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Peptido Polysaccharide Antigens from *Trichophyton mentagrophytes* var. *granulosum*

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Two highly purified peptido polysaccharide antigens have been isolated from surface-grown cultures of *Trichophyton mentagrophytes* var. *granulosum*. Trichloroacetic acid extraction and ethanol precipitation yielded a mixture containing high-molecular-weight components which were first separated on Sephadex G-200. Subsequent fractionation by ion-exchange chromatography on DE-52-cellulose (borate form) yielded the two peptido polysaccharides. Both of the peptido polysaccharides reacted with rabbit antiserum to *T. mentagrophytes* var. *granulosum*. The two peptido polysaccharides contain 73.2% hexoses (mannose-galactose-glucose, 7.5:0.7:1), 8.6% amino acids and 1.8% amino sugars and 77.4% hexoses (mannose-galactose-glucose, 9:0.3:1), 6.2% amino acids, and 0.4% amino sugars, respectively. Each contains 16 different amino acids, threonine, proline, and serine predominating.

A considerable amount of work has been directed toward the isolation of antigenic components from dermatophytes beginning with the studies of Bloch et al. in 1925 (7). In the early investigations, emphasis was on the "trichophytin" activity of crude polysaccharide-containing extracts.

More recently, three groups have been active in preparing purified glycopeptide and polysaccharide antigens from dermatophytes with a view toward a better understanding of the relationship between antigenic structure and immunological reactivity. In 1962, Barker et al. (2) isolated a galactomannan peptide from submerged cultures of *Trichophyton mentagrophytes* by ethylene glycol extraction, followed by fractional precipitation with Cetrimide (cetyltrimethyl ammonium bromide) from a borate solution of increasing pH. Later studies showed that this glycopeptide could be further fractionated (1). No immunochemical criteria of purity were used in their investigations. Their galactomannan peptides elicited both immediate and delayed-type cutaneous hypersensitivity reactions in sensitized guinea pigs. In subsequent studies, such glycopeptide mixtures from *T. mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *Microsporum canis*, *Keratinomyces ajelloi*, and *Epidermophyton floccosum* showed considerable similarities in elicitation of dermal reactivities of guinea pigs sensitized by immunization (3).

Bishop and Blank et al. directed their efforts to the preparation and chemical characterization of purified polysaccharides from nine species of dermatophytes (4-6, 13). Their isolation techniques included tryptic digestion and extraction with hot, dilute alkali. Three polysaccharides, two galactomannans (I and II) and a glucan, were isolated and purified from each species. These were studied immunochemically and compared according to serological reactivities by Grappel et al. (13, 14). The polysaccharide preparations were highly purified but did not elicit hypersensitivity reactions in guinea pigs sensitized by infection (20).

Nozawa et al. (18) isolated polysaccharide-peptide complexes from *T. mentagrophytes* by extraction with phenol. Separation by gel filtration and diethylaminoethyl-cellulose column chromatography resulted in seven fractions. All but one minor fraction were shown to be heterogeneous by immunoelectrophoresis. The biological activity of the purified component was not reported.

The present investigation was initiated due to the need for homogeneous antigens for studies on the specificity of hypersensitivity reactions (trichophytin reactions) associated with dermatophytosis and their relationship to resistance.

MATERIALS AND METHODS

Preparation of mycelium. *T. mentagrophytes* var. *granulosum* was grown in surface cultures at room temperature for 21 to 40 days. The medium consisted of 4% maltose (technical), Pfanstiehl, 1% neopeptone, Difco Manufacturing Co., Detroit, Mich., 0.05% yeast extract, Difco, 0.001% thiamine, and 0.005% inositol.

The fungal mats were harvested by filtration,

thoroughly washed with water, pressed free of excess water, and stored at −20°C until extracted.

Extraction. The method of Boivin and Mesrobeanu (8) was used to extract the washed fungal mats. A 600-ml volume of 0.5 N trichloroacetic acid was added to 200 g (wet weight) of mycelia at room temperature. After 30 min, 600 ml of distilled water was added. The mixture was stirred, left at room temperature for 24 h with occasional stirring, and then filtered. A clear yellow extract was obtained, to which 5 volumes of ethanol (95%) was added. The white precipitate which had formed after 48 h at room temperature was collected by centrifugation. It was washed three times with ethanol and once with diethyl ether and allowed to dry. Finally, the precipitate was dissolved in distilled water, centrifuged to remove insoluble material, and lyophilized.

