Immunochemical Investigation of Four Saprolegnia Species with Parasitic Activity in Fish: Serological and Kinetic Characterization of a Chymotrypsin-Like Activity

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Abstract. The antigenic structure of four aquatic Phycomycetes of the genus *Saprolegnia* with parasitic activity in fish was analyzed with immunoelectrophoresis and double diffusion techniques. It is shown that these serological tests might represent a valuable criterion for the identification of the four *Saprolegnia* species: *S. parasitica, S. ferax, S. delica,* and *S. diclina.* A specific chromogenic reaction revealed the presence of a protease with chymotrypsin-like activity among the precipitation arcs obtained after immunoelectrophoresis. Analysis of this proteolytic activity by kinetic assay methods confirmed the results of the serological tests. Moreover estimates of the quantity of enzyme present in the cellular extract and in the culture medium were obtained. A possible relationship between the proteolytic activity and the host–parasite interaction was discussed.

Introduction

The deterioration of surface water quality has incontestably brought a massive increase in fish mortality, thus explaining the past years' growing scientific interest in aquatic pathogens. Owing to the high incidence of dermatomycosis and its broad propagation [10, 15, 19, 21], we maintain that the study of fish pathogens in natural fresh waters should focus on fungi in particular [5]. All observations seem to point to the fact that this rapid spread must be brought into relation with the ubiquity of fungi in aquatic ecosystems. Thanks to their vast ecological valence as well as to their remarkable capacity to withstand anaerobic conditions, these microorganisms are greatly aided in their saprophytic cycle by aquatic conditions characterized by increased organic load.

Often enough, the pathogens responsible for cutaneous mycoses in fish belong to the fungus genus *Saprolegnia* (Oomycetes, Phycomycetes). Like all

Microbial Ecology *3*, 107–118 (1977)

animal mycosis agents, *Saprolegnia* are not parasites in the strictest sense, so that an etiological study would imply an investigation of their ecological and nutritional behavior in relation to the saprophytic life cycle. An examination of the correlations between the pathogenicity and the ecological demands of the potentially parasitic *Saprolegnia,* including the factors and characteristics which influence and enable the transition from the saprophytic to the parasitic state, would thus be extremely useful.

This paper reports the outcome of the serological approach to the study of four *Saprolegnia* species which are potentially parasitic for fish. The antigenic capacity of the microorganisms was examined and the immune response by animals analyzed.

The parasite's antigenic structure and its biological products give us revealing insights into the physiological behavior of the microorganism. Of particular importance to the understanding of host–parasite relationships is the investigation of proteolytic activities [6]. Our attention has been concentrated on the properties controlling the transformation of the fungus from saprotrophy to biotrophy.

Materials and Methods

Strains

The following *Saprolegnia* strains were used:

Saprolegnia parasitica, Coker,	CBS strain 223.65
Saprolegnia parasitica, Coker,	ATCC strain 22284
Saprolegnia ferax (Gruithuisen) Thuret, Dick,	CBS strain 534.67
Saprolegnia delica, Coker, Seymour,	CBS strain 345.62
Saprolegnia diclina, Dick,	CBS strain 536.67

ATCC (American Type Culture Collection, Rockville, Maryland)
CBS (Centraalbureau voor Schimmelcultures, Baarn, Netherlands)

Growth Conditions and Preparation of the Antigenic Extracts

Medium. Mycophil Broth (BBL, Becton Dickinson), composition peptone 10 gm/liter, dextrose 40 gm/liter, pH 7. The antigenic cultures were prepared under shaking conditions (100 r.p.m.) at 25°C in 500 ml Erlenmeyer flasks containing 150 ml Mycophil Broth. Four-day old mycelial cultures at the filamentous stage, without sporange differentiation, were used for antigen extraction. *Saprolegnia* antigens were prepared according to the method used for *Neurospora* described in detail previously [13].

After separation of the mycelium from the liquid medium by filtration through a Büchner funnel, the extraction was carried out as follows: The mats were frozen in liquid nitrogen and thawed in a water bath at 30°C. After lyophilization and grinding at 5°C with quartz sand in phosphate buffer (0.5 *M,* pH 7.2) containing NaCl 0.018 *M* (according to Sørensen), the homogenate was centrifuged 10 min at 10,000 *g* at 4°C.

