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CHEMICAL AND ULTRASTRUCTURAL STUDIES OF ISOLATED CELL WALLS OF *EPIDERMOPHYTON FLOCCOSUM*

PRESENCE OF CHITIN INFERRED FROM X-RAY DIFFRACTION ANALYSIS AND ELECTRON MICROSCOPY

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SUMMARY

Cell walls of *Epidermophyton floccosum* were isolated in high purity after mechanical breakage in the Ribi fractionator, followed by sonication and sodium dodecyl sulfate treatment. Major carbohydrate components of cell wall hydrolysates were glucose (35.2%) and glucosamine (30.9%), with lesser amounts of mannose and galactose.

After treating isolated cell walls with acid and alkali, the glucosamine polymer was isolated in the form of insoluble residues, and was shown to be be composed of chitin fibers by X-ray diffraction analysis and electron microscopy. The surface architecture of isolated cell walls, observed by scanning and shadowing electron microscopy, revealed some remarkable differences in the length and thickness of the fibrils, and also in the orientation of the network, between the internal and external surfaces of the cell wall. A possible involvement of chitin in cell wall integrity is discussed.

INTRODUCTION

Unlike the intensive studies of the cell walls of bacteria, chemical and ultrastructural investigations of cell walls from filamentous fungi have so far been limited[1–4]. In particular, information about the cell walls of dermatophytes has been meager[5,6]. In our laboratory, attempts have been made to isolate pure cell walls of dermatophytes; biochemical and ultrastructural analyses have been carried out on isolated cell walls[7,8]. Recently, we examined the surface ultrastructure by scanning and shadowing electron microscopy, and also the carbohydrate composition, of cell walls of *Trichophyton mentagrophytes* by gas–liquid chromatography, infrared spectroscopy and X-ray analysis. Glucose and glucosamine (as chitin) appeared to be the predominant components; these chitin fibers may be involved in the integrity of the cell wall[9]. Evidence is increasing that chitin occurs widely in cell walls of different fungi[10–15]. Blank[5] had suggested, from X-ray powder diagrams, the presence of chitin in dermatophyte cell walls.

In the present study, the highly purified cell walls of the filamentous fungus *Epidermophyton floccosum*, which causes some mycoses in humans, were examined for chemical composition and ultrastructure, with regard to evidence for the presence of chitin in the cell wall.

MATERIALS AND METHODS

Organism and growth

E. floccosum TEF-30 was grown for 5 days at 28 °C in shake-flasks containing Sabouraud's medium (4% glucose, 1% BBL Polypeptone, and 0.5% yeast extract). The filamentous mycelia were harvested by filtration and were washed several times with distilled water.

Isolation and purification

The isolation procedure for cell walls was essentially based on that previously described[8,9] except for the addition of sonication and sodium dodecyl sulfate treatment. Mycelia prepared as above were suspended in 10 vol. of distilled water and blended for 15 min in a Waring blendor at full speed, and subsequently disrupted in the Ribi cell fractionator (Sorvall, Inc., Norwalk, Conn.); pressure in the cell was $3 \cdot 10^4$–$3.5 \cdot 10^4$ lb/inch^2. During the entire period of the disruption procedure, care was taken to cool the needle valve of the pressure cell to -50 °C with N_2 gas to keep the temperature of effluent at the outlet below 15°. The efficiency of breakage was estimated from phase-contrast microscopy after each increment in pressure. The disintegrated mycelial material taken from the effluent was centrifuged at $1500 \times g$ for 10 min in a Sorvall refrigerated centrifuge, and the supernatant cytoplasmic debris was discarded. The residue was resuspended in water and sonicated (Branson Sonifier, Branson Sonic Power Company, Danbury, Conn.) to remove adhering cytoplasmic contamination. The sonicated suspension of cell wall fragments was spun down at $1500 \times g$ for 5 min. After this, the sonication and washing procedure was repeated twice more. The final washed residue was suspended in 1% sodium dodecyl sulfate solution, left for 1 h at room temperature, and centrifuged again as above. The sediment was thoroughly washed with water until free of detergent (no turbidity was observed on addition of $BaCO_3$ to the washings), then freeze-dried and kept in a desiccator.

