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STUDIES ON ASPERGILLUS FUMIGATUS; CASEIN PRECIPITATING AND PROTEOLYTIC EFFECTS OF MYCELIAL FILTRATE

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Schäffer (1900) and *Butkewitch* (1903) seem to have been the first to focus attention on the proteolytic activity of micro-fungi. The occurrence and properties of proteolytic enzymes from various fungus species were subsequently studied by several authors. Literature in this field was reviewed by e.g. *Ito* (1950), *Gorbach & Koch* (1955), *Koch & Dedic* (1957), *Hagihara* (1960), *Davies* (1963) and *Roper & Fennell* (1965).

In connection with investigation of the proteolytic activity of several fungus species, a gelatin hydrolyzing effect of Aspergillus fumigatus (AF) was reported to be exerted by living organisms in pure culture (*Jensen* 1931), extracted mycelial material (*Maxwell* 1950, *Dingle & Solomons* 1952), and by fluid culture medium in which AF was cultured (*Dion* 1950a, b, *Dingle & Solomons*). *Ayres & Tobie* (1943) demonstrated moderate casein hydrolyzing activity in extracted mycelial material from 4 AF strains, and *Amatayakul* (1955) observed low fibrinolytic activity in 1 strain.

Proteolytic activity measured by breakdown of gelatin and casein was demonstrated by *Jönsson & Martin* (1964) in culture medium in which AF had been cultured. Three activity optima were observed at pH values around 3, 6.5 and 10. A subsequent study indicated that the activity in neutral and alkaline environment was caused by the same enzyme (*Martin & Jönsson* 1965).

By means of electrophoretic separation combined with re-actions for enzyme characterization, *Tran Van Ky et al.* (1966) demonstrated proteolytic activity in mycelial extracts of 21-day AF cultures. A casein precipitating enzyme (CP enzyme) was

demonstrated by *Sandvik* (1967) in the fluid phase of frozen and thawed skimmilk agar cultures from e.g. AF.

In addition to haemolysin and toxin (*Rutqvist* 1965, 1968, *Rutqvist & Persson* 1966), mycelial filtrates of AF have proved to contain a proteolytic enzyme. An account is given in the following of a study of this enzyme with respect to (1) casein precipitating ability, (2) casein and gelatin hydrolyzing effect, and (3) relation to toxin and haemolysin.

MATERIAL AND METHODS

Strains. The AF strains used derived from a collection previously described (*Rutqvist* 1965).

Media. The strains were maintained at room temperature on agar slants of Sabouraud's agar, and were recultured once a month. Cultures for production of mycelial filtrates were made in fluid medium, consisting of proteose-peptone Difco (1.5 %), yeast extract Difco (0.5 %), dextrose (3 %) and NaCl (0.5 %) in distilled water, pH 7.2.

Filtrate preparation. Strain cultures grown for 7 days at 20°C on Sabouraud's agar in agar slants were suspended with 10 ml of proteose-peptone broth per tube, and 1 ml was transferred to 300 ml of fluid medium in Roux flasks. The mycelial mats, which after 3 days' incubation at 37°C had grown on the surface of the medium, were washed on harvesting with sterile, distilled water, dried between filter paper and kept at —20°C for 2—4 days.

To 100 g of frozen mycelial material was added 75 ml of sterile distilled water, and the mixture was homogenized in a mixer at 11,000 r.p.m. The homogenate was stored at 4°C for 5 days and centrifuged at 2,500 × g for 20 min., after which the supernatant was filtered through a Seitz EK filter. The filtrate was frozen, and stored at —20°C.

The ethanol and acetone precipitates were obtained by addition of cold ethanol and acetone, respectively, to the crude filtrate with a pH of 7.0. When the mixture had been kept for 17 hrs. at 4°C, the precipitate formed was separated by centrifugation (2,500 × g for 20 min.), and dried in an exsiccator for 24 hrs. In ammonium sulphate precipitation, the ammonium sulphate was added in substance to the crude filtrate (pH 7.0). The precipitate formed after 17 hrs.' storage at 4°C was separated off by centrifugation. The precipitate was dissolved in a small amount of sterile distilled water, dialyzed and freeze-dried. All dried preparations were kept at 4°C, and on dissolving in distilled water, butanol to 1 % (v/v) was added as preservative. Butanol (1 %) was also added to thawed crude filtrates and dialysates of crude filtrates intended for activity determinations, immediately after thawing and dialysis, respectively. It is evident from Fig. 7 that butanol had no inhibitory effect on the activities determined.

Toxic activity. The toxic activity of the various filtrate preparations was determined as the LD_{50} titre in mice (denoted here as

MLD$_{50}$) by Kärber's formula. Groups of 10 mice belonging to the NMRI strain (Naval Medical Research Institute, Bethesda, Md., USA), weighing 16—18 g, were inoculated intraperitoneally with each of a series of 10-fold dilutions of the filtrate. The dilution fluid was 0.9 % NaCl solution. The volume of the inoculum was 0.5 ml per mouse. The mice were kept under observation for 10 days.

