SHORT COMMUNICATION

Solubility of (1→3)-β-D/(1→6)-β-D-Glucan in Fungal Walls: Importance of Presumed Linkage between Glucan and Chitin

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In *Saccharomyces cerevisiae, Neurospora crassa, Aspergillus nidulans* and *Coprinus cinereus* most of the alkali-insoluble (1→3)-β-D/(1→6)-β-D-glucan of the wall can be extracted with dimethyl sulphoxide. The same fraction, and in *Saccharomyces cerevisiae* a small additional fraction, can be extracted by a destructive procedure involving 40% NaOH at 100 °C. The small fraction of the glucan which resists this treatment becomes soluble after a subsequent treatment with HNO_2 indicating that it is covalently linked to chitin in the wall. In contrast, in *Schizophyllum commune* and *Agaricus bisporus*, nearly all the (1→3)-β-D/(1→6)-β-D-glucan appears to be held insoluble by linkage to chitin.

INTRODUCTION

The alkali-insoluble wall residue of ascomycetes and basidiomycetes consists mainly of (1→3)-β-D/(1→6)-β-D-glucan and chitin (Rosenberger, 1976). These polymers are generally considered as separate entities; chitin is thought to be present as microfibrils physically embedded in a β-glucan matrix. Contrary to this view it was recently found that in the walls of *Schizophyllum commune* most of the β-glucan may be chemically linked to chitin, explaining the insolubility of the β-glucan in the walls of this organism (Sietsma & Wessels, 1979). The linkage between β-glucan and chitin, involving basic amino acids and *N*-acetylglucosamine, would also prevent crystallization of chitin chains bearing glucan branches thus explaining the low crystallinity of chitin in the native wall or alkali-extracted residues (Sietsma & Wessels, 1977). Only after removal of glucan chains do X-ray diffraction lines of chitin appear, suggesting secondary formation of microcrystals and possibly microfibrils of chitin.

In view of these findings with the wall of *Schizophyllum commune*, we have re-examined the solubility characteristics of (1→3)-β-D/(1→6)-β-D-glucans in the walls of some other fungi for which detailed chemical analyses of the walls are available. As a criterion for linkage of β-glucan to chitin we used the solubilization of the β-glucan after selective depolymerization of deacetylated chitin by HNO_2.

METHODS

Organisms and growth conditions. The isolates of *Schizophyllum commune* strain 1-40 (699) (Raper & Miles, 1958), *Aspergillus nidulans* (*biA1*) (Zonneveld, 1974), and *Coprinus cinereus* (Shaeff.) Fr. (Gooday, 1974) have been described previously and were kindly provided by the authors; *Neurospora crassa* (CBS 327.54) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; *Saccharomyces cerevisiae* was an isolate from pressed baker's yeast (Koninklijke Nederlandse Gist en Spiritusfabriek, Delft, The Netherlands). All organisms were grown in shaken cultures in minimal medium (Raper & Miles, 1958) supplemented with trace elements (Whitaker, 1951) and 0·3 % (w/v) yeast extract (Difco) at 25 °C for 3 d.

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Preparation of hyphal fractions and assay procedures. A mycelial wall preparation of *Agaricus bisporus* was generously provided by Dr D. Rast, Institute of General Botany, University of Zürich, Switzerland. The mycelia of the other organisms were disrupted in an X-press (Biotec, Sweden) at −25 °C and the hyphal walls were isolated by differential centrifugation and extensively washed with buffer and water as has been described previously for *Schizophyllum commune* (Wessels *et al.*, 1972). The microscopically clean hyphal walls were extracted twice with 1 M-KOH under N_2 at 60 °C for 20 min. The residue was washed successively with water, methanol and ether, then dried and treated with dimethyl sulphoxide (DMSO) at room temperature for 16 h with vigorous shaking. The material remaining after the DMSO extraction was washed with water and treated with 40% (w/v) NaOH under N_2 at 100 °C for 1 h. The residue was then treated with HNO_2 and finally extracted with 1 M-KOH as described before (Sietsma & Wessels, 1979). After each extraction procedure a sample was taken from the washed insoluble residue to determine the total amount of carbohydrate with the anthrone reagent (Fairbain, 1953).

