A COMPARISON OF ANTIGENS OF TRICHOPHYTON RUBRUM OBTAINED BY SOME METHODS OF PREPARATION

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Summary

Westphal's and Reyes and Friedman's methods of extraction were used to study the antigens of *Trichophyton rubrum*. Three types of antigens were compared - phenolic (P) and aqueous (A) materials from mechanically ground organisms, precipitated by increasing gradients of ethanol and extracellular (X) metabolites (concentrated medium) precipitated by increasing gradients of acetone. There was no yield after concentration of 75% for both ethanol and acetone. The extracellular material (X) had the maximum nitrogen content. Polyacrylamide gel disc electrophoresis separations stained by PAS and amido black showed a few common PAS positive bands in the three polyvalent extracts. (X) was the richest in protein and showed four bands. Antisera were raised in rabbits using (P), (A) and (X) material. (X) was an effective antigen showing two immuno-precipitates with homologous antibody and one each with (P) and (A) in agar gel double diffusion (AGD) and as many as eight components in two dimensional-crossed immunoelectrophoresis (2D-CIEP.) (A) and (P) materials were poorly antigenic. Antisgera to these had no reactivity in AGD and showed only one and two precipitinogens respectively in 2D-CIEP.

Introduction

Studies of the antigens of fungi are beset with many problems. Fungal antigens are complex mixtures and it is difficult to obtain identity because of the many variables in antigen preparation, which is so important for comparisons to be valid[1,2,3]. The variables are: the method of culture, its age, the medium, the form of the growth used for antigen preparation and the method of extraction and disintegration of the fungal elements. Heating and drying of the growth add to the problem. The medium in which the fungus is grown is a rich source of antigen but may contain materials of the medium, and autolytic and metabolic products of fungal growth. Another problem is on account of peculiar substances in fungal materials. These are haemolytic, haemagglutinating and anticomplimentary components besides proteolytic and other enzymes[4,5]. Finally, carbohydrate fractions of

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some fungi show a cross-reactivity with human serum, like that of the carbohydrate C-substance of *Streptococcus pneumoniae*[6]. Methods of purification by use of sephadex gel filtration, resin and carboxy methyl and DEAE cellulose columns have also been used to obtain pure fractions [7,8,9,10].

A careful survey of the literature indicates the lack of a uniform method of preparation of antigens of dermatophytes. The main effort is directed at isolation and characterisation of cellular carbohydrate antigens for cutaneous hypersensitivity studies[11]. We describe here a comparative study of three different commonly used extraction procedures and an analysis in terms of antigen-antibody systems obtained. Such comparative studies have not been described in the literature.

Materials and Methods

Strain :

A strain of *Trichophyton rubrum* freshly isolated from a human patient with ringworm was used in these studies. It was downy and pigmented. The strain was maintained on neutral Sabouraud's dextrose agar at room temperature with transfers to potato-dextrose agar during the time required for preparation of the different antigens.

Extraction procedures :

The organism was grown in a case in hydrolysate liquid medium[12] at room temperature in static culture for 21 days.

(a) *Mycelial antigens*[13]. The surface growth was harvested and washed thoroughly in sterile distilled water. It was macerated in a sterile mortar and pestle with about 1½ times its volume of sterile distilled water. An equal volume of phenol-water (200g phenol in 65ml distilled water) was added and the mixture stored at 4°C and shaken intermittently. It was then centrifuged at 4°C at 3000g for 20 minutes and till three phases developed. The upper was aqueous, the middle of undissolved solids and the lower of phenol. The aqueous phase was treated with absolute ethanol to give successively 50, 66, 75 and 80% concentrations of the precipitant. A precipitate was obtained only with 50, 66, and 75% concentration of the precipitant. These were soluble in distilled water. The three extracts, designated A_{50}, A_{66}, and A_{75} were dialysed against distilled water and lyophillised to obtain powders. The phenol phase was treated in a similar fashion but the resulting alcoholic precipitates, which dissolved in a small volume of N/10 NaOH, were neutralised with N/10 HCl, dialysed against dilute alkali (pH 7.6 to 7.8) and lyophillised. Three more extracts P_{50}, P_{66} and P_{75} were thus obtained.

(b) *Extra-mycelial antigens*[14]: The casein hydrolysate medium, after harvesting the growth, was also used to prepare antigens. The medium was concentrated in a vacuum flash evaporator, seitz filtered and successively treated overnight with increasing volumes of cold acetone to achieve concentrations of 50, 66, 75 and 80%. There was no precipitate with 80% acetone. Each of the three precipitates was dissolved in distilled water, dialysed and lyophillised. The three antigen powders thus obtained from the culture medium were labelled X_{50}, X_{66} and X_{75}.

