Phytochemistry, 1975, Vol. 14, pp. 2347–2351. Pergamon Press. Printed in England.

STEROL-BINDING POLYSACCHARIDES OF *RHIZOPUS ARRHIZUS, PENICILLIUM ROQUEFORTII* AND *SACCHAROMYCES CARLSBERGENSIS*

C. G. P. PILLAI* and J. D. WEETE†

Department of Botany and Microbiology, Auburn University Agricultural Experiment Station, Auburn, Al 36830, U.S.A.

(*Received* 7 *March* 1975)

Key Word Index—*Rhizopus arrhizus*; Mucorales; *Penicillium roquefortii*, Hyphales; *Saccharomyces carlsbergensis*; Endomycetales; sterol binding polysaccharides.

Abstract—Polysaccharides that bind with sterols and render them water-soluble were isolated from two mycelial fungi, *Rhizopus arrhizus* and *Penicillium roquefortii* and a yeast *Saccharomyces carlsbergensis*. The polysaccharides from *R. arrhizus* and *S. carlsbergensis* were accompanied by small quantities of phosphorus, protein and lipid, none of which significantly influenced the binding of sterol to polysaccharide. The chemical composition and sterol-binding properties of the polysaccharides from the filamentous species were almost identical, but differed significantly from those of the yeast polysaccharide. The principal sterol-binding polysaccharide of *S. carlsbergensis* was identified as a mannan and that of the filamentous fungi as a glucan(s). The binding capacity of the purified yeast polysaccharide was almost two-fold greater than that of *R. arrhizus* and *P. roquefortii*.

INTRODUCTION

Adams and Parks [1] first reported the presence in commercial yeast extract of a material that could bind non-covalently with cholesterol and ergosterol and render them water-soluble. They subsequently reported that this material was present in aqueous extracts of yeast and was polysaccharide in nature [2]. The polysaccharide nature of the sterol-binding substance was later confirmed and it was identified as cell-wall mannan [3]. More recently, a bound form of sterols was found in the protozoan *Euglena gracilis* Z. [4] and higher plants such as maize [5] and *Kalanchoe* [6].

The research reported here was conducted to determine whether sterol-binding polysaccharides are present in non-yeast fungi and, if so, to compare the chemical and physical nature of these substances from mycelial and yeast fungi.

* Post-doctoral Research Fellow.

† To whom reprints requests should be addressed.

RESULTS AND DISCUSSION

Chemical composition of sterol-binding polysaccharides. Chemical analyses showed that the purified hot-water extracts of *S. carlsbergensis* and *R. arrhizus* that bind with cholesterol and ergosterol contained between 83 and 95% polysaccharide. The polysaccharides were accompanied by relatively small amounts of protein, phosphorus, unidentified lipid and sterols. The composition of the polysaccharide preparations from these fungi was very similar to that of *S. cerevisiae* [3] and is typical of similar extracts of isolated fungal cell walls [13].

Acid hydrolysis of the polysaccharide preparation from *R. arrhizus* yielded a mixture of 4 principal monosaccharides. The major sugar was glucose (42·3%) which was accompanied by two unidentified sugars and mannose (10·1%) (Table 1). *R. arrhizus* (class Mastigomycotina, subclass Zygomycete) produce cell walls of the chitin–chitosan type, a distinguishing feature of which is the absence of glucose polymers in the vegetative

Table 1. Principal monosaccharide components of sterol-binding polysaccharides of *S. carlsbergensis* and *R. arrhizus*

Fungus	Mannose	Glucose	Monosaccharides (%)*†‡ 1	2	3	4
Saccharomyces carlsbergensis	53·5	—	34·5	7·2	—	—
Rhizopus arrhizus	10·1	42·3	—	—	14·2	30·1

* The Cifonelli fractions were used in the analytical studies. Each contained additional components which ranged from trace amounts to 5% of the total polysaccharide precipitate.

† Mannose and glucose (as TMS derivatives) were identified by comparison of GLC retention times with known standards on OV-1 and SE-30.

‡ Compounds 1–4 represent unknown constituents.

cell walls [13]. However, the spore walls of *Mucor rouxii*, also of the class Mastigomycotina, subclass Zygomycete, contain glucose at levels almost identical to those reported in this study for water-soluble polysacchardies from vegetative hyphae of *R. arrhizus* [13]. The low proportion of mannose in polysaccharides from *R. arrhizus* is consistant with other species in this group [13].