Preparation of the Antisera

The antisera were prepared on rabbits against four reference *Saprolegnia* CBS strains: *S. parasitica, S. ferax, S. delica,* and *S. diclina.* The weak antigen capacity of the *Saprolegnia*

extracts as compared with those of *Neurospora* of the same age led us to alter the injection procedure.

The antigens were injected into the rabbits' footpads, gradually stepping up the injection frequency according to the following scheme:

1st day	1 ml homogenate + 1 ml Freund's complete adjuvant	*footpad*
22nd day	1 ml homogenate + 1 ml Freund's adjuvant	*intramuscular* and *subcutaneous*
34th day	first bleeding (preliminary bleeding)	
47th day	1 ml homogenate + 1 ml Freund's adjuvant	*intramuscular* and *subcutaneous*
78th day	1 ml homogenate without Freund's adjuvant	*intramuscular*
92nd day	Bleeding	
100th day	Bleeding	

Immunochemical Techniques

Immunoelectrophoresis. This was carried out according to the technique of Grabar and Williams [4] modified by Scheidegger [14]. Slides (76 × 26 mm) were covered with a 1 mm thick layer of 1% agarose (Industrie Biologique française, Gennevilliers, Seine, France) in sodium-diethylbarbiturate at pH 8.2 and ionic strength 0.05. Electrophoretic separation of the antigenic mixture (5 μl) was pursued in a Shandon migration curve at 5°C, using 8 slides under 5 mA for each; 50 μl of antiserum were pipetted in the troughs. The precipitation patterns were stained with thiazine red R (Fluka).

Double diffusion. The usual Ouchterlony technique [11] with the antigen wells arranged circularly around a central antibody well was used.

Detection of a chymotrypsin-like activity after immune precipitation. For the characterization of enzyme–antienzyme complexes from the precipitation patterns obtained by immunoelectrophoresis and double diffusion we referred to Uriel's classic work [18]. In the case of the identification of a chymotrypsin-like activity in antigenic constituents of fungal origin we used the same methods applied to *Aspergillus fumigatus* by Biguet [1].

After development of the precipitation patterns and elution of the nonprecipitated excess proteins in a 9‰ NaCl solution, the presence of the enzyme was revealed by incubating the plate for 40 min at 37°C in the following solution:

5 mg	Acetyl-DL-phenylalanine β-naphthylester (K & K Laboratories, Hollywood)
2 m.	Dimethyl formamide (Serva, Heidelberg)
10 mg	Naphthanil Diazo blue B (K & K Laboratories)
20 ml	TRIS buffer, pH 7.4, 0.05 *M* (Serva)

Measurement of a Chymotrypsin-like Activity

Mycelial extracts. 500 mg of the lyophilized mycelia (obtained as described above for the preparation of antigenic extracts) were ground in a mortar with quartz sand in 5 ml water and centrifuged 15 min at 25,000 *g*. The protein concentration in the supernatant was estimated on the basis of the ratio of the absorbances at 280 and 260 nm according to Warburg and Christian [20] using a Zeiss MN 12 spectrophotometer. The sample subjected to the activity assay consisted of 3 ml of the supernatant diluted to 5 ml with 0.5 *M* sodium chloride. The pH of this solution, originally 5.5, was adjusted to 7.5 by the addition of 50 μl of 1 M sodium hydroxide.

Growth medium. The preparation obtained from the medium after filtration of the mycelia and lyophilization was dissolved in 5 ml of water. For the activity assay, 2.5 ml of this solution

were diluted to 5 ml with 0.4 *M* sodium chloride and its pH adjusted to 7.5 by the addition of 100 μl of 1 *M* sodium hydroxide.

Activity assays. They were carried out using N-acetyl-L-phenyl alanine methyl ester (Schwarz/Mann, New York) as a substrate. Enzymes with chymotrypsin-like activity easily hydrolyze this substrate releasing acetyl-L-phenylalanine, whose carboxylic acid group is fully dissociated at pH 7.5 where the measurements were performed. The amount of sodium hydroxide solution which must be added in order to keep the pH constant during the reaction is recorded as a function of time, thus monitoring the progress of hydrolysis. This continuous titration was carried out with the aid of a pH-stat instrument (Radiometer) consisting of a thermostatted titration cell equipped with a combination calomel–glass electrode, a pH-meter (PHM 26), a titrator (TTT 11b), an automatic buret (ABU 1) and a recorder (SBR 2).