Haemolytic activity. Sheep erythrocytes in Alsever's fluid, kept at 4°C for maximally 4 days, were washed with 1/10 M veronal buffer, pH 7, and diluted in the buffer to a 2 % suspension. Of this suspension, 0.5 ml was added to 0.5 ml of the strain filtrate in 2-fold serial dilutions with 0.9 % NaCl. The haemolytic activity was read after incubation for 30 min. at 37°C and 90 min. at 20°C. The smallest quantity of filtrate producing complete haemolysis was taken as the titre of haemolytic activity, and expressed as 1 haemolytic unit (HU). As in the toxicity determinations, the quantity of dry substance in the various filtrate preparations investigated was 5—50 mg/ml.

Casein precipitating activity. With certain modifications, the casein precipitating activity (CP activity) of the filtrate preparations in fluid and solid casein substrates was determined according to *Sandvik* (1962).

The casein stock solution was prepared by dissolving 4 g of sodium caseinate (Hammarsten grade, Hopkins & Williams Ltd., Chadwell Heath, England) to 100 ml with distilled water, the pH being adjusted to 7.5 with 2 N-NaOH.

To determine the precipitation in casein solution, the stock solution was diluted with distilled water to a casein concentration of 0.2 %, and merthiolate to 0.01 % (w/v) was added. A solution not used immediately was kept at —20°C. Before each determination, the pH of the solution was adjusted to the desired value with 0.1 N-NaOH or 0.1 N-HCl, and 5-ml volumes were transferred to test tubes. After 15 min. equilibration in a thermostat at 37°C, 0.2 ml of mycelial filtrate dilution was added, after which the samples were incubated in a thermostat at 37°C for 2 hrs. The turbidity arising in the sample was recorded in a Beckman Model B spectrophotometer at 600 mμ, after correction against a blank, and given as the percentage change in turbidity in relation to the blank. This differed from the sample only in that the filtrate solution added had been inactivated by boiling for 15 min. By diluting the mycelial filtrate with distilled water, the strength of the CP enzyme was adjusted to 60—140 diffusion units (see the following) per ml reaction mixture in the sample.

In the cases in which the influence of various salts on the casein precipitation was studied, 4-ml volumes of casein substrate and 1 ml of distilled water were used, the content of the various salts being adjusted to the desired concentration.

Quantitative determination of the CP enzyme in filtrate preparations was performed in casein agar, the casein being taken from the aforementioned casein stock solution. The final concentrations of the substances contained in the casein agar were: 0.25 % casein, 1.05 % Bacto agar Difco, 0.004 M-MgCl$_2$ added as 8 % (w/v) solution,

and 0.01 % (w/v) merthiolate. After mixing, the pH was adjusted to 6.2, and the substrate was poured in 2 mm thick layers into Petri dishes 185 mm in diameter. After the agar had set, holes 6 mm in diameter were punched in it. These were filled with 0.025 ml of filtrate, diluted 2-fold with distilled water, and the agar plates were incubated in a moist chamber at 37°C — unless otherwise stated — for 19 hrs. The diameter of the precipitation zones appearing around the holes was measured by means of a loupe (2.5 × magnification) and millimetre paper.

Sandvik's (1962) definition of the diffusion unit (DU) and titre of the CP enzyme (CP titre) were used. Thus, the CP titre was calculated by plotting the diameters of the precipitation zones of the 4—5 greatest filtrate dilutions on the ordinate as a function of the degree of dilution, after which the resulting curve was extrapolated to the abscissa. The point of intersection of the curve and the abscissa gives the filtrate dilution of which 0.025 ml contains 1 DU.

A logarithmic standard curve was obtained by plotting on the ordinate the diameters of the precipitation zones against the logarithms of the number of DU in 0.025 ml of the filtrate dilutions used. Such a standard curve was used only for calculating the residual activity of the CP enzyme after heat inactivation.

All quantitative determinations of the CP enzyme in mycelial filtrates were based on the mean of 2—3 parallel measurements per filtrate.

Casein hydrolyzing activity. Spectrophotometric determination of the casein hydrolyzing activity of the mycelial filtrate was performed by the method of *Kunitz* (1947), as described by *Narayanan & Shanmugasundaram* (1966). The desired quantity of nitrogen per ml was obtained by diluting the dialyzed mycelial filtrate with distilled water. One ml of filtrate dilution was added to 4 ml of 0.15 M acetate buffer, pH 5.8, and 1 ml of 1 % (w/v) casein (Hammarsten grade) in 0.01 N-NaOH solution, so that the reaction mixture had a pH of 6.2. The sample was incubated in a water bath at 45°C for 60 min., after which the reaction was interrupted by addition of 1 ml of 0.6 M trichloroacetic acid (TCA). After 30 min. the precipitate formed was filtered off. Blanks, in which TCA was added to the filtrate-buffer solution before the casein solution, which was also incubated for 60 min. at 45°C, were prepared parallel with each sample. To 2 ml of TCA filtrate were added 4 ml of 0.5 N-NaOH solution and 1 ml of Folin-Ciocalteu phenol reagent (Merck), diluted 1:3 with distilled water. After exactly 20 min. the intensity of blue colour formed by the breakdown products of casein in the sample was read against the relevant blank in a Beckman Model B spectrophotometer at 640 mμ. The increased optical density in the sample in relation to the blank was taken as a measure of the casein hydrolyzing ability of the enzyme, and was denoted as the caseinase activity.