Gel filtration. A column (2.6 by 100 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) was equilibrated with phosphate buffer (0.05 M, pH 7.8) containing 0.02% sodium azide. The crude extract (500 mg/5 ml of buffer) was applied to the column. The column was eluted with the same phosphate buffer, and the eluate was collected in 13-ml portions which were monitored by ultraviolet absorbance at 280 nm. Protein was also measured by the method of Folin-Ciocalteau at 750 nm (15). The sugar content of eluates was determined by the phenol-sulfuric acid method at 480 nm (10). Two fractions, A and B, were obtained. Each fraction was exhaustively dialyzed against distilled water at 4°C and then lyophilized.

DE-52-cellulose ion-exchange chromatography. A column (2.6 by 40 cm) of DE-52-cellulose, microgranular (Whatman Biochemicals, Ltd., Maidstone, Kent, England), borate form, was prepared using 8 to 10 column volumes of a 0.5 M sodium borate solution. Distilled water (4 column volumes) was used to remove any unbound borate (16). A sample (100 to 200 mg) of fraction A was then applied to the column. The column was first eluted with distilled water to remove any unbound components of fraction A. It was then eluted using a stepwise increase in borate concentration from 0.1 to 0.5 M, pH 9.1 to 9.2. Fifty tubes (12 to 15 ml per tube) were used for each step. Finally, the column was eluted with 0.1 M acetate buffer, pH 5.0. The eluates were monitored by ultraviolet absorbance at 280 nm and by the phenol-sulfuric acid method at 480 nm (10). A total of five fractions was obtained. They were each extensively dialyzed against distilled water at 4°C and were lyophilized.

Immunochemical methods. Antiserum to *T. mentagrophytes* var. *granulosum* was prepared in adult, male New Zealand strain rabbits. Rabbits were given four weekly injections, intramuscularly, of 25 mg of powdered acetone-killed mycelium in Freund complete adjuvant. They were bled by cardiac puncture 7 days after the last injection.

The immunodiffusion method of Ouchterlony (19), the immunoelectrophoresis method as modified by Scheidegger for microscope slides (21), and a modification of the two-dimensional immunoelectrophoresis method of Laurell using glass plates (50 by 50 mm) (9) were used for analyses of antigenic components.

Disc electrophoresis. Standard polyacrylamide gels (7%, pH 9.5) were run in tris(hydroxymethyl)aminomethane-glycine buffer in a Canalco disc electrophoresis unit according to the standard method (Canal Industrial Corp., Bethesda, Md.). Large sample sizes (4 to 5 mg/gel) were used due to the high sugar and low peptide content of the extracts. Gels were cut in half longitudinally and stained for protein with amido black and for sugar with periodic acid-Schiff reagent.

Chemical constituents. Total hexose was measured by the phenol-sulfuric acid method (10) with mannose as standard. Both qualitative and quantitative analyses of the neutral sugars were done by paper chromatography. Samples of the fractions (6.0 mg) were hydrolyzed in dilute sulfuric acid (2 ml, 2 N) under nitrogen for 6 h at 105°C. The reaction mixture was neutralized with saturated aqueous barium hydroxide. The precipitated barium sulfate was removed by centrifugation, and the supernatant was lyophilized. The hydrolysates were analyzed by descending paper chromatography (Whatman no. 1) using the Fischer-Nebel solvent system (11). Standards of galactose, glucose, mannose, xylose, rhamnose, and fucose were run with the hydrolysates. The chromatograms were dried and visualized with aniline phthalate.

The amount of each hexose was measured by eluting the aniline phthalate spots with 2 ml of 0.7 N hydrochloric acid in 80% ethanol (vol/vol) and by determining their absorbance at 390 nm. An eluate of the sprayed, unspotted chromatogram was used as a blank. Results were compared to standards made with galactose, mannose, and glucose of known concentration (22) and were found to be highly reproducible.

