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Glucan–glucosaminoglycan linkages in fungal walls

P. C. MOL, C. A. VERMEULEN and J. G. H. WESSELS

Department of Plant Physiology, Biological Centre, University of Groningen, 9751 NN Haren, The Netherlands

SUMMARY

Chemical depolymerization of glucosaminoglycans with nitrous acid solubilized alkali-insoluble glucan from the walls of the basidiomycetes *Schizophyllum commune* and *Agaricus bisporus* but not from the walls of the ascomycetes *Aspergillus nidulans* and *Neurospora crassa*. In all four fungi depolymerization of glucosaminoglycans with chitinase was effective at converting a considerable portion of the alkali-insoluble glucan into a water/alkali-soluble form. This indicates chemical linkage between glucosaminoglycan (chitin) and β-glucan in these fungi.

Key-words: fungal walls, glucan, glucosaminoglycan.

INTRODUCTION

The alkali-insoluble part of the hyphal wall of ascomycetes and basidiomycetes is principally composed of a complex of (1→3)-β/(1→6)-β-D-glucan with chitin (Wessels & Sietsma 1981). This complex is thought to determine the rigidity of the hyphal wall (Wessels 1986). The extreme insolubility of the glucan in this complex of *Schizophyllum commune* has been explained by the presence of covalent linkages between this glucan and chitin (Sietsma & Wessels 1979). Specific depolymerization of chitin, either by enzymatic hydrolysis or by nitrous acid treatment of deacetylated chitin, resulted in the release of glucan in a water- or alkali-soluble form. The latter technique was also applied by Sietsma & Wessels (1981) in an evaluation of the presence of a presumed linkage between β-glucan and chitin in various fungi.

Complementary to this earlier work, we here examine the solubility characteristics of glucan for some of these fungi after nitrous acid treatment of native hyphal walls and after enzymatic depolymerization of chitin. A similar study on the wall of the yeast *Saccharomyces cerevisiae* was recently published (Mol & Wessels 1987).

MATERIALS AND METHODS

Mycelial wall preparations of *Aspergillus nidulans* (b1A1) (ATCC 24704) (Zonneveld 1974), *Neurospora crassa* (CBS 327.54) and *Schizophyllum commune* strain 1–40 (CBS 344.81) were prepared as described (Sietsma & Wessels 1981). Stipe tissue from rapidly elongating fruit bodies of *Agaricus bisporus* strain Horst U3 was passed through an X-press (Biotec, Sweden) at −25°C. The homogenate was thawed to 4°C, after which hyphal walls were washed with phosphate buffer and water according to Wessels *et al.* (1972). Clean cell walls (30 mg) were extracted with 1 M KOH at 60°C for 20 min or first treated with nitrous acid. For the nitrous acid treatment 3 ml freshly prepared 2 M $NaNO_2$

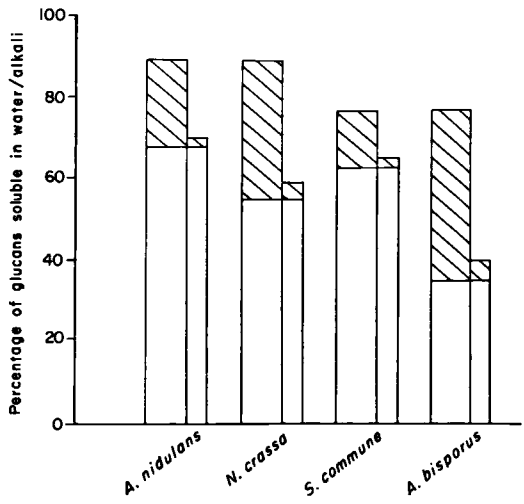

Fig. 1. Solubility of glucans (anthrone-positive material) before and after chitinase treatment from hyphal walls of several fungi. (□) Alkali-soluble glucan; (▨) water/alkali-soluble glucan after chitinase treatment of alkali-treated wall. Narrow bars represent the effects of control treatments.

solution were added to 30 mg cell walls suspended in 2 ml water in a screw-cap tube, followed by 1 ml 2 M HCl, to give a pH of 3. The tube was then closed. After 1·5 h at room temperature, the tube was opened and filtered air was bubbled through the suspension for 30 min, after which the insoluble residue was washed with water. In control treatments, $NaNO_2$ was omitted and replaced by 0·317 M Na_2HPO_4, which gave the same pH as the nitrous acid treatment. Alkali-insoluble fractions and nitrous acid- and alkali-insoluble fractions were digested with chitinase from *Serratia marcescens* (Serva, Heidelberg, FRG), 0·5 mg/ml for three days in 50 mM Na-acetate buffer pH 5·8 at 36°C with 0·02% Na-azide to prevent bacterial contamination. Control treatments without enzyme were carried out simultaneously under the same conditions. After these treatments insoluble residues were extracted with 1 M KOH. Glucan values in residues were determined by the anthrone reagent (Fairbairn 1953).