Gelatin hydrolyzing activity. The ability of the mycelial filtrate to release amino nitrogen by gelatin hydrolysis was determined at different pH values by means of *Sörensen's* (1908) formol titration,

modified according to *Dworschack et al.* (1952). To 10 ml of 10 % (w/v) gelatin solution (Gelatina, Ph. Nord. AKL 653599 P 50, Apote-karnas Droghandel AB, Sweden) with 1 % butanol (v/v) were added 2.5 ml of crude filtrate and 2.5 ml of sterile distilled water. The pH was adjusted to the desired value with 2 N-HCl or 2 N-NaOH, after which the sample was incubated, in a closed vessel, in a water bath at 37°C for 4 hrs. Samples with a pH < 8.5 were adjusted after incubation to 8.5 with 2 N-NaOH, whereas samples with a pH > 8.5 were left at their original pH level. Five ml of neutralized formalin (38 % formal-dehyde) were added to each sample, which was then titrated back to pH 8.5 or to its original pH with 0.07 N-NaOH. One ml of titration fluid was considered to represent 1 mg of released amino nitrogen (*Dworschack et al.*). The difference between the quantity of titration fluid in the sample and that in a blank corresponding to each sample, in which the filtrate was inactivated by boiling for 15 min. gives the amount of amino nitrogen released by 2.5 ml of filtrate. The gelatin hydrolyzing ability was denoted as the gelatinase activity.

Nitrogen content. The nitrogen content of different filtrate pre-parations was determined by Kjeldahl's micro method in solutions with 5—50 mg of dry substance per ml, and related to the dry sub-stance.

pH determination. All pH determinations were made with a glass electrode, using a type 28 pH meter (Radiometer, Copenhagen, Denmark).

Weight determination. The weight of the dry substance in the different filtrate preparations was determined by using a type H 6 analytical scales (E. Mettler, Zürich, Switzerland).

Dialysis. All dialyses were performed against running tap water for 17 hrs., followed by distilled water for 2 hrs.

Presentation of results. Each experiment was made with filtrate preparations from 2 to 14 AF strains, the results obtained being essentially similar. Consequently, representative experiments are presented in the following.

EXPERIMENTAL AND RESULTS

1. *Casein precipitation*

Influence of pH. It is evident from Fig. 1 that the CP enzyme in crude filtrates and dialyzed filtrates gave maximal turbidity in casein solutions at pH 6.2—6.4.

Influence of salts. Low concentrations of phosphate or acetate buffer (0.0025 M), as well as of NaCl (0.0005 M), in the casein solution showed a distinct tendency to raise the casein preci-pitating ability of the CP enzyme in the mycelial filtrate. Rising concentrations of both buffers, as well as of NaCl, produced a marked decrease in the enzymatic turbidity increase, as seen in Figs. 2 and 3.
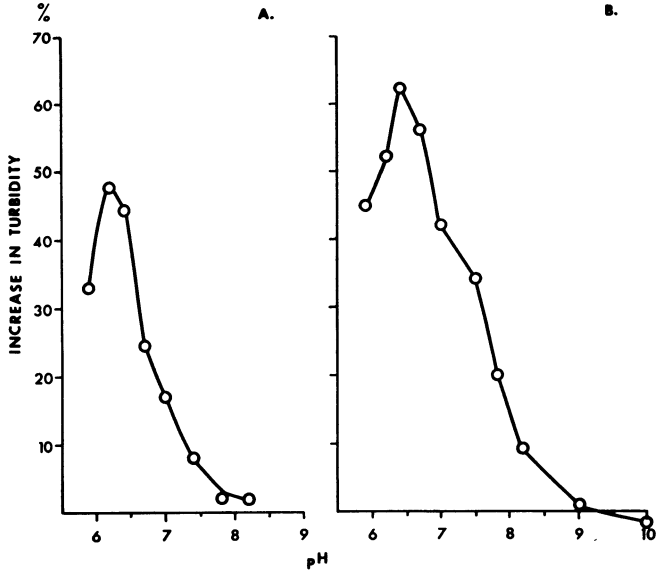
Figure 1. Influence of pH on turbidity of casein solution caused by CP enzyme in A) crude filtrate, B) dialyzed filtrate. Incubation 2 hrs. at 37°C. Aspergillus fumigatus strain 938.
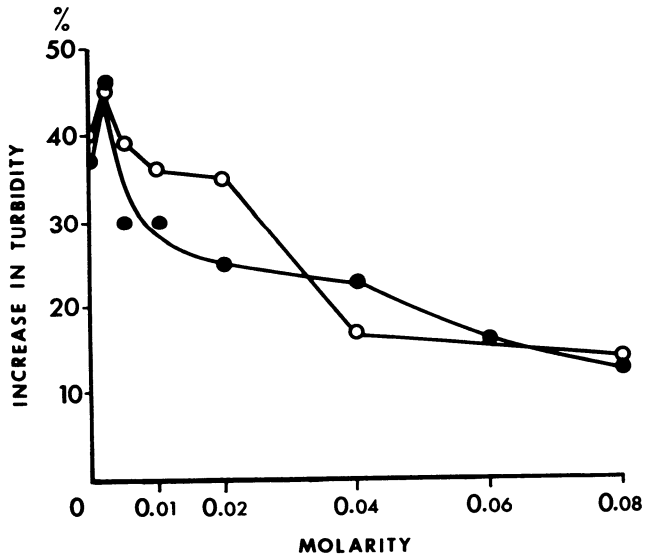
Figure 2. Influence of acetate and phosphate buffers on turbidity of casein solution at pH 6.2 caused by CP enzyme in crude filtrate. Incubation 2 hrs. at 37°C. Aspergillus fumigatus strain 6869.

