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ISOLATION, ULTRASTRUCTURE AND CHEMICAL COMPOSITION OF THE OUTERMOST LAYER ("EXO-LAYER") OF THE *EPIDERMOPHYTON FLOCCOSUM* CELL WALL

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SUMMARY

The outer-most layer ("exo-layer") of the wall was isolated from cell walls of *Epidermophyton floccosum*. The pure cell walls, obtained by disruption in a Ribi cell fractionator, sonication and centrifugation, were digested with snail enzyme for 12 h. Thereafter, the exo-layer preparation was obtained as the fraction resistant to the snail enzyme. Electron microscopy showed that the exo-layer is a thin, stranded network structure 10–20 nm thick. Chemical analysis of the exo-layer showed that the main components are protein (63 %), mannose (10 %) and glucosamine (17 %). Sodium dodecyl sulfate polyacrylamide gel electrophoresis has revealed that the main band is a glycoprotein containing mannose.

INTRODUCTION

Much attention has recently been paid to fungal cell walls from the biochemical and ultrastructural point of view and the results of investigations have been reviewed in some detail [1, 2]. However, information on the chemistry and ultrastructure of the fungal cell walls seems to be limited, especially in pathogenic filamentous fungi, compared with that on bacteria. This may be due to the lack of successful techniques for the isolation of highly purified cell walls and the ability to separate, selectively, their different layers. Thus most of the information so far obtained is based on the analysis of the whole cell wall [1, 2].

We have shown [3] that the whole cell wall of a dermatophyte, *Epidermophyton floccosum*, which is a typical pathogenic filamentous fungus, is composed chemically of chitin (34 %), glucose (35 %), mannose (6 %), protein (10 %) and lipid (5 %) and consists of a thin, electron-dense outer-most layer and a thick, less electron-dense inner layer as seen under the electron microscope. In the present investigation, as a preliminary step toward elucidation of the biological function of the outer-most layer (exo-layer), we have sought to isolate this specific layer exo-layer from the cell wall of *E. floccosum* and we have performed electron microscopic and chemical analysis on the purified preparation of this exo-layer.

MATERIALS AND METHODS

Organism and growth

Epidermophyton floccosum TEF-30 was grown for 7 days at 28 °C in shake flasks containing Sabouraud's medium (4 % glucose, 1 % polypepton and 0.5 % yeast extract). The filamentous mycelia were harvested by filtration and were washed several times with distilled water.

Isolation of the outer-most layer (exo-layer)

The exo-layer was isolated from the purified cell walls of *E. floccosum*. The pure cell walls were obtained by a method somewhat modified to that previously described [3, 4]. Mycelia prepared as above were suspended in 10 vols of 0.85 % NaCl solution, blended for 30 min in a Waring blendor, then subsequently homogenized in a Potter-Elvehjem glass homogenizer, until a fine homogeneous suspension was obtained and subjected to disruption in a Ribi cell fractionator (Sovall, Inc., Norwalk, Conn.). Pressure in the cell was $3–3.5 \cdot 10^4$ 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 °C. This disruption was repeated three times. The disintegrated mycelial material taken from the effluent was centrifuged at $1500 \times g$ for 10 min in a Sovall refrigerated centrifuge and the residue was resuspended in distilled water, followed by subsequent centrifugation at $1500 \times g$ for 10 min to wash. After repeating the washing 5 times, the washed, crude cell wall suspension was sonicated (Branson Sonifier, Branson Sonic Power Company, Danbury, Conn.) to remove adhering cytoplasm and centrifuged at $1500 \times g$ for 10 min. After this, the sonicated cell wall fractions were washed with distilled water 5 times: the residue constituted the isolated wall preparation.

