Taxonomic Significance of Yeast Sphaeroplast Release after Enzymic Treatment of Intact Cells

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(Received 10 November 1978)

Treatment of whole yeast cells with a mixture of a reducing agent and 1,3- β -glucanase isolated from Basidiomycete QM806 led to the production of sphaeroplasts from ascomycetes, from some fungi imperfecti, but not from basidiomycetes. Association of 1,3- β -glucanase with a second enzyme, 1,4- α -glucanase, from *Trichoderma viride*, was required for sphaeroplast release from some, but not all, basidiomycetes and fungi imperfecti. The ability of yeast cells to liberate sphaeroplasts following appropriate enzymic treatment is proposed as a taxonomic criterion for differentiating basidiomycetous from ascomycetous yeasts and for classifying fungi imperfecti yeasts.

INTRODUCTION

While sexual reproduction has long been a criterion for differentiating ascomycetes from basidiomycetes, new taxonomic approaches based on the structure and chemical composition of the yeast wall have recently been reported. Kreger-van Rij & Veenhuis (1971) established structural differences between these classes by electron microscopy. Van der Walt & Hopsu-Havu (1976) used a diazonium blue B staining reaction to separate these two classes. Polysaccharides are the major constituent of the yeast wall (Northcote, 1963; Phaff, 1971). Chemical analysis of wall polysaccharides led Bartnicki-Garcia (1968) to relate them to taxonomy, and Gorin & Spencer (1970) have used physical analysis of mannose-containing polysaccharides as an aid in classification.

Another possible taxonomic character is the sensitivity of these polysaccharides to the lytic action of various enzyme preparations. Indeed, several authors have demonstrated yeast parietal lysis by 1,3- β -glucanases (Bastide *et al.*, 1971; Mann *et al.*, 1972; Yamamoto *et al.*, 1974; Rombouts & Phaff, 1976). Furthermore, Bastide *et al.* (1973) showed that purified 1,3- β -glucanase associated with a reducing agent could liberate sphaeroplasts from whole *Candida* cells. The present communication reports the findings of a study aimed at extending this observation to a greater variety of yeast species in the hope of providing a useful means of separating ascomycetes from basidiomycetes.

METHODS

Strains. All the strains tested, representing 56 different species of yeasts belonging to the Ascomycetes or Basidiomycetes and 26 different species of fungi imperfecti yeasts, were from culture collections; most came from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and others were from the Institut Pasteur (IP), Paris, or from our own collection (IVP).

Strain cultivation. To minimize variations in wall constituents, only one synthetic medium, Yeast Morphology Agar (Difco B-393), was used for strain cultivation. Following three successive 48 h subcultures

at appropriate temperatures, all strains were grown for 15 h immediately prior to enzymic treatment. Agar slants were sown with a loopful of yeast suspension.

Enzymic preparations. Basidiomycete QM806, kindly provided by Elwyn T. Reese, was grown at 26 °C for 14 d in liquid medium containing glucose (1 %, w/v) as the carbon source (Reese & Mandels, 1959). The proteins were precipitated from the culture supernatant by addition of $(\text{NH}_4)_2\text{SO}_4$ at 25 to 50 % saturation. After dialysis against water and against 0.1 M-phosphate/citrate buffer, pH 6, the enzymic extract was fractionated on a Sephadex G-100 gel column (Pharmacia K 15/30).

Trichoderma viride QM6a (United States Army Natick Laboratories, Natick, Mass., U.S.A.) was grown in a liquid medium, pH 5.8, containing (per litre): 5 g glucose, 1 g polypeptone (Bio-Merieux, France), 0.3 g L-glutamic acid, 0.5 g KH_2PO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g CaCl_2 and 1 ml trace metal solution. Following incubation at 28 °C for 10 d, the culture supernatant was treated with 2 vol. acetone and kept for 1 h at -24 °C. The precipitate was suspended in distilled water and dialysed against the same solvent. The residue was discarded and the supernatant was dialysed against a 0.02 M-acetate buffer, pH 3.9. The enzymic preparation was then fractionated on a CM Sephadex C50 column (Pharmacia K 15/30) using a NaCl gradient in the same acetate buffer.

Enzyme assays. Enzyme fractions were assayed against the following substrates: laminarin (Koch-Light); soluble starch (Calbiochem); 1,3- α -glucan extracted from the wall of *Aspergillus niger* NRRL 326 by the method of Hasegawa & Nordin (1969); 1,6- β -glucan extracted from the wall of *Penicillium aculeatum* by the method of Nakamura & Tanabe (1963). Appropriately diluted enzyme fractions were added to 2.5 mg of each substrate suspended in 0.2 ml 0.1 M-phosphate/citrate buffer, pH 6. After 1 h incubation at 37 °C for starch and at 50 °C for the other substrates, reducing sugar was estimated by the Nelson-Somogyi method (Somogyi, 1952). One unit (u) of enzyme activity liberated 0.5 mg glucose equivalent in 1 h. Protease activity was assayed against the charcoal gelatin disc (Oxoid).