Samples for amino acid analyses were hydrolyzed in 6 N hydrochloric acid for 24 h at 110°C in degassed, sealed ampoules. The hydrochloric acid was removed by evaporation in a Büchi flash evaporator, and the samples were dissolved in citric acid buffer, pH 5.24. They were analyzed on a Beckman 120C automatic amino acid analyzer.

Lipid analysis. The quantity of fatty acid esters was determined by the hydroxylamine method (17), using methyl stearate as the standard.

Elemental analysis. The analyses for C, H, N, O, P, and B were done by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

RESULTS

A flow chart showing the products of each step in the extraction and purification procedures is given in Fig. 1. The crude extract contained a relatively small number of components when analyzed by immunodiffusion, immunoelectrophoresis, and two-dimensional immunoelectrophoresis.

Disc electrophoresis did not prove very useful for analysis of the components of the crude extract. They remained for the most part as a broad band at the top of the separating gel. Figure 2 shows the immunoelectrophoretic pat-

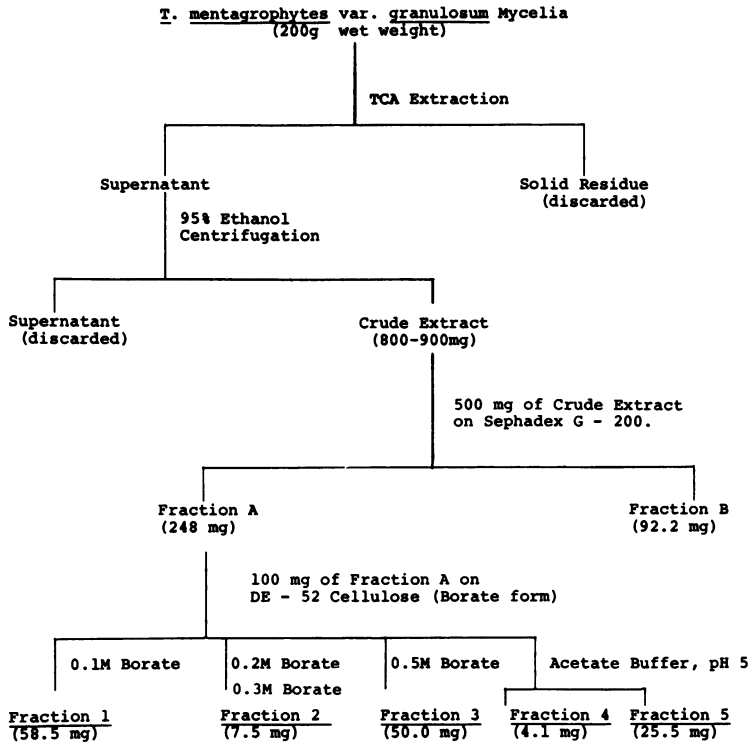

FIG. 1. *Extraction and purification of the peptido polysaccharide antigens from T. mentagrophytes var. granulosum.*

tern obtained. Three components were detected with the antiserum to *T. mentagrophytes* var. *granulosum*. Two-dimensional immunoelectrophoresis also revealed three components, two of which migrated toward the anode.

The first step in the separation of the crude extract on a Sephadex G-200 column is shown in Fig. 3. Fraction A, which was eluted in the volume corresponding to the void volume of the column, was the major fraction, containing the bulk of the trichloroacetic acid-extractable material. These components had a molecular weight of at least 200,000, as determined by gel filtration, and contained both carbohydrate and peptide. A smaller fraction of lower molecular weight, fraction B, was also eluted.

Disc electrophoresis of fraction A showed two strong polysaccharide bands with two corresponding faint protein bands. With a similar sample size on the gel, fraction B stained only faintly for sugar and protein at the top of the separating gel.

Whereas immunodiffusion analyses with rabbit antiserum to *T. mentagrophytes* var. *granulosum* revealed several antigenic components in fraction A, fraction B did not react well with this antiserum.

