6.0 (—●—), and 0.2M NaOH followed by adjustment to pH 6.0 but with mixed glucanase in place of zymolyase (—■—).

RESULTS

The susceptibility of scleroglucan to hydrolysis by zymolyase 20T, which possesses (1→3)-β-D-glucanase activity, and mixed glucanase derived from novozyme 234, which possesses both (1→3)- and (1→6)-β-D-glucanase activity, was examined.

The addition of zymolyase to a solution of scleroglucan in 0.16M MES (pH 6) caused no appreciable hydrolysis at 37° during 3 h (Fig. 1). Under similar conditions, curdlan was converted completely within 1 h into glucose, laminaribiose, and laminaritriose (results not shown). However, scleroglucan was susceptible to the mixed glucanase present at the same level of (1→3)-β-D-glucanohydrolase activity,

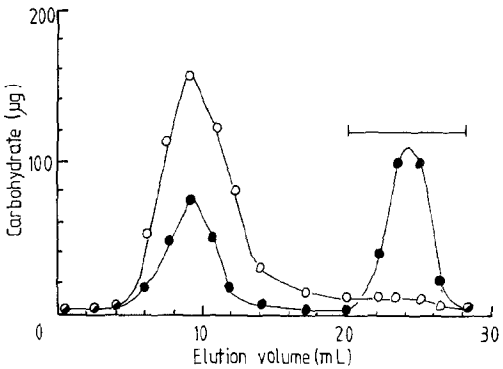

Fig. 2. Gel-permeation chromatography (see Experimental) of scleroglucan exposed to alkali before adjustment to pH 6.0 followed by addition of inactivated zymolyase (—○—) and digestion with zymolyase for 180 min to completion of hydrolysis (Fig. 1) (—●—). Fractions (peak 1) indicated by the bar were combined for fractionation by h.p.l.c. (Fig. 3).

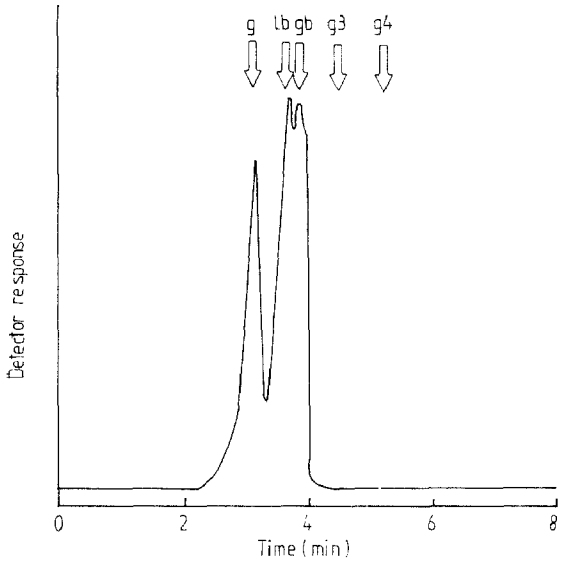

Fig. 3. H.p.l.c. of products of low M_r in Fig. 2 (peak 1) produced on digesting alkali-treated scleroglucan with zymolyase: g, glucose; lb, laminaribiose; gb, gentiobiose; g3, maltotriose; and g4, maltotetraose.

and 40% of the bonds were hydrolysed to give mainly glucose and gentiobiose together with smaller amounts of laminaribiose and laminaritriose.

Exposure of scleroglucan to 0.2M NaOH for 20 min at 22° under N_2 followed by adjustment of pH to 6.0 with solid MES gave a product that was hydrolysed by zymolyase (Fig. 1) to an extent of 38% after 3 h. The susceptibility to the mixed glucanase was also increased to 65% (Fig. 1) with glucose and gentiobiose as the products. The results of gel-permeation chromatography of the products obtained by digestion with zymolyase are shown in Fig. 2; 58% of the carbohydrate emerged in the low M_r fraction, the remainder being in the excluded peak. The elution profile of polysaccharide after treatment with alkali is also shown. Analysis of the low M_r fraction by h.p.l.c. (Fig. 3) and p.c. (results not shown) revealed only glucose, gentiobiose, and laminaribiose with a glucose and disaccharide ratio of ~1:2.