o ———— o phosphate buffer
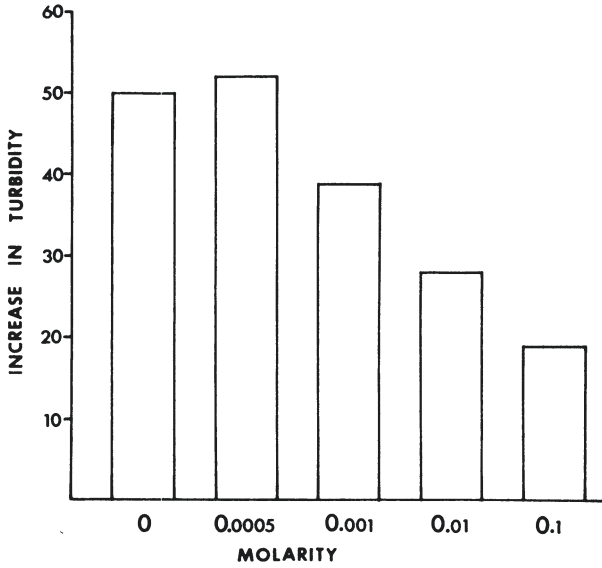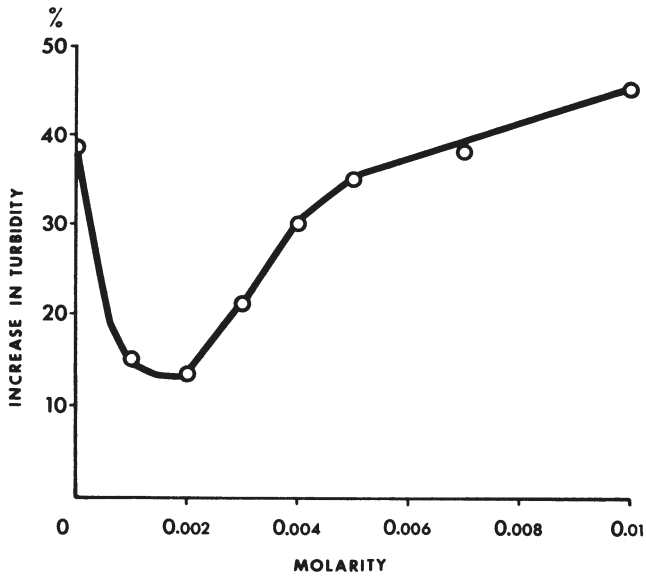● ———— ● acetate buffer

Figure 3. Influence of NaCl on turbidity of casein solution at pH 6.2 caused by CP enzyme in crude filtrate. Incubation 2 hrs. at 37°C. Aspergillus fumigatus strain 6869.

Figure 4. Influence of $MgCl_2$ on turbidity of casein solution at pH 6.2 caused by CP enzyme in crude filtrate. Incubation 2 hrs. at 37°C. Aspergillus fumigatus strain 6869.

As can be inferred from Fig. 4, the turbidity produced by the CP enzyme in the mycelial filtrate decreased in casein solutions containing 0.001—0.002 $M-MgCl_2$, after which rising concentration was associated with increasing turbidity. It could be noted that a 0.004 M concentration of $MgCl_2$ in the casein agar produced more distinct precipitation zones caused by the enzyme, concurrently with the lytic effect of the CP enzyme on the inner edge of the precipitation zones becoming more conspicuous. Concentrations of $MgCl_2$ exceeding 0.004 M in the casein agar resulted in increasing opacity of the agar, which made it more difficult to read the precipitation zones.