The measurements were done as previously described for kinetic investigations of α-chymotrypsin [2]: 5 ml of the solution to be assayed were placed in the titration cell, maintained at 25°C and stirred under nitrogen. The pH of the solution was adjusted manually at exactly 7.50 and the reaction started on the addition of 1 ml of a 9.15 *M* solution of the substrate by simultaneously turning on the titrator. The titrant solution in the buret was 0.045 *M* sodium hydroxide.

Results

Immunochemistry

Analysis of the cellular extracts. The soluble antigenic constituents of the mycelial extracts of four *Saprolegnia* species most frequently found as fish parasites, i.e., *S. parasitica, S. ferax, S. delica,* and *S. diclina,* were investigated by immunoelectrophoresis and double-diffusion techniques. On the basis of this immunological analysis an attempt was made to establish a distinction between the various species by comparing their antigenic composition by means of both homologous and heterologous reactions. The homologous reactions gave reproducible immunoelectrophoregrams characteristic for each of the four species. From the serological comparisons obtained with the heterologous reactions it was possible to evaluate the degree of relationship between the species. Figure 1 illustrates, as an example, the homologous and heterologous reactions of anti-*S. diclina* antiserum with the antigens of *S. diclina* and *S. parasitica* and *S. ferax* and *S. delica,* respectively. The results of a detailed examination of these homologous and heterologous reactions are summarized in Table 1.

The double-diffusion precipitation patterns obtained from the reaction of anti-*S. parasitica* antiserum with the antigenic extracts of the four *Saprolegnia* species are given in fig. 2.

Analysis of the culture medium. The culture media collected after separation of the mycelia and containing the metabolic antigens released by the growing microorganisms were also subjected to immunological analysis as described above.

Figure 2 shows the results of the serological reactions of these metabolic products with the rabbit antibodies induced against the cellular antigens. This figure clearly shows the antigenic behavior of the metabolites released in the