Since some polysaccharides form borate complexes at pH 9 to 10 (12), further fractionation of fraction A was carried out on a DE-52-cellulose column, borate form. Figure 4 shows the stepwise elution of three fractions with increasing borate concentration at pH 9.1 to 9.2. The amounts of the minor fractions eluted with 0.2 M and 0.3 M sodium borate varied considerably. They were pooled and referred to as fraction 2. When no further material could be eluted at the highest concentration of sodium borate, 0.5 M, the eluent was changed to acetate buffer, pH 5.0. Two additional fractions were obtained, fractions 4 and 5.

The major fractions, designated as 3 and 5, appeared to be highly purified and similar in reactivity with antiserum to *T. mentagrophytes* var. *granulosum*. Both fractions had a molecular weight of at least 200,000, as determined by gel filtration. Figure 5 shows precipitin lines of identity obtained with fractions 3 and 5 prepared by extraction and purification of three different lots of mycelia. As shown in Fig. 6, single precipitin arcs of similar mobility were obtained with each of the fractions.

A mixture of equal amounts of fractions 3 and 5 at a concentration of 2 mg/ml, when subjected

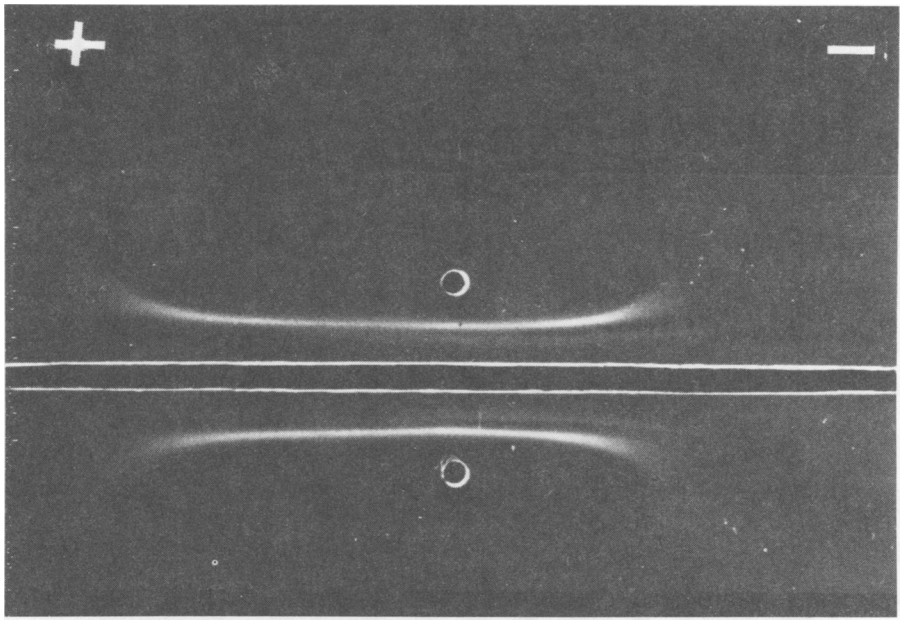

FIG. 2. *Immunoelectrophoretic pattern of the crude extract. Top well: crude extract, 10 mg/ml. Trough: rabbit antiserum to T. mentagrophytes var. granulosum. Bottom well: crude extract, 5 mg/ml.*

