Thus for the active catabolism of citrate, very high levels of isocitrate dehydrogenase are required to remove the substrate isocitrate which may otherwise be converted to citrate by the reversible aconitase reaction. If the activity of isocitrate dehydrogenase decreases, citrate will accumulate in the tissues. In the present study the activities of aconitase and isocitrate dehydrogenase were found to be much lower in the infected tissues compared with the healthy ones (table 2). This indicates that the decrease of citrate catabolizing enzymes is one of the reasons for the accumulation of citrate in infected tissues.

The activities of fructose biphosphate aldolase, malate dehydrogenase and succinate dehydrogenase decreased in the infected tissues (table 2) indicating that the rate of respiration may be affected. A decrease in the activity of malate dehydrogenase has also been shown by Boser[18] in potato leaf roll. Since there was much lower succinate dehydrogenase activity, mitochondrial integrity might be affected after infection leading to a disturbance in the enzymes of the citric acid cycle. This consequently would cause an imbalance in the relative concentrations of metabolites of the tricarboxylic acid cycle. On the contrary the concentrations of monocarboxylic and dicarboxylic ketoacids were found to be higher in infected tissues compared with healthy tissues (table 2). This may be due to immobilisation of these metabolites in subsequent reactions or to low activities of specific transaminases. Furthermore, the overall protease activity was found to be higher in infected mango fruits than in healthy ones (table 2). This observation is in agreement with the postulation of Friedman[19], that greater virulence in the case of *Erwinia carotovora* can be attributed to an increase in proteolytic activity. The high overall protease activity may be responsible for the observed decrease in the activities of certain enzymes in the infected tissues.


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## Trypsinogen-kinase from *Aspergillus fumigatus*


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*Summary.* The activation of bovine trypsinogen by an extracellular acid proteinase from *A. fumigatus* is described. The enzyme activates trypsinogen optimally at pH 3.5 and 32 °C. The effect of substrate and enzyme concentrations on the activation has been studied and the $K_m$-value has been determined.


It has been reported that several fungal acid proteinases have the unique ability to activate pancreatic trypsinogen to trypsin. This property was first discovered by Kunitz[2] for an unidentified strain of *Penicillium*. The activity was later demonstrated in *Aspergillus oryzae*[3], in *Aspergillus saitoi*[4,5] and in *Rhizopus chinensis*[6]. Hofmann[7] described the presence of this activity in *Aspergillus carbonarius* and in a number of *Penicillia* including 2 strains of *Penicillium janthinellum*. In an earlier communication[8], the production of extracellular acid proteinase by *Aspergillus fumigatus* has been reported. The object of the present study is to investigate the trypsinogen-activating property of the acid proteinase.

*Materials and methods.* Bovine trypsinogen (pure) and bovine trypsin (twice crystalline) were obtained from Sigma Chemical Co., USA, and vitamin-free casein was purchased from ICN Pharmaceuticals, Inc., USA. All other chemicals and reagents were of analytical grade.

Partial purification of the acid proteinase from *A. fumigatus:* Culture filtrate (3 l) obtained by culturing *A. fumigatus* in a liquid medium at room temperature (30–32 °C) for 3 days and by filtering off the mat, as described earlier[8], was centrifuged at 5000 × g. The centrifugate was collected and the pH of the centrifugate was adjusted to 4.8. 45 g of DEAE cellulose which had been equilibrated to pH 4.8 by repeated washing with 0.1 M citrate of pH 4.8 were added to the centrifugate with stirring for 1 h at 4 °C. After this period the slurry was packed in a column, and the enzyme was collected in a 500 ml fraction by passing through the column 0.2 M citrate of pH 4.0 containing 0.2 M NaCl.

To the eluate, solid ammonium sulphate was added with gentle stirring till 60% saturation was obtained. The precipitate thus formed was collected by centrifugation, and was dialyzed against bi-distilled water at 4 °C for 40 h. The dialyzed enzyme solution was freeze dried in a Virtis-lyophilizer. The dried acid proteinase thus obtained was used for the present investigation.

Trypsinogen activation assay. The assay for the acid proteinase, based on its ability to activate trypsinogen at pH 3.0–4.0 was determined according to the method described by Kunitz[9] using casein as substrate with slight modifications as suggested by Hofmann and Shaw[10]. The trypsin standard curve was also prepared according to the same method.