DISCUSSION

Scleroglucan occurs in fungal cell walls[2-5], as an extracellular fungal polysaccharide[6], and is isolated on an industrial scale from the culture filtrate of *Sclerotium rolfsii*[7]. The structure is similar to that of curdlan, namely, a (1→3)-β-D-glucan backbone with a 6-linked single β-D-glucosyl group on each third residue[6,8]. It is this modification which makes scleroglucan soluble at neutral pH. Studies using ultracentrifugation and viscometry showed that the polymer is present as a triple helix in solution[9]. The 6-linked glucosyl groups, which are on the external face of the rod-like triplex, prevent further polysaccharide–polysaccharide association and

promote interaction with surrounding water molecules. The intrinsic viscosity and optical rotation change markedly[10] as the pH of solution is raised beyond 12. Concentrations of NaOH of >0.01M are considered to cause dissociation of the triple helix to constituent single polymers that assume a random coil conformation. The viscosity transition is not reversible[10], which suggests that re-association of an ordered triple helix is either limited or does not take place, so that neutralisation may alter the conformation of scleroglucan and, consequently, the susceptibility to enzymic hydrolysis. Common methods for isolating β-D-glucans from the cell walls of yeast and fungi comprise extraction with aqueous alkali followed by neutralisation.

Periodate oxidation provides a measure of the proportion of 3- and 6-linked units in the solubilised components, and the susceptibility to zymolyase may be used to determine the proportion of 3-linkages. Zymolyase is a commercial product and, although it is not a pure protein, it contains (1→3)- but not (1→6)-β-D-glucanohydrolyase activity[11]. Nevertheless, not all (1→3)-β-D-glucosidic bonds are susceptible to zymolyase. Thus, the 3-linkages in scleroglucan are resistant (Fig. 1), possibly due to shielding by neighbouring 6-linked units. On the other hand, the mixed glucan hydrolyase can hydrolyse scleroglucan, apparently leaving the 6-linkages untouched but cleaving the majority of the 3-linkages. Studies of model substrates such as these can give evidence of their fine structure, but account must be taken of possible changes in tertiary structure during the isolation from cellular debris. Thus, the susceptibility to enzymic hydrolysis of the model substrates should be examined after similar treatment with alkali.

Exposure of scleroglucan to alkali followed by readjustment of the pH to 6 before digestion with enzyme increased its susceptibility to attack by glucanases (Fig. 1). The increase was marginal (40→65%) with the mixed glucanase and appeared to involve more complete hydrolysis of products of low M_r since laminaribiose and laminaritriose were now almost absent. This result is probably due to increased accessibility of bonds adjacent to the gentiobiosyl moiety. However, for the attack by zymolyase, the difference was profound with more than one third of the resistant bonds becoming susceptible (Fig. 1). Analysis of the products of low M_r revealed glucose, gentiobiose, and laminaribiose. Gel-permeation chromatography (Fig. 2) revealed that 42% of the scleroglucan either remained intact or was present as large (excluded) fragments.

The partial susceptibility of scleroglucan to attack by zymolyase may reflect changes in tertiary structure of the polysaccharide. Brief exposure to alkali at room temperature and under nitrogen prevents oxidation. However, dissolution in 0.1M alkali will disrupt the hydrogen bonds that maintain the triple helix and, on neutralisation, the single chains will attempt to re-associate. Measurements of viscosity and birefringence indicate that the uniform triple helix does not reappear[10] but partial association may occur. Thus, the susceptibility to zymolyase may arise from the availability of unhindered domains within the polysaccharide and the resistant part may comprise domains where some re-association has occurred. The

high M_r ($\sim 5 \times 10^6$) of the original polymer[10] means that resistant domains released by zymolyase could still be large enough to be excluded from Sephadex G-200.

Thus, exposure of polysaccharides to alkali may not only hydrolyse bonds but also modify their tertiary structures and hence their susceptibility to enzymic attack.

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