Chemical treatment with hot alkali and acid of the isolated cell walls

The purified cell walls were treated in a sealed ampoule with 1 M NaOH for 5 h at 100 °C. The insoluble residue after this alkali treatment was washed with water, and subsequently treated in a sealed ampoule with 1 M HCl for 2 h at 100 °C. Finally, this alkali- and acid-insoluble fraction was further extracted with 1 m NaOH for 5 h at 75 °C, and a yellow-brownish residue was obtained.

Chitinase digestion of cell walls

Purified cell walls and alkali–acid-treated wall residue were digested with chitinase (Calbiochem, Los Angeles) without any purification at a concentration of 6 mg/ml in 0.05 M citrate buffer (pH 5.1) at 37 °C for 1 week. To this incubation mixture were added a few drops of toluene to prevent bacterial contamination.

Electron microscopy

For transmission electron microscopy the cell wall materials at each stage during the preparation procedure were fixed for 3 h in 1% potassium permanganate buffered to pH 7.4 with veronal acetate, dehydrated in a graded series of acetone solutions, and embedded in epoxy resin. Ultrathin sections were cut with glass knives using the Ultrome (LKB), then double-stained with uranium acetate and lead hydroxide. Observations were made at 50 kV with an electron microscope (Hitachi HS-8 type).

For the observation of surface structure, chemically treated cell walls were dried on Formvar-coated grids and shadowed at a 35° angle with platinum and palladium (4:1). The grids were observed with an electron microscope (Hitachi HS-8 type).

For scanning electron microscopy, samples were dried in air or with acetone, and mounted on the specimen holder. The mounts were allowed to dry under a high vacuum and coated with gold to a thickness of about 150 Å. Specimens were examined by a scanning electron microscope (JSM-U3, Japan Electron Opticals Laboratory Co., Ltd) at an accelerating voltage of 10 kV.

CHEMICAL ANALYSIS

Neutral monosaccharides

The total carbohydrate content was determined by the anthrone method[18] using glucose as the standard.

Instead of the aqueous acid hydrolysis usually employed, methanolysis was employed to hydrolyze cell wall materials in the present study, since this method with methanolic HCl causes less degradation of sugar components than does the aqueous HCl method[19,20]. Quantitative analysis of the individual monosaccharides was performed by gas chromatography (Hitachi K-53 type). 10 mg of the cell wall were refluxed with 3 ml of absolute methanol containing 10% dry HCl gas in sealed ampoules at 100 °C for 4 h. To the methanolyzate, containing mannitol as internal standard, was added 0.3 ml of silylating reagent [a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1)]. Aliquots of 1–2 μl of each sample mixture were used for injection onto a column of 3% Silicone OV-17 (Gaschro Kogyo, Tokyo) at 170 °C; the flow rate of carrier gas of N_2 was 43 ml/min. Corrections for losses due to degradation of sugar components during hydrolysis were calculated and applied to the values obtained from the cell wall hydrolyzates[23].

Amino sugars

The weighed standard glucosamine and 10 mg of the wall material were refluxed with 2 ml of 6 M HCl in sealed ampoules at 100 °C for 4 h. To the hydrolyzate of the cell wall was added 10 ml of 2 M HCl solution. The hydrolyzate suspension was placed on an Amberlite resin column (CG-120 Type 1) from which neutral sugars and amino acids were eluted with distilled water. Amino sugars were then eluted from the ion-exchange column with 10 ml of 2 M HCl. Total hexosamine was estimated by the method of Blix[22]. Also, the fractions eluted with 2 M HCl were examined by an amino acid autoanalyzer (Hitachi KLA-3 type) with glucosamine, galactosamine, and mannosamine as standards for qualitative analysis. In this case too, corrections were made for losses due to decomposition during hydrolysis[8].