The monosaccharide composition of the soluble sterol-binding polysaccharides from *S. carlsbergensis* differed from that of *R. arrhizus* but was similar to that reported previously for *S. cerevisiae* [3]. Mannose (53·5%) was the principal sugar in the yeast polysaccharide which was accompanied by two unidentified sugars. Mannan is one of the principal structural components of yeast cell walls [14] and the polysaccharides isolated from *S. carlsbergensis* in this study are probably associated with the cell wall. This is supported by the phosphorus content of the polysaccharide preparation (see below).

Other components of the water-soluble polysaccharide preparations from *R. arrhizus* and *S. carlsbergensis* included phosphorus, protein and lipid. It is well known that phosphopolysaccharides are components of fungal cell walls. For example, Stewart and Ballou [15] showed that the cell walls of several yeasts contain a phosphomannan and that the phosphorus content varies considerably with species and culture age. The value obtained in this study for the molar ratio of mannose to phosphorus for the polysaccharide from *S. carlsbergensis* (24·0) agrees closely with that (21·0) reported previously for this species [13]. The phosphorus content is considered high when compared to *S. cerevisiae* (120·0) [2,15]. The phosphorus content of polysaccharide from *R. arrhizus* was two-fold greater than that of the yeast (Table 2). The protein contents of the polysaccharides of *S. carlsbergensis* and *R. arrhizus* (Table 2) were in the expected range for fungal cell walls [13].

If the sterol–polysaccharide complex occurs *in vivo* and possibly has some physiological significance, bound sterols should be present in the polysaccharide preparations. The polysaccharides (non-defatted) from each species were refluxed in alkaline-pyrogallol and the sterols recovered in hexane. Although sterols were detected in the hexane wash from each species, they comprised a low proportion of the polysaccharide which suggests that the sterol–polysaccharide complexes are of little physiological significance in the cell (Table 2). On the other hand, bound sterols were present in the aqueous extracts of several higher plants [5,6] and were almost as abundant as the free sterols in *Euglena gracilis* [5]. Pryce [6] showed that total sterols and squalene were present in low relative amounts (0·16 and 0·15%, respectively) in aqueous extracts of *Kalanchoe blossfeldiana*.

Table 2. Minor constituents of sterol-binding polysaccharides isolated from *S. carlsbergensis* and *R. arrhizus*

Fungus	Protein	μg/mg Polysaccharide precipitate Phosphorus	Sterols	Lipid	Total sugars*
Saccharomyces carlsbergensis	127·2	5·2	0·64	—	830
Rhizopus arrhizus	33·5	9·5	0·23	49·5	946

* Estimated by the anthrone method [21].

Although the polysaccharide preparations contained very low amounts of bound sterol, the polysaccharide preparation from *R. arrhizus* contained 4·95% of lipid (Table 2). The cell wall lipid content of over 40 fungal species has been reported and the value reported here for *R. arrhizus* falls within the expected range for fungi (1–18·9%), particularly Phycomycetes (3·1–12·0) [16]. Little is known about the lipids present in fungal cell walls although polyprenols have received considerable attention because of their role in polysaccharide biosynthesis [17], particularly in yeast [18]. Dolichols from yeasts did not have the same R_f values as the lipids from *R. arrhizus* polysaccharide.

Water-soluble polysaccharide from another mycelial fungus, *Penicillium roquefortii*, was also examined. While analysis of this polysaccharide was not as complete as for the other species, the monosaccharide and phosphorus content was very similar to that of *R. arrhizus*. The GLC separation pattern of monosaccharides of the two mycelial species was identical. In *P. roquefortii*, glucose was the principal sugar (50·4%) and it was accompanied by two unidentified sugars and mannose (10·1%). The phosphorus content of the polysaccharide preparation was 11·4 μg/mg. The sugar and phosphorus content of *P. roquefortii* water-soluble polysaccharide agree closely with that reported previously for the cell wall of this species [19].

Binding properties of the fungal polysaccharides. It is difficult to obtain quantitative recoveries of sterols dispersed in water, but the recovery can be raised to 100% by adding phosphate to the water [1]. We obtained similar results in this study, except that only 85% recovery was obtained with the addition of phosphate. Also, the recovery was affected very little by pH in the range 5—8. Binding of ergosterol by the polysaccharides from *R. arrhizus*, *P. roquefortii*, and *S. carlsbergensis* was not significantly influenced by pH in this range.