Thus a total of nine antigen powders were obtained. Three each were from the phenolic and watery phases of the mycelial matt and three from gradient extracts of the culture medium.

Estimation of yield :

The powders were weighed. The yield was expressed as a weight/volume

ratio of the original unconcentrated liquid culture medium or as a weight/weight ratio of the moist weight of the fungal matt. The nitrogen content was determined by the micro-Kjeldahl method[16].

Physico-chemical characterization :

(a) The solubility of each extract in water, salt, and concentrated acids and alkalies, ether, acetone and ethanol was noted.

(b) *Polyacrylamide gel disc electrophoresis analysis :* Each extract was studied in duplicate in polyacrylamide gel disc electrophoresis with a bromophenol blue marker in a miniaturized system[16,17]. One gel was stained by the Periodic Acid Schiff method and the other by the use of Amido black B[18] for carbohydrates and proteins respectively. Bands were inspected visually and the Ef values carefully calculated by the use of the formula :

$$Ef = \frac{\text{Distance of the band from origin}}{\text{Distance travelled by bromophenol blue marker}}$$

Antisera :

Three antisera were raised. The powders of each of the extracts of the culture medium (X series) and of the aqueous (A series) and phenolic (P series) phases of the fungal matt were mixed and used to immunize three different rabbits weighing one to 1.5 kg. One ml of the dissolved mixtures containing 25 mg/dL in terms of nitrogen content of 'X' and 'P' antigens and 15 mg/dL of 'A' antigen was emulsified in an equal volume of Freund's complete adjuvant and injected at multiple subcutaneous sites. This course of immunization was repeated twice, at monthly intervals. Each rabbit was bled repeatedly starting 15 days after the last immunization. A serum pool of 50 ml representing antibody to each one of the 'X', 'A' and 'P' antigens was thus prepared. It was stored frozen in small aliquots.

Agar gel double diffusion :

This was performed as described earlier[19]. Each antibody was studied with the extracts and the pooled mixtures from 'A', 'P' and 'X' phases.

Two dimensional crossed antigen-antibody electrophoresis :

Each antigen mixture (from each of the three phases) was studied with each of the three rabbit antisera in agarose gels using the method of Clarke and Freeman[20] as modified by Kelkar and Jad[21].

Results

Yield of antigen :

Table 1 gives the details of yields in terms of dry powder obtained by the different sources and methods of extraction.

Nitrogen content :

Table 1 gives the details of the values of total nitrogen content in the antigenic extracts from the mycelial and broth phases.

Physico-chemical characteristics :

1. (a) *Mycelial phenolic antigens* were light brown, insoluble in water, saline, organic solvents (acetone, ether and ethanol) and mild acids and alkalies. They were only soluble in strong acids and alkalies.

(b) *Mycelial aqueous antigens* were flaky and white and soluble in all the agents used except the organic solvents – acetone, ether and ethanol.

(c) *Extramycelial antigens* were brown and granular. These were soluble in all the agents used with the exception of the organic solvents – acetone, ether and ethanol.

TABLE 1

Yield expressed in terms of dry weights of powders (mg/100g we'ght of wet fungal matt or dL of culture medium) and nitrogen content (mg/100mg dry powder) obtained by the different methods of extraction. For nitrogen content, 10 mg dry powder was studied by the micro-Kjeldahl method.

Type of extract	Yield of dry powder	Total	N_2 content mg/100mg	Total
A. Mycelial				
—Phenolic;				
P_{50} (50% ethanol)	2 mg/100g		1.52	
P_{66} (66% ethanol)	18 mg/100g		0.72	
P_{75} (75% ethanol)	4 mg/100g	46 mg/100g	1.35	3.59
—Aqueous;				
A_{50} (50% ethanol)	52 mg/100g		0.53	
A_{66} (66% ethanol)	30 mg/100g		0.64	
A_{75} (75% ethanol)	6 mg/100g	88 mg/100g	0.70	1.87
Total of matt		134 mg/100g		5.46
B. Extra-mycelial				
X_{50} (50% acetone)	3.2 mg/dL		1.95	
X_{66} (66% acetone)	4.8 mg/dL		2.09	
X_{75} (75% acetone)	2.5 mg/dL		2.86	
Total of culture filtrate	10.5 mg/dL	10.5 mg/dL		6.90

2. *Polyacrylamide gel disc electrophoresis analysis:* Figure 1 gives the details of the bands which were PAS positive and those which stained for proteins, and their Ef values. Results of analyses of composite mixtures of the aqueous-mycelial (A), phenolic-mycelial (P) and the culture-filtrate (X) antigens are also presented.

