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STUDIES ON ASPERGILLUS FUMIGATUS;
HYDROLYSIS OF POLYAMINO ACIDS
BY MYCELIAL FILTRATE AND CHROMATO-
GRAPHIC FRACTIONATION OF THE
FILTRATE

By

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The properties of intracellular proteinase from *Aspergillus fumigatus* (AF) have been described in a previous paper (*Rutqvist 1969 b*).

In the present work, the ability of mycelial filtrate from AF to hydrolyze poly-L-lysine and poly-L-glutamine at various pH values has been investigated by means of a viscosimetric method that was initially worked out by Hultin (*Hultin 1946, 1948, Hultin & Lundblad 1955, Lundblad & Hultin 1966, Hultin & Wanntorp 1966*) and was later applied by Söder (1967) in his studies of poly- α -amino acid hydrolysis caused by proteinase in dental plaque material. The hydrolysis of polyglutamine at optimum pH was also investigated spectrophotometrically following gel filtration.

It has been shown earlier (*Rutqvist 1965, 1968, 1969 a*) that mycelial filtrate from AF possesses haemolytic, toxic and casein precipitating properties. It has also been shown that the proteolytic activity of dialyzed filtrate has 3 activity optima at different pH values (*Rutqvist 1969 b*). To illustrate the mutual relations of these activity optima and their relations to casein precipitating enzyme, haemolysin and toxin, mycelial filtrate has been fractionated by means of gel filtration. In addition, purified proteinase-positive fractions have been studied from an immunochemical point of view.

MATERIALS AND METHODS

Strain. The strain used in these experiments was the AF strain 6869 from the AF collection previously described (*Rutqvist* 1965).

Media. The strain has been cultivated at room temperature on Sabouraud agar slants, with transfers made once a month. A culture for producing mycelial filtrate has been 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.

Filtrate preparation. A stock culture grown for 7 days on Sabouraud agar slants was suspended in 10 ml of liquid culture medium for each test tube, 1 ml of the resultant suspension then being transferred to 300 ml liquid medium in Roux flasks. At the harvest, the mycelial growths 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 being homogenized for 20 min. in a mixer running at 11,000 r.p.m. The supernatant liquid was subsequently centrifuged at 2,500 × g and filtered through a Seitz EK filter. The filtrate (pH 6.7) was then stored at -20°C*.

Antiserum. Antiserum against mycelial filtrate from AF strain 6869 was prepared as described by *Rutqvist* (1969 a). On 4 occasions at 1-week intervals rabbits were injected intramuscularly 2, 3, 4, and 5 ml, respectively, of a mixture of equal parts formalin-treated filtrate and Difco Bacto adjuvant complete. Two weeks later, series of 0.5, 0.6, 0.7, 0.8, and 1 ml formalin-treated filtrate were injected intravenously, and 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 MLD₅₀ of toxin. The intravenous injections were made at 3-4 days' 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.

Fractionation of mycelial filtrate

The mycelial filtrate was fractionated on a chromatographic column using either of Sephadex G-75 and Sephadex G-100 (the Sephadex gel used in these experiments was manufactured by AB Pharmacia Fine Chemicals, Uppsala, Sweden). Portions of the Sephadex gel were treated according to the maker's instructions and filled into columns that were buffered with 0.2 M acetate buffer (pH 5.2), containing 1.5% (v/v) butanol. This buffer solution was also used as an elution agent. The gel filtration was carried out at 5°C.

*) The filtrate titres for toxic activity measured as LD₅₀ for mice (MLD₅₀) and haemolytic activity (HU) were 10-2.6 ml and 2-10 ml, respectively. The determinations of MLD₅₀ and HU were made according to descriptions published previously (*Rutqvist* 1968).

The Sephadex G-75 column, measuring 2.8×172 cm, was charged with 8 ml mycelial filtrate that had been concentrated from 100 ml to 20 ml by Sephadex G-25 Coarse. An addition of sucrose to a concentration of 5% was made to the concentrated filtrate in order to increase its density before it was entered into the column. The flow rate was 22–25 ml/hr., the effluent being collected in 5-ml fractions.

The Sephadex G-100 column, measuring 5.2×172 cm, was charged with 40 ml mycelial filtrate containing 5% sucrose. The flow rate dropped gradually from an initial 45 ml/hr. to a final 25 ml/hr. The effluent was collected in 10-ml fractions.

In both cases the mycelial filtrate was poured on top of the Sephadex gel, between the gel surface and the covering buffer solution, using a special syringe.

The optical density of each fraction was determined on a type DU Beckman spectrophotometer at 260 and 280 m μ and the fractions were stored at -20°C until activity determinations could be performed.

Concentration of mycelial filtrate

Twenty g Sephadex G-25 Coarse was added to 100 ml of the crude filtrate and the mixture was left at 4°C overnight. Centrifugation at $5,000 \times g$ yielded 20 ml concentrated filtrate. The titre for haemolytic activity (HU; cf. footnote on p. 256) showed an increase from 2^{-10} ml in the crude filtrate to 2^{-13} ml in the concentrate.

