Nature of the Nets Produced by Protoplasts of *Schizosaccharomyces pombe* During the First Stage of Wall Regeneration in Liquid Media

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The microfibrillar nets formed during the first stage of wall regeneration by protoplasts of *Schizosaccharomyces pombe* in a liquid medium contain, in contrast to normal walls, (1→3)- β -linked glucan of high crystallinity, like the nets produced by protoplasts of *Saccharomyces cerevisiae* under the same conditions. During wall regeneration by protoplasts of *Schizosaccharomyces*, β -glucan synthesis precedes the formation of (1→3)- α -glucan.

INTRODUCTION

The microfibrillar nets produced by protoplasts of *Saccharomyces cerevisiae* in liquid media are structurally and chemically different from those in normal walls (Kopecká *et al.*, 1967). The latter are fine networks (Houwink & Kreger, 1953; Kreger & Kopecká, 1973, 1976a; Kopecká *et al.*, 1974a) and consist mainly of (1→3)- β -linked, (1→6)- β -branched glucan (Manners *et al.*, 1973). The former have much wider meshes and microfibrils with characteristic straight parts and fasciations. They contain about 40% (dry wt) unbranched (1→3)- β -linked D-glucan and 10% chitin, both conspicuously crystalline as is apparent from their X-ray patterns (Kreger & Kopecká, 1973, 1976a). The crystallinity and high degree of polymerization of the unbranched glucan may explain the ultrastructural features of the nets (Kreger & Kopecká, 1976b), while the differences between the protoplast nets and the glucan nets in normal walls have been attributed to selective removal or inhibition of (1→6)- β -branching enzyme by the liquid medium (Kreger & Kopecká, 1973, 1976a).

The fission yeasts of the genus *Schizosaccharomyces* have cell walls containing a branched glucan with (1→3)- β -linked chains (Kreger, 1954, 1967; Bush *et al.*, 1974; Manners & Meyer, 1977), like those of *Sacch. cerevisiae*. The walls of *Schizosaccharomyces* differ from the latter in that they also contain (1→3)- α -glucan (Bacon *et al.*, 1968). This glucan is easily identified by its X-ray pattern, even in untreated walls, and in *Schiz. octosporus* it constitutes over 40% of the wall dry weight (Kreger, 1954, 1967). In *Schiz. pombe* the percentage is lower: 28% (Bush *et al.*, 1974) or 18% (Manners & Meyer, 1977).

Protoplasts of *Schiz. pombe*, cultivated in liquid medium, form a network of microfibrils which, in contrast to that of the protoplasts of *Sacch. cerevisiae*, increases in density. A new wall is formed underneath, and subsequently cell division takes place (Nečas *et al.*, 1968a; Havelková, 1972). The network formed within the first 2 h cultivation (Nečas *et al.*, 1968a, Fig. 10; this paper, Fig. 2) strongly resembles, in its structural features, the nets formed by protoplasts of *Sacch. cerevisiae* described above, suggesting that they have the same constitution. In this paper, we present evidence from X-ray diffraction and ancillary electron microscopy which supports this suggestion.

METHODS

Schizosaccharomyces pombe (strain 44-1-3, Czechoslovak Collection of Microorganisms, Institute of Chemistry, Slovak Academy of Sciences, Bratislava) was cultivated, protoplasts were obtained, cultivated and lysed, and the nets were cleaned, according to the methods described for *Sacch. cerevisiae* (Kreger & Kopecká, 1976a). Protoplasts were cultivated for 4 h. Walls of *Schizosaccharomyces* species are incompletely degraded by snail enzyme and frequently adhere as empty envelopes to the released protoplasts. Apart from the place of adherence, the surface of such protoplasts is clean (Nečas *et al.*, 1968a). Two samples of nets were prepared: (a) a sample in which about 35 % of the nets adhered to old walls; (b) a sample of which the proportion of nets that adhered to old walls had been reduced to about 10 % by careful shaking and centrifugation. Methods for complete digestion of old walls (Kopecká *et al.*, 1974b; Kopecká, 1975) were considered to be unnecessary for our purpose. Normal walls of *Schiz. pombe* and *Schiz. octosporus* were from type strains of the yeast culture collection at Delft of the Centraal Bureau voor Schimmelcultures, Baarn, and were prepared from suspensions of cells broken by shaking with ballotini glass beads in a Mickle homogenizer. Electron micrographs, X-ray diagrams and densitometer curves were obtained as indicated by Kreger & Kopecká (1976a).