Influence of incubation temperature. The influence of the incubation temperature on the size of the precipitation zones in casein agar produced by the CP enzyme in mycelial filtrate is evident from Fig. 5. The largest precipitation zones were obtained on incubation at 45°C, whereas incubation at 50°C resulted in
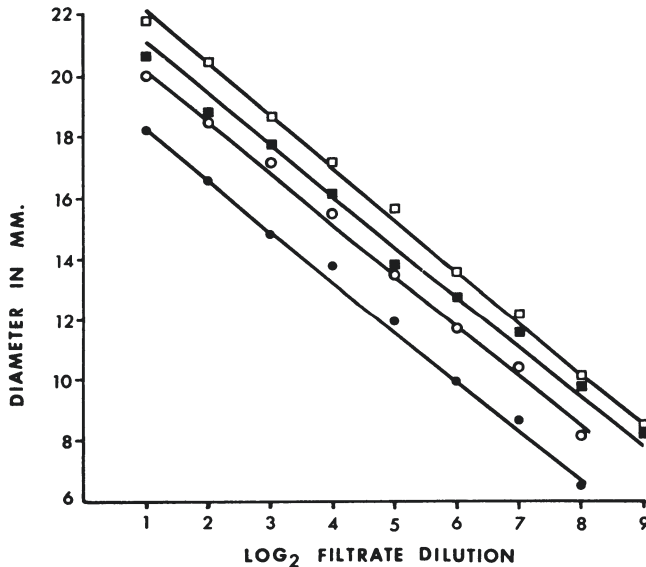
Figure 5. Diameter of precipitation zones in casein agar at pH 6.2 caused by CP enzyme in crude filtrate at different incubating temperatures. Incubation time 19 hrs. Aspergillus fumigatus strain 6869.

●———● 30°C
○———○ 37°C
□———□ 45°C
■———■ 50°C

smaller zones, which were nevertheless larger than after in-
cubation at 37°C.

Influence of heat. Crude filtrates with a pH of 6.7 were heated
in 0.5-ml volumes in thin-walled, stoppered glass tubes for 15
min. in a water bath at various temperatures. After heating, the
tubes were cooled under running water, and kept at 4°C until the
activity was determined, which took place within 2 hrs. of heat-
ing. The residual activity of the CP enzyme in the heated filtrate
was determined by comparing the diameter of the precipitation
zones in casein agar with a logarithmic standard curve, plotted
for unheated filtrate preparations. The inactivation curve is seen
in Fig. 6. Complete inactivation of the CP enzyme in crude fil-
trate was obtained after heating at 80°C.

When crude filtrate, pH 7, was incubated in a thermostat at
37°C for 14 days, it was observed that the CP enzyme had the
greatest stability, and the haemolysin the lowest (Fig. 7 A). After
the initial reduction of activity, the CP enzyme showed a ten-
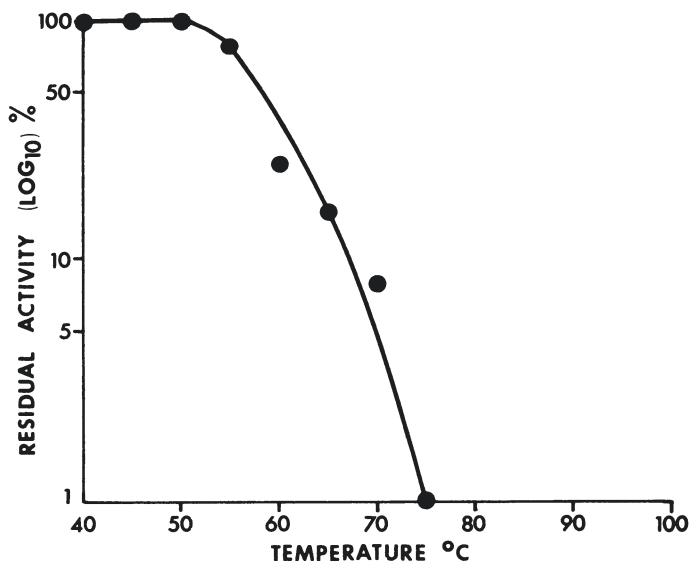dency, after incubation for 3—5 days, to be reactivated or, at
any rate, stabilized.

Figure 6. Inactivation at pH 6.7 of CP enzyme in crude filtrate at
different temperatures. Aspergillus fumigatus strain 6869.

As is apparent from Fig. 7 A and B, no definite effect of butanol on the stability of the haemolytic, toxic or casein precipitating activity could be observed.

2. *Casein and gelatin hydrolysis*

To determine whether casein precipitating filtrate also hydrolyzed protein substrate, the caseinase activity was investigated in 14 strain filtrates and the gelatinase activity in 2. In every case, caseinase and gelatinase activity, respectively, was demonstrated. As seen in Fig. 8, the caseinase activity in dialyzed filtrate, pH 6.2, increased with rising nitrogen concentration in the filtrate.

The optimal gelatinase activity in crude filtrate was obtained when the filtrate affected the gelatin at pH 6—8 (Fig. 9).