Determination of proteolytic activity

(A) *Hydrolysis of poly- α -amino acids.* The ability of filtrate from AF mycelium to break down poly-L-lysine, HBr, mol.wt. 90,000, and poly-L-glutamic acid, Na salt, mol.wt. 75,000 (both compounds from Pilot Chemicals Inc., Watertown, Mass., USA), was determined by a viscosimetric process devised by *Hultin* (1946). The reaction mixture, comprising 0.5 ml crude filtrate, 1 ml buffer solution (0.2 M McIlvaine and 0.2 M glycine buffer, respectively) and 1 ml 4% (w/v) polyamino acid in distilled water, was incubated at 35.5°C . The determinations were performed in an Ostwald viscosimeter at 35.5°C and the enzymatic activity of the filtrate was then calculated in accordance with the formula given by *Hultin* (cf. *Lundblad & Johansson* 1968), viz.

$$A = \frac{a+b}{a} \times C_s^2 \times \frac{d \left(\frac{1}{\eta_{sp}} \right)}{dt} = \frac{a+b}{a} \times C_s^2 \times \alpha$$

in which a = filtrate (ml)

b = liquid medium (ml)

C_s = concentration of medium in reaction mixture (g medium per g solution)

η_{sp} = specific viscosity

t = time (sec.)

α = inclination of line obtained by plotting $\frac{1}{\eta_{sp}}$ against time.

$$\eta_{sp} = \frac{\text{efflux time of mixture} - \text{efflux time of water}}{\text{efflux time of water}} =$$

$$= \frac{\tau_{\text{mix}} - \tau_{\text{H}_2\text{O}}}{\tau_{\text{H}_2\text{O}}}$$

and hence

$$\frac{1}{\eta_{sp}} = \frac{\tau_{\text{H}_2\text{O}}}{\tau_{\text{mix}} - \tau_{\text{H}_2\text{O}}}$$

The activity values (A) thus obtained were multiplied by 10^9 and were denominated "Hultin units" (H.U.) as suggested by *Lundblad* (1962).

The degradation of poly-L-glutamine at pH 5 was also followed spectrophotometrically. To this end we incubated a reaction mixture comprising 2 ml 4 % (w/v) poly-L-glutamine, 2.70 ml 0.2 M McIlvaine buffer (pH 5.0), and 1.30 ml of filtrate that was dialyzed against 0.02 M phosphate buffer (pH 6.8) at low temperature for 20 hrs. During dialysis the dialyzing medium was changed several times. One-ml samples of the reaction mixture were withdrawn after 0, 2, 4, and 6 hrs. incubation at 35.5°C in a shaker incubator and gel filtrated on a Sephadex G-25 Fine column measuring 1.1×163 cm. Elution was carried out using a 0.05 M Tris-HCl buffer (pH 7.5) + 0.2 M-NaCl as the eluant. Butanol was added to the solution in a concentration of 2 % (v/v). Filtration was done at 6°C and at a flow rate of 8 ml/hr. Fractions of 2.5 ml were collected in a fraction collector fitted with a special device (by Mr. L.-G. Falksveden, Department of Chemistry, National Bacteriological Laboratory, Stockholm) to enable constant volume fractions to be taken. The optical density of the fractions was determined at wavelengths 240 m μ and 280 m μ on a Beckman type DU spectrophotometer.

(B) *Hydrolysis of haemoglobin*. Haemoglobin medium was prepared in accordance with a modification to the original (1938) *Anson* method. Four g bovine haemoglobin (enzyme substrate powder, Armour Pharmaceutical Company Ltd., Eastbourne, England) was 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 haemoglobin solution was kept for 4–5 hrs. at room temperature and under frequent stirring. With all ingredients dissolved pH was adjusted to 7.5 by means of 2 N-HCl and the solution was then stored at – 20°C.

The determination of haemoglobin hydrolyzing activity was performed in a reaction solution made up of 0.3–1 ml enzyme solution, 2 ml buffer solution and 1 ml 4 % substrate solution. (Further details are to be found under Figures). Following incubation at 37°C the

digestion 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 m μ . The increase in optical density of the sample as compared to that of a blank has been used as a measure of the enzymatic activity of the fraction solution. The blank differed from the sample in that buffer solution mixed with enzyme solution was incubated separately, and the substrate solution, which had been incubated during the same length of time, was not incorporated until the TCA solution had been added. The hydrolytic effect of mycelial filtrate on haemoglobin is denominated 'haemoglobinase activity'.

The hydrolysis was performed at values of pH that were known (Rutqvist 1969b) to yield optimum haemoglobin hydrolysis. The buffer solutions used in these experiments were all 0.45 M. A citrate buffer was used when hydrolyzing at pH 2.9, an acetate buffer at pH 4.6 and a glycine buffer at pH 10.

(C) *Casein precipitation.* The casein precipitating activity of various fractions obtained by gel filtration on Sephadex G-100 was investigated by means of casein agar that was prepared according to a method previously described (Rutqvist 1969a). The casein solution was thus obtained by dissolving 4 g sodium caseinate (Hammarsten grade, Hopkins & Williams Ltd., Chadwell Heath, England) in 100 ml distilled water, adjusting the pH to 7.5. The definite composition of the casein agar was as follows: 0.25 % sodium caseinate, 1.5 % Bacto-Agar Difco, 0.004 M-MgCl₂, added as an 8 % (w/v) solution, and 0.01 % (w/v) merthiolate. After mixing the ingredients the pH was adjusted to 6.2 and the agar was poured into Petri dishes of 185 mm diameter to a depth of 2 mm and allowed to set. Following solidification, holes of 6 mm diameter were punched in the agar. Each hole was filled with 0.025 ml fraction solution and the agar plates were incubated for 19 hrs. in a moist chamber at 37°C. The precipitation zones occurring around the holes were then read and measured by means of millimetre sectional paper and a magnifier (2.5 \times).

Determination of haemolytic activity.

The various Sephadex fractions were examined for haemolytic activity by mixing 0.5 ml fraction solution with 0.5 ml of a 2 % suspension of sheep erythrocytes in $1/10$ M veronal buffer (pH 7). The mixture was incubated at 37°C for 30 min. and at 20°C for an additional 90 min. prior to reading.