Preparation of yeast sphaeroplasts. Yeast cells from cultures grown for 15 h were suspended in 0.1 M-phosphate buffer, pH 6, at 2×10^8 cells ml^{-1} ; 0.2 ml of the suspension was exposed to each lytic system. The first system was composed of an enzyme [either 0.2 ml of Basidiomycete 1,3- β -glucanase II (200 u ml^{-1}) or 0.2 ml of *T. viride* 1,3- β -glucanase (40 u ml^{-1})] associated with a reducing agent (0.2 ml of 0.03 M-2-mercaptoethylamine in 0.1 M-phosphate buffer, pH 6). The second system was composed of two *T. viride* enzymes [0.2 ml of 1,3- β -glucanase (40 u ml^{-1}) and 0.2 ml of 1,4- α -glucanase (25 u ml^{-1})]. In both test systems, cells were osmotically stabilized with 0.5 M-KCl and incubated in haemolysis tubes on a reciprocal shaker at 26 °C for 24 h. Sphaeroplast formation was assessed by light microscopy, monitoring cell lysis on addition of water.

RESULTS

Enzyme activities

Basidiomycete QM806 produced two constitutive 1,3- β -glucanases (Fig. 1). These enzymes differed not only in molecular weight but also in their lytic activity against the wall of ascomycetous yeasts as demonstrated by sphaeroplast release: 1,3- β -glucanase I was very active against laminarin and inactive against the ascomycetous wall, whereas 1,3- β -glucanase II was less active against laminarin but highly active against the ascomycetous wall.

Trichoderma viride also produced two glucanases (Fig. 2): a 1,3- β -glucanase which was very active against laminarin as well as against the ascomycetous wall, and a 1,4- α -glucanase which alone could not liberate sphaeroplasts from either ascomycetous or basidiomycetous yeast cells. However, together the enzymes did release sphaeroplasts from basidiomycetous cells. No 1,3- α -glucanase, 1,6- β -glucanase or protease activity was detected in the enzyme preparations described above.

Yeast differentiation using two lytic systems

A total of 91 yeast species were examined in the lytic systems (Table 1). In the first lytic system, all ascomycetous yeasts tested yielded sphaeroplasts, while all basidiomycetous yeasts were resistant to this treatment, even after exposure for 48 h. This 'all-or-none' response was not observed with the fungi imperfecti yeasts tested: four genera (*Brettanomyces*, *Kloeckera*, *Trigonopsis* and *Candida*) yielded sphaeroplasts and appeared to respond like ascomycetes, while the three other genera (*Cryptococcus*, *Rhodotorula* and *Sterigmatomyces*) were insensitive in this system, like basidiomycetes.

Fig. 1. Gel filtration of the enzyme preparation from Basidiomycete QM806: 2 mg of the fraction obtained between 25 and 50% $(\text{NH}_4)_2\text{SO}_4$ saturation were fractionated on Sephadex G-100, using 0.1 M-citrate/phosphate buffer, pH 6, as eluant. Fractions of 2 ml were collected. ○, Enzyme activity on laminarin; —, protein (transmission at 280 nm).

Fig. 2. CM Sephadex C50 chromatography of the enzyme preparation (3 mg protein) from *T. viride*. Fractions of 2 ml were collected. ○, Enzyme activity on laminarin; ●, enzyme activity on soluble starch; ---, NaCl concentration; —, protein (transmission at 280 nm).

In the second lytic system, 26 basidiomycetous species, as well as the 13 species of fungi imperfecti which responded like basidiomycetes in the first system, were examined. Among the basidiomycetes, only three species, namely *Sporobolomyces roseus*, *Sp. gracilis* and *Sp. singularis*, were insensitive in this system, while within the fungi imperfecti all *Cryptococcus* species were sensitive and all *Rhodotorula* and *Sterigmatomyces* species were resistant.

DISCUSSION

Structural studies on walls of ascomycetous yeasts have revealed the presence around the glucan layer of an envelope composed of a mannan-protein complex rich in disulphide bridges (Lampen, 1968; Kidby & Davies, 1970). Our results seem to be in agreement with this finding since the addition of a reducing agent was required for maximum sphaeroplast release from ascomycetous yeasts.