RESULTS AND DISCUSSION

The densitometer curves of the X-ray patterns from normal walls of *Schiz. octosporus* and *Schiz. pombe*, the net samples (a) and (b) from *Schiz. pombe* protoplasts and a net sample from *Sacch. cerevisiae* protoplasts are shown in Fig. 1 as curves A, B, C, D and E, respectively. Curve E in Fig. 1 is the same as Fig. 11 (b) B of Kreger & Kopecká (1976a). These nets from *Sacch. cerevisiae* had been extracted with cold 30 % (w/v) HCl to remove chitin, which is not produced by *Schizosaccharomyces* (Roelofsen & Hoette, 1951; Kreger, 1954).

The curves from X-ray patterns of native walls (Fig. 1, A and B) show only one peak attributable to β -glucan, which is broad and low, indicating a low crystalline order of this chain type. The α -glucan peaks are higher in the curve from *Schiz. octosporus* walls than in the one from walls of *Schiz. pombe*, thus confirming the data (see Introduction) on the difference in α -glucan content between these walls. The peaks represent lattice spacings of 0.917, 0.497 and 0.423 nm, respectively. The β -glucan spacings represented by the peaks in curve E were reported by Kreger & Kopecká (1976a).

The samples of protoplast nets of *Schiz. pombe* were clearly different from each other: sample (a), with the higher content of old walls, produced considerably higher intensity peaks attributable to α -glucan (Fig. 1, curve C) than did sample (b) (Fig. 1, curve D), while in the latter the β -glucan peaks were more pronounced. Both samples differed markedly from the native walls by the high crystallinity of the β -glucan; this is evident from the sharp inner peak (β_1) and the appearance, in particular in curve D, of nearly all β -glucan reflections observed in the diagram from the nets of *Sacch. cerevisiae* protoplasts (Fig. 1, curve E).

Comparison of curves C and B in Fig. 1 shows that the specimen of protoplast nets with many old walls gave much more pronounced α -glucan peaks than did the native walls of *Schiz. pombe*. This may be explained by selective digestion of the non- α -glucan components of the old walls during the incubation for protoplast production.

The X-ray patterns of the samples of young protoplast nets of *Schiz. pombe* showed that they had a high content of (1 $\rightarrow$ 3)- β -glucan, which was well crystallized, as it is in the nets of *Sacch. cerevisiae*. This glucan must have been formed *de novo* by the protoplasts since (i) the normal walls only produced a diffuse β -glucan ring and (ii) the sample with the smaller contamination by old walls gave the better resolution of β -glucan peaks. We may conclude that during the first stage of wall regeneration *Schiz. pombe* protoplasts produce only or mainly (1 $\rightarrow$ 3)- β -glucan chains, perhaps with a fraction branched in C-6, as do *Sacch. cerevisiae* protoplasts (Kreger & Kopecká, 1976a). Hence, the structural similarity of the microfibrillar pattern of the young nets of *Schiz. pombe* protoplasts (Fig. 2) to that of the protoplast nets of *Sacch. cerevisiae* may be attributed to the concomitant correspondence

Fig. 1. Radial density distribution curves of X-ray patterns taken at 40 mm from the specimen: α 1 to 3, α -glucan peaks; β 1 to 10, β -glucan peaks. A, Normal walls of *Schiz. octosporus*; B, normal walls of *Schiz. pombe*; C, net sample (a), about 35 % of nets adhering to old walls; D, net sample (b), about 10 % of nets adhering to old walls; E, nets from protoplasts of *Sacch. cerevisiae* extracted with cold 30 % (w/v) HCl.

in constitution. The effect of the liquid medium on synthesis and ultrastructure of the β -glucan appeared, therefore, to be the same as in *Sacch. cerevisiae* (Kreger & Kopecká, 1976a, b).