3. *Relation to haemolysin and toxin*

On fractionation of mycelial filtrate with acetone, ethanol or ammonium sulphate, it was found that the highest CP activity per mg nitrogen was obtained in the ethanol precipitate when 1 part filtrate was precipitated with 3 parts ethanol (Table 1). No haemolytic and only inappreciable toxic activity could be demon-
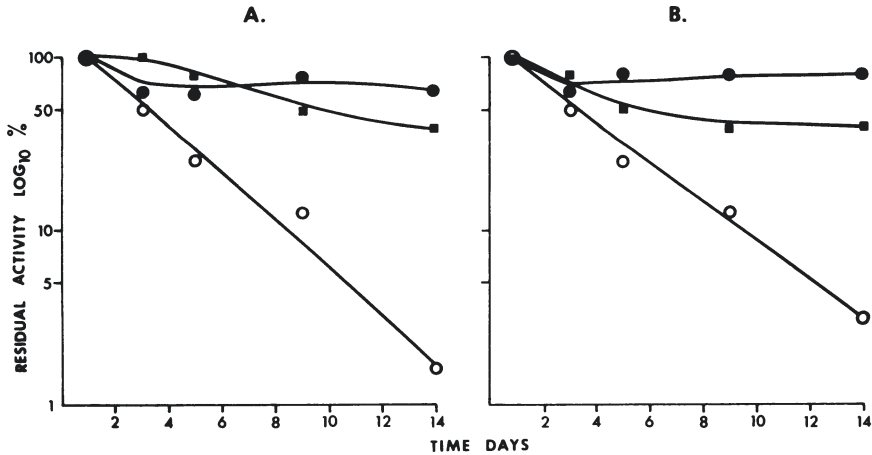

F i g u r e 7. Inactivation at 37°C and pH 7.0 of toxin (MLD_{50}), haemolysin (HU) and CP enzyme (DU) in crude filtrate. A) without and B) with 1 % butanol. Aspergillus fumigatus strain 938.
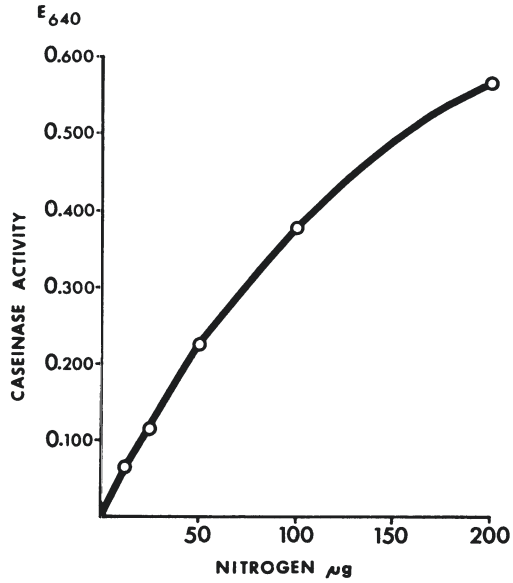
■———■ MLD_{50}
o———o HU
●———● DU

Figure 8. Casein hydrolyzing effect at pH 6.2 of dialyzed filtrate in relation to nitrogen content of the filtrate. Digestion: 1 hr. at 45°C. Aspergillus fumigatus strain 6869.
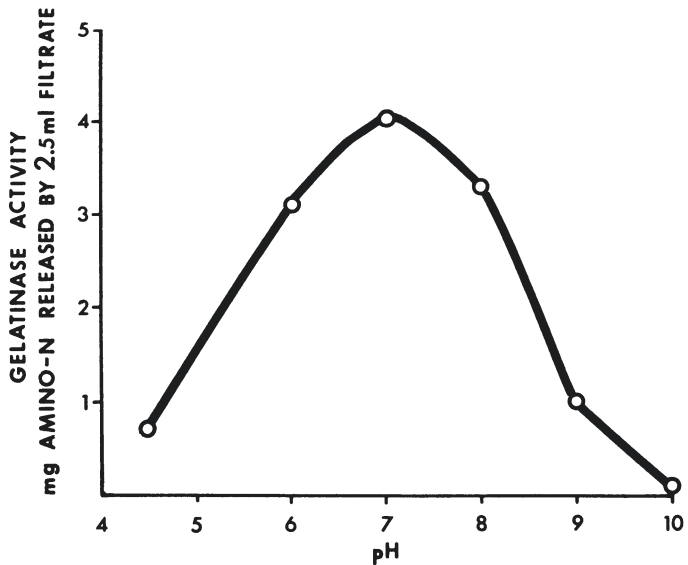

Figure 9. Gelatin hydrolyzing effect of crude filtrate at different pH values. Digestion: 4 hrs. at 37°C. Aspergillus fumigatus strain 6869.

T a b l e 1. Activity at pH 7.0 of CP enzyme (DU), toxin (MLD_{50}) and haemolysin (HU) in various filtrate preparations. Aspergillus fumigatus strain 938.