Proteins and amino acids

Protein was measured with the Folin–Ciocalteu reagent using bovine serum albumin as standard[15]. After samples were hydrolyzed for 20 h in 6 M HCl at 105 °C in sealed ampoules. the hydrolysates were analysed with the amino acid analyzer.

Lipids

Lipids were extracted by the method of Bligh and Dyer[23]. Silica Gel G thin-layer chromatography for neutral lipids and phospholipids was carried out by the method described previously[24]. For the neutral lipids, light petroleum–ethyl ether–acetic acid (70:30:1, by vol.) and, for phospholipids, chloroform–methanol–acetic acid–water (75:25:5:2.2, by vol.) were used.

X-ray diffraction analysis

The dried intact and acid–alkali-resistant cell wall fractions were crushed in a mortar. X-ray diagrams were obtained by use of a copper (CuK) radiation tube with a nickel filter (Toshiba Diffpet ADG-301 type), operating at 30 kV and 13 mA. An authentic sample of lobster chitin (Seikagaku Fine Chemicals, Tokyo) was treated identically.

RESULTS

Isolation of pure cell walls and electron microscopy

Mycelial cells of *E. floccosum* were mechanically disintegrated by a combination of Waring blendor, Ribi fractionator and sonication with a breakage efficiency

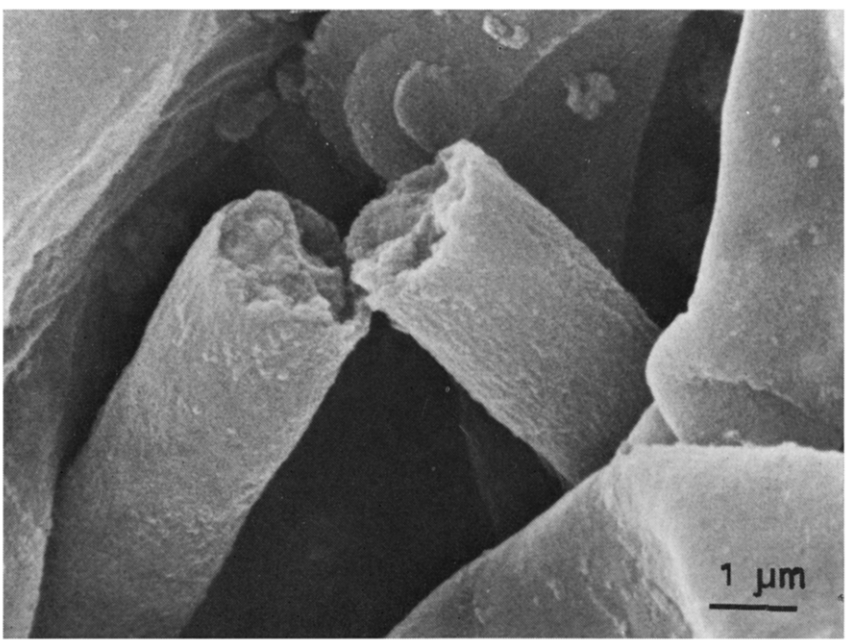

Fig. 1. Portion of *E. floccosum* mycelium mechanically disrupted. Note cytoplasmic contents remaining within the mycelium.