While very little is known about the wall structure of basidiomycetous yeasts, the presence of a starch-like substance in the growth medium of some *Bullera* and *Leucosporidium* species has been reported by Phaff (1970) and Fell *et al.* (1969). Our results suggest that, except for

Table 1. *Yeast sphaeroplast release after enzymic treatment**

Species	Strain no.	1,3- β - Glucanase and reducing agent	1,3- β - Glucanase and 1,4- α - glucanase
Ascomycetes			
<i>Dipodascus aggregatus</i>	CBS 17553	+	
<i>Dip. albidus</i>	CBS 15257	+	
<i>Endomyces lactis</i>	CBS 17853	+	
<i>E. geotrichum</i>	IVP 603	+	
<i>Nematospora coryli</i>	CBS 2608	+	
<i>Nadsonia elongata</i>	CBS 114 T	+	
<i>Saccharomycodes ludwigii</i>	CBS 821	+	
<i>Sacch. ludwigii</i> var. <i>bisporus</i>	CBS 2624	+	
<i>Hanseniaspora apuliensis</i>	CBS 1972	+	
<i>Hans. valbyensis</i>	IVP 399	+	
<i>Lipomyces lipofer</i>	CBS 944	+	
<i>L. starkeyi</i>	IVP 405	+	
<i>Saccharomyces carlsbergensis</i>	IVP 386	+	
<i>S. cerevisiae</i>	IVP 387	+	
<i>S. ellipsoideus</i>	IVP 389	+	
<i>S. fragilis</i>	IVP 390	+	
<i>S. rosei</i>	IVP 391	+	
<i>S. uvarum</i>	IVP 599	+	
<i>Kluyveromyces africanus</i>	CBS 2517	+	
<i>K. polysporus</i>	CBS 2163	+	
<i>Endomycopsis bispora</i>	CBS 5515	+	
<i>End. capsularis</i>	CBS 2519	+	
<i>End. javanensis</i>	CBS 2555	+	
<i>End. chodati</i>	IVP 601	+	
<i>Pichia fermentans</i>	CBS 187	+	
<i>P. bovis</i>	IVP 400	+	
<i>P. membranaefaciens</i>	CBS 107	+	
<i>P. fibuligera</i>	CBS 2521	+	
<i>Hansenula anomala</i>	CBS 112	+	
<i>H. angusta</i>	CBS 1976	+	
<i>H. californica</i>	CBS 5760	+	
<i>H. beckii</i>	CBS 1890	+	
<i>Citeromyces matritensis</i>	CBS 2764	+	
<i>Debaryomyces japonicus</i>	CBS 2334	+	
<i>Deb. kloeckeri</i>	CBS 5770	+	
<i>Deb. kloeckeri</i>	CBS 770	+	
<i>Deb. hansenii</i>	CBS 767	+	
<i>Metschnikowia pulcherrima</i>	IVP 604	+	
<i>Dekkera intermedia</i>	IVP 598	+	
Basidiomycetes			
<i>Leucosporidium antarcticum</i>	CBS 5942	—	+
<i>Leu. nivale</i>	CBS 5266	—	+
<i>Leu. gelidum</i>	CBS 5272	—	+
<i>Leu. frigidum</i>	CBS 5270	—	+
<i>Leu. scottii</i>	CBS 614	—	+
<i>Leu. capsuligenum</i>	CBS 1906	—	+
<i>Sporobolomyces salmonicolor</i>	CBS 490	—	+
<i>Sp. odoratus</i>	CBS 2636	—	+
<i>Sp. holsaticus</i>	CBS 1522	—	+
<i>Sp. albidus</i>	CBS 2839	—	+
<i>Sp. gracilis</i>	CBS 71	—	—
<i>Sp. roseus</i>	CBS 1486	—	—

Table 1 (cont.)