Preparation	Dry substance mg/100 ml	Nitrogen in dry substance %	DU/mg nitrogen	MLD_{50}/mg nitrogen	HU/mg nitrogen	Recovery %			Relative activity		
						DU	MLD_{50}	HU	DU	MLD_{50}	HU
Crude filtrate	4972	8.3	4.3×10^3	3.8×10^1	1.2×10^2				1	1	1
Dialyzed filtrate	594	6.1	2.9×10^4	8.2×10^2	1.7×10^3	60	190	120	6.6	21.6	13.8
Acetone precipitation											
Filtrate: acetone 1:3	2378	7.1	1.1×10^4	2.3×10^1	2.9×10^2	100	30	100	2.5	0.6	2.4
,, ,, 1:6	2503	6.6	1.7×10^4	3.0×10^1	1.6×10^2	160	30	50	4.0	0.8	1.3
,, ,, 1:8	3371	6.7	1.1×10^4	3.0×10^1	1.5×10^2	130	40	70	2.4	0.8	1.2
Ethanol precipitation											
Filtrate: ethanol 1:3	549	5.9	4.8×10^4	3.0×10^0	$<1.4 \times 10^0$	90	0.6	<0.1	11.0	0.1	<0.02
,, ,, 1:6	983	5.9	3.7×10^4	4.0×10^0	$<1.4 \times 10^0$	120	1.5	<0.2	8.5	0.1	<0.02
,, ,, 1:8	1113	6.2	2.7×10^4	4.0×10^0	1.0×10^1	100	1.8	1	6.1	0.1	0.1
Ammonium sulphate precipitation											
Saturation 0.5	204	8.8	4.4×10^4	1.1×10^3	2.3×10^3	50	130	80	10.0	30.0	18.9
,, 0.7	222	9.0	4.1×10^4	8.8×10^2	2.3×10^3	50	110	90	9.5	23.3	18.5
,, 1.0	366	7.8	2.5×10^4	1.3×10^3	2.6×10^3	40	240	150	5.7	33.9	21.9

strated in this fraction. When the filtrate was precipitated with increasing amounts of ethanol, the CP activity per mg nitrogen fell.

On precipitation of 1 part filtrate with 8 parts ethanol, small amounts of haemolysin appeared in the precipitate. No marked difference could be demonstrated between the toxic activity of precipitates obtained by precipitating filtrate with varying amounts of ethanol.

The highest toxic and haemolytic activities per mg nitrogen were measured in the ammonium sulphate-precipitated filtrate fractions. The CP activity in these fractions coincided closely with that of the ethanol fractions. Saturation of filtrate with ammonium sulphate to 50 % or 100 % produced no definite difference in the toxic or haemolytic activity of the precipitate per mg nitrogen. The CP activity per mg nitrogen was, on the other hand, lower in the latter filtrate than in the former.

In relation to crude filtrate, the CP and haemolytic activities per mg nitrogen were somewhat increased in precipitates from acetone-treated filtrates, whereas the toxic activity tended to be lower. As can be inferred from Table 1, dialysis alone resulted in considerable purification of toxin, haemolysin and CP enzyme.

DISCUSSION

The principles of the casein precipitating reaction (CP reaction) induced by bacterial proteolytic enzymes were thoroughly investigated and discussed by *Sandvik* (1962). On the basis of this reaction, he also devised a method for quantitative determination of the CP enzyme.

To study the relations between CP enzyme, haemolysin and toxin in various preparations of AF mycelial filtrates, the CP reaction was used, in the present work, in the first place for quantitative determination of the CP enzyme. The influence of salts used in producing the filtrate preparations, or in performing the CP reaction, was also studied. This is because it could not be assumed unconditionally that these salts did, in fact, influence the CP reaction induced by CP enzyme from AF in the same way as the CP reaction caused by bacterial CP enzymes. Moreover, when studying the CP enzyme from AF, the quality of the casein was not the same as that in *Sandvik*'s (1962) investigations of bacterial CP enzymes.

In the presence of the salts investigated, the CP reaction caused by CP enzyme from AF proved to be influenced in essentially the same way as that produced by most bacterial CP enzymes. Thus, the reaction was stimulated by low concentrations of acetate buffer, phosphate buffer or sodium chloride, whereas rising concentrations resulted in its successive inhibition. The CP reaction was, on the contrary, inhibited by low concentrations of $MgCl_2$, and stimulated by high ones. Although the CP enzyme from AF gave a positive CP reaction in casein agar without the presence of $MgCl_2$, the reaction was more distinct, and the lytic effect acting on the inner edge of the precipitation zone was more conspicuous, when $MgCl_2$ was present. A concentration of $MgCl_2$ higher than 0.004 M did, however, produce increasing opacity of the agar, with a resulting difficulty in reading. In accordance with these results, the mycelial material was extracted with distilled water, instead of with physiological saline as earlier (*Rutqvist* 1965, *Rutqvist & Pehrsson* 1966). Precipitated or freeze-dried filtrate preparations were dissolved in distilled water instead of in buffer solution, and 0.004 M-$MgCl_2$ was used in the casein agar in quantitative determination of the CP enzyme.

The optimal incubation temperature for the CP reaction induced by CP enzyme from AF after 19 hrs.' incubation proved to be 45°C. However, since it was observed that the precipitation zones became diffuse at temperatures above 37°C, incubation at 37°C was preferred.