The crude polysaccharide extract from each species was initially precipitated from EtOH (EtOH precipitate fraction) and purified further by a second precipitation from Benedict's solution with KOH (Cifonelli fraction). When the degree of ergosterol-binding by these two fractions was compared, the Cifonelli fractions of *S. carlsbergensis* and *R. arrhizus* possessed the greater capacity for binding; containing 39·3 and 24·3% more sterol, respectively, than the corresponding EtOH precipitate fractions. There was no apparent difference in the sterol-binding capacity of the two polysaccharide fractions from *P. roquefortii*.

The effects of temperature on ergosterol binding were also determined. At temperatures of 35° and 4°, the degree of sterol-binding was decreased when compared to the degree of binding at 25°. For example, sterol-binding was decreased about 50% for each species at the lower temperature and 6–20% at the higher temperature. The decrease in binding at 4° was probably due to the lower solubility of the two constituents, particularly sterols.

The sterol-binding capacity of polysaccharides from the three fungi used in this investigation was determined by maintaining the polysaccharide concentration constant (1·0 mg/ml) and increasing the ergosterol content from 25–400 μg (Fig. 1). The binding capacity for the polysaccharides from *S. carlsbergensis* was 97 μg ergosterol per mg. Thompson *et al.* [3] showed that the sterol-binding capacity for polysaccharide isolated from *S. cerevisiae* was dependent on the mannan content (linear with respect to mannan). The binding capacity of mannan from this yeast was 185 μg ergosterol per mg of the monosaccharide. When calculated on the basis of mannose content, the binding capacity for polysaccharides of *S. carlsbergensis* was 218. Both of these values are higher

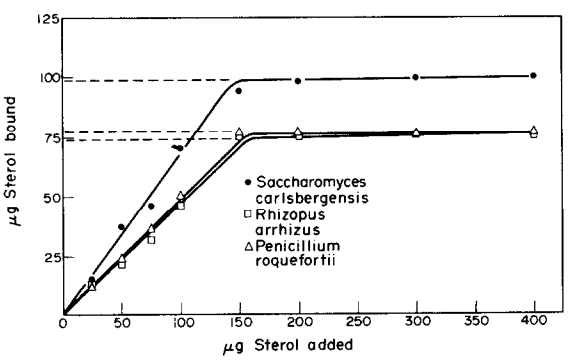

Fig. 1. Saturation curve showing the sterol-binding capacities for polysaccharide from *S. carlsbergensis*, *R. arrhizus*, and *P. roquefortii*. In each case, 25–400 μg ergosterol was added to 1·0 mg/ml of the polysaccharide material.

than that (160 μg/mg) previously published for commercial mannan [3].

The ergosterol-binding capacity of polysaccharides isolated from both *R. arrhizus* and *P. roquefortii* was about 76 μg ergosterol per mg (Fig. 1). Because of the low mannose content of polysaccharides from these fungi and the reported sterol-binding capacity for purified mannan [3], the binding of sterols by polysaccharides from these species cannot be attributed to the mannan content. This, combined with the fact that the sterol-binding capacity for purified polysaccharide extracted from *S. cerevisiae* and *S. carlsbergensis* is higher than that of commercial yeast mannan, strongly suggest that non-mannan polysaccharides also bind sterols. Comparison of the ratio of the moles of ergosterol bound per mole of the principal monosaccharides present shows that the binding efficiency of polysaccharide from *S. carlsbergensis* ($8{\cdot}3 \times 10^{-2}$ Moles ergosterol/mole mannan) is almost two-fold greater than that from *R. arrhizus* ($4{\cdot}5 \times 10^{-2}$ Moles ergosterol/mole glucose).