1 g (wet weight) of the purified cell walls was suspended in 5 ml of 0.1 M citrate buffer (pH 5.8) containing 500 mg (dry weight) of snail enzyme (Industrie Biologique Française, Paris) and 25 μg of chloramphenicol and incubated at 28 °C for 12 h in a shaking water bath. After incubation, the reaction mixture was centrifuged at $4000 \times g$ for 8 min and the undigested residue was washed with distilled water. This wash was repeated three times and the resultant pellet was resuspended in distilled water and centrifuged at $400 \times g$ for 10 min. The turbid supernatant was centrifuged again at $4000 \times g$ for 10 min and the exo-layer was isolated as a pellet. The digestion of the cell walls and the purity of exo-layer fractions were assessed visually by phase contrast and electron microscopy after each step.

Electron microscopy

For electron microscopy, the exo-layer materials were fixed for 3 h in 1 % potassium permanganate, buffered to pH 7.4 with Veronal acetate [5], dehydrated in a graded series of acetone solution and embedded in epoxy resin [6]. Ultrathin sections were cut with glass knives using Ultrome I (LKB), then stained doubly with uranyl acetate [7] and lead citrate [8]. The sections were examined under the electron microscope (Japan Electron Opticals Laboratory Co., JEM-100 U type).

For negative staining, exo-layer suspensions were stained with an equal volume of 2 % (w/v) phosphotungstic acid, adjusted to pH 6.8 with KOH. Aliquots

of the stained exo-layer suspensions were deposited on Formvar-coated copper grids, excess sample removed with small pieces of filter paper and after drying at room temperature the specimens were examined in a JEM-100 U type electron microscope.

Chemical analysis

Neutral monosaccharides. Qualitative and quantitative determination of the individual monosaccharide components was performed by gas-liquid chromatography, according to a method previously described [9]. 10 mg of the exo-layer were refluxed at 100 °C for 4 h with 2 ml of absolute methanol containing 5 % dry HCl gas in sealed ampoules. To the dried esterified preparation, containing mannitol as internal standard, was added 0.3 ml of silylating reagent, a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane (10 : 2 : 1, by volume). Aliquots of 1–2 μl of each sample mixture were used for injection onto a column 3 % silicone OV-17 (Gaschro Kogyo, Tokyo) at 170 °C, the flow rate of N_2 gas was 45 ml/min. Corrections for losses due to degradation of sugar components were calculated [9] and applied to the value obtained from the exo-layer methanolysate.

Total carbohydrate. This was determined by the anthrone method using glucose as the standard [10].

Amino sugars. 10 mg of the exo-layer material was refluxed at 100 °C for 4 h, with 2 ml of 6 M HCl in sealed ampoules and thereafter the reaction mixture was diluted to 1 M HCl by addition of 10 ml of distilled water. The hydrolysate suspension was placed on an Amberlite resin column (CG 120 type 1) from which neutral sugar and amino acids were eluted with distilled water. Amino sugars were then recovered from the ion-exchange column with 10 ml of 2 M HCl. Total hexosamine was estimated by the method of Blix [11]. The fraction eluted with 2 M HCl was also examined in 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 [4].

Proteins and amino acids. Protein was measured by the method of Lowry et al. [12]. After samples were hydrolyzed for 20 h in 6 M HCl at 105 °C in sealed ampoules, the hydrolysates were dried in boiling water and dissolved in deionized water. The hydrolysates thus obtained were analyzed with an amino acid autoanalyzer (Hitachi KLA-3 type).

Lipids. Lipids were extracted by the method of Bligh and Dyer [13]. The amount of phosphorus was determined colorimetrically at 820 nm, with molybdate as the chromophore reagent [14].

Gel electrophoresis

Prior to polyacrylamide gel electrophoresis, 2 mg of the exo-layer preparation were dissolved at 37 °C for 10 min in 1 ml of a solution containing 1 % sodium dodecyl sulfate, 10 % sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 40 mM dithiothreitol in 10 mM Tris · HCl, pH 8, with 10 μg/ml of pyronin Y as tracking dye. The sample solution was centrifuged at 16 000 $\times g$ for 10 min and the clear supernatant solution was analyzed on 5.6 % polyacrylamide gel (8 cm long $\times$ 0.7 cm thick) containing 0.1 % sodium dodecyl sulfate, prepared by the method described by Fairbanks et al. [15]. Electrophoresis was performed at a current of 4 mA in total current for 3 h using 0.1 M Tris/acetate buffer (pH 7.4). Proteins were stained with Coomassie Blue [15], and carbohydrates were stained with Schiff reagent [15].