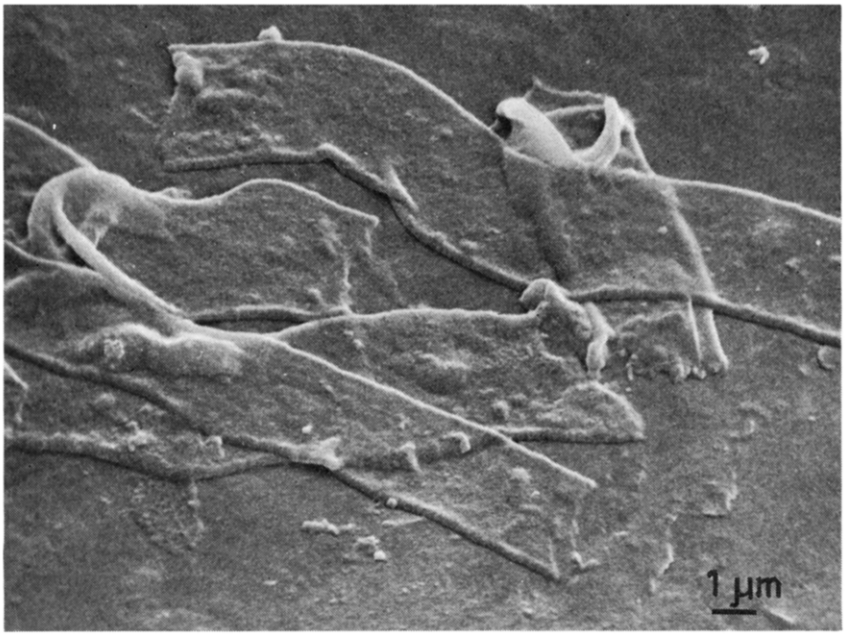

Fig. 2. Purified cell walls observed by scanning electron microscopy. Samples air-dried.

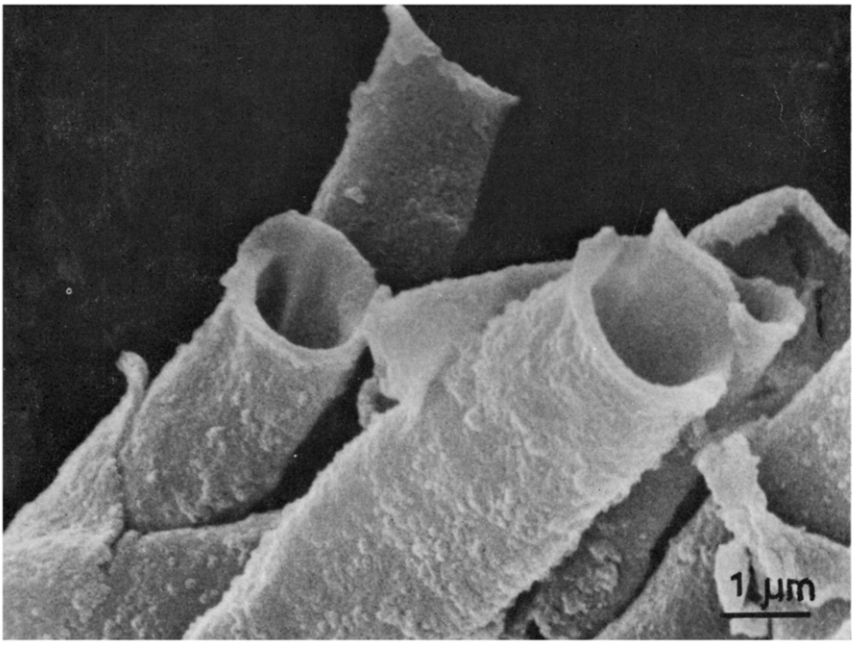

Fig. 3. Scanning electron micrograph of purified cell walls, showing complete absence of cytoplasmic contents. Samples dried with acetone.