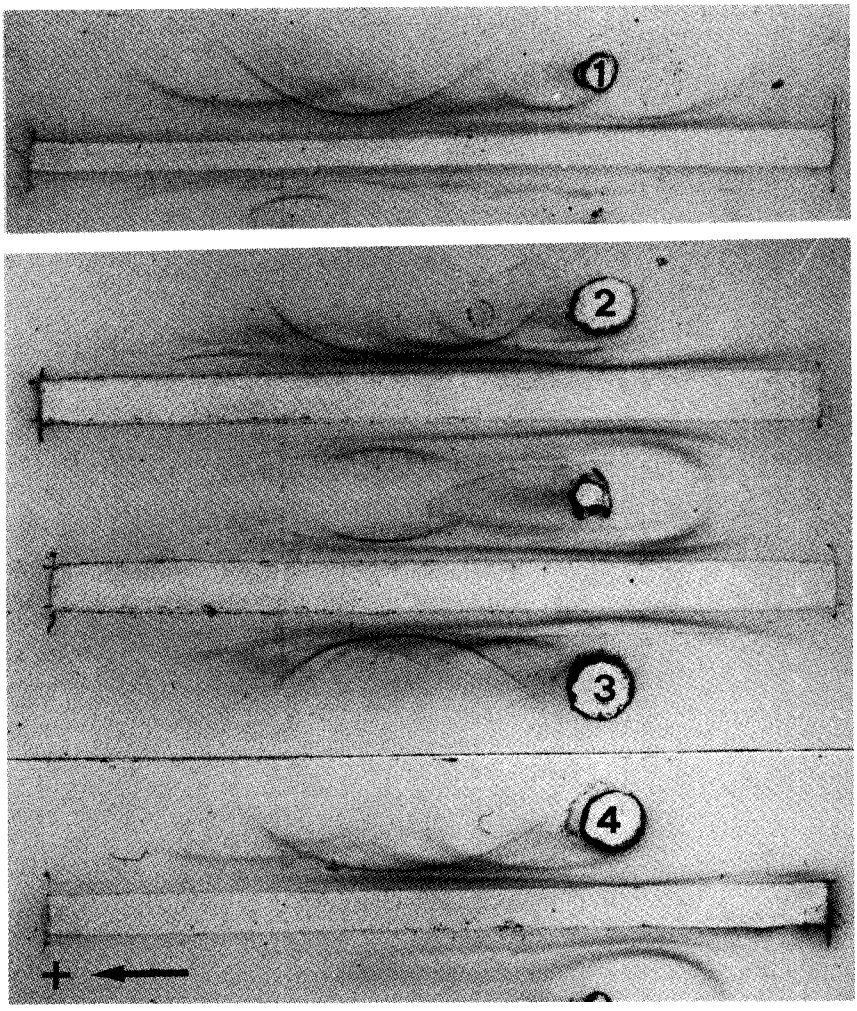


Fig. 1. Comparative immuno-electrophoretic analysis between antigenic extracts of: *Saprolegnia diclina* (1), *S. parasitica* (2), *S. ferax* (3), and *S. delica* (4). Antiserum: *anti-S. diclina*. Protein staining Thiazine red R.

culture medium during the growth of the microorganism. It also indicates that there are several common antigenic constituents among the proteins of the mycelial cell extract and the proteins secreted into the outside medium.

Immunochemical enzyme assay. The presence of an enzyme with chymotrypsin-like activity among the antigenic constituents of the cellular extracts precipitated as antigen–antibody complexes after immunoelectrophoresis and double diffusion was detected using acetyl-DL-phenylalanine β-naphthylester as a substrate. This substrate is hydrolyzed by chymotrypsin-

Table 1

Antigens Common to the 4 Saprolegnia *Species, Strains CBS, revealed by the Homologous and Heterologous Reactions in Immunoelectrophoresis Analysis. Number of Precipitation Arcs*

	Antisera			
Antigens	*S. parasitica*	*S. ferax*	*S. delica*	*S. diclina*
S. parasitica	11	4	4	7
S. ferax	5	7	4	6
S. delica	7	3	8	5
S. diclina	7	5	4	12

like enzymes releasing β-naphtol which can be easily revealed by chromogenic reactions.

The results of the positive chymotryptic reactions obtained after double-diffusion analysis can be summarized as follows:

(1) The four antisera contain at least one antibody capable of precipitating antigens exhibiting positive chymotryptic activity.
(2) The most evident enzymatic reactions are shown by the patterns of the homologous reactions of *S. parasitica* and *S. diclina*.

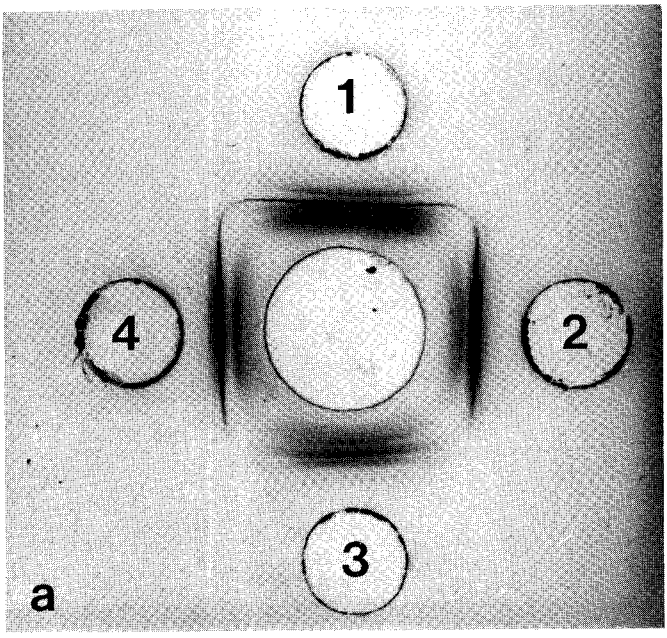
Figure 3a shows the precipitate arc with chymotrypsin-like activity. The double-diffusion patterns are obtained with the antiserum anti-*S. parasitica* reacting against the four antigenic solutions from CBS *Saprolegnia* strains.

On the immunopherograms the test for chymotryptic activity gave positive chromogenic reactions with the precipitates of low anodic electrophoretic mobility (Fig. 3b). These results are obtained with anti-*S. parasitica* antiserum reacting with two antigen solutions from *S. parasitica* and *S. diclina*.

With similar experiments it was possible to demonstrate the presence of the enzyme in the culture media. One of their main metabolic constituents exhibits an evident chymotryptic activity. A quantitative evaluation of this activity is reported in the following section.

