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STUDIES ON ASPERGILLUS FUMIGATUS; PROPERTIES OF INTRACELLULAR PROTEINASE

By

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Mycelial filtrate from Aspergillus fumigatus (AF), following incubation at 37°C for 3 days, showed not only haemolytic and toxic but also casein precipitating and proteolytic activity (cf. *Rutqvist* 1969, with references). The present work has been devoted to an investigation of the latter 2 activities.

MATERIALS AND METHODS

Strains. In all these experiments, AF strain 6869 from a previously described collection (*Rutqvist* 1965) was used. In the serological investigations the 13 additional AF strains included in the collection were also utilized.

Media. The strains were cultivated at room temperature on Sabouraud agar slants, with transfers being made once a month. Cultures for producing mycelial filtrate were grown in a liquid medium, comprising proteose-peptone Difco (1.5 %), yeast extract Difco (0.5 %), dextrose (3 %), and NaCl (0.5 %) in distilled water pH 7.2.

Buffer solutions. The buffer solutions used in protein hydrolysis were all 0.45 M, viz. citrate buffer (pH 1.5—4.2), acetate buffer (pH 4.6), phosphate buffer (pH 5.4—8.1), and glycine buffer (pH 9—11.9). Other buffer solutions that were used will be mentioned in connection with the individual experiments. The pH values given in the figures are those of the reaction mixtures.

Filtrate preparation. Stock cultures grown for 7 days on Sabouraud agar slants were suspended in 10 ml proteose-peptone broth for each tube, 1 ml of the resultant suspension then being transferred to 300 ml liquid medium in Roux flasks. At the harvest, the mycelial mats present on the surface of the medium after 3 days' incubation at 37°C were washed with sterile distilled water, dried between filter papers, and stored at — 20°C during 2—4 days.

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

Antiserum. Immunization of rabbits against mycelial filtrate from AF strain 6869 was initiated by a series of injections of filtrate that was 0.06 M with respect to formaldehyde and had been stored in an incubator at 37°C for 5 days (*Rutqvist* 1968). The formalin-treated filtrate was mixed with the same amount of Difco Bacto adjuvant complete. The mixture was injected on 4 occasions in doses of 2, 3, 4, and 5 ml intramuscularly at weekly intervals. Two weeks after the last injection in this series another series of 0.5, 0.6, 0.7, 0.8, and 1 ml formalin-treated filtrate was injected intravenously at 3—4-day intervals. The immunization was completed by 4 intravenous injections of 0.01, 0.1, 0.2, and 0.5 ml, respectively, of untreated filtrate, corresponding to 4—200 MLD_{50} of toxin*), which were administered at 3—4-day intervals. Serum was collected 14 days after the last injection. After heating the serum at 56°C for 30 min., merthiolate to 0.01 % (w/v) was added. The serum was then stored at 4°C.

Normal serum. This was obtained from apparently healthy rabbits and stored at 4°C, following heating at 56°C for 30 min. and addition of 0.01 % (w/v) merthiolate (final concentration).

Determination of proteolytic activity. Determinations of the proteolytic activity of the mycelial filtrate were carried out using casein and haemoglobin as substrates. The hydrolytic effect of the filtrate on the 2 substrates was denominated "caseinase activity" and "haemoglobinase activity", respectively.

The determinations of caseinase activity were performed by a modification of the *Kunitz* (1947) method. Four g sodium caseinate (Hammarsten grade, Hopkins & Williams Ltd., Chadwell Heath, England) were mixed with 4 ml 1 N-NaOH, 36 g urea (*Wallenfels* 1950, *Dworschack et al.* 1952), 1 ml 1 % (w/v) merthiolate, and distilled water to make 100 ml. Haemoglobinase activity was determined in accordance with a modification to the original (1938) *Anson* method. Four g bovine haemoglobin (enzyme substrate powder, Armour Pharmaceutical Company Ltd., Eastbourne, England) were mixed with 8 ml 1 N-NaOH, 36 g urea and 1 ml 1 % (w/v) merthiolate. Distilled water was added to make 100 ml. The substrate mixtures were kept under frequent stirring at room temperature during 4—5 hrs. With all ingredients dissolved pH was adjusted to 7.5 by means of 2 N-HCl and the substrate solutions were then stored at —20°C.

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*) The filtrate preparation of AF strain 6869 used in these experiments had titres for toxic activity measured as LD_{50} for mice (MLD_{50}) and for haemolytic activity (HU) of $10^{-2.6}$ ml and 2^{-10} ml, respectively. Determinations of MLD_{50} und HU were made according to earlier descriptions (*Rutqvist* 1968).

The determination of protein hydrolyzing activity was performed in a reaction mixture made up of 2 ml 0.45 M buffer solution, 1 ml 4 % substrate solution and 0.5—1 ml dialyzed filtrate solution. Following incubation (further details are to be found under Figures) the hydrolysis was discontinued by addition of 5 ml 0.3 M trichloroacetic acid solution (TCA). The mixture was allowed to stand at room temperature for 1 hr. after which the precipitate was removed by filtration. The amount of TCA-soluble degradation products was determined by measuring the optical density of the TCA filtrate, using a Beckman, type DK-2 spectrophotometer operating at 280 mμ. The increase in optical density of the sample as compared to that of a blank for each sample was used as a measure of the enzymatic activity of the filtrate. The blank differed from the sample in that buffer solution mixed with filtrate solution was incubated separately and the substrate solution, which was incubated during the same length of time, was not incorporated until the TCA solution had been added.

Determination of the effect of normal serum and antiserum on the casein hydrolyzing activity of mycelial filtrate

To determine the inhibitory effect of serum on the casein hydrolyzing activity of mycelial filtrate samples of 5 ml dialyzed filtrate were mixed with 2, 3, 4, and 5 ml, respectively, of normal serum or antiserum, sterile distilled water then being added to make 10 ml. Five ml filtrate mixed with sterile distilled water to 10 ml was used as a reference. The filtrate-serum mixtures and the reference sample were incubated for 60 min. at 37°C and the caseinase activity of 1 ml filtrate-serum mixture and of the reference sample were then determined at pH values 2.9, 6.2 and 10. The hydrolysis was carried out at 37°C during 90 min.