The molecular weight of the polypeptides was estimated from a calibration curve in which the logarithm of the molecular weights of standard proteins was plotted agains tthe migration distance [16]. The calibration curve was prepared with bovine serum albumin (M_r 67 000), catalase (M_r 60 000), egg albumin (M_r 45 000), chymotrypsinogen A (M_r 25 000) and cytochrome *c* (M_r 12 500). These standards (Combithek Protein Calibration Kit, Size II) were purchased from Boehringer Mannheim GmbH (West Germany).

RESULTS

Isolation and ultrastructure of the exo-layer

The procedure employed in this study for the isolation of the exo-layer from the cell wall of *Epidermophyton floccosum* is based on the difference in sensitivity between the exo-layer and the inner layer of the cell wall to the snail enzyme. Since it was observed by electron microscopy that the cytoplasmic membrane was also highly resistant to the snail enzyme, it was necessary to isolate the cell walls prior to the snail enzyme digestion procedure in order to prevent contamination with cytoplasmic membrane components. The electron microscopy of the thin sections of the intact cell wall of *E. floccosum* revealed that the cell wall consists of the thin, electron-dense outer-most layer, which we tentatively designate "exo-layer" in this paper, and the thick, less electron-dense inner layer (Figs 1a and 1b). The exo-layer is highly resistant to the snail enzyme, although the inner layer was readily dissolved after incubation for 12 h with this enzyme.

The purity of the exo-layer fraction was determined by electron microscopy and a typical preparation is shown in Fig. 2. No contamination was observed. Thin sections of the isolated exo-layer showed identical electron density and thickness (10–20 nm) with those of the intact exo-layer shown in Fig. 1a. The unit membrane structure and vesicle formation usually observed in the cell membrane preparations were not found in this preparation.

With the negative staining technique, the structural features of the isolated exo-layer are clearly established. The exo-layer is seen to exhibit a marked difference from the intact cell wall in appearance and rigidity (Figs 3a and 3b), although the exo-layer maintained the original tubular shape of the hyphal cell. The structure of the negatively stained exo-layer is a randomly arranged fine stranded, thin reticulum (Fig. 3a), whereas the intact cell wall is of a rigid and solid appearance (Fig. 3b). At a higher magnification, the thin, randomly stranded network can clearly be seen (Fig. 3a Insert).

Chemical analysis of the exo-layer

The chemical composition of the exo-layer is summarized in Table I.

Neutral and amino sugars. The total carbohydrate content was 11 % of dry weight by the anthrone method. By gas-liquid chromatography, it was revealed that the only significant monosaccharide contained in the exo-layer was mannose: this was almost 90 % w/w of the total neutral sugar content. A trace amount of glucose and galactose was also detected by gas-liquid chromatography.

The qualitative analysis of hexosamine was performed by using the amino acid autoanalyzer and only glucosamine was detected. The glucosamine content was