The chemical basis for the binding between sterols and polysaccharides of fungal origin has not been established. Although sterols are bound with considerable tenacity, covalent bonding is apparently not responsible for the sterol-polysaccharide association [2]. Comparisons of the phosphorus, protein, and lipid content and the sterol-binding capacity of the polysaccharides suggest that there is no relation between these constituents in the sterol–polysaccharide complex. In addition, removing the lipid and reducing the protein content of the polysaccharide does not result in corresponding reductions in the degree of sterol binding. Specific aspects of the polysaccharide structure may account for its sterol-binding ability. For example, cell wall mannan from *S. cerevisiae* is a highly branched mannose polymer with α-(1 → 2)-and α-(1 → 3)-linked side chains attached to an α-(1 → 6)-linked backbone [15]. The proportions of these linkages vary in different mannans and may be characteristic of different strains or species of yeast [15]. Branching within the mannan molecule may account for its sterol-binding properties while the degree and type of branching may determine the sterol-binding capacity. This may explain the slight variations in the sterol-binding capacity obtained in this study for polysaccharides from *S. carlsbergensis* and those previously reported for *S. cerevisiae* [3] and commercial mannan [3].

Although the cell wall mannan content of the filamentous fungi was relatively low, this study shows that soluble polysaccharides other than mannan are present which are capable of binding with sterols. Also, although the two filamentous fungi are classified differently according to their cell wall structure, the monosaccharide composition of the the soluble polysaccharides from each species was quantitatively and qualitatively very similar. In fungal cell wall analyses, preparations are initially separated into two or three crude fractions according to their solubility in water and/or alkali. The soluble fractions from fungi that have cell walls of the chitin-glucan type are usually mixtures composed of linear and branched α- or β-(1 → 4)-, (1 → 3)-, and (1 → 6)-linked glucans [13,14,20] and the alkali-insoluble residues of chitin and/or chitosan. The ability of the non-mannan polysaccharides from the filamentous fungi to bind sterols and the degree of binding may also be dependent on the presence, extent, and type of branching within the polysaccharide molecule. Whether the binding of sterols by fungal cell wall polysaccharides is only a transistory phenomenon or whether it has physiological significance is not known.

EXPERIMENTAL

Growth conditions. *Saccharomyces carlsbergensis* ATCC 2345, *Rhizopus arrhizus* AU 544 (Auburn University Culture Collection) and *Penicillium roquefortii* NRRL 849 were grown in vats containing 7 l. of medium as previously described [7]. Yeast cells were collected by low speed centrifugation (1000 *g*) and washed 2 × with dist. H_2O. Mycelia of *R. arrhizus* were collected on cheesecloth in a Buchner funnel, washed 2 × with dist. H_2O, and dried by lyophilization. While *P. roquefortii* was grown as described above, the mycelia was not grown in our laboratory and only a limited supply was available for this study.

Extraction and purification of polysaccharides. Yeast cells and the powdered mycelia were each refluxed for 2 hr in 0·01 M citrate buffer at pH 7·0 [8]. Insoluble fungal material was removed from the aq extract by centrifugation and the polysaccharide precipitated by the addition of absolute EtOH to a final concn of 50% and allowing it to stand at 4° for 2 hr. The polysaccharides were collected by centrifugation (2500 *g*) and re-pptd 2 × from 60% EtOH. Prior to use in the binding experiments or chemical analysis, the partially purified polysaccharides were either dried by lyophilization or further purified by a procedure modified from that of Cifonelli and Smith [9]. In the second purification step, the freshly-precipitated or lyophilized polysaccharide material was suspended

in Benedict's soln and precipitated by addition of 60% KOH to a final conc. of 10%. Blue ppt was collected by centrifugation (2500 *g*) and resuspended in 0·1 N HCl until the soln became clear. Purified polysaccharides were then recovered by precipitation from 60% EtOH as described above. Prior to purification the samples were defatted by washing first for 12 hr with *n*-hexane and 12 hr with $CHCl_3$: MeOH (2 : 1). The lipid extraction was aided by gentle heating of the extraction mixture and magnetic stirring.

Sterol–polysaccharide binding. Polysaccharide preparations from each species (0·5 mg) in 2 ml dist. H_2O were mixed in 0·4 ml absolute EtOH containing 0·2 mg ergosterol. After thorough mixing using a Vortex mixer, the soln was brought to pH 8 with 0·2 ml of 1·0 M Pi buffer. Unbound (freely extractable) ergosterol was removed by washing the soln 3× with 10 ml each of *n*-hexane. The combined hexane phases were evaporated under N_2 and the unbound ergosterol was assayed by a modified Liebermann–Burchard reaction [10]. The quantity of ergosterol bound to the polysaccharide was determined by difference. The effect of pH on the degree of sterol-polysaccharide binding was determined similarly, but the soln containing ergosterol and polysaccharide was maintained at the appropriate pH with Pi buffer.