Determination of the effect of normal serum and antiserum on the casein precipitating activity of mycelial filtrate

The effect of various serum fractions on the casein precipitating activity of mycelial filtrate was investigated in compliance with the method of *Sandvik* (1962, 1967). Following serum electrophoresis the strips of wet paper were applied to the surface of a 2 mm layer of casein agar in plexiglas dishes measuring $12 \times 22 \times 0.5$ cm. After incubation at 37°C for 3 hrs. the strips were removed and replaced by 0.5×18 cm strips of filter paper that were moistened with mycelial filtrate solution. Following incubation at 37°C during 4—12 hrs. these paper strips were also removed and the precipitation was determined.

The casein agar was prepared according to a method previously described by *Rutqvist* (1969), involving dissolution of 4 g sodium caseinate (Hammarsten grade) in 100 ml distilled water while adjusting pH to 7.5. The definite composition of the casein agar was as follows: 0.25 % casein, 1.5 % Bacto-Agar Difco, 0.004 M-$MgCl_2$, added as an 8 % (w/v) solution, and 0.01 % (w/v) merthiolate; pH 6.2.

Paper electrophoresis of serum. A number of paper strips (Schleicher & Schüll No. 2043 B) 4 cm wide were wetted with 10 µl serum each along a 3 cm line perpendicular to the length of the strip and then fractionated electrophoretically in an electrophoresis apparatus (DeLuxe Electrophoresis Chamber, Gelman Instrument Company, Ann Arbor, Mich., USA), using an 0.05 M phosphate buffer of pH 6.4. Electrophoresis was continued for 17 hrs. at a current of 0.25 mamp./cm and a potential gradient of 5 v/cm. Each serum fractionation was duplicated, 1 paper strip being transferred to casein agar and the other being dried at 100°C for 30 min. and stained. The staining solution was prepared by dissolving 7 g Amidoschwarz 10 B (Merck) in 1 l af a mixture of 45 % distilled water, 45 % methanol and 10 % acetic acid. The staining time was 10 min. Decolorization was then effected in a series of 4 baths containing the water-methanol-acetic-acid mixture. The entire decolorization procedure was completed in 2 hrs.

The relative amounts of the various serum fractions were determined photometrically by a scanner (Evans Electroselenium Ltd., Harlow, Essex, England) after rendering the paper strips transparent by means of liquid paraffin. The absolute amount of γ-globulin in normal serum and antiserum was calculated from the relative amount and the protein content of each individual serum.

Dialysis. The mycelial filtrate was dialyzed against ordinary running tap water for 17 hrs. and subsequently against sterile distilled water for 2 hrs. The dialyzed filtrate was mixed with 1 % butanol (v/v, final concentration).

pH determination. All pH determinations were performed by means of a glass electrode, using a pH meter of the type 28 made by Radiometer, Copenhagen, Denmark.

Nitrogen determination. Determinations of the nitrogen content in filtrates and sera were carried out according to the micro-Kjeldahl technique. The factor, 6.25 was used in converting nitrogen content into protein content.

EXPERIMENTS AND RESULTS

Absorption spectrum. Fig. 1 shows the u.v. absorption spectrum of dialyzed filtrate that had been diluted with 0.15 M phosphate buffer (pH 6) to a final nitrogen concentration of 0.004 %. Maximum absorption occurred at 275 mµ.

Effect of pH on proteinase activity. Dialyzed filtrate when acting upon casein that had been buffered at various pH values resulted in 3 clearly different activity optima at pH 2.9, 6.2 and 10, with maximum activity at pH 6.2. With haemoglobin as substrate, maximum activity was recorded at pH 4.6. Moderate activity optima were recorded at pH 3.6 and 10. The activity curves are reproduced in Fig. 2.

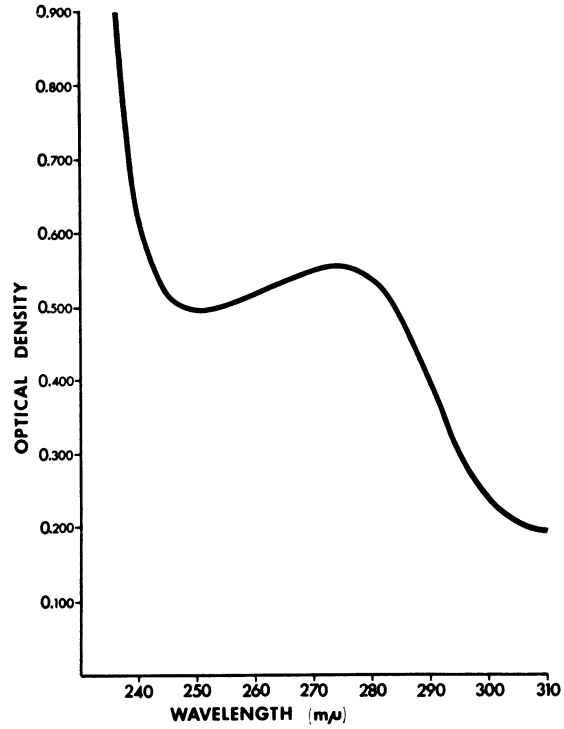

Figure 1. Ultraviolet absorption spectrum of dialyzed mycelial filtrate diluted with 0.15 M phosphate buffer, pH 6, to a nitrogen content of 0.004 %. Aspergillus fumigatus strain 6869.

Effect of pH on the inactivation of proteinase activity. As can be seen from Fig. 3, maximum caseinase activity at pH 2.9, following storage at 4°C, was found in filtrates stored at pH 6—7, whereas at pH 6.2 and pH 10 maximum activity was recorded in filtrates that had been stored at pH 4. In the latter case the activity at pH 2.9 and 6.2, proved to possess great stability against i.e. close to the original pH value (7.3) of the dialyzed filtrate. The caseinase activity at pH 10, in contrast to the corresponding activity at pH 2.9 and 6.2, proved to possess great stability against high pH values. There was no noticeable decrease in caseinase activity at pH 10 in filtrates that had been stored at pH 5—11. The activity at pH 2.9 and pH 6.2 dropped in filtrates stored at pH >7.5. The caseinase activity at pH 2.9 and pH 6.2 showed a greater stability against low pH values than the corresponding activity at pH 10.

