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Characterization of types of enzymatic activity in somatic extracts of selected fungi, thermophilic actinomycetes and pollen by immunoelectrophoresis

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Introduction

Pollen grains, fungi and actinomycetes are known to readily release substances into extracting fluids, many of which are capable of inducing an immune response in man as well as in animals. There is, however, no correlation between immunogenicity of these extracts in an experimental animal and the capacity of eliciting hypersensitivity reactions in humans (allergenicity). Whole ragweed pollen for example, while capable of inducing precipitating antibody against a wide range of antigens within its makeup, contains the major portion of its allergenic activity within a single protein, which represents only 0·5% of its extractable solids (King, Norman & Connell, 1964). It is therefore important to examine aeroallergens for properties other than immunogenicity. One property which may play a role in pollen and fungal spore allergenicity is that of enzymatic activity. It has been demonstrated that pollen cell wall proteins from different species as well as metabolic products of fungi and actinomycetes possess enzymatic activity and there is evidence for rapid release of enzymes when pollen grains contact biological surfaces (Knox & Heslop-Harrison, 1970; Walbaum, Biguet & Tran Van Ky, 1969). It is possible that the activity of such enzymes on tissue substrates plays an important role in pollen and mold spore allergenicity by regulating absorption and penetration of proteins across respiratory tract mucosal surfaces. The ability to detect enzymatic activity in proteins when complexed to antibodies has permitted the identification of enzymes in mixtures using immunoelectrophoresis. In the current report we have used this technique to study somatic antigens prepared from ragweed pollen and selected fungi and actinomycetes.

Materials and methods

Preparation of antigens

The selected fungi, actinomycetes and pollen chosen for study included the thermophilic actinomycetes, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari*, a deuteromycete *Fusarium solani*, a basidiomycete *Puccinia coronata* var. avenae (oat rust) and the pollen giant ragweed (*Ambrosia trifida*).

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The actinomycete species selected for analysis were chosen on the basis of their clinical importance as rich sources of antigen in production of certain hypersensitivity pneumonitides, notably farmer's lung and bagassosis. *Fusarium solani* and *Puccinia coronata* were chosen as representative of the many deuteromycete and basidiomycete species present in the local atmosphere and associated with production of atopic respiratory disease (Salvaggio & Seabury, 1971; Salvaggio, Seabury & Schoenhardt, 1971). *Ambrosia trifida* pollen was chosen for study because of its local abundance and known importance as an aeroallergen.

Whole, undefatted dried ragweed pollen and *Puccinia coronata* spores (lot S8-GL) were obtained from Greer Laboratories (Lenoir, N.C.). *Puccinia coronata* was harvested directly from the plant in July 1971, without defatting, dried for 6 days at room temperature, and mechanical sieved with a 200 mesh sieve. *Fusarium solani* (ATCC No. 12823) was grown in L-M* synthetic broth for 15 days at 25°C. The mould mat was ground $\times 1$ min in a Waring blender followed by drying $\times 24$ hr and powder milling for 2 hr in a ball mill jar.

* *L-M culture medium*

Dextrose	900·0 g	$FeSO_4.H_2O$	30·0 ml
K_2HPO_4	90·0 g	$ZnSO_4 \cdot H_2O$	6·0 ml
KH_2PO_4	30·0 g	$CuSO_4.5\ H_2O$	6·0 ml
NH_4NO_3	90·0 g	$MnSO_4.5\ H_2O$	6·0 ml
$MgSO_4.H_2O$	15·0 g	$NaMoO_4.2\ H_2O$	6·0 ml
$CaCl_2$	15·0 g	Vitamin B complex Betalin (Lilly)	30·0 ml
Distilled H_2O	30 l		

Thermoactinomyces vulgaris and *Thermoactinomyces sacchari* were isolated from samples of bagasse (dried sugar cane fibre from which sucrose content had been extracted followed by storage outdoors in southern Louisiana for a period of months). These isolates were identified morphologically on thin layer slide culture and biochemically by cell wall amino acid and amino sugar composition as previously described (Seabury *et al.*, 1973). Actinomycete isolates were grown in trypticase soy broth at 55°C and harvested as previously described (Seabury *et al.*, 1973).