Recovery of bound ergosterol. Polysaccharide-bound ergosterol was recovered by refluxing in methanolic-pyrogallol as previously described [1]. Pyrogallol (0·5%) in MeOH, 60% KOH, and absolute MeOH (3 : 2 : 3) were added to the dry polysaccharide and refluxed for 1·5 hr. Refluxing medium was cooled and washed 3× with 30 ml *n*-hexane. Combined hexane extracts were taken to dryness and the recovered sterols determined quantitatively as before.

Hydrolysis of the polysaccharides. Polysaccharides were refluxed with 20 ml 1·5 M H_2SO_4 for 5 hr and allowed to cool to room temp. overnight. Hydrolysate was neutralized with $BaCO_3$ and centrifuged (2500 *g*). Supernatant was filtered and taken to dryness by flash evaporation.

GLC. The monosaccharides obtained by acid hydrolysis of the fungal polysaccharides were converted to their trimethylsilyl ether (TMS) derivatives using Tri-Sil and separated by GLC on a 3 m × 0·2 mm glass column packed with 3% OV-1 or a 3 m × 0·4 mm stainless steel column packed with 3% SE-30. TMS derivatives were prepared by dissolving 10 mg or less of the monosaccharides in 1 ml Tri-Sil, heating and allowing to stand at least 5 min prior to injection.

Phosphorus and protein determinations. Phosphorus content of the fungal polysaccharides were determined by the method of Chen *et al.* [11] and protein contents by the method of Groves *et al.* [12].

Acknowledgements—The authors thank Dr. W. Tanner for his gift of yeast dolichols. This work was supported in part by the Research Corporation and by Auburn University Grant-in-Aid No. 73–36.

REFERENCES

1. Adams, B. G. and Parks, L. W. (1967) *Biochem. Biophys. Res. Commun.* **28**, 490.
2. Adams, B. G. and Parks, L. W. (1968) *J. Lipid Res.* **9**, 8.
3. Thompson, E. D., Knights, B. A. and Parks, L. W. (1973) *Biochim. Biophys. Acta* **304**, 132.
4. Brandt, R. D., Ourisson, G. and Pryce, R. J. (1969) *Biochem. Biophys. Res. Commun.* **37**, 399.
5. Anding, C., Brandt, R. D., Ourisson, G., Pryce, R. J. and Rohmer, M. (1972) *Proc. Roy. Soc. (Lond) B.* **180**, 115.
6. Pryce, R. J. (1971) *Phytochemistry* **10**, 1303.
7. Weete, J. D. and Laseter, J. L. (1975) *Lipids* **9**, 575.
8. Kocourek, J. and Ballou, C. E. (1969) *J. Bacteriol.* **100**, 1175.
9. Cifonelli, J. A. and Smith, F. (1955) *Am. Chem. Soc.* **77**, 5682.
10. Starr, P. R. and Parks, L. W. (1962) *J. cell Comp. Physiol.* **59**, 107.
11. Chen, P. S. Torbara, T. Y. and Warner, J. (1956) *Anal. Chem.* **28**, 1758.
12. Groves, W. E., Davis, F. C. and Sells, B. S. (1968) *Anal. Biochem.* **22**, 195.
13. Bartnicki-Garcia, S. (1968) *Ann. Rev. Microbiol.* **22**, 87.
14. Bartnicki-Garcia, S. (1970) *in Phytochemical Phylogeny* (Harborne, J. B. ed.), p. 81, Academic Press, New York.
15. Stewart, T. S., Mendershausen, P. B. and Ballou, C. E. (1968) *Biochemistry* **7**, 1843.
16. Weete, J. D. (1974) *Fungal Lipid Biochemistry*. p. 28–29, New York.
17. Hemming, F. W. (1974) *in Biochemistry of Lipids* (Goodwin, T. W. ed.) p. 39–97, University Park Press, Baltimore.
18. Jung, P. and Tanner, W. (1973) *European J. Biochem.* **37**, 1.
19. Applegarth, D. A. and Bozoian, G. (1968) *Can. J. Microbiol.* **14**, 489.
20. Aronson, J. M. (1965) in *The Fungi*, Vol. I. p. 49 Academic Press, New York.
21. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 459.