Determination of toxic activity

The various Sephadex fractions were examined for toxic activity by intraperitoneal injections into groups of 2–5 mice for each frac-

tion. The mice (NMRI strain, Naval Medical Research Institute, Bethesda, Md., USA) weighed 16—18 g and were injected 0.5 ml each. The animals were observed for a period of 10 days.

Immuno-electrophoresis

Immuno-electrophoretic investigations of dialyzed mycelial filtrate and of proteinase purified by gel filtration were performed by means of adapted microtechniques as described by *Grabar & Burtin* (1964). The purified proteinase comprised the combined proteinase-positive fractions 185—248 (cf. Fig. 4) obtained after gel filtration on Sephadex G-100 which, following dialysis, had been freeze-dried and subsequently dissolved in distilled water to yield the same dry-matter content as the dialyzed filtrate.

The electrophoresis was carried out in a layer of buffered Noble agar about 1 mm thick (1 g Noble agar Difco + 75 ml distilled water + 25 ml 0.05 M phosphate buffer, pH 7) on slides (2.5 × 7.5 cm) that were arranged in 5.3 × 26.6 cm frames. At the electrophoresis a 0.05 M phosphate buffer (pH 7) was employed. The fractionation was performed in an electrophoresis apparatus (De Luxe Electrophoresis Chamber, Gelman Instrument Company, Ann Arbor, Mich., USA) at room temperature during 1 hr. at a current of about 10 mamp. per frame and at a potential gradient of about 10 v/cm.

Following fractionation, antiserum was introduced into a trough cut out in the agar layer parallel to the fractionated antigens. The precipitation arcs thus produced were read after storing the slides for 24 hrs. in a moist chamber at room temperature.

Dialysis

The mycelial filtrate was dialyzed, unless otherwise stated, at room temperature against ordinary running tap water for 17 hrs. and against sterile distilled water for 2 hrs.

pH determination

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

Protein determination

Determinations of nitrogen content in mycelial filtrate were carried out according to the micro-Kjeldahl technique. The factor, 6.25 was used in converting N content into protein content. In fractions collected after gel filtration the protein content was calculated after measuring the optical density at 260 m μ and 280 m μ , using nomograms devised by *Warburg & Christian* (1942).

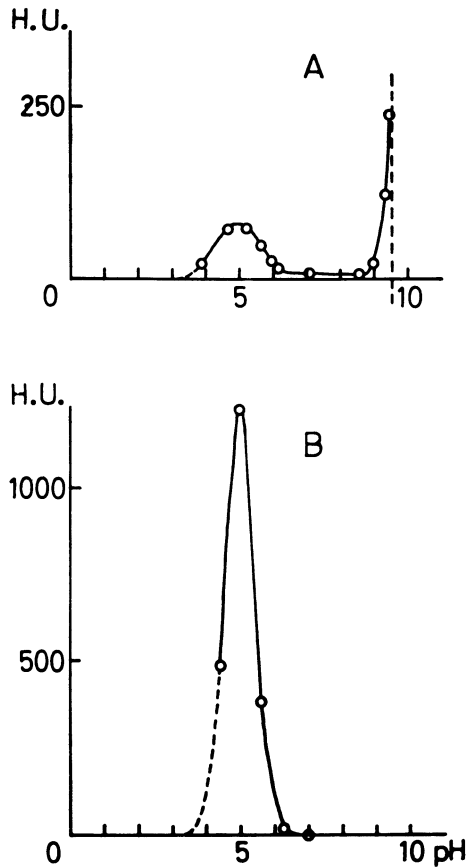


Figure 1. Influence of pH upon the hydrolysis of poly-L-lysine (A) and poly-L-glutamine (B) by mycelial filtrate from *Aspergillus fumigatus* strain 6869. H.U. = Hultin unit.

EXPERIMENTS AND RESULTS

Hydrolysis of poly- α -amino acids

The effect of pH on the hydrolysis of poly-L-lysine and poly-L-glutamine caused by crude filtrate of mycelial material from AF, is illustrated in Fig. 1. Optimum activity against the 2 substrates occurred at pH 5. A certain hydrolytic activity on poly-L-lysine starting at pH 9 could also be observed.

Fig. 2 illustrates fractionation by gel filtration of poly-L-glutamine after hydrolysis caused by dialyzed mycelial filtrate from AF. The absorption spectrum of the dialyzed filtrate per se at the same concentration as in the reaction mixture can be seen

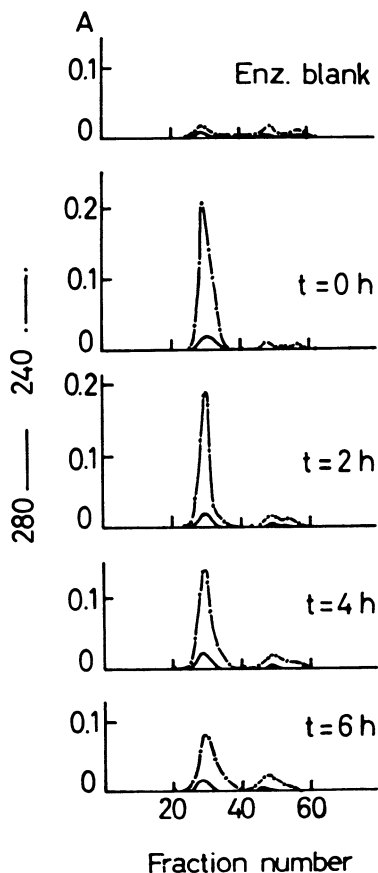


Figure 2. Fractionation by gel filtration on Sephadex G-25 Fine of the products obtained when hydrolyzing poly-L-glutamine by a mycelial filtrate from *Aspergillus fumigatus* strain 6869.

at the top of the figure. At this concentration the hydrolytic effect of the filtrate upon poly-L-glutamine was 2990 H:U./ml. The substrate reduction following prolonged incubation and the increase in low-molecular degradation products are illustrated in the chromatograms.