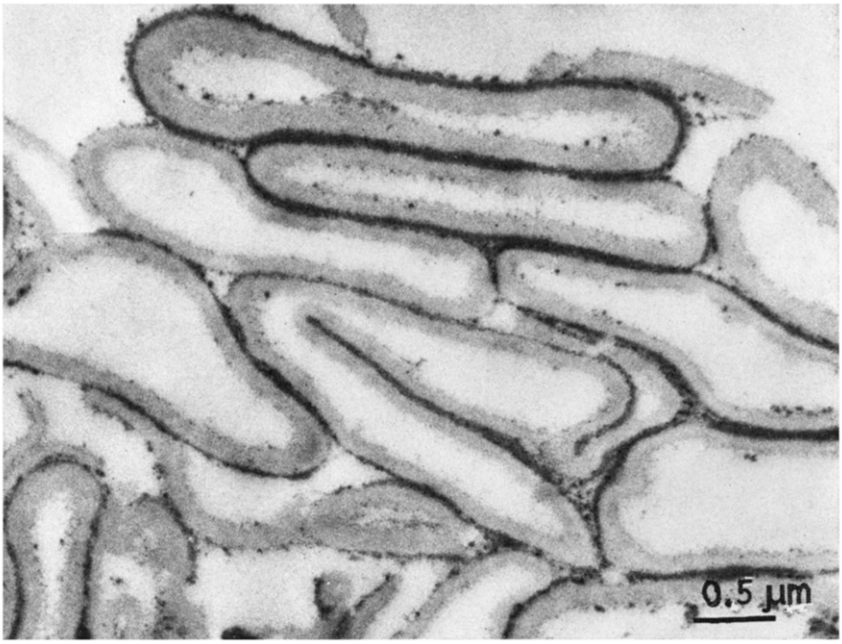

Fig. 4. Electron micrograph of a thin section of purified cell walls after sodium dodecyl sulfate treatment.

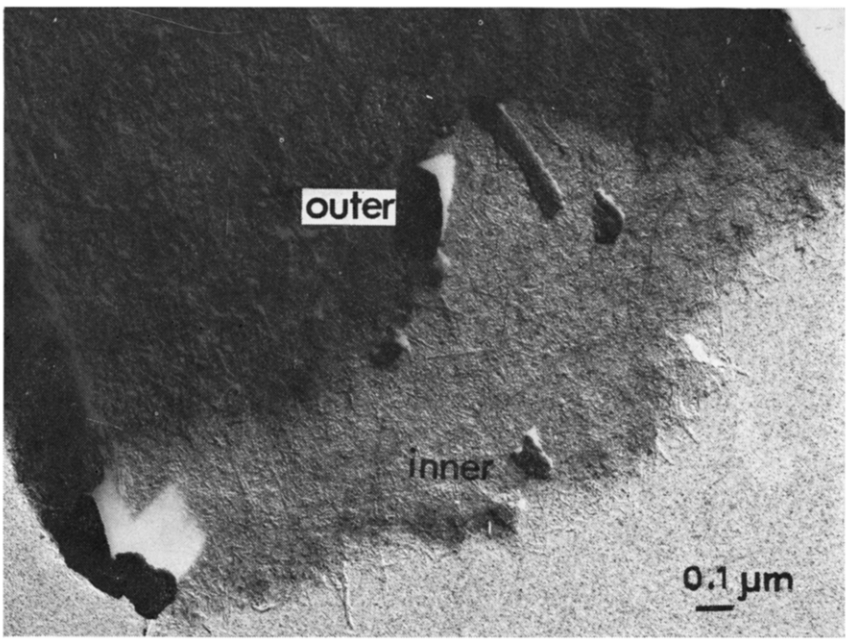

Fig. 5. Segment of purified cell wall showing difference in the fibrous texture between the outer and inner surfaces. Shadowed with Pd–Pt.