Changes in optical density of the suspensions during chitinase and control incubations were monitored with a Vitatron colorimeter at 762 nm. Optical densities were between 0·450 and 0·550 at the start of incubation.

RESULTS AND DISCUSSION

Alkali extraction of hyphal walls of *Aspergillus nidulans* rendered 68% of the glucan soluble (Fig. 1). This fraction consisted mainly of (1→3)-α-D-glucan (Bull 1970, Zonneveld 1971). As can be seen from Fig. 2, treatment of walls with nitrous acid, which causes specific depolymerization of 2-amino-2-deoxyglycans (Shively & Conrad 1970), does not accomplish any solubilization of glucan. Because measurements on glucosaminoglycan degradation were not included in this experiment, it is not known whether nitrous acid-sensitive sites were actually absent from the native walls. Yet it is evident that non-acetylated glucosaminoglycans are not involved in keeping glucan insoluble. Enzymatic depolymerization of chitin by chitinase results in a rapid decrease in optical density

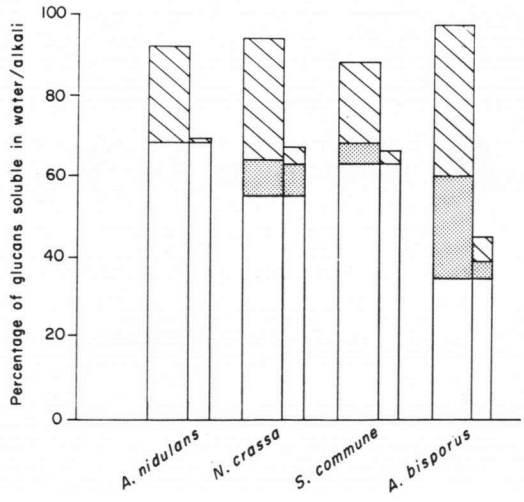

Fig. 2. Effects of nitrous acid treatment and subsequent chitinase treatment on alkali-solubility of glucans (anthrone-positive material) from hyphal walls of several fungi. (□) Alkali-soluble glucan; (▨) additionally water/alkali-soluble glucans after nitrous acid treatment of native wall; (■) water/alkali-soluble glucan after chitinase treatment of nitrous acid and alkali-treated wall. Narrow bars represent the effects of control treatments.