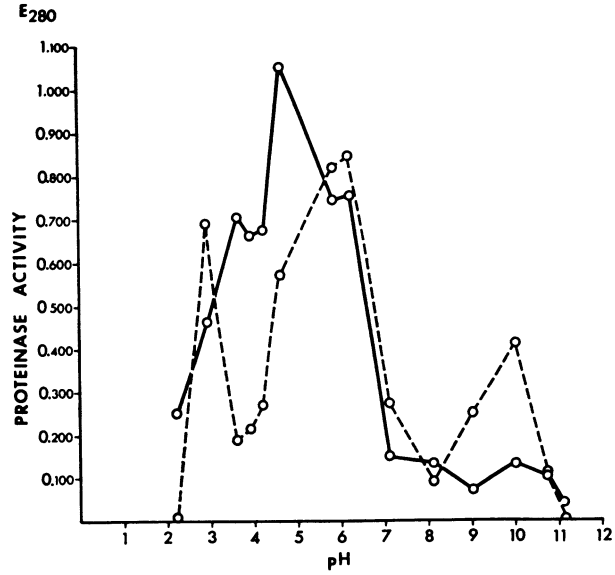

Figure 2. Effect of pH on the proteinase activity of mycelial filtrate from Aspergillus fumigatus strain 6869. Basic procedure of protein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % substrate solution and 2 ml of buffer was incubated at 37°C for 90 min.

o————o haemoglobinase activity.
o- - - - - - -o caseinase activity.

Effect of incubation temperature on proteinase activity. The hydrolysis of casein at pH 2.9 showed a maximum after incubation at 40°C (Fig. 4A), at pH 6.2 after incubation at 45°C (Fig. 4B) and at pH 10 after incubation at 35°C; in the latter case, however, maximum hydrolysis after 30 min. incubation occurred after incubation at 40°C (Fig. 4C). Casein hydrolysis at pH 2.9 and pH 10 was low in both cases at incubation temperatures above 40°C, and no definite caseinase activity was recorded at 50°C. As can be seen in Fig. 4B caseinase activity at pH 6.2 occurred over a broad temperature range and could be recorded even after incubation at 65—70°C.

Caseinase and haemoglobinase activity in relation to nitrogen content. When dialyzed mycelial filtrates containing 50—250 μg N/ml were allowed to act upon casein that was buffered at pH 6.2 or on haemoglobin buffered at pH 4.6 there was an increase

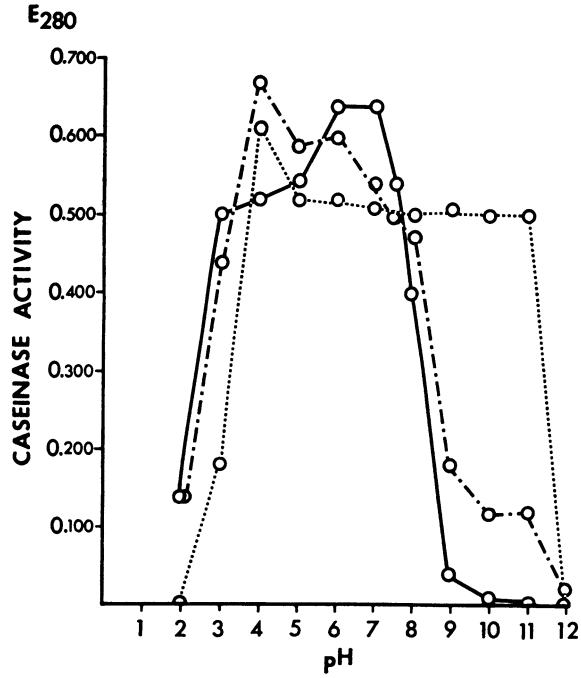

Figure 3. pH stability of the caseinase activity of mycelial filtrate from Aspergillus fumigatus strain 6869.

Dialyzed filtrate in volumes of 4 ml was adjusted to different pH values with 0.1 N—1 N-HCl and 0.1 N—1 N-NaOH, respectively. After being kept at 4°C for 90 min. the filtrate was neutralized. The volumes were corrected with distilled water and the caseinase activity at pH 2.9, pH 6.2 and pH 10, respectively, was determined. Basic procedure of casein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % casein solution and 2 ml of buffer was incubated at 37°C for 90 min.

o———o caseinase activity at pH 2.9.
o—·—·—·—o „ pH 6.2.
o o „ pH 10.

in caseinase and haemoglobinase activity, respectively, at increasing nitrogen content, as can be seen from Fig. 5. Under the prevailing experimental conditions the graph representing the correlation between caseinase activity and nitrogen content of the filtrate was rectilinear whereas no rectilinear correlation could be found for the haemoglobinase activity of filtrates containing more than 100 μg N/ml.

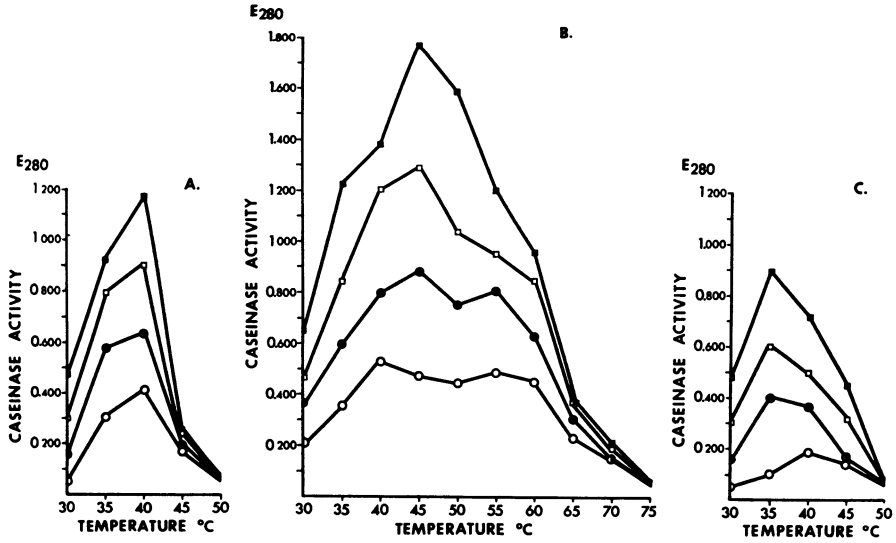

Figure 4. Optimum incubating temperature for casein hydrolysis at (A) pH 2.9, (B) pH 6.2, and (C) pH 10 caused by mycelial filtrate from Aspergillus fumigatus strain 6869. Basic procedure of casein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % casein solution and 2 ml of buffer was incubated in a water bath at different temperatures for 30, 60, 90, and 120 min.