Species	Strain no.	1,3- β - Glucanase and reducing agent	1,3- β - Glucanase and 1,4- α - glucanase
<i>Sp. hispanicus</i>	CBS 2873	—	+
<i>Sp. singularis</i>	CBS 5109	—	—
<i>Bullera alba</i>	CBS 501	—	+
<i>B. dendrophila</i>	CBS 6074	—	+
<i>B. tsugae</i>	CBS 5038	—	+
<i>B. piricola</i>	CBS 6754	—	+
	(Stadelman 141 L)		
<i>Rhodospiridium diobovatum</i>	CBS 6085	—	+
<i>Rhodosp. toruloides</i>	CBS 14	—	+
<i>Rhodosp. sphaerocarpum</i>	CBS 5939	—	+
<i>Rhodosp. capitatum</i>	CBS 6358	—	+
<i>Rhodosp. infirmominiatum</i>	CBS 323	—	+
<i>Rhodosp. malvinellum</i>	CBS 6082	—	+
<i>Filobasidium capsuligenum</i>	CBS 1906	—	+
<i>Sporidiobolus johnsonii</i>	CBS 522-50	—	+
Fungi Imperfecti			
<i>Brettanomyces anomalus</i>	CBS 77	+	±
<i>Br. bruxellensis</i>	CBS 72	+	±
<i>Kloeckera apiculata</i>	IVP 408	+	±
<i>Kl. africana</i>	CBS 277	+	±
<i>Kl. javanica</i>	CBS 282	+	±
<i>Trigonopsis variabilis</i>	CBS 1040	+	±
<i>Candida albicans</i>	IP 628	+	±
<i>C. krusei</i>	IP 208	+	±
<i>C. macedoniensis</i>	CBS 600	+	±
<i>C. pelliculosa</i>	IP 606	+	±
<i>C. pseudotropicalis</i>	IP 513	+	±
<i>C. tropicalis</i>	IP 857	+	±
<i>C. utilis</i>	CBS 621	+	±
<i>Cryptococcus albidus</i> var. <i>diffluens</i>	CBS 160	—	+
<i>Cr. ceraneus</i>	CBS 6644	—	+
<i>Cr. flavus</i>	CBS 331	—	+
<i>Cr. gastricus</i>	CBS 2288	—	+
<i>Cr. laurentii</i>	CBS 139	—	+
<i>Cr. neoformans</i>	CBS 132	—	+
<i>Rhodotorula aurantiaca</i>	CBS 317	—	—
<i>Rh. glutinis</i>	CBS 124 T	—	—
<i>Rh. lactosa</i>	CBS 5826	—	—
<i>Rh. marina</i>	CBS 2365	—	—
<i>Rh. pallida</i>	CBS 320	—	—
<i>Sterigmatomyces indicus</i>	CBS 5628	—	—
<i>St. polyborus</i>	CBS 6072	—	—

* +, Release of sphaeroplasts; ± weak release of sphaeroplasts; —, no sphaeroplasts formed.

the three species of *Sporobolomyces*, all other basidiomycetous yeasts as well as the species of *Cryptococcus* tested possess 1,4- α -glucan associated with 1,3- β -glucan in their walls.

Using the wall lytic systems described here, we have not only been able to separate ascomycetous from basidiomycetous yeasts but have also been able to place some of the fungi imperfecti in one or other of these two groups. This latter finding is consistent with the results of other investigations. Kreger-van Rij & Veenhuis (1971) showed the presence of a typical basidiomycetous lamellar structure in the walls of *Cryptococcus diffluens*, *Rhodotorula glutinis* and *Sterigmatomyces halophilus*. Van der Walt & Hopsu-Havu (1976) showed that

Cryptococcus albidus, *Cr. laurentii* and *Cr. neoformans* as well as *Rhodotorula minuta*, *Rh. rubra*, *Sterigmatomyces elviae* and *St. polyborus* reacted like basidiomycetes to diazonium blue staining. Earlier studies of DNA base content of fungi imperfecti yeasts revealed that, having < 50% G+C content, *Candida* species, *Kloeckera apiculata*, *Brettanomyces bruxellensis* and *Trigonopsis variabilis* could be grouped with the ascomycetes, while *Rhodotorula glutinis*, *Rh. rubra* and *Sterigmatomyces elviae* could be grouped with the basidiomycetes which have > 50% G+C content (Nakase & Komagata, 1968, 1971; Meyer & Phaff, 1969). By DNA hybridization studies, Stenderup & Leth Bak (1969) identified *Kluyveromyces fragilis* as the perfect form of *Candida pseudotropicalis*, and indeed, this species grouped with the ascomycetes in our tests. Furthermore, Kwon-Chung (1975) and Kwon-Chung & Popkin (1976) identified the basidiomycete *Filobasidiella neoformans* as the perfect form of *Cryptococcus neoformans* and, because of the similarity in their extra-cellular polysaccharides, Slodki *et al.* (1966) suggested that *Tremella* and *Cryptococcus* might be related.

While the selective sensitivity to the first lytic system developed provides a convenient means for separating the two major classes, the separation of species within the basidiomycetes can be achieved by the use of the second lytic system. So far, *Bullera* and *Sporobolomyces* have been distinguished only by their ballistospore forms; our results suggest that five of the eight species of *Sporobolomyces* are related to *Bullera* and that the three species which were not sensitive to the second lytic system are true *Sporobolomyces* (*Sp. roseus*, *Sp. gracilis* and *Sp. singularis*). Using the second lytic system we have also been able to separate the genus *Cryptococcus* from the genus *Rhodotorula*. These two fungi imperfecti have been shown by chemical analysis to differ in the nature of their wall polysaccharides (Elinov *et al.*, 1974). Our results strongly suggest that sphaeroplast release by means of the two lytic systems investigated could be useful in improving the classification of fungi imperfecti yeasts.

We thank Drs A. and S. L. Salhi for helpful discussion during the preparation of this manuscript. The technical assistance of Miss S. Jouvert is greatly appreciated. We also thank the United States Army Natick Laboratories and Dr E. T. Reese for generously supplying the micro-organisms.

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