Fractionation of mycelial filtrate

Gel filtration on Sephadex G-75 of mycelial filtrate that was first concentrated by means of Sephadex G-25 Coarse can be seen in Fig. 3. The u.v. absorbing material produced 5 peaks the first of which was elongated with diffuse borders. Most of the u.v.

absorbing material was present in fractions 180—260 where it produced a peak, which was divided at the end of the chromatogram into a large fourth peak and a smaller yet well defined fifth peak. The u.v. absorbing material present in the last 2 peaks, as distinguished from that of the previous peaks, showed greater absorption at 260 $m\mu$ than at 280 $m\mu$.

Proteolytic activity, using haemoglobin as substrate, at pH 2.9, pH 4.6 and pH 10 occurred within the same area of the chromatogram, viz. in fractions 95—125. In fractions 84—94 there was also slight activity at pH 2.9. Maximum haemoglobinase activity at pH 2.9 was found in fraction 111, at pH 4.6 in fractions 111 and 113, and at pH 10 in fractions 113 and 114. Fractions 111, 113 and 114 all contained 120 μg protein per ml (Table 1).

The haemoglobinase activity was as follows. At pH 2.9 in fraction 111: 0.670 $E_{280}/0.3$ ml, corresponding to an activity of 18.600 E_{280}/mg protein. At pH 4.6 in fraction 113: 0.880 $E_{280}/0.3$ ml, corresponding to an activity of 24.400 E_{280}/mg protein. At

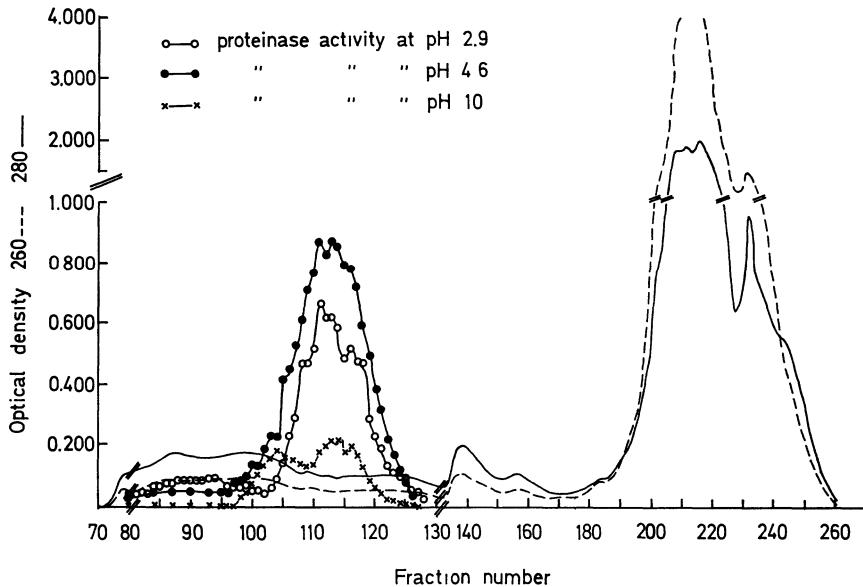


Figure 3. Gel filtration on Sephadex G-75 of mycelial filtrate from *Aspergillus fumigatus* strain 6869. Basic procedure of haemoglobin hydrolysis: a reaction mixture of 0.3 ml of fraction solution, 1 ml of 4 % haemoglobin solution and 2 ml of buffer was incubated at 37°C for 4 hrs.

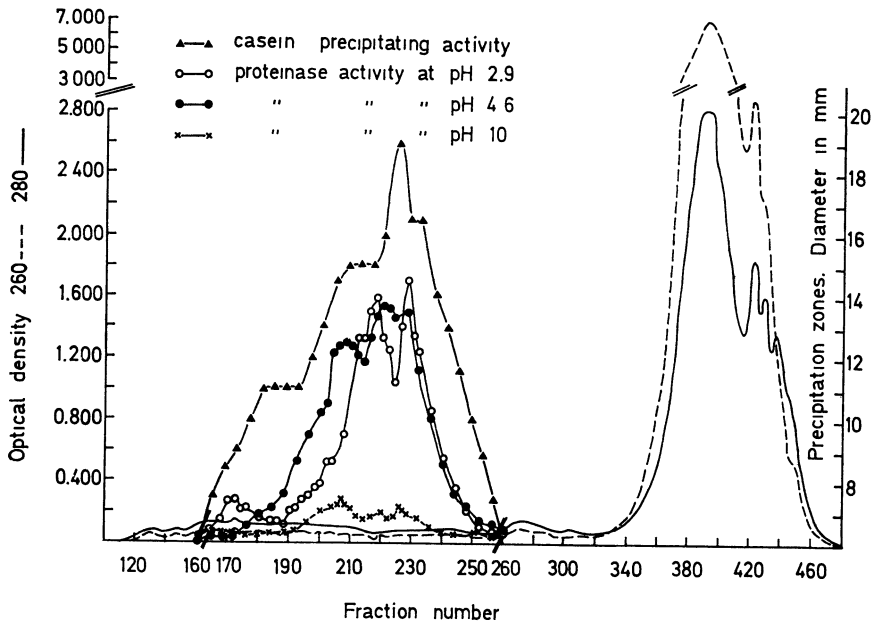


Figure 4. Gel filtration on Sephadex G-100 of mycelial filtrate from *Aspergillus fumigatus* strain 6869. Basic procedure of haemoglobin hydrolysis: a reaction mixture of 1 ml of fraction solution, 1 ml of 4 % haemoglobin solution and 2 ml of buffer was incubated at 37°C for 4 hrs.