o——o 30 min.
●——● 60 min.
□——□ 90 min.
■——■ 120 min.

Effect of heat in relation to pH on proteinase activity. Filtrates of pH 7.3, when heat treated for 2 hrs. at temperatures in the 40—70°C range, showed a caseinase activity at pH 10 that was more heat stable than the caseinase activity at pH 2.9 or pH 6.2 (cf. Fig. 6). So, at pH values 2.9 and 6.2, respectively, no caseinase activity could be detected after heat treatment at 55°C whereas the filtrate still retained 19 % residual activity at pH 10 following heat treatment at 70°C.

In filtrates that had been heat treated at pH 3, pH 8 or pH 10 the caseinase activity determined at pH 2.9 and pH 10 showed clearly divergent inactivation curves (Figs. 7A, C, D). The strongest residual caseinase activity as determined at pH 2.9 occurred in filtrates that had been heat treated at pH 3 (83 %

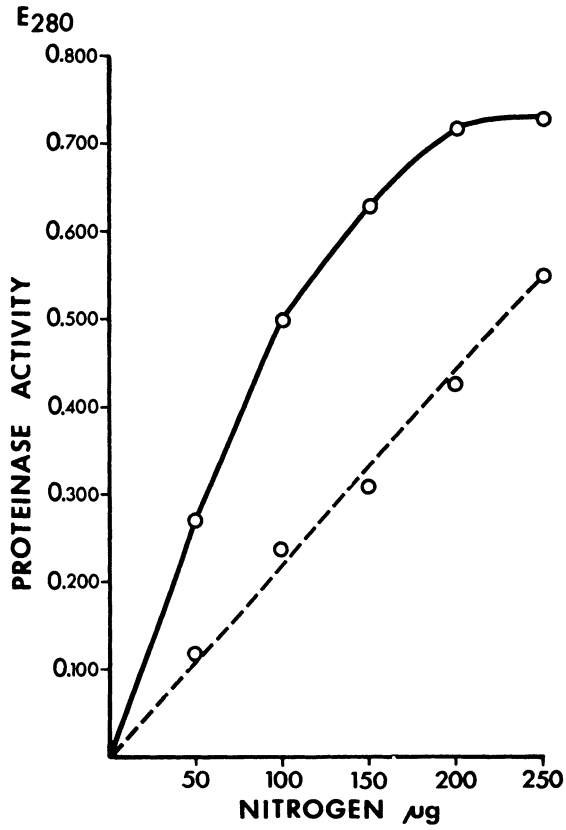

Figure 5. Proteinase activity in relation to nitrogen content. Aspergillus fumigatus strain 6869. Basic procedure of protein hydrolysis: a reaction mixture of 1 ml of filtrate dilution, 1 ml of 4 % substrate solution and 2 ml of buffer was incubated in a water bath at 45°C for 60 min.

o———o haemoglobinase activity at pH 4.6.
o- - - - - -o caseinase activity at pH 6.2.

following heat treatment at 50°C, Fig. 7A) and the weakest activity in filtrates that had been heat treated at pH 10 where no residual activity at all could be recorded after heat treatment at 40°C (Fig. 7D). On the other hand, the strongest residual caseinase activity at pH 10 was recorded in filtrates that had been heat treated at pH 10 (42 % following heat treatment at 50°C, Fig. 7D) and the weakest activity in filtrates that had been heat treated at pH 3 where only 2 % residual activity could be de-

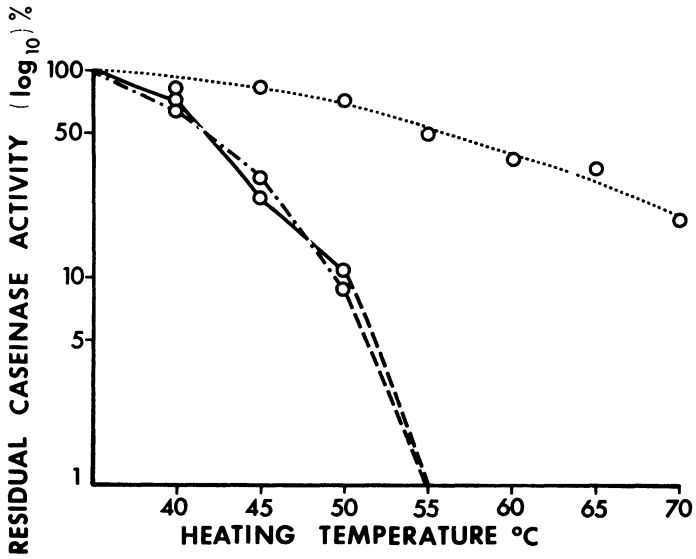

Figure 6. Thermal inactivation of caseinase activity of mycelial filtrate, pH 7,3, Aspergillus fumigatus strain 6869.