Kinetic assay of the chymotrypsin-like activity. The successful detection of serological methods of enzyme with chymotrypsin-like activity encouraged the attempt to evaluate this activity quantitatively. Preliminary experiments showed that enough activity was present in the extracts to be easily evaluated by a conventional kinetic assay method. This consists in following the hydrolysis of a typical chymotrypsin substrate, acetyl-L-phenylalanine methylester (APHME), with the aid of a pH-stat.

Fig. 2. Immune precipitates obtained by double-diffusion analysis. The central wells contained rabbit antiserum against *S. parasitica*. (a) The surrounding wells contained: antigenic cellular extracts of *S. parasitica* (1), *S. ferax* (2), *S. delica* (3), and *S. diclina* (4). (b and c) Comparison between the antigenic cellular extracts (b) and the antigenic medium extracts (c) from *S. parasitica* (1 and 1a), *S. ferax* (2 and 2a), and *S. diclina* (4 and 4a).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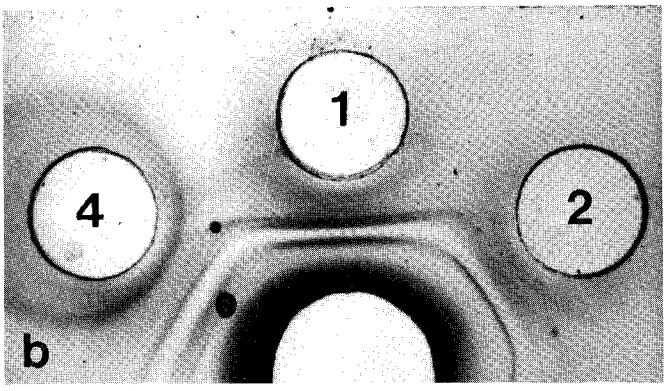
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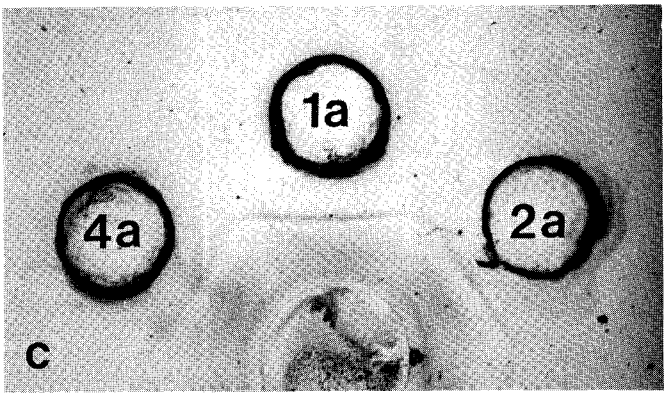
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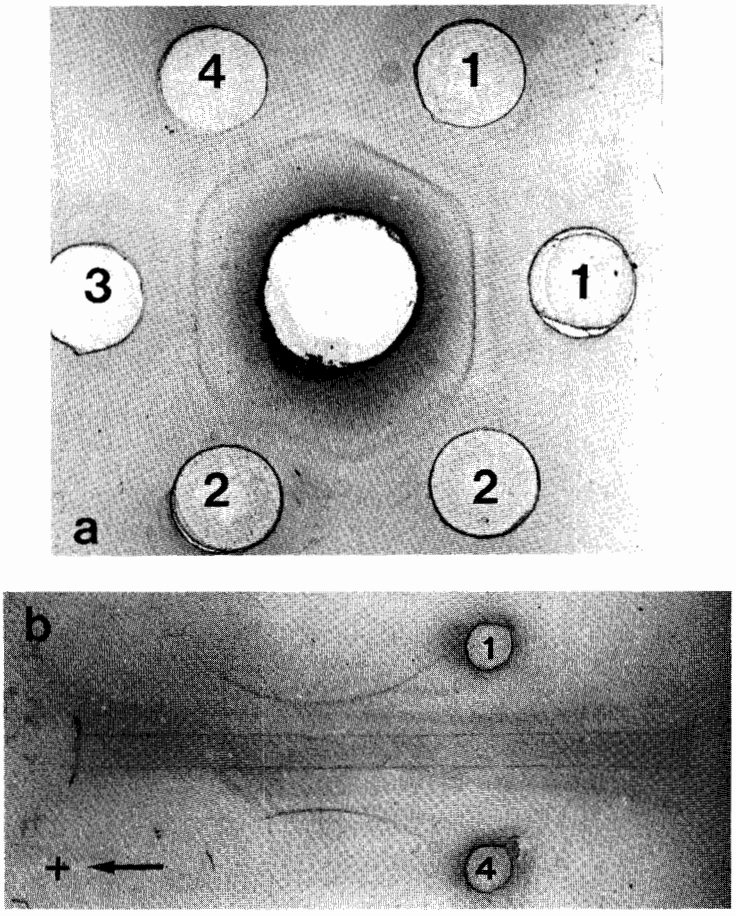