pH 10 in fraction 113: $0.210 E_{280}/0.3$ ml, corresponding to $5.800 E_{280}/\text{mg}$ protein. Under the same conditions the dialyzed filtrate, which contained 0.064 % nitrogen, corresponding to 4 mg protein per ml, showed the following activity. At pH 2.9: $0.590 E_{280}$, at pH 4.6: $1.330 E_{280}$, and at pH 10: $0.320 E_{280}$, in each case per 0.3 ml, corresponding to $0.490 E_{280}$ at pH 2.9, $1.100 E_{280}$ at pH 4.6 and $0.270 E_{280}$ at pH 10, in each case per mg protein. Hence, when using dialyzed mycelial filtrate as a standard, the purification factors were 38, 22 and 22 at pH values 2.9, 4.6 and 10, respectively.

In the chromatogram obtained after Sephadex G-75 filtration there was a certain tendency towards separation of the haemoglobinase activity curves at pH 2.9, pH 4.6 and pH 10. For this reason mycelial filtrate was filtered on a Sephadex G-100 column in an attempt at separating from each other any possible activity peaks at the same pH value. The result is reproduced in Fig. 4.

As for the u.v. absorbing material the same basic picture was obtained as in the Sephadex G-75 filtration. The first peak was

even less distinct, however, and the third peak was not quite so conspicuous as in the Sephadex G-75 filtration. At 280 m μ the fifth peak was subdivided into 3 small peaks.

The haemoglobinase activity at pH 2.9 had been divided into 3 distinct activity peaks. The first, and smallest, peak occurred in fractions 164—186, with an activity maximum in fraction 172. The other 2 activity peaks occurred in fractions 190—222 and 226—256, with activity maxima in fractions 218 and 228, respectively.

A certain tendency towards separation was also noticed for the haemoglobinase activity at pH 4.6 and pH 10, but the activity peaks were less marked and displayed less clear-cut borders than the peaks for the haemoglobinase activity at pH 2.9. Haemoglobinase activity occurred in fractions 176—260 and 184—252 at pH 4.6 and pH 10, respectively. At pH 4.6 2 activity peaks were noticed. The first, and smaller, peak extended from fraction 176 to fraction 214 with an activity maximum in fraction 208. The second, larger, activity peak, of a blunt shape, extended from fraction 214 to fraction 260, showing maximum activity in fraction 220. However, almost as strong activity was found in fractions 222, 224 and 228. The haemoglobinase activity occurring at pH 10 showed a division in 3 moderately marked peaks.

Table 1. Protein content of fractions obtained by gel filtration of mycelial filtrate on Sephadex columns. *Aspergillus fumigatus* strain 6869.

Protein μ g/ml	Fractions			
	Sephadex G-75		Sephadex G-100	
		Number		Number
10—40	74, 171—173	4	117—121	5
50—90	75—76, 128—133, 161—170, 174—187	32	122—130, 134—147, 150—155, 213—236, 246—260, 291—302, 304—329	106
100—140	77—82, 107—127, 134, 146—160, 188—191	47	131—133, 148—149, 156—162, 170—171, 173—212, 237—245, 261—264, 279—290, 303	80
150—190	83—97, 100—106, 135, 143—145, 192—193	28	163—169, 172, 265—278	22
200—240	98—99, 136, 142	9		

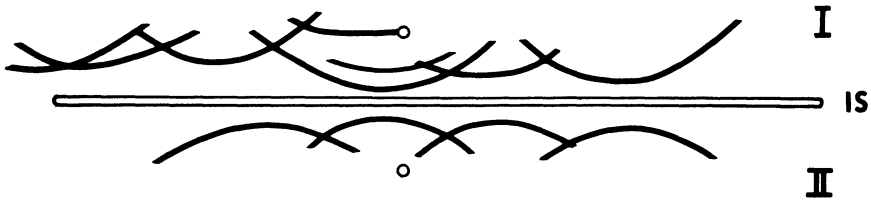


Figure 5. Immuno-electrophoretic patterns of dialyzed mycelial filtrate and proteinase-positive fractions obtained by gel filtration of crude mycelial filtrate on Sephadex G-100 (fractions 185—248, Fig. 4). *Aspergillus fumigatus* strain 6869.

I = Dialyzed mycelial filtrate.

II = Proteinase-positive fractions' obtained by filtration of mycelial filtrate on Sephadex G-100.

Casein precipitating activity was recorded in fractions 165—257 (Fig. 4). Maximum activity occurred in fraction 225, where 0.025 ml fraction solution in casein agar resulted in a precipitation zone of 19 mm diameter. There was no evident separation of the casein precipitating activity.

No haemolytic or toxic activity could be found in 0.5 ml samples of fraction solution from any of the fractions obtained after gel filtration on Sephadex G-75 or G-100.

The protein contents of fractions 74—190 from the gel filtration on Sephadex G-75 and of fractions 117—335 from the gel filtration on Sephadex G-100 are summarized in Table 1.

In fractions obtained after gel filtration on Sephadex G-75 the protein content was found to be 30—230 $\mu\text{g}/\text{ml}$ and in fractions from gel filtration on Sephadex G-100, 10—170 $\mu\text{g}/\text{ml}$.