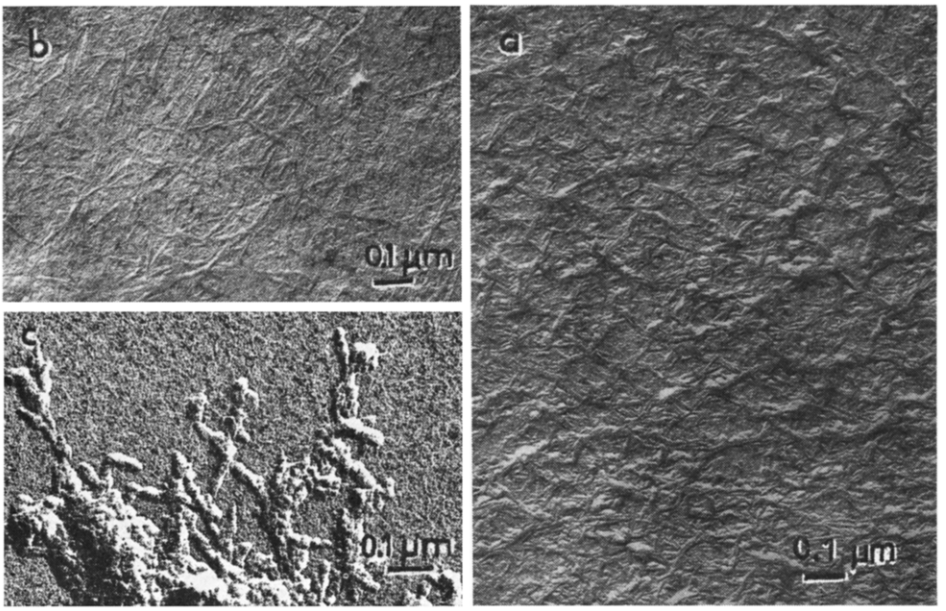

Fig. 6. Shadowed electron micrographs of outer (a) and inner (b) surfaces of the isolated cell wall from *E. floccosum*, and also the alkali–acid-resistant wall fraction (c). Randomly arranged short fibrils in bundles lie on the outer surface (a), while thin fibrils with some regular orientation lie on the inner surface. Short rodlet-type fibers are suggestive of chitin (c).

>95%. Also, sodium dodecyl sulfate treatment was very effective for the removal of concomitant cytoplasmic debris from the isolated cell walls. The purified cell wall preparations thus obtained were creamy and fluffy in appearance.

Fig. 1 shows a portion of mycelium broken by Waring blendor disruption, as revealed by scanning electron microscopy. The cytoplasmic contents inside the mycelium are still observable. However, after cycles of the combination of the disruption procedures as described above, cell walls could be isolated in a highly pure state and in good yield. Scanning electron microscopy observation of the air-dried cell wall specimen displayed a high homogeneity (Fig. 2). The complete absence of cytoplasmic contents from inside the mycelia is more clearly demonstrated in Fig. 3, where, instead of air-drying, the samples were dried with acetone before examination. Thus the acetone-drying procedure used by us proved highly suitable for the observation of the internal features of the cell wall of filamentous fungi. In general, rough external and smooth internal surface structures are observed. The edges of disrupted mycelia are also clearly seen. Electron microscopy of sections (Fig. 4) of cell walls after dodecyl sulfate sodium treatment show that they are homogeneous and free from cytoplasmic materials. These cell walls, from examination of shadowed preparations, clearly showed a fibrous texture with random orientation (Figs 5, 6a and 6b). Short and thick fibrils in bundles were observed on the outer surface (Fig. 6a), whilst long and thin fibrils were seen on the inner surface (Fig. 6b). On the other hand, the cell wall fraction obtained after hot alkali- and acid-treatment, is composed mostly of short rodlet-like fibers (Fig. 6c). The fact that these fibrils

were largely digested by chitinase strongly suggests that the released glucosamines are present as a chitin polymer.

Chemical composition of the purified cell walls

The cell wall materials, hydrolyzed as described above, yielded the components summarised in Table I.

TABLE I

CHEMICAL COMPOSITION OF THE INTACT CELL WALLS AND THE ALKALI-ACID-RESISTANT FRACTION OF *E. FLOCCOSUM*

Results are given as percentage of dry weight.

Constituents	*E. floccosum*		*T. mentagrophytes** *intact cell walls*
	Intact cell walls	*Alkali-acid-resistant residue*	
Total carbohydrate**	46.3	3.1	51.9
Glucose	35.2	2.9	36.2
Mannose	5.3	0	11.7
Galactose	trace	0	trace
N-Acetylglucosamine***	30.9	95.3	30.4
Protein*	10.3	0.3	3.7
Lipid**	4.6	0	6.6
Recovery	92.1	98.4	92.6

* Data from the previous paper[8].

** Anthrone method with glucose as standard.

*** Morgan–Elson's method.

* Folin–Ciocalteu's reagent.