Fig. 3. Characterization of antigen–antibody complexes with chymotrypsin-like activity. Proteolytic activity is exhibited by the precipitation lines obtained by double diffusion (a) and immunoelectrophoresis (b). (a) The central well contained antiserum anti-*S. parasitica* and the surrounding wells antigenic solutions from *S. parasitica* (1), *S. ferax* (2), *S. delica* (3), and *S. diclina* (4). (b) The central trough contained antiserum anti-*S. parasitica* and the lateral wells antigenic solutions from *S. parasitica* (1) and *S. diclina* (4).

The main experiments were conducted at pH 7.5, 25°C in a 0.2*M* sodium chloride solution. For all species, both the extract of the cellular material and the culture medium showed a pronounced enzymatic activity as can be seen from Fig. 4.

The reaction observed can be attributed unambiguously to the hydrolysis of the substrate, since a blank experiment, performed in absence of substrate, showed a much slower reaction (Fig. 4a), probably due to the hydrolysis of proteins present in the sample. Moreover, the progress curves clearly level off

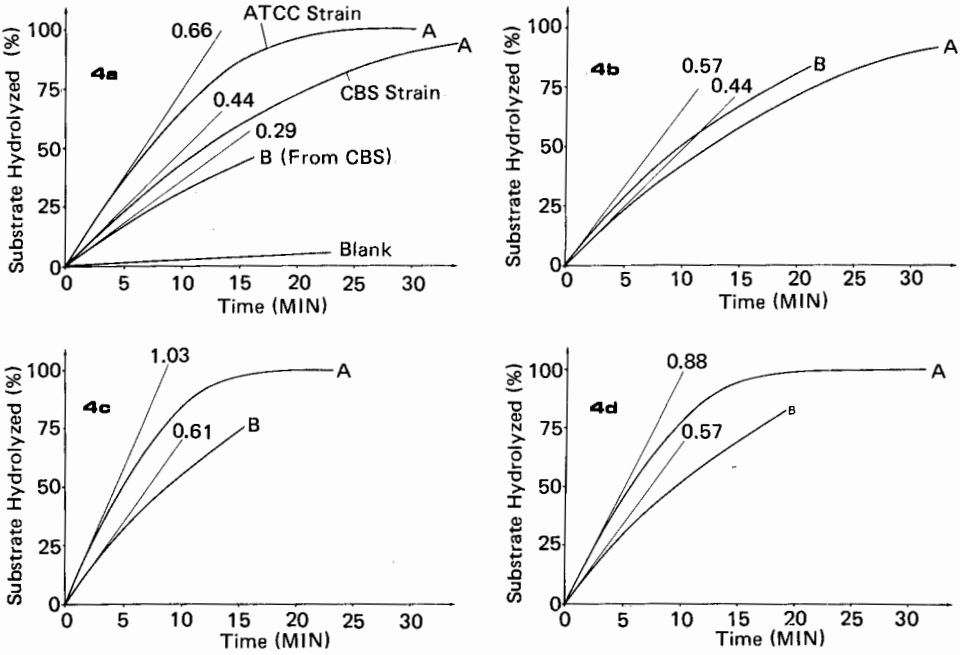

Fig. 4. Progress curves of the hydrolysis of acetyl-L-phenylalanine methylester catalyzed by a proteolytic enzyme present in the mycelial extracts (curve A) and in the culture media (curve B) for the various species of *Saprolegnia:* (a) *S. parasitica* CBS and ATCC strain; (b) *S. ferax;* (c) *S. delica;* (d) *S. diclina.* The values indicated beside each tangent are the initial rates, expressed in μmol min^{-1}, of the corresponding reaction.

when the amount of sodium hydroxyde used equals the amount of substrate initially present in the sample.