As can be seen in Fig. 5, dialyzed mycelial filtrate and proteinase purified by gel filtration on Sephadex G-100 produced, after electrophoretic separation, 8 and 4 precipitation arcs, respectively, with antiserum prepared against crude filtrate.

DISCUSSION

It has been shown by applying a viscosimetric procedure that mycelial filtrate from AF hydrolyzes poly-L-lysine and poly-L-glutamine. Earlier work by *Martin & Jönsson* (1965) has shown that proteolytic enzyme recovered from broth used for AF cultivation hydrolyzes poly-L-glutamine at pH 4 and poly-L-lysine at pH 10. This was substantiated by a quantitative ninhydrin

method devised by *Matheson et al.* (1961). The mycelial filtrate used in the present investigations proved to hydrolyze the 2 amino acids in the pH range, 4.6—5.3. As can be seen in Fig. 1 the filtrate showed stronger activity against poly-L-glutamine than against poly-L-lysine. An incipient activity against poly-L-lysine was noted at pH 9 but, owing to spontaneous hydrolysis of the polyamino acid at pH values beyond 10, it was impossible to trace the activity peak any further.

As in a previous investigation (*Rutqvist* 1969b) crude AF filtrate showed strong absorption at 280 m μ . For this reason dialyzed filtrate was used when investigating spectrophotometrically the degradation of poly-L-glutamine after gel filtration. The hydrolyzing effect of the dialyzed filtrate appeared as a distinct decrease in substrate associated with a simultaneous increase in the amount of low-molecular degradation products (Fig. 2).

The pronounced drop in viscosity that was observed when AF filtrate acted upon poly-L-glutamine or poly-L-lysine seems to indicate that the filtrate hydrolyzes central bonds in the molecule, i.e., that it acts as an endopeptidase.

Gel filtration of mycelial filtrate from AF on Sephadex G-75 or G-100 columns proved the filtrate to contain a considerable amount of low-molecular substances. The major part of the proteinase activity using haemoglobin as substrate at pH values 2.9, 4.6 and 10 appeared within the same area on the chromatograms. In gel filtration on Sephadex G-100, however, it was observed that the haemoglobinase activity reached a first peak, of moderate size, at pH 2.9 and that this peak included also fractions of which no haemoglobinase activity could be determined at pH values 4.6 and 10 (Fig. 4). It is interesting to note that casein precipitating activity occurred also in fractions where haemoglobin hydrolyzing activity occurred only at pH 2.9 but not at pH 4.6 or 10 (Fig. 4). This seems to indicate that the casein precipitating activity is linked not only to the protein hydrolyzing activity with pH 6.2 as the optimum, using casein as the substrate (cf. *Rutqvist* 1969 a), but also to the protein hydrolyzing activity at pH 2.9. Since it was possible to measure casein precipitating and protein hydrolyzing activity in identical fractions, following gel filtration on Sephadex G-100, the present investigations, like those of *Sandvik* (1962), have produced no evidence against the assumption that the casein precipitating

enzyme is identical with the protein hydrolyzing enzyme. It should be remembered, however, that the enzymatic casein precipitation may probably express at the same time the activity of 2 or more protein hydrolyzing enzymes that may occur simultaneously in the test specimen. It is a confusing fact that the strongest casein precipitating activity, following filtration on Sephadex G-100, was found within an area of the chromatogram (fractions 221—229) where at the same time there was a marked inhibition of the haemoglobinase activity at pH 2.9 (fractions 220—226). There also seemed to be a certain inhibition of the haemoglobinase activity at pH 4.6 and pH 10 in this area of the chromatogram. It may be suggested as an explanation that the fractions in question may contain an inhibitor against haemoglobinase activity and that this inhibitor may be particularly active at pH 2.9 without any apparent effect on the casein precipitating activity at pH 6.2.

The above mentioned gel filtrations of mycelial filtrate from AF have not yielded any definite information enabling us to solve the question whether the haemoglobinase activity at pH values 2.9, 4.6 and 10 is actually associated with the activity of a single enzyme with several pH optima or a number of enzymes acting at different pH values. It might be possible to further elucidate this problem by means of isoelectric focussing. Following gel filtration on Sephadex G-100, however, the haemoglobinase activity has a peak at pH 2.9, which includes fractions that do not seem to show any haemoglobinase activity at pH 4.6 or pH 10. Apart from this the haemoglobinase activity curve for pH 2.9 seems to differ slightly from the corresponding curves for pH 4.6 and pH 10. Also, the purification factor obtained after gel filtration on Sephadex G-75 in fractions with maximum haemoglobinase activity was greater at pH 2.9 than the purification factors — both of the same size — associated with the haemoglobinase activity at pH 4.6 and pH 10. These conditions may suggest that the haemoglobinase activity at pH 2.9 is caused by an enzyme, which is not identical with the enzyme(s) causing the haemoglobinase activity at pH 4.6 and pH 10. As for the haemoglobinase activity at the latter 2 pH values the present investigation has revealed nothing that would be inconsistent with its being caused by one and the same enzyme in both cases. This would also be in line with the suggestion by *Martin & Jönsson* that the protein hydrolyzing activity at pH 6.6 and at pH 10 with

casein as substrate of broths employed for growing AF might well be caused by the same enzyme.