Each crude antigen to be used for rabbit immunization was prepared in the following manner. Crude fungal growth, actinomycete growth or ambrosia pollen mixed with an equal volume of distilled water, were initially disrupted with glass beads (0·25–0·30 mm diameter) in a model MSK Braun Cell Homogenizer (Bronwell Scientific Company, Rochester, N.Y.) at 400 cycles for 4 min. The supernatant was decanted, the beads washed twice with distilled water and the combined washings and supernatant lyophilized, followed by extraction with 0·018 M NaCl at 4°C using a teflon rotating rod (Salvaggio *et al.*, 1969). Suspensions were centrifuged at 2000 rev/min for 20 min, the supernatant dialysed for 48 hr against distilled water and re-lyophilized. Final working antigen preparations were prepared in veronal buffer, pH 8·2 in a concentration of 20 mg/ml. Best results were obtained when antigens were prepared and used within 2–3 weeks.

Preparation of antisera

Twenty-five 2–3 kg white female New Zealand rabbits were divided into groups of five for immunization with *F. solani*, *P. coronata*, *T. vulgaris*, *T. sacchari*, and *Ambrosia trifida* antigen.

Animals were inoculated subcutaneously into the shoulder and flank regions with 1·0 ml of an emulsion containing 8·0 mg of lyophilized antigen in Freund's complete adjuvant. Subsequent inoculations were made in incomplete adjuvant at 2-week intervals for a minimum period of 6 months or until no further precipitin bands appeared on immunoelectrophoresis against the respective immunizing antigen. Animals were bled at monthly intervals from the central ear artery and sera harvested and frozen at −20°C. Monthly bleedings from each group were pooled. One aliquot of pooled sera from each group was precipitated by the ammonium sulphate technique (Kawai *et al.*, 1973) and the precipitated globulins concentrated five times followed by resuspension in phosphate buffer, pH 7·4.

Electrophoresis

Standard gel electrophoresis was performed on 103 by 83 mm Kodak glass slides covered with 15·0 ml of 0·80% agarose (Sigma Chemical Company, St Louis, Mo.) in veronal buffer pH 8·2 using a previously described semi-macromethod (Arquembourg, Salvaggio & Bickers, 1970). Antigens solubilized in veronal buffer (7·0 μl) were subjected to electrophoresis using a potential gradient through the gel of 5·6 V/linear cm of agar for approximately 90 min. Following gel electrophoresis plates were immediately stained with either a general protein stain or the appropriate enzymatic stain. They were then fixed in 2% acetic acid, and dried under filter paper for 1 hr at 60°C.

Immunoelectrophoresis was performed using the same electrophoretic conditions and semi-macromethod (Arquembourg *et al.*, 1970). Troughs were filled with 0·20–0·25 ml of rabbit serum or globulin prepared against the respective fungal, actinomycete, or pollen antigen. Immunodiffusion was carried out for 48 hr at room temperature in a humidified chamber. Gels were subsequently wrapped in wet filter paper and washed for 72 hr in 0·9% saline followed by drying, staining, clarification in 2% acetic acid solution and preservation with a decanted shellac solution.

Ouchterlony double diffusion analysis for somatic antigen cross reactivity was performed using 0·80% agarose agar and pooled antisera against each respective isolate. Templates employed were as previously described (Salvaggio *et al.*, 1966).

Characterization techniques

For general characterization of proteins, plates were immersed in a solution of Light green (Light green SF 0·5 g, 1·0 M sodium acetate 50·0 ml, glacial acetic acid 10·0 ml, q.s. distilled water to 500·0 ml). After 15 min plates were clarified with 2% acetic acid.

Specific reactions for characterizing enzymes in immunodiffusion precipitates were performed in duplicate according to the methods outlined by Uriel (1971). The enzymes and substrates employed together with the commercial supplier are listed in Table 1.

Results

Table 2 shows the results of detectable enzymatic activities in the somatic antigen

Table 1. Enzymes and substrates employed in characterization techniques

Enzymes	Substrates	Commercial source
Acetylcholinesterase	Acetylthiocholine iodide	Sigma Labs
Alanine dehydrogenase	L-Alanine	NBC Labs
Aldolase	D-fructose diphosphate	NBC Labs
α-amylase	Soluble starch	Difco Labs
Carboxylic ester hydrolase	2-naphthyl acetate	Sigma Labs
Carboxypeptidase	Carbonaphthoxy-D, L-phenylalanine	Schwarz/Mann Research
Carboxypeptidase	Hippuryl-L-arginine	Schwarz/Mann Research
Catalase	H_2O_2	HLH Products
Chymotrypsin	N-acetyl-D, L-phenylalanine beta-naphthyl ester	Schwarz/Mann Research
Glucose-6-phosphatase	Glucose-6-PO_4, dipotassium	NBC Labs
Glucose-6-phosphate dehydrogenase	Glucose-6-PO_4, dipotassium	NBC Labs
Leucine aminopeptidase	L-lecyl-4-methoxy-2-nephthylamide	NBC Labs
Lipase	2-naphthyl nonanoate	NBC Labs
Peroxidase	Benzidine NCl	Merck and Company
Ribonuclease	Cytitidine 2′3′ PO_4, monobarium	Schwarz/Mann Research
Trypsin	Benzoyl-D, L-Arginine-2-naphthylamide HCl	Schwarz/Mann Research
Carbonic anhydrase	H_2CO_3	Mallincroat Labs
Lactic dehydrogenase	Na Lactate—60%	NBC Labs

extracts after gel electrophoresis and immunoelectrophoresis followed by enzyme characterization stains.