Agar gel double diffusion:

Table 2 summarises the results. Antibody to the mixed culture filtrate antigen showed two lines of immune precipitation with the homologous extracts and a single line with the antigens of the phenolic and aqueous extracts of the fungal matt (Figure 2). In contrast, the antisera raised to the mixtures of antigens of the fungal matt failed to show any precipitins.

Two dimensional crossed antigen-antibody electrophoresis:

Table 3 gives the details. Figure 3 shows a two dimensional CIEP separation of the pooled extracellular antigens with the use of the homologous antibody. As many as eight distinct components can be made out. Two lines of immune precipitation were observed with the homologous antigen-antibody system of the phenol phase

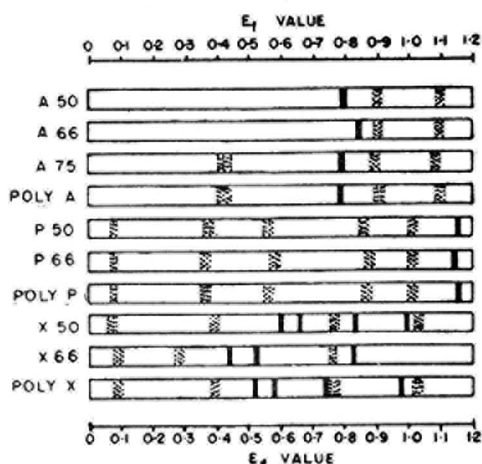

Fig. 1 Diagrammatic representation of results of polyacrylamide gel disc electrophoresis of six extracts and three composite mixtures stained by PAS (dotted areas) and Amido black (Blank bands)

TABLE 2

Results of agar gel double diffusion (AGD) analysis of nine antigenic extracts of *T. rubrum* and the three mixtures using antibodies to mixtures of aqueous (A) and phenolic (P) antigens and the extracellular antigen (culture filtrate) (X)

Antibodies (mixtures)	Antigens											
	A_{50}	A_{66}	A_{75}	Poly-A	$(P)P_{50}$	P_{66}	P_{75}	(P)-P	X_{50}	X_{66}	X_{75}	(P)-X
Anti—A	—	—	—	—	—	—	—	—	—	1	—	—
Anti—P	—	—	—	—	—	—	—	—	1	1	—	—
Anti—X	—	—	—	—	—	—	—	—	2	2	—	—

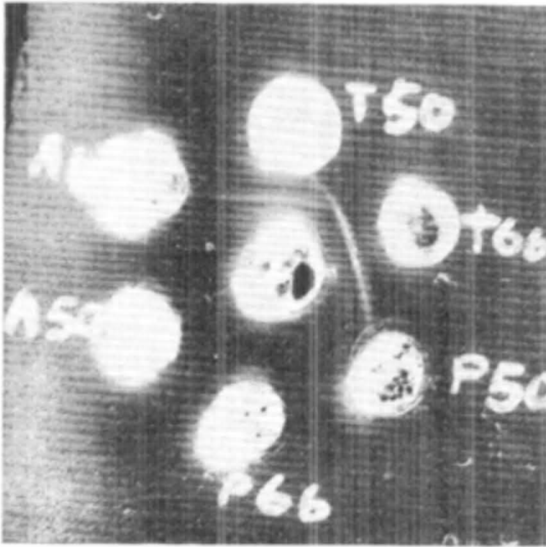

Fig. 2
Dark ground illumination photograph of an AGD study of antibody to extracellular antigen (in central well) and six extracts (A_{50}, A_{66}, P_{50}, P_{66}, X_{50} and X_{66}) in peripheral wells, Note two components with the homologous antigen and one component each with two other extracts One of the components shows reaction of identity.

TABLE 3

Results of two dimensional crossed antigen-antibody electrophoresis (2D-CIEP) analysis of phenolic (P) and aqueous (A) (fungal matt) and extracellular culture filtrate (X) antigens and homologous antibodies. Each antibody was incorporated into the agarose gel at a dilution of 1 : 5.