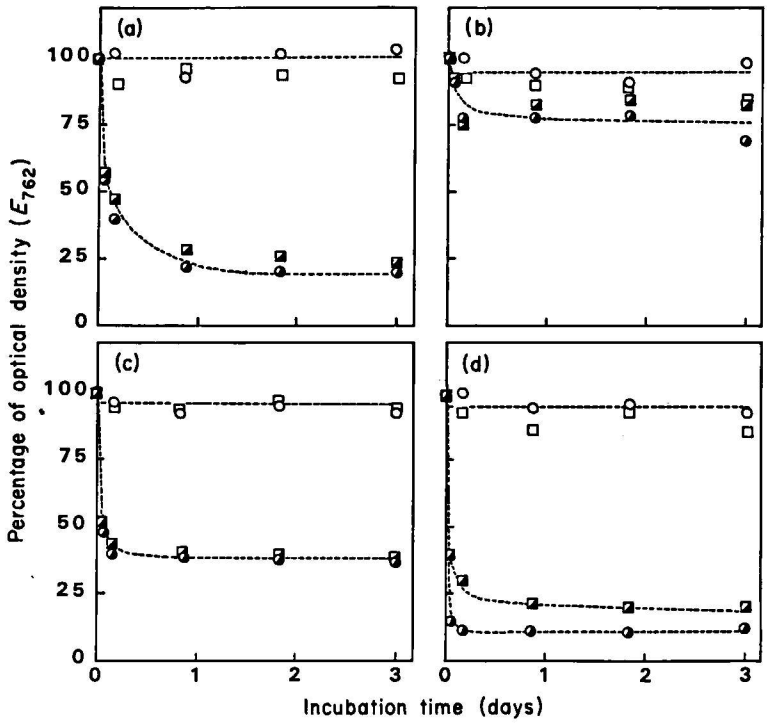

Fig. 3. Changes in optical density of suspensions of alkali-insoluble wall fractions (◪) and nitrous acid and alkali-insoluble wall fractions (◕), respectively, during incubation with 0·5 mg/ml chitinase in 50 mM Na-acetate buffer (pH 5·8). Open symbols represent control treatments in buffer. (a) *Aspergillus nidulans*, (b) *Neurospora crassa*, (c) *Schizophyllum commune* and (d) *Agaricus bisporus*.

(Fig. 3a). Only 9% of the glucan in the alkali-insoluble complex is directly solubilized by this treatment (data not shown). Therefore, a decrease in optical density of suspensions during chitinase incubation must be attributed mainly to degradation of chitin which can make up 45–46% of the complex (Bull 1970, Zonneveld 1971). The larger part of the glucan is solubilized by subsequent alkali extraction (Figs 1 and 2). Close association between glucan and chitin has also been demonstrated for cell walls of *Aspergillus niger* (Stagg & Feather 1973). The alkali-resistant wall fraction (10% NaOH, 36 h 25°C) appeared to be largely insoluble in 40% of NaOH as well as in dimethyl sulphoxide. But after nitrous acid treatment, the alkali-insoluble residue was completely soluble in dimethyl sulphoxide and 50% of the fraction could be solubilized in 10% NaOH. This implies that at least some of the glucosaminoglycan was depolymerized by the nitrous acid treatment. However, deacetylation of *N*-acetylglucosamine residues in glucosaminoglycan could have occurred during preparation of the alkali-insoluble fraction (Sietsma & Wessels 1979).

The alkali-soluble part of *Neurospora crassa* hyphal walls not only contains (1→3)-α-D-glucan (De Vries 1974) but also glycoproteins (Mahadevan & Tatum 1965, 1967). In addition to the glucan released by alkali treatment alone, another 9% was released by the combined nitrous acid and alkali extractions (Figs 1 and 2). However, control treatments with Na_2HPO_4/HCl and alkali remove a similar amount of glucan. We therefore conclude that solubilization of this glucan is due to exposure to the low pH in the nitrous acid treatment. Apparently in hyphal walls of *N. crassa*, glucan is not linked to glucosaminoglycan containing non-acetylated glucosamine residues. Depolymerization of chitin by chitinase, which accounts for 12% of the dry weight of the alkali-insoluble complex, does not result in concomitant release of glucan in water. This may explain the only slight decrease in optical density as seen in Fig. 3b. However, after chitinase treatment 76–84% of the glucan becomes soluble in alkali (Figs 1 and 2). Alkali extraction performed on material that was incubated in acetate buffer results in solubilization of only 9% of the glucan; hence solubilization of the glucan is actually due to depolymerization of chitin. Because a portion of the alkali-insoluble glucan of *Aspergillus nidulans* and *Neurospora crassa* can be extracted by dimethyl sulphoxide, it was earlier concluded that part of the alkali-insoluble glucan is not associated with chitin (Sietsma & Wessels 1981). However, it was not proved that the glucosaminoglycan–glucan complex was completely refractory to dimethyl sulphoxide extraction. The present findings strongly suggest that all the glucan is linked to chitin.

Hyphal walls of the basidiomycetes *Schizophyllum commune* and *Agaricus bisporus* are different from the ascomycetes examined in that they both contain non-acetylated glucosamine residues.

After nitrous acid treatment 5% of the total glucan in *S. commune* is solubilized in alkali in addition to (1→3)-α-D-glucan (Fig. 2). An identical result was reported previously (Sietsma & Wessels 1979). Incubation of the alkali-insoluble complexes with chitinase causes a considerable decrease in optical density (Fig. 3c). During incubation with chitinase 38% of the glucan in the alkali-insoluble complex of *S. commune* becomes soluble in alkali. Pretreatment with nitrous acid renders a total of 63% of the glucan in the alkali-insoluble complex soluble in water and alkali. Sietsma & Wessels (1979) found that during complete hydrolysis of chitin in the chitin–glucan complex by a purified chitinase preparation from *Streptomyces satsumaensis*, 70% of the alkali-insoluble glucan became water-soluble and 20% became alkali-soluble. Considering that we used chitinase from a different source for a shorter time, it is likely that some of the glucan remained insoluble as a result of incomplete chitin digestion.