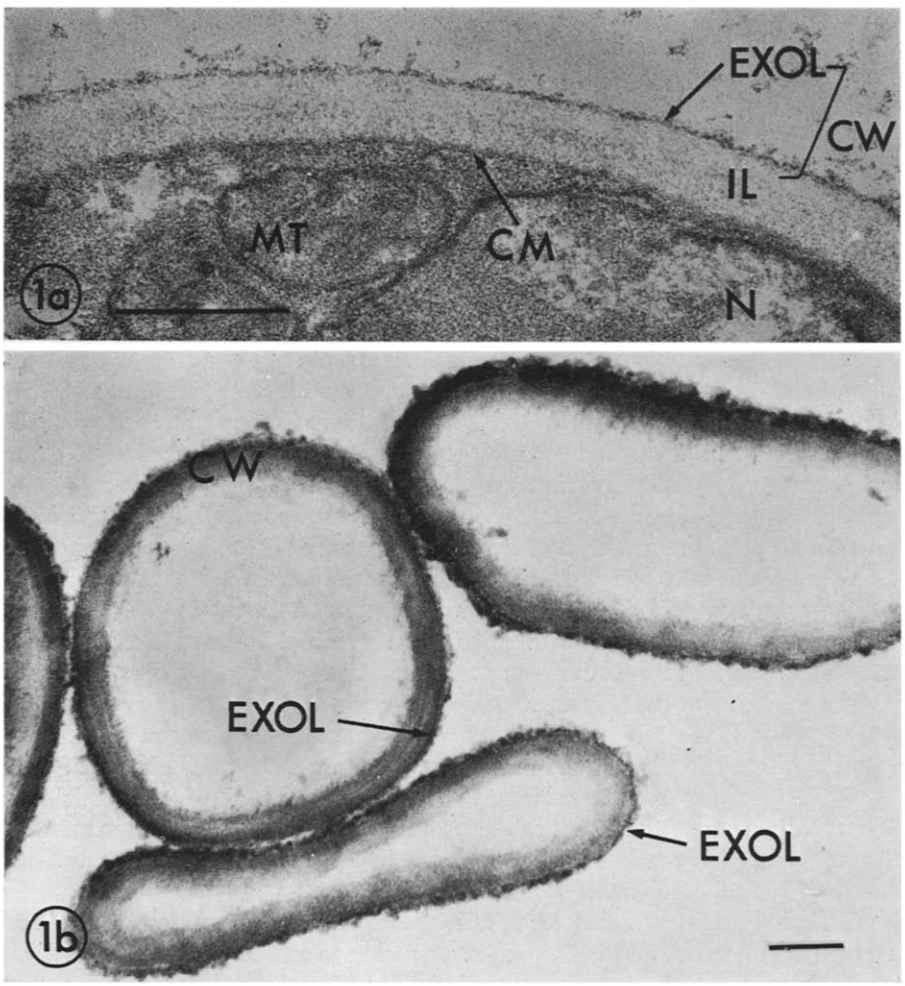

Fig. 1. (a) Thin section of *Epidermophyton floccosum* showing a part of a hyphal cell and the cell wall. A thin, electron-dense layer, exo-layer (EXOL) is seen on the thick, less electron-dense inner layer (IL) of the cell wall (CW). The thickness of the exo-layer is slightly larger than the cytoplasmic membrane (CM). MT, mitochondria; N, nucleus. Bar indicates 0.5 μm. (b) Thin section of the purified cell wall fraction. It can be seen that the exo-layer is well preserved even after drastic procedures for isolating the pure cell walls. Bar indicates 0.5 μm.

determined according to Blix's modification of the Elson-Morgan reaction [11]. The cell walls contained a large amount of glucosamine (34 %) while the exo-layer contained a smaller amount (17 %).

The extraction method of Bligh and Dyer [13] revealed almost no lipid.

Characterization of protein components by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis. The exo-layer preparation contained 63 % protein by weight. We further analyzed the amino acid composition and the protein components by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fig. 2. Thin section of the purified exo-layer fraction from the *Epidermophyton floccosum* cell walls. It is indicated at the portion pointed out by arrows that the thickness of the exo-layers ranges from 10 to 20 nm. Bar indicates 0.5 μm.