** Bligh–Dyer's extraction procedure.

Carbohydrate. Before quantitative analysis of sugar components by gas–liquid chromatography, it was necessary to establish the most suitable conditions for hydrolysis: *i.e.*, maximum release of sugars from cell walls and minimum degradation of the released sugar components. Of the hydrolytic conditions tested, methanolysis in 10% HCl in anhydrous methanol for 4 h at 100 °C proved to be most satisfactory. Values obtained by gas–liquid chromatography were corrected for loss due to decomposition during methanolysis.

The data presented here that the major monosaccharides are glucose (35.2%), mannose (5.3%) and glucosamine (30.9%), largely agree with those by Shah and Knight[6] for one strain of *E. floccosum*, in which there was a higher content of glucose (45.8%) as compared with our present result. This discrepancy may reflect variations in carbohydrate composition between different strains even in the same species of fungi. Interestingly, the monosaccharide composition of *E. floccosum* shown here resembles that from our previous data from hydrolyzates of *Trichophyton mentagrophytes* cell walls[8], although this fungus belongs to different genus of dermatophyte.

Amino sugars. Acid hydrolysates of the cell walls were analyzed for hexosamine according to Blix's modification[24] of the Elson–Morgan reaction. As shown in Table I, only glucosamine was detected but not galactosamine and mannosamine. The hydrolytic conditions used – 6 M HCl for 4 h at 100 °C – were based on previous data for the stability of glucosamine during acid hydrolysis[8]. Corrections were made for decomposition during hydrolysis.

As for the hexosamine content, intact cell walls contain 30.9% glucosamine, while the alkali- and acid-resistent fraction is chiefly glucosamine (95.3%). This indicates that the insoluble cell wall residue might consist of chitin fibrils as revealed by shadow-cast electron microscopy (Fig. 6c). Furthermore, confirmation of the presence of chitin was made by X-ray analysis as shown in a later section.

Protein and amino acids. The involvement of protein in cell wall integrity has not been clarified, yet it can be considered that protein molecules may serve to link chitin to carbohydrate. The amino acid composition of *Epidermophyton* cell walls (Table II) shows a relatively significant proportion of threonine, serine, aspartic acid and glutamic acid, which may contribute to linkages between carbohydrate and protein moieties in the cell wall. Also noteworthy is the high content of alanine and glycine, since both amino acids are involved in the cross-linking of peptidoglycans in the bacterial cell wall[26,27].

Lipids. The total lipid content of the cell wall amounted to 4.6% of the weight of wall material. Thin-layer chromatographic analysis on a silica gel G plate showed

TABLE II

OVERALL AMINO ACID ANALYSIS OF CELL WALLS OF *E. FLOCCOSUM*

Amino acid	*μmoles/10 mg cell wall*	*Percentage of total amino acids**
Lysine	0.50	5.6
Histidine	0.20	2.2
Arginine	0.40	4.4
Aspartic acid	0.84	9.4
Threonine	0.72	8.2
Serine	0.76	8.5
Glutamic acid	0.78	8.7
Proline	0.80	8.7
Glycine	0.90	10.0
Alanine	0.84	9.4
Cystine	0.30	6.6
Isoleucine	0.42	4.7
Leucine	0.68	7.6
Tyrosine	0.16	1.7
Phenylalanine	0.30	3.3
Total	8.92	99.0

* Total amount of amino acids was 10.3% of the initial dry weight of cell walls.

the usual lipid constituents: sterol, triglyceride, free fatty acid, and phospholipids (phosphatidylcholine, trace amount of phosphatidylserine, and ethanolamine). Quantitative determinations of these components are in progress.

X-ray diffraction analysis

The presence of chitin in the cell walls of *E. floccosum* was suggested by the high glucosamine content and by the appearance of short rod-like fibers in the alkali–acid-insoluble residue as revealed electron microscopically (Fig. 6c). There was other evidence that appreciable amounts of *N*-acetylglucosamine was released from the alkali–acid-resistant fraction and from the intact cell walls by chitinase digestion. Furthermore, to confirm the presence of cell wall chitin, we investigated cell wall materials by X-ray diffraction. As expected, the diffraction patterns of authentic commercial chitin and the alkali–acid-resistant residue are almost identical to one another (Fig. 7). On the other hand, intact cell walls did not show the sharp reflections of chitin.