Judging from their position in the chromatogram following gel filtration on Sephadex G-100 the components producing the first activity peak at pH 2.9 (fractions 164—186) seem to have a molecular weight beyond 150,000 whereas the molecular weight of the components causing enzymatic activity at pH 2.9, pH 4.6 and pH 10 in fractions 190—260 seems to range between 100,000 and 30,000. It would thus seem possible that the enzymatically active components encountered in the first activity peak at pH 2.9 may comprise a so-called multiple enzyme, i.e. aggregated molecular entities. It is also possible that the enzymatic activity occurring at pH 2.9, pH 4.6 and pH 10 in fractions 190—260 may have been caused, at least in part, by such multiple forms. Further, the presence of aggregated molecular entities may have been responsible for the separation into 2 distinct active peaks (fractions 190—222 and 226—256) that was observed in connection with the haemoglobinase activity at pH 2.9. The fact that the casein precipitating activity shows a very distinct activity peak in the area between the 2 peaks of haemoglobinase activity at pH 2.9 (fractions 220—226) suggests, however, that the separation of the haemoglobinase activity at that pH does not depend on the presence of multiple molecular entities. As mentioned previously it would rather seem probable that the separation may be associated with an inhibiting agent present in the fractions in question (i.e. 220—226), which counteracts the haemoglobinase activity, especially at pH 2.9, but has no apparent effect on the casein precipitating activity at pH 6.2.

Haemolysin and toxin present in mycelial filtrate from AF could not be recovered in any fraction following gel filtration on Sephadex G-75 and G-100 columns, respectively. The titres for haemolytic and toxic activity of the dialyzed filtrate were 2^{-10} ml and $10^{-2.6}$ ml, respectively, the nitrogen content being 0.064 %, corresponding to a protein content of 4 mg/ml. It is found under these conditions that a content of 40 μ g of protein per ml will yield 2 MLD₅₀ of toxin per 0.5 ml filtrate and that 10 μ g of protein per ml will yield > 1 HU per 0.5 ml filtrate. As can be seen from Table 1 most of the fractions showing a stronger absorption at 280 m μ than at 260 m μ contain more than 40 μ g protein per ml. Provided that the haemolysin (and toxin) retains unchanged its relation to the protein content after gel filtration,

these activities, and the haemolytic activity in particular, should have been recovered in 0.5 ml fraction solution. After the gel filtration there should also have been a certain purification effect, resulting in stronger activity per mg protein than could actually be found in the dialyzed filtrate. Hence, it is not very probable that haemolytic or toxic activity could have escaped discovery in these fractions, owing to the protein concentration being too low in the fractions. Fractions characterized by strong absorption, stronger at 260 m μ than at 280, probably contain the greater part of the low-molecular substances that disappear in ordinary dialysis and thus do not show any relationship to the haemolytic or toxic activity (cf. *Rutqvist* 1969 a). No haemolytic or toxic activity was expected in these fractions, nor could any such activity be established in samples of 0.5 ml fraction solution from any of the fractions. The reason why no haemolysin or toxin could be found after fractionating the mycelial filtrate remains a matter of speculation. One explanation may be that activating substances necessary for haemolytic and toxic activity have simply been separated off during fractionation. There is also a slight possibility of the haemolysin and the toxin being adsorbed by the Sephadex gel despite the comparatively high concentration of the eluant. A third possibility, and perhaps one more probable than the other two, would be that the haemolysin or toxin may have lost its stability and become inactive, following purification. It should be mentioned here that *Wynston & Tilden* (1963) seem to have been unsuccessful in recovering haemolysin or toxin from mycelial filtrate of AF after chromatography on a Duolite CS-101 column. Both haemolysin and toxin were found, however, in fractions obtained after chromatography on DEAE cellulose columns (*Rau et al.* 1961, *Wynston & Tilden*). The fractions with haemolytic and toxic activity in these cases seem to have been less pure and have probably contained a considerably higher percentage of protein than the fractions obtained during our gel filtration, which may have had a stabilizing effect on the haemolysin and toxin.

Biguet et al. (1965) state that cellular antigen from AF produces 16 precipitation arcs with homologous rabbit antiserum. The number of arcs shows certain variations, however, depending on the length of time during which the mycelial material used for preparing the cellular antigen has been grown (cf. *Biguet et al.* 1964). It has been the sole object of the immuno-

electrophoretic investigation reported by the authors in the present paper to study (a) how many precipitating antigens that could be found in the protein hydrolyzing fractions obtained after gel filtration on Sephadex G-100 and (b) the degree of purification that could be achieved as compared to dialyzed mycelial filtrate. It should be pointed out in this connection that the fractions forming the first small activity peak at pH 2.9 after gel filtration have not been included among the fractions investigated.

Our investigation shows that, when using antiserum to a crude filtrate from a homologous AF strain, the purified proteinase-positive fractions with protein hydrolyzing activity at pH 2.9, pH 4.6 and pH 10 contained 4 precipitating antigens whereas the dialyzed filtrate contained 8 such antigens.

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SUMMARY

Mycelial filtrates from *Aspergillus fumigatus* (AF), shown to possess haemolytic, toxic, casein precipitating, and protein hydrolyzing activity, hydrolyzed poly-L-lysine and poly-L-glutamine in the pH range 4.6—5.3. Incipient activity against poly-L-lysine was observed

at pH 9. Owing to spontaneous hydrolysis of the polyamino acid at pH > 10, no activity optimum could be traced.

Gel filtration of mycelial filtrate on Sephadex G-75 or G-100 columns offered no definite information whether the protein hydrolyzing activity, using haemoglobin as substrate, at the optimum pH values, 2.9, 4.6 and 10, shows the activity of a single enzyme with more than 1 pH optimum or of more than 1 enzyme active at different pH values. Certain results of the investigations seem to indicate that the protein hydrolyzing activity at pH 2.9 was not caused by enzymes identical with the enzyme(s) causing the protein hydrolyzing activity at pH 4.6 or pH 10.