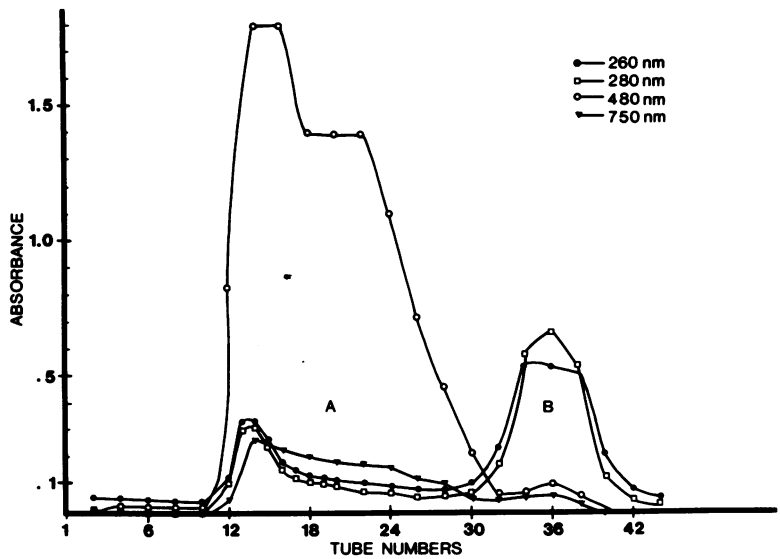

FIG. 3. *Elution profile of the crude extract on Sephadex G-200. Tube volume, 13 ml. Fractions are indicated by A and B.*

to immunoelectrophoresis, also gave a single precipitin arc with the rabbit antiserum to *T. mentagrophytes* var. *granulosum*, further indicating similarities in the antigenic determinants and mobilities of these two fractions.

These fractions were not contaminated with other antigens. However, the shape and length of the precipitin arcs could indicate a certain degree of heterogeneity.

Two-dimensional immunoelectrophoresis also showed a single precipitin arc for the purified fractions. The precipitin arc formed close to the point of origin since these fractions do not migrate toward the anode.

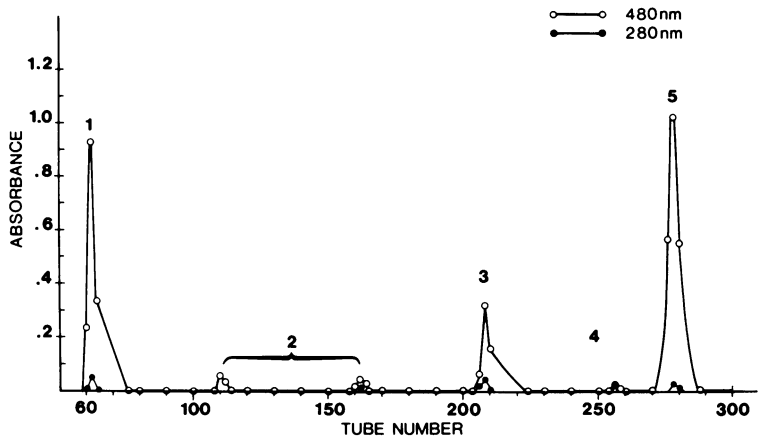

FIG. 4. *Elution profile of fraction A on DE-52 cellulose (borate form). Distilled water: tubes 1 to 50. Sodium borate: 0.1 M, pH 9.1 to 9.2, tubes 51 to 100; 0.2 M, tubes 101 to 150; 0.3 M, tubes 151 to 200; 0.5 M, tubes 201 to 250. Acetate buffer, pH 5.0: tubes 251 to 300. Fractions are indicated by number. Tube volume, 12 to 15 ml.*

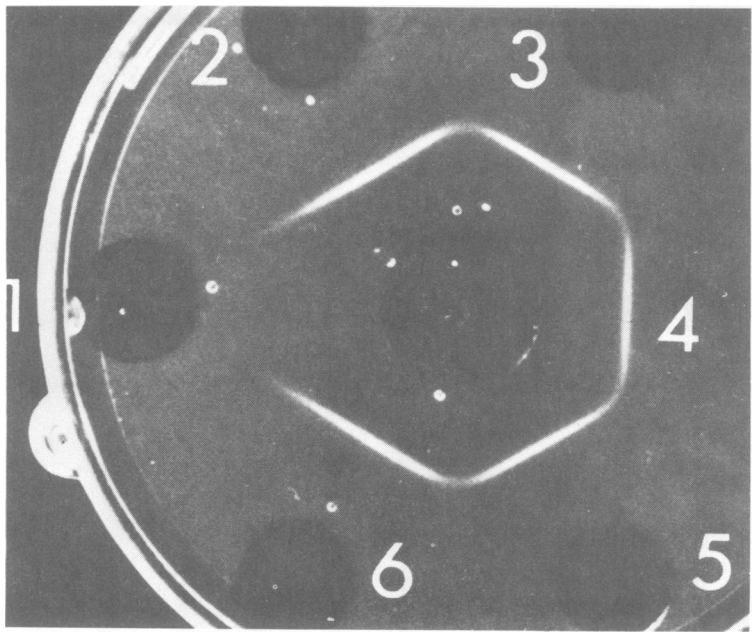

FIG. 5. *Immunodiffusion analysis of fractions 3 and 5. Wells: (1) saline; (3), (5) fraction 3, 2 mg/ml; (2), (4), (6) fraction 5, 2 mg/ml.*

The elemental analyses as well as the total carbohydrate and amino acid contents of fractions 3 and 5 are given in Tables 1 and 2.