RESULTS AND DISCUSSION

Figure 1 shows the effect of the various chemical treatments on the removal of anthrone-positive material from the walls of a number of fungi. The residues after extraction with 1 M-KOH essentially contain glucose and *N*-acetylglucosamine. Because hexosamines do not react with the anthrone reagent, the anthrone-positive material in this alkali-resistant fraction refers to glucan. However, the material extracted with 1 M-KOH may contain other sugars, in addition to glucose, which do react with the anthrone reagent but with a different sensitivity. The values obtained for this fraction should thus be taken as approximations of the amount of polysaccharides removed.

From *Saccharomyces cerevisiae* walls, 1 M-KOH probably extracts all the mannan and mannoprotein and also a small amount of the (1→3)-*β*-D/(1→6)-*β*-D-glucan (Fleet & Manners, 1976). Most of the insoluble (1→3)-*β*-D/(1→6)-*β*-D-glucan can then be extracted with dimethyl sulphoxide (cf. Bacon *et al.*, 1969). Subsequent treatment with 40% NaOH at 100 °C further reduces the amount of this glucan. This latter treatment may involve not only extraction but also degradation of the *β*-glucan (Horton & Wolfrom, 1963). The remaining *β*-glucan becomes soluble when the deacetylated chitin in the residue is depolymerized with HNO_2.

From these results it can be concluded that about 15% of the *β*-glucan in the yeast wall is linked to chitin chains, probably through the reducing ends of the glucan chains because of the resistance to alkaline degradation. Most of the chitin in the wall of *Saccharomyces cerevisiae* is known to be localized in the bud scar region (Houwink & Kreger, 1953; Bacon *et al.*, 1966; Beran *et al.*, 1972), probably as a remains of the primary septum (Cabib & Bowers, 1971). From our results we conclude that at least some chitin chains in this region are linked to *β*-glucan. This would explain the low crystallinity of chitin in native or alkali-extracted walls of *Saccharomyces cerevisiae* and the appearance of sharp X-ray reflections after treatment with hot mineral acid (Houwink & Kreger, 1953; Beran *et al.*, 1972) which disrupts the linkages between the chitin and glucan chains (Sietsma & Wessels, 1979). In addition, Houwink & Kreger (1953) have shown that the acid treatment not only generates granular chitin particles but also microfibrillar crystalline (1→3)-*β*-D-glucan, suggesting that not only the liberated chitin chains but also the liberated glucan chains, from which (1 → 6)-*β*-linked glucose residues may have been removed by the acid treatment, line up to form crystallites in the bud scar region.

In *Neurospora crassa* the alkali-soluble part of the wall includes both glycoproteins (Mahadevan & Tatum, 1965, 1967) and (1→3)-α-D-glucan (de Vries, 1974); in *Aspergillus nidulans* the alkali-soluble part is mainly (1→3)-α-D-glucan (Zonneveld, 1974). In both cases at least some of this glucan is present in a crystalline form similar to that reported for the (1→3)-α-D-glucan present in the wall of *Schizophyllum commune* (Wessels *et al.*, 1972). The alkali-insoluble portion of the wall in these ascomycetes consists of (1→3)-*β*-D/(1→6)-*β*-D-glucan and 12% and 43% glucosamine in *Neurospora crassa* and *Aspergillus nidulans*, respectively. Which part of this glucosamine is actually contained in chitin is difficult to say but a considerable part may be present in polymers other than chitin (Troy & Koffler, 1969). As can be seen in Fig. 1, most of the *β*-glucan is soluble in dimethyl sulphoxide and