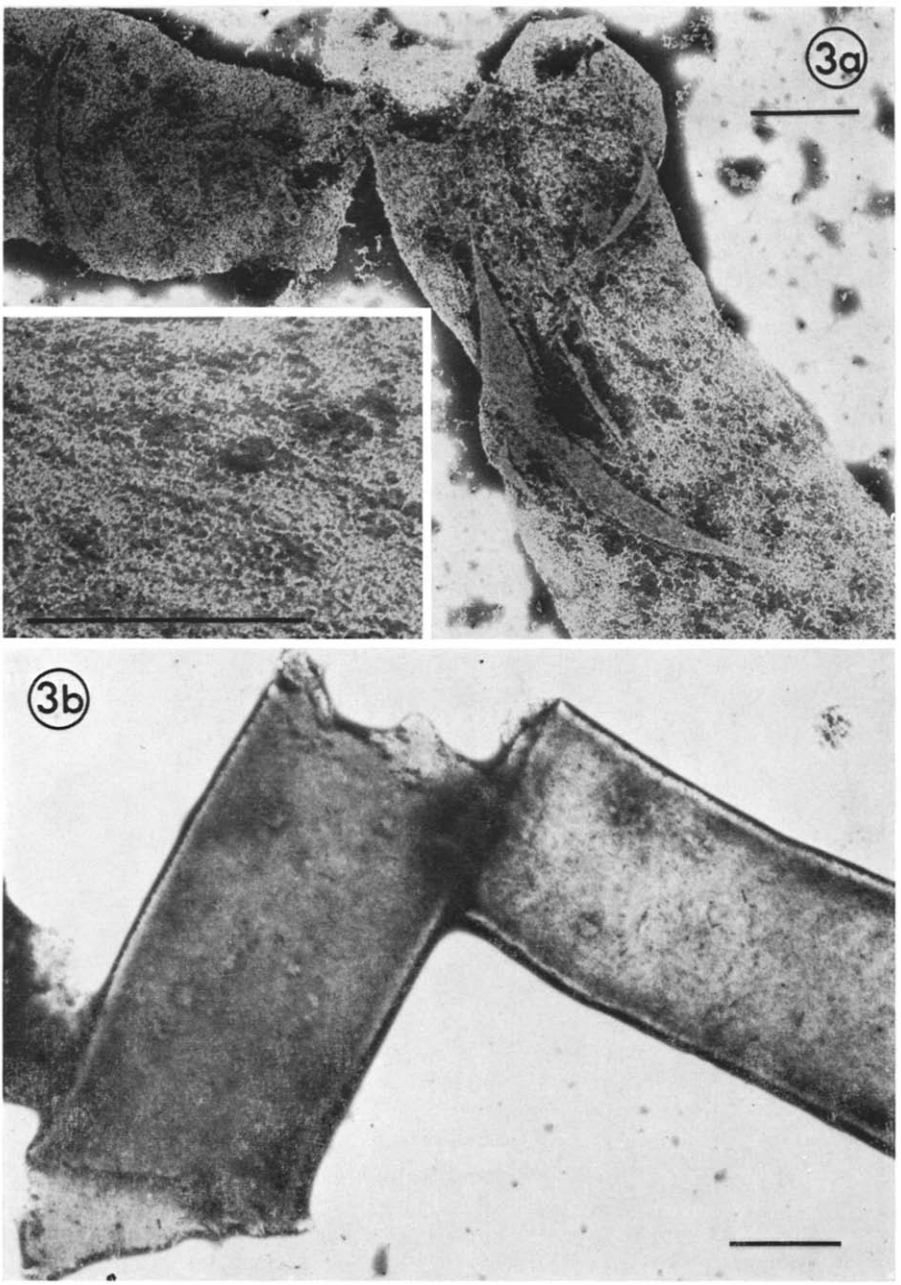

Fig. 3. (a) Electron micrograph of the negatively stained, purified exo-layer preparations. The isolated exo-layers are seen to retain the original tubular shape of hyphae even after dissolving the thick, inner layers with snail enzyme. When compared with the intact cell wall (b) which appears very rigid, the exo-layer is seen to be a very thin, elastic network structure. Insert: a higher magnification of the negatively stained exo-layer. Bars indicate 1 μm. (b) Electron micrograph of a negatively stained intact purified cell wall, showing thick and rigid tubular appearance. Bar indicates 1 μm.