The constituent sugars for each fraction were mannose, galactose, and glucose. They were present at ratios of 7.5:0.7:1 and 9:0.3:1 in fractions 3 and 5, respectively. Fraction 3 had a higher amino acid and amino sugar content than fraction 5. The quantities, relative to leucine, are given in Table 3. Both fractions contained the same amino acids, the threonine, proline, serine, and methionine values being higher in fraction 3 and the tyrosine value being higher in fraction 5.

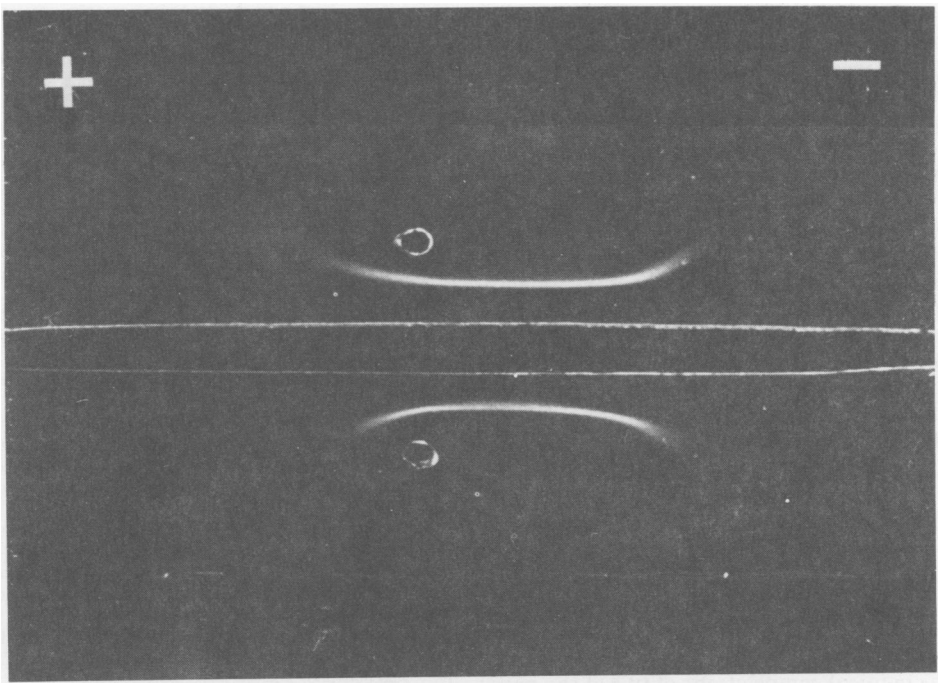

FIG. 6. *Immunoelectrophoretic analysis of fractions 3 and 5. Top well: fraction 3, 2 mg/ml. Trough: rabbit antiserum to T. mentagrophytes var. granulosum. Bottom well: fraction 5, 2 mg/ml.*

TABLE 1. *Elemental analyses of the purified peptido polysaccharides*[a]

Element	Fraction 3	Fraction 5
C	38.12 ± 0.6	36.38 ± 0.6
H	6.35 ± 0.02	6.17 ± 0.02
N	1.84 ± 0.16	1.53 ± 0.2
O	50.49 ± 2.2	43.55 ± 2.2
B	0.15	0.40
P	1.22	0.35
Ash	6.2 ± 1.1	11.9 ± 2.0

[a] Data given as percentage of total weight.