The formation of nets by *Sacch. cerevisiae* protoplasts is inhibited by the action of snail enzyme added to the culture liquid (Kopecká *et al.*, 1967; Svoboda & Nečas, 1968). Apparently the (1 $\rightarrow$ 3)- β -glucan is digested before microfibril formation takes place. Similarly, the young nets of *Schiz. pombe* protoplasts discussed here were not formed if snail enzyme was added to the medium (Havelková, 1972); this supports the preceding conclusion that they consist of (1 $\rightarrow$ 3)- β -glucan. Nevertheless, with protoplasts of *Schiz. pombe*, in contrast to those of *Sacch. cerevisiae*, net formation is not completely inhibited by the action of snail enzyme (Havelková, 1972). After 24 h cultivation an incomplete wall was formed with a structure (Havelková, 1972, Fig. 7) which, though not well resolved, was different from that of the (1 $\rightarrow$ 3)- β -glucan nets shown in Fig. 2. This material was obviously not attacked by snail enzyme, as is also the case with (1 $\rightarrow$ 3)- α -glucan (de Vries & Wessels, 1973). We repeated this experiment with 14 h cultivation. Examination of the nets obtained at higher magnification (Fig. 3) revealed that they only contained rather short microfibrils with a nodose, rope-like appearance, characteristic of specimens rich in (1 $\rightarrow$ 3)- α -glucan (Carbonell *et al.*, 1970; San-Blas & Carbonell, 1974). No trace of the microfibrils like those in Fig. 2 could be detected. However, when sample (a), containing both α - and β -glucan (Fig. 1, curve C), was dissolved in dilute alkali and precipitated, both the nodose, short type and the long microfibrils were visible (Fig. 4). The latter are similar to those of the (1 $\rightarrow$ 3)- β -glucan of the nets of *Sacch. cerevisiae* after the same treatment (Kreger & Kopecká, 1976a, Fig. 9). These observations are evidence that the polysaccharide of the incomplete wall formed in a medium with snail enzyme is (1 $\rightarrow$ 3)- α -glucan.

Figs. 2, 3 and 4. For legend see opposite.

The present X-ray data combined with our electron micrographs and those published by Nečas *et al.* (1968*a*) and Havelková (1972) lead to the conclusion that in wall regeneration by *Schiz. pombe* protoplasts the formation of β -glucan precedes α -glucan synthesis. This result differs from the observation made in a study of the chemistry of wall regeneration in *Schizophyllum commune* by de Vries & Wessels (1975). These authors showed that synthesis of the β -glucan of these walls lagged behind for a considerable time after the formation of α -glucan and chitin had begun. Obviously the kinetics of wall regeneration are different in these fungi. Such a difference is also indicated by the observation that cycloheximide inhibits β -glucan formation in *Schizophyllum commune* protoplasts (de Vries & Wessels, 1975) whereas in yeast protoplasts (*Sacch. cerevisiae* in a solid medium) it had the effect that only the microfibrillar fraction of the wall (i.e. the β -glucan fraction) was formed and formation of the matrix (i.e. the mannan-protein fraction) was inhibited (Nečas *et al.*, 1968*b*; Elorza & Sentandreu, 1969). Since cycloheximide inhibits protein synthesis, the latter difference might be explainable, as noted by de Vries & Wessels (1975), if peptides form part of the β -glucan complex in the walls of *Schizophyllum commune*.

Another example of a young microfibrillar net, reminiscent on structural grounds of those produced by *Sacch. cerevisiae* and *Schiz. pombe* protoplasts, is the net produced by protoplasts of *Nadsonia elongata* (Havelková, 1969, Figs 9 and 10). Since its formation was also inhibited by snail enzyme [under these conditions a different net was formed, which was soluble in cold 30% (w/v) HCl and therefore chitinous] (Havelková, 1972, Fig. 6) and the normal walls of *Nadsonia elongata* contain the β -glucan found in *Sacch. cerevisiae* walls (Kreger, 1954), these microfibrils are most probably also of the crystalline (1 $\rightarrow$ 3)- β -glucan type. It seems, therefore, that in wall regeneration by protoplasts of yeast species whose walls contain a (1 $\rightarrow$ 3)- β -linked glucan branched in C-6, a liquid culture medium generally effects a deficiency in (1 $\rightarrow$ 6)- β -branching enzyme, which gives rise to the synthesis of long chains with (1 $\rightarrow$ 3)- β -links only and concomitant structural features of the microfibrils and nets due to a preponderance of this chain type.

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All bar markers represent 1 μ m.

Fig. 2. Net formed after 2 h on protoplast of *Schiz. pombe*, with adhering old wall on right side. Long microfibrils of (1 $\rightarrow$ 3)- β -glucan.

Fig. 3. Net formed after 14 h on protoplast of *Schiz. pombe* in culture medium with snail enzyme. Short, nodose type of microfibrils of (1 $\rightarrow$ 3)- α -glucan only; old wall, bottom right.

Fig. 4. Precipitate from neutralized alkali extract from nets of sample (a), with short, nodose and long microfibrils.

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