Dialyzed filtrate in volumes of 4 ml in thin-walled, stoppered glass tubes was heated in a water bath at different temperatures for 2 hrs. After heating, the tubes were immediately cooled under running cold water and the residual caseinase activity at pH 2.9, pH 6.2, and pH 10, respectively, was determined. Basic procedure of casein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % casein solution and 2 ml of buffer was incubated at 37°C for 90 min.

o———o caseinase activity at pH 2.9.
o - · — · — o „ pH 6.2.
o o „ pH 10.

tected after heat treatment at 45 °C and no activity at all after heat treatment at 50°C (Fig. 7A). The caseinase activity at pH 6.2 showed greater stability in filtrates that had been heat treated at pH 3 than did the caseinase activity at pH 10 (Fig. 7A). Greater stability of caseinase activity at pH 6.2 than that at pH 2.9 was found in filtrates that had been heat treated at pH 8 or pH 10. (Figs. 7C, D). The strongest residual caseinase activity at any of the investigated pH values occurred in filtrates that had been heat treated at pH 5 (Fig. 7B). The inactivating effect of pH per se on the caseinase activity at the pH values examined shows a basic similarity to previous investigations (Fig. 3).

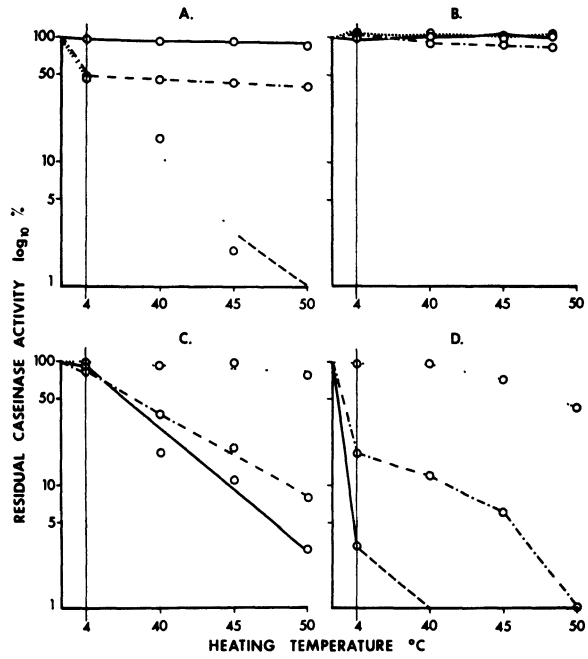

Figure 7. Thermal inactivation of caseinase activity of mycelial filtrate heated at (A) pH 3, (B) pH 5, (C) pH 8, and (D) pH 10 at different temperatures. Aspergillus fumigatus strain 6869.

The filtrate in volumes of 4 ml was adjusted to pH 3, pH 5, pH 8, and pH 10, respectively, transferred to thin-walled, stoppered glass tubes and heated in a water bath at different temperatures for 60 min. After heating, the tubes were immediately cooled under running cold water. The filtrate was neutralized and the volumes corrected with distilled water. The residual caseinase activity of the filtrate at pH 2.9, pH 6.2, and pH 10, respectively, was then determined. 0.1 N—1 N-HCl or 0.1 N—1 N-NaOH was used for pH adjustments. Basic procedure of casein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % casein solution and 2 ml of buffer was incubated at 37°C for 90 min.

o———o caseinase activity at pH 2.9.
o—·—·—o „ pH 6.2.
o o „ pH 10.

The divergent stability of the caseinase activity at pH values 2.9, 6.2 and 10 of dialyzed mycelial filtrates that had been heat treated at 45°C for 60 min. can be seen in Fig. 8.

Effect of normal serum and antiserum on caseinase activity. It is seen (Table 1) that 0.2 ml normal serum inhibited some 50 %

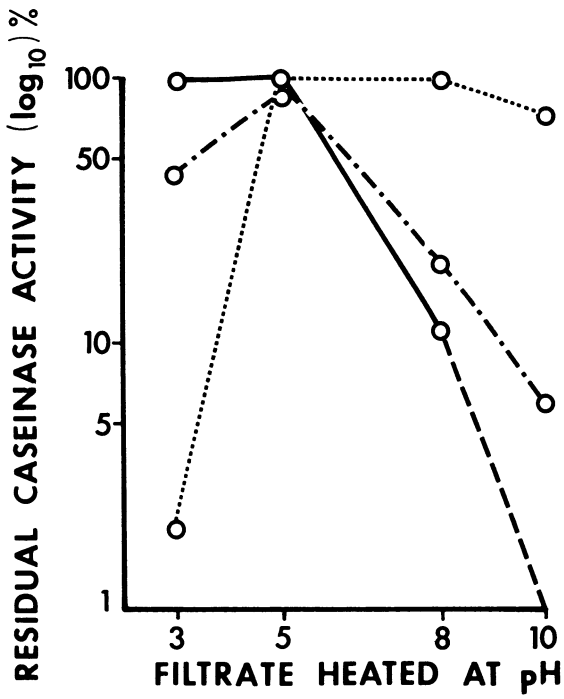

Figure 8. Comparison of thermal inactivation of caseinase activity at pH 2.9, pH 6.2, and pH 10, respectively, of mycelial filtrate heated at pH values 3, 5, 8, and 10, respectively, at 45°C for 60 min. Aspergillus fumigatus strain 6869. Basic procedure of casein hydrolysis: a reaction mixture of 0.5 ml of filtrate, 1 ml of 4 % casein solution and 2 ml of buffer was incubated at 37°C for 90 min.

o——————o caseinase activity at pH 2.9.
o—·—·—·—o „ pH 6.2.
o o „ pH 10.

of the caseinase activity of 0.5 ml filtrate at pH 2.9 and pH 6.2. Caseinase activity at pH 10 was almost completely inhibited. By increasing the amount of serum it was possible to achieve an additional, though slower, inhibition of the caseinase activity at pH values 2.9 and 6.2.

Antiserum produced no definite inhibition beyond that obtained by normal serum.

Effect of normal serum and antiserum on the casein precipitating activity. Electrophoretic separation of the normal sera and antisera used in these experiments showed that the γ-glo-

Table 1. Inhibitory effect of normal serum and antiserum from the rabbit on the casein hydrolyzing activity of mycelial filtrate. Aspergillus fumigatus strain 6869.