TABLE 2. *Carbohydrate and amino acid analyses of the purified peptido polysaccharides*[a]

Substance	Fraction 3	Fraction 5
Hexoses	73.2 ± 7	77.4 ± 8
Amino acids	8.6 ± 0.4	6.2 ± 0.3
Amino sugars	1.8 ± 0.09	0.4 ± 0.02

[a] Data given as percentage of total weight.

No lipids were detected in either the crude extracts or the purified fractions by the method used.

DISCUSSION

Trichloroacetic acid proved to be an excellent extracting solvent for peptido polysaccharides from mycelia of *T. mentagrophytes* var. *granulosum*. A mixture with only a few antigenic components was obtained.

The purified peptido polysaccharides, fractions 3 and 5, eluted differently from an ion-

TABLE 3. *Amino acid analyses of the purified peptido polysaccharides*[a]

Amino acid	Fraction 3		Fraction 5	
	nmol/mg[a]	Relative to leucine	nmol/mg[a]	Relative to leucine
Lysine	9.78	0.67	9.28	0.84
Histidine	5.68	0.39	2.72	0.24
Arginine	6.81	0.48	3.33	0.30
Aspartic acid	22.25	1.54	22.00	1.98
Threonine	160.84	11.11	98.40	8.86
Serine	117.57	8.15	77.90	7.02
Glutamic acid	51.14	3.55	49.90	4.50
Proline	145.02	10.06	92.50	8.29
Glycine	82.18	5.70	62.70	5.65
Alanine	49.48	3.43	39.60	3.57
Half-cystine				
Valine	36.00	2.50	28.10	2.53
Methionine	10.88	0.75	1.60	0.15
Isoleucine	16.62	1.15	12.80	1.15
Leucine	14.42	1.00	11.10	1.00
Tyrosine	8.91	0.62	16.10	1.45
Phenylalanine	8.00	0.55	5.90	0.53
Glucosamine	62.07	4.30	19.20	1.73
Galactosamine	39.17	2.72	4.40	0.39

[a] Uncorrected for ash.

exchange column. This may be due to the differences in their chemical composition. However, they were not antigenically distinguishable with our antisera.

The glycopeptides isolated by Barker et al. from *T. mentagrophytes* were considerably lower in molecular weight and also differed from ours in their carbohydrate composition (1, 2). These included two from submerged cultures, a glucomannan (1:1) peptide with a molecular weight of 20,000 to 30,000 and a galactomannan peptide with a sugar ratio varying from 1:3 to 1:8 (galactose-mannose) and a molecular weight of about 40,000 (2); and three from surface cultures, each a galactomannan peptide designated GPI, II, and III (1). GP II and III had a somewhat higher percentage of amino acids (11.71% and 12.18%) as compared with our peptido polysaccharides (8.6% and 6.2%). However, their amino acid compositions were very similar. The mannose-galactose ratio for GP II (8.8:1) was similar to that of fraction 3. Glucose was also present in our antigens. Amino sugar content was not reported for their preparations.

The chemical compositions of two of the polysaccharide-peptide fractions obtained by Nozawa et al. (18) were more similar to ours. However, both of those fractions contained several components when analyzed by immunoelectrophoresis.

The peptido polysaccharides, fractions 3 and 5, have been isolated from several different lots of mycelia and the procedure has been shown to be highly reproducible. Studies with these highly purified antigens should contribute to a better understanding of the immune response in dermatophytosis.