It can be seen that up to four bands contained enzymatic activity in the case of *Ambrosia trifida* and six in *Thermoactinomyces sacchari*. Enzymatic activity was detectable in five bands after gel electrophoresis of *Puccinia coronata* but some of these were no longer detectable after immunoelectrophoresis (Fig. 1) possibly due to lack of optimal enzyme antibody concentration ratios under the experimental conditions employed. Two areas of major enzymatic activity were detected in *Thermoactinomyces vulgaris* and one in *Fusarium solani*.

Composite immunoelectrophoretic drawings of major precipitin arcs developed with hyperimmune serum against each respective crude somatic antigen are illustrated in Fig. 1. *Ambrosia trifida* pollen was highly immunogenic in the rabbit, and enzymatic activity was detected in six of the ten major antigen-antibody precipitin arcs. Seven antigens were demonstrated in *Thermoactinomyces vulgaris* cellular extracts after immunoelectrophoresis, four with enzymatic activity. The major arcs developed, corresponded roughly in mobility to those of the A, B, C, and D zones as defined by Jenkins and co-workers for mouldy hay extracts (Pepys, 1969). The number of precipitin arcs developed against our *T. vulgaris* cellular extract is also similar to that noted by Walbaum and co-workers using hyperimmune rabbit sera against somatic *T. vulgaris* antigen (Walbaum *et al.*, 1969).

Results with the newly described species *T. sacchari* indicate the presence of twelve major somatic antigens after immunoelectrophoresis. Six of these were identified as enzyme-antibody complexes by reactions specific for enzyme characterization.

Five major arcs were identified in *Puccinia coronata* and six in *Fusarium solani* somatic extracts on immunoelectrophoretic analysis against specific hyperimmune

Table 2. Demonstrable enzymatic activities by gel electrophoresis and immunoelectrophoresis

	Fusarium solani		*Puccinia coronata*		*Thermoactinomyces sacchari*		*Ambrosia trifida*		*Thermoactinomyces vulgaris*	
	EP	IEP	EP	IEP	EP	IEP	EP	IEP	EP	IEP
Acetylcholinesterase	−		+		+	−	−		−	
Alanine dehydrogenase	−		−		−	−	+	+ (2)*	−	
Aldolase	−		−		−	−	−		−	
Amylase	−		−		−	−	−		−	
Carboxylic ester hydrolase	+	+ (3)*	+		+	+	+	+ (4)*	+	+ (3)*
Carboxypeptidase	−		−		−	+	−		−	
Carboxypeptidase	−		−		±	+	−		−	
Catalase	−		−		+	+	−		−	
Chymotrypsin	−		−		+		−		+	+
Glucose-6-phosphatase	−		+		−	−	−		−	
Glucose-6-phosphate dehydrogenase	−		+	+	−	−	−		−	
Leucine aminopeptidase	−		−		−	−	+		−	
Lipase	−		−		−	−	−		−	
Peroxidase	−		−		+	+	−		−	
Ribonuclease	−		−		−	−	−		−	
Trypsin	−		−		−	−	−		−	
Carbonic anhydrase	−		−		−	−	−		−	
Lactic dehydrogenase	−		+		−	−	+		−	

* Parentheses indicate number of arcs with enzymatic activity.