Amount of serum µl	E_{280} (caseinase activity)					
	Normal serum pH			Antiserum pH		
	2.9	6.2	10	2.9	6.2	10
0	0.695*)	0.775	0.490	0.695	0.775	0.490
200	0.335	0.415	0.055	0.335	0.285	0.040
300	0.265	0.360	0.035	0.310	0.255	0.045
400	0.215	0.320	0.035	0.320	0.260	0.050
500	0.195	0.275	0.000	0.300	0.245	— **)

*) All values are the means of 2 estimates.
**) Not investigated.

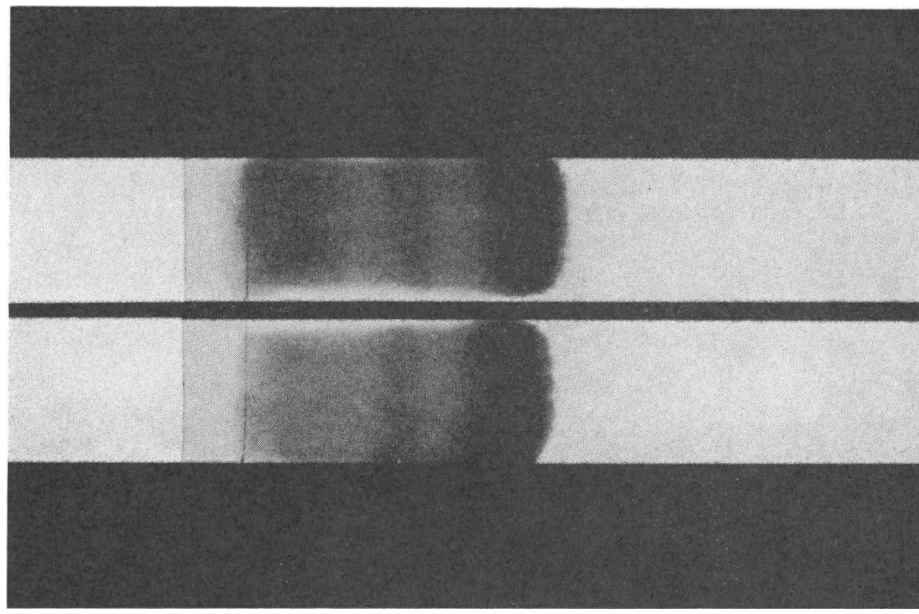

Figure 9. Electrophoretic patterns of antiserum (upper electrophorogram) and normal serum (lower electrophorogram) of rabbit. Electrophoresis was run in 0.05 M phosphate buffer for 17 hrs. with a current strength of 0.25 mamp./cm.

The antiserum was prepared against mycelial filtrate from Aspergillus fumigatus strain 6869.

bulin content of the antiserum was more than twice that of the normal serum (viz. 2.58 g/100 ml in the antiserum and 1.05 g/100 ml in the normal serum). Under the prevailing conditions of electrophoresis and using a phosphate buffer of pH 6.4, only 2 distinct globulin fractions were obtained (Fig. 9).

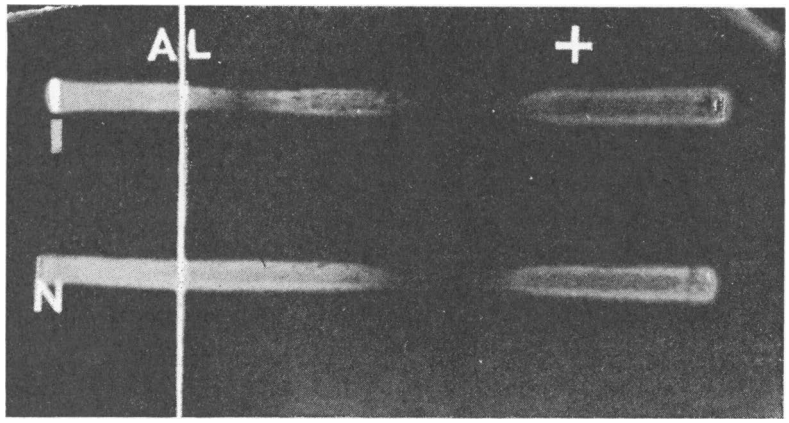

Figure 10. Electrophoretic patterns of normal serum and antiserum from rabbit after being transferred to the surface of casein agar and influenced by casein precipitating enzyme of mycelial filtrate from Aspergillus fumigatus strain 6869, which was homologous to the antiserum.
AL = application line, I = antiserum, N = normal serum, + = anode end.

The corresponding zones of inhibition of casein precipitating enzymes as seen in both the normal serum and the antiserum reflect natural inhibitors in the sera. The zone of inhibition near AL observed in the antiserum is caused by specific antienzyme.

As can be seen from Fig. 10 the casein precipitating enzymatic activity was inhibited by normal serum as well as antiserum within a range starting over the albumin fraction and extending towards the most mobile globulin fraction. With antiserum there was also a clear inhibition zone, corresponding to the position of the γ-globulin. Such inhibition of the casein precipitating ability of mycelial filtrate as caused by the γ-globulin fraction of the antiserum was also recorded when studying filtrates from each of the other 13 AF strains included in the investigations.

DISCUSSION

Since the crude mycelial filtrate from AF showed very strong absorption at 280 mμ, dialyzed filtrate was used in all digestion tests. Maximum absorption of the dialyzed filtrate occurred at 275 mμ. This is the case with many simple proteins and amino acids (*Hagihara* 1954).

It is a quite common observation in the study of proteinase from various species of fungi that the proteolytic activity of a particular species shows more than one pH optimum at the same time (cf. *Ito* 1950a, b, *Ghosh* 1952, *Crewther & Lennox* 1953a, b, *Yamamoto* 1957, *Matsushima* 1958, 1959 a, b, *Singh & Martin* 1960, *Jönsson & Martin* 1965, *Narayanan & Shanmugasundaram* 1966, and others). *Bergkvist* (1963a,b), using Aspergillus oryzae, succeeded in isolating 3 different proteolytic enzymes, showing optimum activity at pH 4.3—4.5, pH 6.3—6.8 and pH 7.6—9.5, respectively. On casein and gelatin hydrolysis, nutrient broth used for growing AF displayed 3 pH optima, viz. at pH 2.9, pH 6.6 and pH 10 (*Jönsson & Martin* 1964).