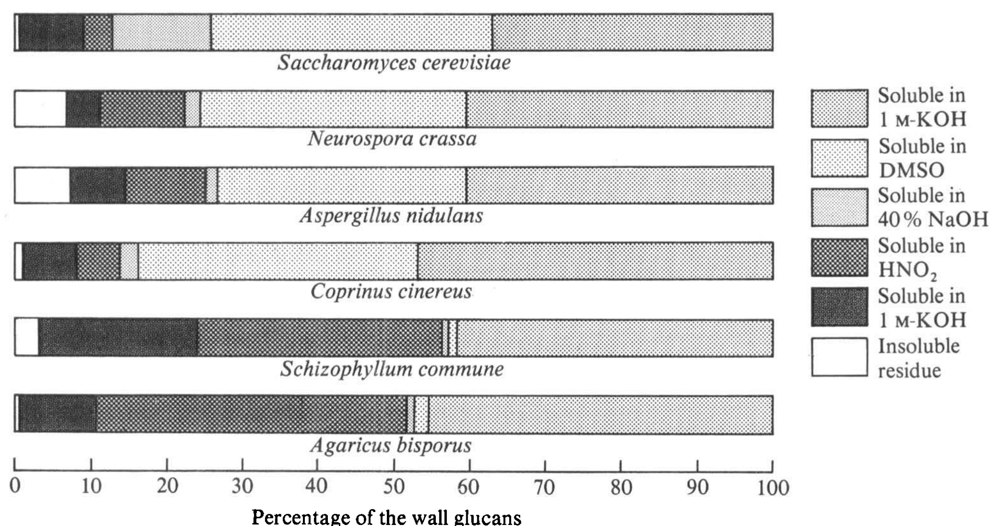

Fig. 1. Effect of several consecutive treatments on the extraction of glucans from the hyphal walls of various fungi.

subsequent treatment of the residue with 40% NaOH at 100 °C causes little reduction in the amount of β-glucan. However, a large part of the remaining glucan becomes water- or alkali-soluble after depolymerization of the deacetylated chitin with HNO_2. The alkali-soluble fraction of *Coprinus cinereus* walls consists of (1→3)-β-D/(1→6)-β-D-glucan (Schaefer, 1977). The alkali-resistant portion also contains a glucan with at least (1→3)-β-linkages, for after a short acid treatment a hydroglucan X-ray diffraction pattern was obtained (unpublished); this wall fraction also contains glucosamine (25%) at least partly in a homopolymeric form because the presence of chitin in these walls has been confirmed by X-ray diffraction methods (Frey, 1950). A large part of the glucan is extractable with DMSO. However, almost 15% of the alkali-insoluble glucan appears to be linked to chitin for it becomes water- and alkali-soluble after HNO_2 treatment.

The walls of *Schizophyllum commune* and *Agaricus bisporus* appear much the same, both chemically and structurally (Wessels *et al.*, 1972; van der Valk *et al.*, 1977; Michalenko *et al.*, 1976). In both cases we have found that the alkali-soluble part is mainly (1→3)-α-D-glucan at least partly present as microcrystals. In addition to (1→3)-β-D/(1→6)-β-D-glucan, the alkali-insoluble part was reported to contain 51% and 22·5% glucosamine in *Agaricus bisporus* and *Schizophyllum commune*, respectively. In *Schizophyllum commune* it was found that only 18·7% of the glucosamine could be present in chitin (Sietsma & Wessels, 1977). As shown in Fig. 1, dimethyl sulphoxide extracts very little β-glucan from the alkali-insoluble residues of the walls of these basidiomycetes. Also, the subsequent treatment with 40% NaOH at 100 °C causes little extraction or degradation of β-glucan. In both cases, however, depolymerization of chitin is accompanied by solubilization of all the β-glucan in water or alkali. In accordance with results obtained previously with *Schizophyllum commune* walls (Sietsma & Wessels, 1977), we found that chitin in the walls or alkali-insoluble residues of *Agaricus bisporus* is only weakly crystalline but becomes highly crystalline after acid treatment. This may be due to hydrolysis of the linkage between chitin and β-glucan, allowing the chains of the former to crystallize (Sietsma & Wessels, 1979).

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