TABLE I

CHEMICAL COMPOSITION OF EXO-LAYER OF *E. FLOCCOSUM* CELL WALL

Results are given as percentage of dry weight.

	Whole cell wall	Exo-layer
Neutral sugar[a]	42	11
Glucose[b]	35	trace
Mannose	7	10
Galactose	trace	trace
Glucosamine[c]	34	17
Peptide[d]	10	63
Lipid[e]	6	0
Total	92	91

[a] By anthrone method [10].
[b] By gas-liquid chromatography [9].
[c] By the method of Blix's modification of Elson-Morgan reaction [11].
[d] By the method of Lowry et al. [12].
[e] By the method of Bligh and Dyer [13].

TABLE II

AMINO ACID COMPOSITION OF EXO-LAYER OF *E. FLOCCOSUM* CELL WALL

Exo-layer material was hydrolyzed for 20 h in 6 M HCl at 100 °C in sealed ampoules and then the hydrolysate was analyzed with an amino acid autoanalyzer (Hitach KLA-3 type). Individual figures are expressed as mol %.

Lysine	2.4	Glycine	17.0
Histidine	0.0	Alanine	10.1
Arginine	0.9	Valine	4.1
Aspartic acid	10.5	Cystine	0.0
Threonine	9.4	Leucine	9.6
Serine	8.0	Isoleucine	4.7
Glutamic acid	13.3	Tyrosine	4.0
Proline	0.0	Phenylalanine	5.4

The amino acid composition of the exo-layer (Table II) shows, unexpectedly, a significantly large amount of glycine and alanine, which are known as the integral components of the peptidoglycan of some bacteria [17, 18]. It should also be noted that threonine, serine, aspartic acid and glutamic acid were found in high proportions and these may be contributing to linkages between carbohydrate and protein moieties [19–21].

Fig. 4 shows the electrophoretic pattern of exo-layer polypeptides of *E. floccosum* in polyacrylamide gels containing 0.1 % sodium dodecyl sulfate. Visual inspection of the gels revealed 5 major bands; one main band (I), two sharp bands (II, III) and others (IV, V). Since the electrophoretic patterns were highly reproducible in different runs and with different batches of the exo-layer preparations, this pattern seems to be characteristic of the exo-layer of *E. floccosum* cell wall. In the periodate-Schiff-stained gels, which had been subjected to electrophoresis in parallel, only one band was observed and the mobility corresponded to Band I, suggesting that the Band I

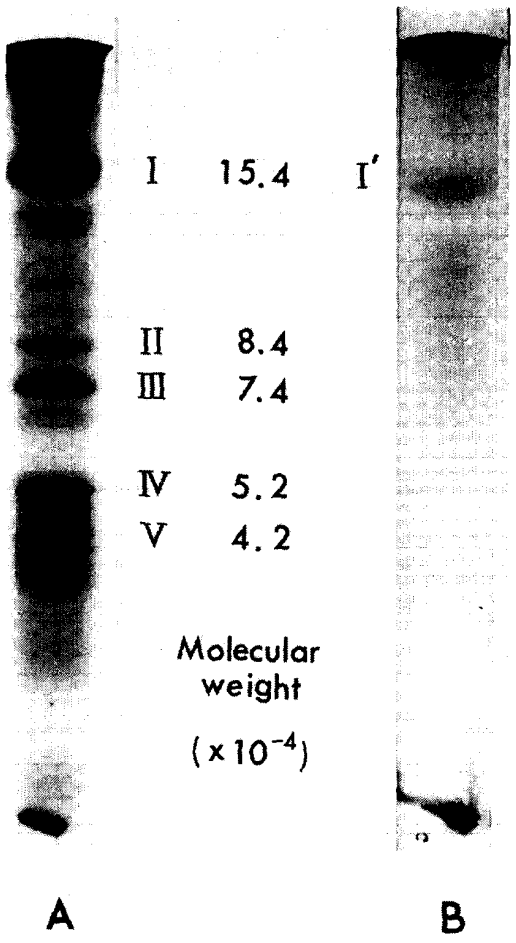

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis of isolated exo-layers. Gel A was stained for protein with Coomassie Blue and gel B was stained for carbohydrate with periodic acid Schiff reagent. The polypeptides are designated I, II, III, IV and V from top to bottom. The band detected in gel B corresponds to band I of gel A in relative mobility.