In the present investigation also, concentrating on the proteolytic activity of mycelial filtrate from AF, casein hydrolysis revealed 3 pH optima (at pH values 2.9, 6.2 and 10; Fig. 2). There is thus a clear coincidence in this respect between the proteolytic activity of mycelial filtrate and the proteolytic activity of culture media used for cultivation of AF as reported by *Jönsson & Martin* (1964) and by *Martin & Jönsson* (1965). There was a distinct difference between the culture medium and the mycelial filtrate in that the caseinolytic activity of the former constantly had its maximum at pH 10 whereas the caseinolytic activity of the latter was at its peak at pH 6.2. It would seem close at hand to assume that the enzyme produced by the mycelium is transferred to the culture medium in which the mycelium is grown, by migration or autolysis. On this assumption the employment of culture media of various compositions may have resulted in the production of slightly different proteinase varieties in the 2 cases. This would account for the discrepancy noticed here. It may also be possible that the proteolytic activity at the 2 pH values (pH 6.2 and 10 according to the present investigation, pH 6.6 and 10 according to *Martin & Jönsson* 1965) may derive from different enzymes. That being the case the discrepancy may be explained by one enzyme being released from

the mycelium and migrating into the culture medium to a greater extent than the other.

Like *Singh & Martin* (1960) in their studies of proteinase from Penicillium cyano-fulvum, *Bergkvist* (1963a) in his studies of proteinase from Aspergillus oryzae, and others, the present author observed the lacking coincidence between the pH optima for casein hydrolysis caused by mycelial filtrate from AF and the pH optima for hydrolysis of haemoglobin (Fig. 2). In a previous paper (*Rutqvist* 1969) the author demonstrated that the optimum pH value for gelatin hydrolysis caused by mycelial filtrate from AF was about 7, which does not coincide with the optimum pH value for the casein hydrolysis nor for the haemoglobin hydrolysis. This may very probably be attributed to different properties of the various substrates. It is interesting to note, however, that one of the pH optima for casein hydrolysis (pH 6.2) is identical with the pH optimum for the enzymatic precipitation of the casein complex (cf. *Rutqvist* 1969).

The caseinase activity at the pH values giving optimum casein hydrolysis (pH 2.9, pH 6.2 and pH 10) showed variations of stability after storing (at 4°C) and subsequently neutralizing mycelial filtrate that had been adjusted to various pH values (Fig. 3). Following storage, the caseinase activity at pH 6.2 and pH 10 displayed maximum stability at pH 4, a value common to both, whereas the caseinase activity at pH 2.9 showed its greatest stability at pH values 6—7. It is interesting to note that maximum stability occurred at pH values other than the optimum pH values for protein hydrolysis.

A clear divergence of stability between the caseinase activity at pH 2.9, pH 6.2 and pH 10 could also be demonstrated in filtrates that had been heated at pH 3, pH 8 and pH 10, respectively (Figs. 7A,C,D and Fig. 8). *Martin & Jönsson*, however, report that the caseinase activity at pH 6.6 and pH 10 of enzyme extracted from nutrient broth used for growing AF showed identical inactivation curves when being heated. These authors unfortunately do not state the pH value at which the heating took place. Assuming, like *Martin & Jönsson*, that the same enzyme is responsible for the casein hydrolyzing activity at pH 6.2 (pH 6.6 according to *Martin & Jönsson*) and pH 10, the discrepancy between the results obtained by the present author and those of *Martin & Jönsson* in this respect may well be explained by the latter authors carrying out the heating at a pH value that by coin-

cidence happened to cause identical inactivations of the caseinase activity at pH 6.6 and that at pH 10.

Martin & Jönsson, under the influence of the uniformity of inactivation of the caseinase activity at pH 6.6 and that at pH 10, together with the results of their chromatographic and electrophoretic investigations, put forward a hypothesis according to which these two pH optima for casein hydrolysis were caused by a single enzyme and would reflect the hydrolysis of different groups of peptide bonds which, to be attacked by the enzyme, would be dependent on different electronic charges within the substrate molecule and hence also on the pH value of the medium in which the reaction takes place.

The difference between pH inactivation and heat inactivation of the caseinase activity at pH 6.2 and pH 10 as demonstrated under various conditions in the present investigation, however, would rather speak in favour of the caseinase activity at these pH values reflecting different proteolytic principles.

Even if assuming that the proteolytic activity at pH 6.2 and pH 10 may be caused by the same enzyme it would seem possible that the proteolytic activity at these pH values may derive from different active sites of the enzyme, with different stability against heat and/or hydrogen ion concentration.

The degradation of a substrate by an enzyme is influenced by 2 temperature factors acting simultaneously, viz. an increase in the initial degradation of the substrate per unit time associated with the rise in temperature, on one hand, and a gradual destruction of the enzyme, which is also linked up with the rise in temperature, with a resultant continuous drop in the concentration of the active enzyme, on the other.

Investigations of the effect of the incubation temperature on the casein hydrolyzing activity of mycelial filtrate from AF seem to indicate the existence of a broad temperature range of the caseinase activity at pH 6.2, with an optimum at 45°C (Fig. 4B).

The optimum incubation temperature for casein hydrolysis at pH 2.9 and pH 10 was 40°C and 35°C, respectively. At these pH values the caseinase activity had narrow temperature ranges, with very little or no caseinase activity even at incubation temperatures around 45 or 50°C (Figs. 4A, C). It does not seem possible here to suggest thermal destruction of the enzyme as an explanation, knowing that (as may be seen from Figs. 7A and D) considerable caseinase activity at the relevant pH values was de-

tected in mycelial filtrates that had been heated at 50°C at pH 3 and pH 10, respectively. The decreased or lacking ability of the proteolytic enzyme of breaking down casein at pH 2.9 or pH 10 during incubation at temperatures above 40°C and 35°C, respectively, was not investigated. It is possible that under these environmental conditions the enzyme may be incapable of attaching itself to the substrate.