Antigens	Antibody in gel		
	A	P	X
A	1	—	—
P	—	2	—
X	1	1	8

Fig. 3
Dark ground illumination photograph of a antibody to extracellular antigen and its homologous antigen. The antibody, diluted 1 : 10 in buffer, is incorporated in the agarose gel. Note the presence of eight components (precipitinogers).

antigen. Only one line could be observed in the aqueous phase antigen-antibody system. Unimmunized, normal rabbit serum did not show any reactivity against the antigens by both the procedures.

Discussion

The results described above analyse some facets of extraction of dermatophyte antigens. Information of this type is not readily available and becomes useful when undertaking a study of fungal antigens. In the procedures followed the fungal matt was extracted in aqueous and phenolic phases[13] and treated with a gradient of alcohol. Increasing the gradient of alcohol upto 66 per cent increased the yield of antigen but beyond this there was little yield. The extracellular material (culture filtrate) was extracted with an increasing gradient of another organic solvent-acetone. Here again, there was no yield when the concentration of the precipitant exceeded 75 per cent. Extraction from a soluble phase using these two reagents is known[10].

The nitrogen content of the products obtained by the three methods showed considerable variation, being the least in the aqueous phase and maximum in the extracellular fluid. The solubility of these powders, however, showed considerable difference. Phenolic extracts were insoluble except in strong acids and alkalies while the other two were soluble except in organic solvents like acetone, ether and alcohol.

Electrophoretic analysis of the three types of extracts showed both similarities and differences. Extraction with ascending grades of the precipitant did not lead to any major difference in the components as seen in PAGDE (Fig. 1). This analysis was the basis for pooling extracts when raising antisera. The phenolic and aqueous-phase antigens had similar PAS positive components with Ef values 0.4, 0.9 and 1.1. The dissimilar components were a polysaccharide of Ef 0.08 in the phenolic extract and a protein of Ef 0.8 in the aqueous phase and one of Ef 1.2 in the phenolic phase. The extracellular extract had PAS positive material similar to the other phases, with Ef 0.4 and 0.8. The PAS positive material (Ef 0.8) resembled that seen in the phenolic phase. However, with the exception of the protein of Ef 0.8 which resembled similar material in the aqueous phase, the extracellular material (pooled) had three more protein staining bands of Ef 0.5, 0.6 and 0.97. None of the extracts showed protein and polysaccharide staining at identical positions.

AGD analysis of the antigens and their antisera showed the extracellular material to be the richest source of antigens. The antiserum not only showed two lines of immune precipitation with the homologous antigen but reacted even with the extracts obtained from the mycelial matt. This warrants the conclusion that for raising antisera, extracellular material is better than that obtained from the fungal matt. In AGD, antisera to the phenolic and aqueous extracts of the matt failed to show any reactivity whatsoever. CIEP studies further confirmed the superiority of extracellular material for raising antibodies. Two dimensional CIEP analysis of this homologous antigen-antibody system showed as many as eight antigens. The other two antigens studied with their homologous antibodies showed only one component for aqueous and two components for phenolic antigens. Casein hydrolysate medium did not yield any precipitate when subjected to an identical process of extraction. The antigens obtained were, therefore, fungal and not from the medium.

Philpot[22] extensively studied antigens of dermatophytes and their cross reactivity. Antigens were prepared from

disintegrated organisms grown for 21 days in static liquid cultures. Two types of antigens were prepared. One was a concentrate of the sonicate and the other a precipitate-extract from a 66 per cent gradient of acetone. Antisera were also raised by two methods: injection of the sonicated material treated with formalin or by injection of the acetone-extracted antigen. As many as 11 different antigens could be made out in AGD.

Christiansen and Svejgaard[23] also studied antigen-antibody systems of four dermatophytes. The antigen was a concentrate of mechanically disrupted organism. *Trichophyton rubrum* showed, in a two dimensional CIEP, as many as 35 precipitating antigens. However, the authors immunized their animals for a period of one year.

The results of the present study and those of the other workers indicate that dermatophytes have many precipitating antigen - antibody systems which are marked by a great deal of serological cross reactivity[22,23,24]. The present study clearly points out that the extracellular material (concentrated medium) provides the richest source of antigen. This correlates well with the protein and nitrogen content of these extracts. In *in vivo* clinical infection also such extra mycelial metabolic products of the kera tinophilic dermatophytes would be important in triggering antibody formation. Extracellular material (concentrated medium) appears to be well suited for the study of antigens or when antibodies in patients' sera are being looked for. When specific antigenic components unique to individual organisms are required further procedures of purification and fractionation would have to be adopted.

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