CP enzyme in crude filtrate was inactivated after heating for 15 min. at 80°C. After heating at higher temperatures, no reactivation of inactivated enzyme could be observed. On the other hand, on incubation at 37°C, it was observed that — after an initial reduction in activity — the CP enzyme showed a tendency, after 3—5 days' incubation, to be reactivated, or at any rate for the activity to be stabilized.

In the environment constituted by a crude filtrate, the causes of such a phenomenon may be complex. So-called active fragments arising by autolysis or inactivation of inhibitor substances with greater heat sensitivity than the enzyme can, for instance, either separately or together be conceivable explanations.

The ability to precipitate casein and to hydrolyze gelatin coincided in all the bacterial CP enzymes studied by *Sandvik* (1962). The author presumed that, in the individual case, gelatin

hydrolysis and CP reaction could be ascribed to an identical enzyme. In the present investigation as well, it was found that AF mycelial filtrates with a positive CP reaction also hydrolyzed casein at a pH optimal for the CP reaction, i.e., 6.2. I did not investigate whether this pH value is also an optimal pH for casein hydrolysis. The optimal pH for gelatin hydrolysis seems to lie around pH 7.

In relation to crude filtrate, the CP activity per mg nitrogen increased in precipitates from ethanol-treated filtrates, concurrently with disappearance of the haemolytic activity and a marked decrease in the toxic activity (Table 1). This shows that the CP enzyme constitutes a principle independent of haemolysin and toxin. The completely different inactivation curves for CP enzyme, haemolysin and toxin, arising when crude filtrate was stored for any lengthy time at 37°C, also indicate that the CP enzyme differs essentially from both toxin and haemolysin.

I did not investigate whether the inappreciable presence or absence of toxin and haemolysin in precipitates from ethanol-treated filtrates is due to these activities not being precipitated by ethanol, or whether they are inactivated on ethanol treatment. The presence of haemolysin in the precipitate when the filtrate was treated with large amounts of ethanol (1 part filtrate to 8 parts ethanol) seems to indicate that inactivation does not occur. In any event, it seems possible to use ethanol precipitation to distinguish, to a great extent, the CP enzyme in AF mycelial filtrates from haemolysin and toxin.

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SUMMARY

In filtrates of mycelial material from Aspergillus fumigatus, cultured in proteose-peptone-yeast extract broth for 3 days at 37°C, the presence of proteolytic enzyme could be demonstrated in addition to haemolysin and toxin. A study was made of the casein precipitating ability of the enzyme, as well as of its casein and gelatin hydrolyzing effect.

On precipitation of filtrate with ethanol, acetone or ammonium sulphate, the highest casein precipitating activity per mg N was demonstrated in the precipitate of filtrates precipitated with 3 parts ethanol. This precipitate contained no measurable haemolytic and only inappreciable toxic activities. The haemolytic and toxic activities per mg N were greatest in precipitates of ammonium sulphate-treated filtrates.

ZUSAMMENFASSUNG

Studien über Aspergillus fumigatus; Kasein-präzipitierende und proteolytischen Eigenschaften der Filtrate von Myzelienkulturen.

In Myzelienfiltraten von Aspergillus fumigatus-Kulturen konnte neben Toxin und Hämolysin auch ein proteolytisches Enzym nachgewiesen werden. Das Kasein-präzipitierende Vermögen des Enzymes sowie seine Kasein- und Gelatine-hydrolysierende Wirkung wurden untersucht.

Nach Präzipitation der Filtrate mit Äthylalkohol, Azeton oder Ammoniumsulphat erwies sich, dass die Kasein-präzipitierende Aktivität per mg N höchsten in mit Äthylalkohol hergestellten Fällungen war. Keine messbare hämolytische und nur geringe toxische Aktivität war in diesen Präzipitaten nachweisbar. Das proteolytische Enzym dürfte demnach eine von den bereits früher beschriebenen Kompo-

nenten, dem Hämolysin und dem Toxin, ein weiterer, selbständiger Bestandteil der Filtrate sein. Die hämolytische und toxische Wirkung war per mg N am stärksten in den mit Ammoniumsulphat hergestellten Fällungen.

SAMMANFATTNING

Studier av Aspergillus fumigatus; kaseinprecipiterande och proteo-
lytisk verkan hos myceliefiltrat.

I filtrat från myceliematerial från Aspergillus fumigatus, som kultiverats i proteos-pepton-jästextraktbuljong under 3 dagar vid 37°C påvisades förutom hämolysin och toxin även förekomst av proteolytiskt enzym. Enzymets kaseinprecipiterande förmåga samt dess kasein- och gelatinhydrolyserande verkan har undersökts.

Vid precipitation av filtrat med etanol, aceton eller ammoniumsulfat påvisades den högsta kaseinprecipiterande aktiviteten per mg N i precipitat från filtrat som fällts med 3 delar etanol. I detta precipitat påvisades samtidigt ingen mätbar hämolytisk och endast ringa toxisk aktivitet. De hämolytiska och toxiska aktiviteterna var per mg N störst i precipitat från ammoniumsulfatbehandlat filtrat.