polypeptide might be a glycoprotein. The molecular weights were determined by sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis using a calibration curve prepared from standard proteins [16] as described in Materials and Methods. Mobilities were measured relative to the distance of the tracking dye from the top of the gel. The molecular weights of polypeptide bands I, II, III and V are thus found to be 154 000, 84 000, 74 000, 52 000 and 42 000, respectively.

DISCUSSION

We have been studying the chemical and ultrastructural architecture of the

cell wall of *Epidermophyton floccosum* [3] and other pathogenic fungi [4, 5, 21]. In the previous paper [3] we reported an improved procedure for the isolation of pure cell walls from this filamentous fungus, *E. floccosum*, and the chemical composition of the isolated cell walls was also presented together with an electron microscopical analysis.

The cell wall of *E. floccosum* is a lamellar structure [3, 22] similar to that of other fungal cell walls. Therefore, it is significant and interesting to isolate the individual layers from the cell wall for the detailed investigation of the cell wall structure. In the present study, we have isolated the outer-most layers (exo-layer) from the *Epidermophyton* cell walls and showed the proteinous nature of the exo-layer. It is of some interest to note that the isolated exo-layer material is rich in protein (63 %), while the remainder of the cell wall comprises principally polysaccharides (76 %). Little has been reported about the fungal cell wall proteins and most studies reported so far have been limited to analysis of total amino acid composition of the cell walls [1, 2, 23] with exceptions of Wrathall & Tatum's work on the cell wall peptide components of *Neurospora crassa* [24, 25]. Therefore, we analysed the exo-layer preparation by sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis and five distinct bands were found. Since the band revealed by the periodate-Schiff staining corresponds to Band I in mobility and the only monosaccharide found is mannose, it is clearly suggested that Band I is a glycoprotein, containing mannose. A glycoprotein containing only mannose as the monosaccharide component has not been extracted from other dermatophyte cell walls but similar glycoproteins were found in yeast cell walls [19, 20].

Our results indicate that the outer-most layer of the *Epidermophyton* cell wall is mostly composed of a glycoprotein containing mannose and some protein components and that its chemical nature differs largely from that of the whole cell wall. Ethylendiamine-soluble glycoproteins were extracted from a dermatophyte, *Microsporum gypseum* hyphal walls and their amino acid composition largely differs from that of *E. floccosum* exo-layer [26], while the *Neurospora crassa* cell wall proteins show closely similar amino acid composition to this exo-layer amino acid composition. It is also reported that the surface component of the spore wall of *Penicillium* contains protein, which has an amino acid composition almost identical with that of this exo-layer [27]. It should be mentioned that the amino acid constituents of the surface protein components are different from these of whole cell wall proteins. These differences appear to be a characteristic feature of the surface layer [25] and are also seen in the present study of the *Epidermophyton* cell wall exo-layer.

The amino acid analysis of the exo-layer revealed that glycine, alanine, glutamic acid, aspartic acid, threonine, serine and leucine are predominant. These amino acids may be involved in the linkage between carbohydrates and polypeptides because of the possibility of *O*-mannosyl bonds from mannose or mannose oligosaccharides to serine and threonine, and that of a nitrogen glycosyl bond to aspartic acid and glucosamine which have been reported by Santandreu and Northcote [19]. Glucosamine found in the exo-layer seems likely to be involved in linkages between carbohydrate and peptide as found in the yeast glycoproteins [20], rather than being due to chitin microfibrils of the inner cell wall, since glucosamine remains even after treatment with snail enzyme capable of easily digesting chitin components.

The biological functions of the exo-layer remains to be clarified by further experiments.

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