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LITERATURE CITED

1. **Barker, S. A., O. Basarab, and C. N. D. Cruickshank.** 1967. Galactomannan peptides of *Trichophyton mentagrophytes*. Carbohydr. Res. 3:325–332.
2. **Barker, S. A., C. N. D. Cruickshank, J. H. Morris, and S. R. Wood.** 1962. The isolation of trichophytin glycopeptide and its structure in relation to the immediate and delayed reactions. Immunology 5:627–632.
3. **Basarab, O., M. J. How, and C. N. D. Cruickshank.** 1968. Immunological relationships between glycopeptides of *Microsporum canis, Trichophyton rubrum, Trichophyton mentagrophytes,* and other fungi. Sabouraudia 6:119–126.
4. **Bishop, C. T., M. B. Perry, and F. Blank.** 1966. The water-soluble polysaccharides of dermatophytes. V. Galactomannans II from *Trichophyton granulosum, Trichophyton interdigitale, Microsporum quinckeanum, Trichophyton rubrum,* and *Trichophyton schönleinii*. Can. J. Chem. 44:2291–2297.
5. **Bishop, C. T., M. B. Perry, F. Blank, and F. P. Cooper.** 1965. The water-soluble polysaccharides of dermatophytes. IV. Galactomannans I from *Trichophyton granulosum, Trichophyton interdigitale, Microsporum quinckeanum, Trichophyton rubrum,* and *Trichophyton schönleinii*. Can. J. Chem. 43:30–39.
6. **Bishop, C. T., M. B. Perry, R. K. Hulyalkar, and F. Blank.** 1966. The water-soluble polysaccharides of dermatophytes. VI. Glucans from *Trichophyton granulosum, Trichophyton interdigitale, Microsporum quinckeanum, Trichophyton rubrum* and *Trichophyton schönleinii*. Can. J. Chem. 44:2299–2303.
7. **Bloch, B., A. Labouchère, and F. Schaaf.** 1925. Versuche einer chemischen Charakterisierung und Reindarstellung des Trichophytins (des aktiven, antigenen Prinzips pathogener Hautpilze). Arch. Dermatol. Syph. 148:413–424.
8. **Boivin, A., and L. Mesrobeanu.** 1938. Recherches sur les antigènes somatiques et sur les endotoxines des bactéries; sur la nature chimique et sur les propriétés biologiques des antigènes O et Vi du bacille typhique. Rev. Immunol. 4:469–480.
9. **Clarke, H. G. M., and T. A. Freeman.** 1967. A quantitative immunoelectrophoresis method (Laurell electrophoresis), p. 503–509. *In* H. Peeters (ed.), Protides of the Biological Fluids, vol. 14. Elsevier, Amsterdam.
10. **Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356.
11. **Fischer, F. G., and H. J. Nebel.** 1955. Nachweis und Bestimmung von Glucosamin und Galaktosamin auf Papierchromatogrammen. Hoppe-Seyler's Zs. Physiol. Chem. 302:10–19.
12. **Foster, A. B.** 1953. Ionophoresis of some disaccharides. J. Chem. Soc. p. 982–986.
13. **Grappel, S. F., F. Blank, and C. T. Bishop.** 1969. Immunological studies on dermatophytes. IV. Chemical structures and serological reactivities of polysaccharides from *Microsporum praecox, Trichophyton ferrugineum, Trichophyton sabouraudii,* and *Trichophyton tonsurans*. J. Bacteriol. 97:23–26.
14. **Grappel, S. F., C. A. Buscavage, F. Blank, and C. T. Bishop.** 1970. Comparative serological reactivities of twenty-seven polysaccharides from nine species of dermatophytes. Sabouraudia 8:116–125.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
16. **Neukom, H., and W. Kuendig.** 1965. Fractionation on diethylaminoethylcellulose columns. Fractionation of neutral and acidic polysaccharides by ion-exchange column chromatography on diethylaminoethyl (DEAE)-cellulose, p. 14–17. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.
17. **Nowotny, A.** 1969. Basic exercises in immunochemistry, p. 127–131. Springer-Verlag, New York.
18. **Nozawa, Y., T. Noguchi, Y. Ito, N. Sudo, and S. Watanabe.** 1971. Immunochemical studies on *Trichophyton mentagrophytes*. Sabouraudia 9:129–138.
19. **Ouchterlony, O.** 1948. In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta Pathol. Microbiol. Scand. 25:186–191.
20. **Saferstein, H. L., A. A. Strachan, F. Blank, and C. T. Bishop.** 1968. Trichophytin activity and polysaccharides. Dermatologica 136:151–154.
21. **Scheidegger, J. J.** 1955. Une micro-méthode de l'immunoélectrophorèse. Int. Arch. Allergy Appl. Immunol. 7:103–110.
22. **Wilson, C. M.** 1959. Quantitative determination of sugars on paper chromatography. Anal. Chem. 31:1199–1201.