The alkali-insoluble complex of hyphal walls from *Agaricus bisporus* fruit body stipes consists of about 45% glucan and 53% hexosamine (Novaes-Ledieu & Garcia-Mendoza 1981). Nitrous acid treatment causes solubilization of 18% of the glucosaminoglycans (Mol, unpublished). Simultaneously a noticeable fraction of the total glucan is released in addition to (1→3)-α-D-glucan (Fig. 2). This glucan, which constitutes about 38% of the glucan in the alkali-insoluble complex, must be linked to glucosaminoglycan containing non-acetylated glucosamine residues. Nearly all of the glucan in the complex can then be released in alkali by treatment with chitinase. Also, the decrease in optical density by chitinase is somewhat more severe after prior treatment of the alkali-insoluble complex with nitrous acid (Fig. 3d).

The present study shows solubilization of glucans by depolymerization of glucosaminoglycans in two ascomycetes and two basidiomycetes. This suggests that this phenomenon, which was interpreted to indicate covalent linkage between the two polymers (Sietsma & Wessels 1979), may be a general feature among these fungi.

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REFERENCES

Bull, A.T. (1970): Chemical composition of wild-type and mutant *Aspergillus nidulans* cell walls. The nature of polysaccharide and melanin constituents. *J. Gen. Microbiol.* **63:** 75–94.

Fairbairn, N.J. (1953): A modified anthrone reagent. *Chem. Ind.* **72:** 86.

Mahadevan, P.R. & Tatum, E.L. (1965): Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. *J. Bacteriol.* **90:** 1073–1081.

— & — (1967): Localization of structural polymers in the cell wall of *Neurospora crassa*. *J. Cell Biol.* **35:** 295–302.

Mol, P.C. & Wessels, J.G.H. (1987): Linkages between glucosaminoglycan and glucan determine alkali-insolubility of the glucan in walls of *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **41:** 95–99.

Novaes-Ledieu, M. & Garcia-Mendoza, C. (1981): The cell walls of *Agaricus bisporus* and *Agaricus campestris* fruiting body hyphae. *Can. J. Microbiol.* **27:** 779–787.

Shively, J.E. & Conrad, H.E. (1970): Stoichiometry of the nitrous acid deaminative cleavage of model aminosugar glycosides and glycosaminoglycuronans. *Biochemistry* **9:** 33–43.

Sietsma, J.H. & Wessels, J.G.H. (1979): Evidence for a covalent linkage between chitin and β-glucan in a fungal wall. *J. Gen. Microbiol.* **114:** 99–108.

— & — (1981): Solubility of (1→3)-β-D-/(1→6)-β-D-glucan in fungal walls: importance of presumed linkage between glucan and chitin. *J. Gen. Microbiol.* **125:** 209–212.

Stagg, C.M. & Feather, M.S. (1973): The characterization of a chitin-associated D-glucan from the cell walls of *Aspergillus niger*. *Biochim. Biophys. Acta* **320:** 64–72.

Vries, O.M.H. de (1974): *Formation and cell wall regeneration of protoplasts from* Schizophyllum commune. PhD Thesis, University of Groningen, The Netherlands.

Wessels, J.G.H. (1986): Cell wall synthesis in apical growth. *Int. Rev. Cytol.* **104:** 37–39.

—, Kreger, D.R., Marchant, R., Regensburg, B.A. & de Vries, O.M.H. (1972): Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. *Biochim. Biophys. Acta* **273:** 346–358.

— & Sietsma, J.H. (1981): Fungal cell walls: a survey. In: W. Tanner & F.A. Loewus (ed.): *Encyclopedia of Plant Physiology, New Series,* Vol. 13 B, pp. 352–394. Springer-Verlag, Berlin.

Zonneveld, B.J.M. (1971): Biochemical analysis of the cell wall of *Aspergillus nidulans*. *Biochim. Biophys. Acta* **249:** 506–514.

— (1974): α-1,3 Glucan synthesis correlated with α-1,3 glucanase synthesis, conidiation and fructification in morphogenetic mutants of *Aspergillus nidulans*. *J. Gen. Microbiol.* **81:** 445–451.