It has been known for a long time that normal serum from various animal species is capable of inhibiting bacterial proteinases (cf. *Sandvik* 1962, and others) and proteinases from microscopic fungi (cf. *Stefanini & Karaca* 1963, *Bergkvist* 1963c, *Sandvik* 1967, and others). It may be assumed that the concentrations of the normal serum inhibitors may vary from one individual to another even whithin the same animal species. In the present investigation it was found that the casein hydrolysis caused by mycelial filtrate from AF at pH 2.9, pH 6.2 and pH 10 was subjected to strong inhibition by normal rabbit serum. It was not possible, however, to demonstrate any definite inhibition of the casein hydrolysis, in addition to that caused by normal serum, by using rabbit antiserum in which the γ-globulin content exceeded that of normal serum by more than 240 %.

By applying the immuno-electrophoretic technique for serological differentiation of proteinases devised by *Sandvik* (1962, 1967) the present author succeeded in demonstrating, in the γ-globulin fraction of antiserum to mycelial filtrate from an AF strain, the presence of antibodies against casein precipitating enzymes in mycelial filtrates from the homologous strain as well as from 13 heterologous AF strains. This suggests immunological uniformity of the intracellular casein precipitating enzyme of the investigated AF strains and corresponds to the results published by *Sandvik* (1967), demonstrating close serological relationship between extracellular casein precipitating enzymes from 2 AF strains.

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SUMMARY

Mycelial filtrates from Aspergillus fumigatus (AF) hydrolyzed protein substrate buffered at various pH values. Using casein as substrate there were distinct activity optima at pH 2.9, pH 6.2, and pH 10, with maximum activity at pH 6.2. Using haemoglobin as substrate there were activity optima at pH 3.6, pH. 4.6, and pH 10, with the biggest activity peak at pH 4.6.

The pH stability at 4°C of the caseinase activity at pH 6.2 and pH 10 was strongest at pH 4, common to both, whereas the caseinase activity at pH 2.9 showed maximum pH stability at pH 6—7.

The casein hydrolyzing activity at pH 2.9, pH 6.2, and pH 10 showed different optimum incubation temperatures and irregular heat inactivation.

Normal rabbit serum inhibited the caseinase activity at pH 2.9 and pH 6.2 to some extent. The caseinase activity at pH 10 was almost completely inhibited. Antiserum against mycelial filtrate showed no definite inhibition beyond that exerted by normal serum.

Following electrophoresis of antiserum, the presence of specific neutralizing antibodies against the casein precipitating enzyme of mycelial filtrate from AF could be established. Investigations of 14 AF strains showed immunological uniformity with respect to the casein precipitating enzyme.

ZUSAMMENFASSUNG

Studien über Aspergillus fumigatus; Eigenschaften der intracellulären Proteinase.

Die Myzelienfiltrate von Aspergillus fumigatus (AF) hydrolysierten gepufferte Proteinlösungen bei verschiedenen pH-Werten. Mit Kasein als Nährboden traten deutliche Aktivitätsoptima bei pH 2,9, pH 6,2 und pH 10 auf, wobei die Aktivität bei pH 6,2 am stärksten war. Bei Anwendung von Hämoglobin als Nährboden traten die Aktivitäts-

optima bei pH 3,6, pH 4,6 bzw. pH 10 mit dem höchsten Aktivitätswert bei pH 4,6 auf.

Die pH-Stabilität für die Kaseinase-Aktivität bei pH 6,2 bzw. pH 10 war am stärksten bei ein und demselben pH-Wert nämlich pH 4, während die Kaseinase-Aktivität bei pH 2,9 die grösste pH-Stabilität bei pH 6—7 aufwies.

Die Kasein-hydrolysierende Aktivität bei pH 2,9, pH 6,2 bzw. pH 10 hatte verschiedene optimale Inkubationstemperaturen und verschiedenartige Wärme-Inaktivierung.

Kaninchennormalserum hemmte teilweise die Kaseinase-Aktivität bei pH 2,9 bzw. 6,2. Die Kaseinase-Aktivität bei pH 10 wurde praktisch vollständig inhibiert. Ein gegen Myzelienfiltrat hergestelltes Antiserum gab keine stärkere Hemmung als Normalserum.

Nach Elektrophorese des Antiserums konnten in der Gammaglobulinfraktion spezifische neutralisierende Antikörper gegen das Kasein-präzipitierende Enzym im AF-Myzelienfiltrat nachgewiesen werden. Die Untersuchung von 14 AF-Stämmen zeigte immunologische Einheitlichkeit in bezug auf das Kasein-präzipitierende Enzym.

SAMMANFATTNING

Studier av Aspergillus fumigatus; egenskaper hos intracellulärt proteinas.

Myceliefiltrat från Aspergillus fumigatus (AF) hydrolyserade proteinsubstrat buffrat vid olika pH-värden. Med kasein som substrat uppträdde distinkta aktivitetsoptima vid pH 2,9, pH 6,2 och pH 10, varvid aktiviteten vid pH 6,2 var störst. När hämoglobin användes som substrat uppträdde aktivitetsoptima vid pH 3,6, pH 4,6 respektive pH 10 med den största aktivitetstoppen vid pH 4,6.

pH-stabiliteten för kaseinasaktivitet vid pH 6,2 respektive pH 10 var störst vid ett och samma pH-värde nämligen pH 4, medan kaseinasaktiviteten vid pH 2,9 visade största pH-stabiliteten vid pH 6—7.

Kaseinhydrolyserande aktivitet vid respektive pH 2,9, pH 6,2 och pH 10 hade olika optimala inkuberingstemperaturer och olikformig värmeinaktivering.

Normalserum från kanin inhiberade partiellt kaseinasaktiviteten vid respektive pH 2,9 och pH 6,2. Kaseinasaktiviteten vid pH 10 inhiberades praktiskt taget fullständigt. Antiserum till myceliefiltrat gav ingen säker inhibition utöver normalserumets.

Efter elektrofores av antiserum kunde i gamma-globulinfraktionen påvisas specifika neutraliserande antikroppar mot det kaseinprecipiterande enzymet i AF-myceliefiltrat. Undersökning av 14 AF-stammar visade immunologisk uniformitet för det kaseinprecipiterande enzymet.

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