Casein precipitating and protein hydrolyzing activity occurred, following gel filtration on a Sephadex G-100 column, in identical fractions whereas neither haemolysin nor toxin could be found in samples of 0.5 ml fraction solution from any of the fractions after filtration on Sephadex G-75 or G-100 columns.

By using antiserum to a crude filtrate from a homologous AF strain it could be shown, applying immuno-electrophoresis, that dialyzed mycelial filtrate contained 8 precipitating antigens whereas proteinase purified by gel filtration and displaying protein hydrolyzing activity at pH 2.9, pH 4.6 and pH 10 contained 4 such antigens.

ZUSAMMENFASSUNG

Studien über Aspergillus fumigatus; Hydrolyse von poly- α -Aminosäuren durch Myzelienfiltrat sowie chromatographische Fraktionierung des Filtrates.

Myzelienfiltrate von *Aspergillus fumigatus* (AF), die hämolytische, toxische, Kasein-präzipitierende und Protein-hydrolysierende Aktivität zeigten, hydrolysierten poly-L-lysin und poly-L-glutamin in dem pH-Gebiet von 4,6—5,3. Der erneute Beginn einer Aktivität gegen poly-L-lysin konnte bei pH 9 beobachtet werden. Auf Grund der spontanen Hydrolyse der Aminosäure bei höheren pH-Werten als 10 konnte das Aktivitätsoptimum nicht bestimmt werden.

Die Gelfiltration von Myzelienfiltraten mit Sephadex G-75 bzw. G-100 Kolonnen gab keinen sicheren Hinweis darauf in wie weit die Protein-hydrolysierende Aktivität mit Hämoglobin als Nährboden bei den optimalen pH-Werten 2,9, 4,6 bzw. 10 die Aktivität von nur einem Enzym mit mehreren pH-Optima oder die mehrerer bei verschiedenen pH-Werten aktiven Enzymen widerspiegelt. Gewisse Untersuchungsergebnisse deuten jedoch darauf hin, dass die Protein-hydrolysierende Aktivität bei pH 2,9 nicht von einem Enzym verursacht wird, dass mit dem oder den Enzymen identisch ist, die die Protein-hydrolysierende Aktivität bei pH 4,6 bzw. pH 10 bewirken.

Kasein-präzipitierende und Protein-hydrolysierende Aktivität trat nach der Gelfiltration mit Sephadex G-100 Kolonnen in identischen Fraktionen auf, während weder Hämolysin noch Toxin in 0,5 ml Fraktionslösung nach Filtrierung durch Sephadex G-75 bzw. Sephadex G-100 Kolonnen in keiner der Fraktionen nachgewiesen werden konn-

ten. Die Möglichkeit, dass Kasein-präzipitierende und Protein-hydrolyserende Aktivität von identischen Enzymen hervorgerufen werden kann, sowie die Ursachen, dass weder Hämolysin noch Toxin nach den Gelfiltrationen nachgewiesen werden konnten, werden besprochen.

Unter Anwendung eines Immunserums gegen Rohfiltrat von einem homologen AF-Stamm wurde in Immunoelktrophorese nachgewiesen, dass dialysierte Myzelienfiltrate 8 präzipitierende Antigene enthalten, während durch Gelfiltration gereinigte Proteinase mit Protein-hydrolyserender Aktivität bei pH 2,9, pH 4,6 bzw. pH 10, 4 präzipitierende Antigene enthält.

SAMMANFATTNING

Studier av Aspergillus fumigatus; hydrolys av poly- α -aminosyror förorsakad av myceliefiltrat och kromatografisk fraktionering av filtratet.

Myceliefiltrat från *Aspergillus fumigatus* (AF), som visats ha hämolytisk, toxisk, kaseinprecipiterande och proteinhydrolyserande aktivitet hydrolyserade poly-L-lysin och poly-L-glutamin i pH-intervallet 4,6—5,3. En begynnande aktivitet mot poly-L-lysin kunde iakttagas vid pH 9. På grund av aminosyrans spontanhydrolys vid pH-värden högre än pH 10 kunde aktivitetsoptimum ej bestämmas.

Gelfiltrering av myceliefiltratet på Sephadex G-75 respektive G-100 kolonner gav ingen säker upplysning om huruvida den proteinhydrolyserande aktiviteten med hämoglobin som substrat vid respektive optimala pH-värden 2,9, 4,6 och 10 reflekterar aktiviteten hos endast ett enzym med flera pH-optima eller flera enzym aktiva vid olika pH-värden. Vissa undersökningsresultat antydde dock att den proteinhydrolyserande aktiviteten vid pH 2,9 icke förorsakades av enzym identisk med det eller de enzym, som förorsakade den proteinhydrolyserande aktiviteten vid respektive pH 4,6 och pH 10.

Kaseinprecipiterande och proteinhydrolyserande aktivitet uppträdde efter gelfiltreringen på Sephadex G-100 kolonn i identiska fraktioner medan varken hämolysin eller toxin kunde påvisas i 0,5 ml fraktionslösning från någon av fraktionerna efter filtrering på Sephadex G-75 respektive Sephadex G-100 kolonner. Möjligheterna för att kaseinprecipiterande och proteinhydrolyserande aktivitet förorsakas av identiskt enzym liksom orsakerna till att hämolysin respektive toxin ej kunde påvisas efter gelfiltrationerna diskuteras.

Med användande av immunserum mot råfiltrat från homolog AF-stam kunde vid immunelektrofores visas att dialyserat myceliefiltrat innehöll 8 medan genom gelfiltrering renat proteinas med proteinhydrolyserande aktivitet vid respektive pH 2,9, pH 4,6 och pH 10 innehöll 4 precipiterande antigen.

(Received January 13, 1969).