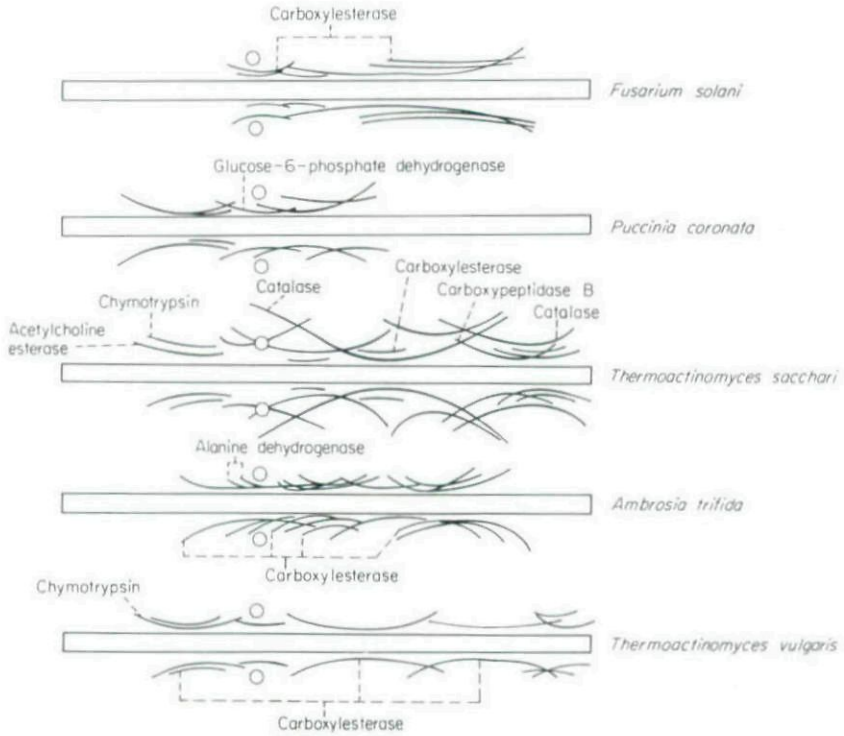

Fig. 1. Demonstrable enzymatic activities in antigen antibody precipitates by immunoelectrophoresis.

rabbit serum. Of the precipitin arcs of *P. coronata*, enzymatic activity was limited to a single beta zone arc which contained glucose-6-P dehydrogenase activity.

On Ouchterlony double diffusion analysis employing rabbit antiserum specific for each somatic antigen analysed in this study there was no demonstrable cross reactivity between *Puccinia coronata*, *Fusarium solani*, *Ambrosia trifida*, or *Thermoactinomyces vulgaris* antigens with *Mycobacterium tuberculosis* strain H 37 RA (Difco Labs). Thus it would appear unlikely that any of the arcs demonstrable on immunoelectrophoresis represented reactions between antigens common to our isolates and *M. tuberculosis* incorporated in the Freund's adjuvant employed for rabbit immunization.

Discussion

These results indicate that some of the many antigenic proteins present in somatic extracts of selected thermophilic actinomycetes, fungi and pollen possess a variety of enzymatic activities. Due to the experimental conditions employed it is likely that these represent only few of the somatic enzymes present. It is, for example, possible that enzymatic activity of many proteins may rapidly diminish when in solution or that activity may be lost during the process of immunodiffusion as noted after immunoelectrophoresis of our *Puccinia coronata* extracts. Furthermore enzyme-antibody reactions not present in optimal antigen-antibody ratios may precipitate poorly or produce no precipitin arcs in gel, thus remaining undetected. Our studies were limited

to analysis of somatic antigens and it is also likely that further analysis of 'metabolic' antigens in our isolates would detect the presence of additional antigen-enzymatic activities. In this regard, previous analyses of the antigenic structure of *Micropolyspora faeni* (*Thermopolyspora polyspora*) metabolic fractions (Walbaum *et al.*, 1969) have identified ten enzymatic activities which exhibited a high degree of reactivity against sera from patients with farmer's lung.

It is well known that fungi and actinomycetes produce a variety of extracellular and endocellular enzymes and that there are advantages in characterizing these antigenic constituents for purposes of classification of organisms and of disease diagnosis. Pollen grains when moistened, also rapidly release proteins with a variety of enzymatic activities (Knox & Heslop-Harrison, 1970). These proteins are localized primarily in the intine in close association with the apertures and their function is mainly connected with early nutrition of the pollen tube and penetration of the stigma (Mäkinen & Brewbaker, 1967; Mäkinen & McDonald, 1968).

Presumably these proteins with enzymatic activity are among those readily released onto human respiratory tract mucosal surfaces after initial contact, and into extracting fluids during the preparation of pollen and mould allergenic extracts. If this is the case, the role of these enzymes in determining 'allergenicity' of a particular spore or pollen grain is deserving of consideration. For example, enzymatic activity of a protein, acting on a tissue substrate, may be a key factor in regulating its absorption across respiratory tract mucous membranes with resultant stimulation of submucosally located IgE producing cells. Conversely secretory IgA or other substrates in mucosal secretions might play an as yet undetermined role in neutralizing such enzymatic activity at a local level. Such a function might be similar to the known effect of IgA in preventing gastrointestinal mucosal adherence and replication of cholera bacilli (Freter, 1972; Fubara & Freter, 1973). The fact that antigenic proteins of pollen, fungi, and actinomycetes possess enzymatic activities which can serve as markers should allow this hypothesis to be tested by appropriate *in vitro* procedures.

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