From the tangent to the progress curves at zero time the initial rates of the reaction can be determined. These initial rates can be taken as a measure of the enzymatic activity of the samples. For the cellular extracts this data is given in Table 2. From this table it can be seen that the variation of the specific activity from sample to sample is comprised within a factor of 2: *S. delica* and *S. diclina* have the same specific activity which is about two times larger than that of *S. parasitica* CBS and *S. ferax,* whereas that of *S. parasitica* ATCC lies inbetween.

The presence of a considerable activity in the culture medium, as can be seen from the values reported in Fig. 4, deserves further attention. In Table 3 the total activity released in the medium is compared with that of the cellular extract of the corresponding culture. The activity found in the medium is two to five times higher than that found in the mycelial extract. This seems to indicate an extracellular destination of these proteolytic enzymes suggesting that they could be related to the physiological function of degrading the host tissues.

Table 2
Chymotrypsin-like Activity of the Cellular Extracts of the Different Saprolegnia *Species.*

Species	Protein concentration (mg/ml)	Activity[a] (U)	Specific activity (mU/mg protein)
S. parasitica ATCC	15.2	0.66	14
S. parasitica CBS	14.1	0.44	10
S. ferax	15.2	0.44	10
S. delica	18.2	1.03	19
S. diclina	15.2	0.88	19

[a]1 unit of activity, U, is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol APHME/min under the conditions used in these experiments.

Table 3
Comparison of the Total Activity Found in the Mycelial Extracts and in the Culture Media.

Species	Total activity (U)	
	Mycelium	Medium
S. parasitica CBS	0.7	1.7
S. ferax	0.7	3.4
S. delica	1.7	3.7
S. diclina	1.5	3.4

Discussion

The following considerations can be derived from the results obtained by comparing the antigenic structures of *Saprolegnia:* The four species examined, *S. parasitica, S. ferax, S. delica,* and *S. diclina,* can be characterized on the basis of their individual, easily reproducible immunophoregrams. This offers the basis of a serological diagnosis of species found in the parasitic state after isolation and culture.

The immunophoregrams, analyzed by the number of common constituents and by the precipitation distribution, confirm that the antigenic structures of the four species are remarkably homogeneous. In analogy to a previous study of another group of fungi [3, 17] we observed that this lack of divergency in structure could be indicative of a parallel protein evolution of these potentially parasitic species.

In the study of mycoses the immunological approach to characterization of enzymatic activities takes on particular importance in shedding light on the host–parasite relationship. This approach enables a determination of the chemical nature of synthesized biological products and it is a criterion for estimating the importance of enzymes released by the microorganism.

The extensive work carried on in the past 10 years on proteases of microbial origin has been recently reviewed [7, 8]. Different authors have also discussed the production by pathogenic fungi of antigenic proteases with chymotrypsin-like activity in connection with the remarkable parasitic adaptability of some species [1, 9, 16].

In the case of *Saprolegnia,* the function of an enzyme like chymotrypsin in parasitism seems evident in the mechanism of tissue digestion necessary to the penetration by the hyphae. A deep penetration of natural and induced infection in fish has been reported by other authors [10, 15].

In general, the secretion of hydrolytic enzymes into host tissue is considered an important criterion (characteristic) to differentiate between biotrophic and necrotrophic pathogens [6]. Therefore, a high production capacity of exo-enzymes in mycosis would increase their power to invade tissues.

It should be mentioned that the enzyme which is secreted into the medium reacts with the antisera formed against the cytoplasmatic soluble constituents. This suggests that the enzyme originally synthesized in the cytoplasm is released in large quantities into the medium without changing its antigenic properties.

It would be premature to conclude that this chymotrypsin-like activity is linked to the phylogenetic transformation of metabolism during the adaptation to a parasitic life.

Finally, we believe that this enzymatic approach provides new elements for the understanding of the etiology of mycosis, in particular of saprolegniosis where the transformation from saprotrophy to biotrophy seems to be controlled by environmental factors.

Acknowledgment

The authors wish to express their sincere thanks to Professor Dr. K. Wuhrmann for his helpful comments and suggestions.

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