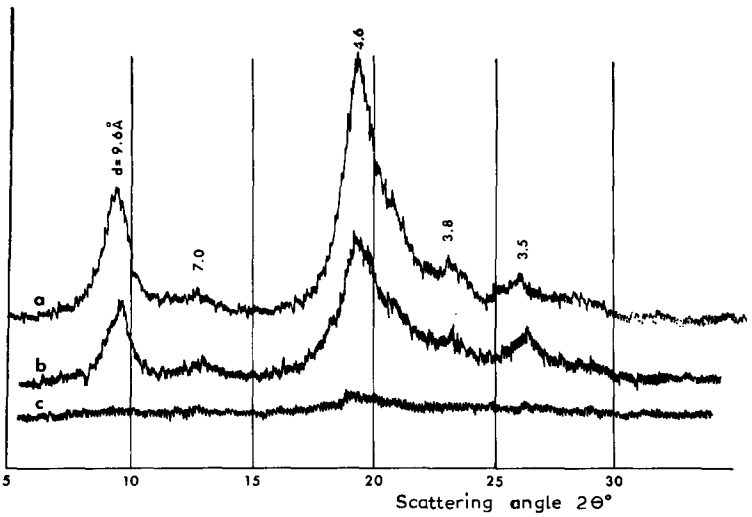

Fig. 7. X-ray diffraction spectra of the intact cell walls of the alkali-acid-resistant wall fraction of *E. floccosum*. a commercial chitin; b alkali–acid-resistant wall fraction; c intact cell walls.

DISCUSSION

In this study we investigated the chemical composition by gas–liquid chromatography and X-ray diffraction, and ultrastructures by scanning and shadow-cast electron microscopy, of the isolated cell walls of *E. floccosum*, with special reference to the presence of chitin. For this purpose, we established an improved procedure for the isolation of cell walls in a highly pure state from mycelia of this filamentous fungus. Carbohydrate composition is the subject of special interest in our work on the fungal cell wall. Glucose and glucosamine are predominant (35.2 and 30.9%, respectively), together with lesser amounts of mannose (5.3%) and galactose (trace). The results are compatible with those for *Trichophyton* cell walls (glucose 36.2%,

glucosamine 11.7% and a trace of galactose) reported previously[8], and also with those of Shah and Knight[6] for a strain of *E. floccosum*, except for their higher glucose content (45.8%). For the quantitative determination of carbohydrates, appropriate corrections must be made for the decomposition of individual sugar components due to hydrolysis with acid, since the stability of various monosaccharides differs from one sugar to another under different hydrolysis conditions (acid concentration, temperature and duration) (Nozawa *et al.*[21], Chambers and Clamp[20]).

Insufficient information has been available on the presence of chitin in the dermatophyte cell wall, although the existence of chitin in a great number of fungi has been indicated by chemical[28,29], X-ray diffraction[15,30–33], and enzymatic[34,35] analysis. In this study we isolated short rodlet fibrils, suggestive of chitin, which were not dissolved by hot alkali-acid treatment. In the literature[1,3,33,36,37], two types of chitin fibers have been described: long, interwoven microfibrils, and short, rodlet fibrils. By shadow-cast electron microscopy, the fibrils isolated by us were not opposed directly to the internal and external surfaces of the cell wall, and were masked by other fibrous structures (probably glucan and galactomannan). On the other hand, X-ray diffraction analysis confirmed the presence of chitin in the *E. floccosum* cell wall.

Digestion of cell walls by chitinase released large amounts of *N*-acetylglucosamine and at the same time strikingly altered the shape of the cell wall. From this one may conclude that there exists a chitin core apparently masked by glucan and galactomannan fibrils, and that it might be "skeletal" material for maintenance of cell wall integrity in this fungus.

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