Activation of trypsinogen. 1.5 ml of $10^{-5}$ M trypsinogen solution which was prepared by dissolving 5.0 mg of trypsinogen in 10 ml to 0.0025 M HCl and mixing with 20 ml of 0.1 M citrate of pH 3.4 was warmed at 36 °C and mixed with 0.5 ml of enzyme solution (5 mg/100 ml solution of 0.1 M $KH_2PO_4$) to start the activation reaction.

Trypsin assay. After exactly 10 min of reaction, 2 ml of casein solution (prepared by mixing equal volumes of 2%

casein and 0.37 M Tris buffer) was added to the trypsinogen solution. After a further period of 10 min the undigested casein was precipitated by 2 ml of 8.5% trichloroacetic acid. After 10 min of standing at 36 °C, the precipitate was filtered through Whatman No.42 filter paper. The filtrate was analyzed in a Pye-Unicam Spectrophotometer and the extinction values at 280 nm were determined against a blank containing the reagents. The number of nmoles of trypsinogen activated was calculated from the trypsin standard curve.

*Results and discussion.* The effect of pH on the activation of trypsinogen was studied using trypsinogen in 0.1 M citrate of varying pH range. The trypsin assay was carried out as usual and the results are presented in figure 1. The activation of bovine trypsinogen by the acid proteinase of *A. fumigatus* was found to be maximal at pH 3.5. A similar result was reported for other microbial acid proteinases. The pH of maximum activation was 3.4 in the cases of *P. janthinellum*[10] and *A. oryzae*[3,11], 3.5 in case of *A. saitoi*[5]. In the case of neutral proteinase, however, a very weak trypsinogen-activation activity of *A. oryzae* at pH 5.5 and not at pH 3.4 was reported[12]. The optimum pH for the hydrolysis of different protein substrates viz. hemoglobin, egg albumin and casein by acid proteinase of *A. fumigatus* was found to be between 2.8 and 3.1.

To study the effect of temperature on the activation, the same procedure was followed except that the pH of the trypsinogen solution was maintained at 3.5 and the reaction of activation was carried out at different temperatures. The results are presented in figure 2. The rate of activation of trypsinogen by the acid proteinase was found to differ at various temperatures. The maximum activation was observed at pH 3.5 and 32 °C. The gradual fall in activation at higher temperatures may be due to the combined effect of heat denaturation and temperature dependence. The optimum activation temperatures with respect to acid proteinases of *P. janthinellum* and *A. saitoi* were reported to be 35 and 30 °C respectively.

The Michaelis-Menten constant ($K_m$) was found to be $3.6 \times 10^{-5}$ M. The $K_m$-values of acid proteinase of *P. janthinellum* and *A. saitoi* were reported to be $7.6 \times 10^{-6}$ M and $1.3 \times 10^{-5}$ M respectively. Considering the trypsinogen-activating properties of different acid proteinases, it was observed that *P. janthenellum* peptidase A[10,13,14], *Paecilomyces varioti* acid proteinase[15], *Rhizopus chienensis* proteinase[6], *Trametes sanguinea* acid proteinase[16] and Aspergillopeptidase A[17] resemble the acid proteinase of *A. fumigatus*. The mechanism of activation of trypsinogen by acid proteinase and that of autocatalytic activation are said to be identical, since both mechanisms involve the cleavage of the lysine-6-isoleucine-7 bond with the liberation of the hexapeptide - valyl $(aspartyl)_4$ lysine[3,5,18,19]. In addition, the liberation of different breakdown products of the hexapeptide by the action of different microbial acid proteinases has also been reported. This has, however, been contradicted by Mains and Hofmann[20], claiming that highly purified enzyme liberates only the hexapeptide, $Val.Asp.Lys_4$ and does not produce smaller peptides. The production of the smaller peptides has been attributed to the action of contaminant peptidases. The trypsinogen-activating property of these acid proteinases is very specific and provides a convenient assay for the proteinase in presence of other acid and alkaline peptidases. The assay is very sensitive and allows the detection of a few ng of the enzyme.

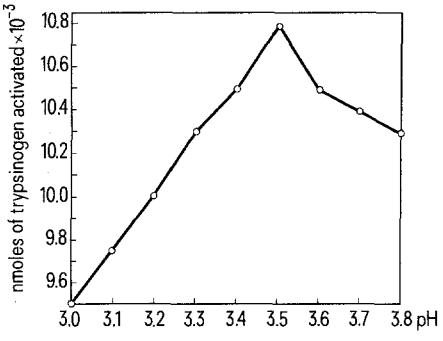


Fig. 1. pH-Trypsinogen activation relationship. Trypsinogen $10^{-5}$ M, acid proteinase 50 γ/ml, 0.1 M citrate, temperature of activation 36 °C.

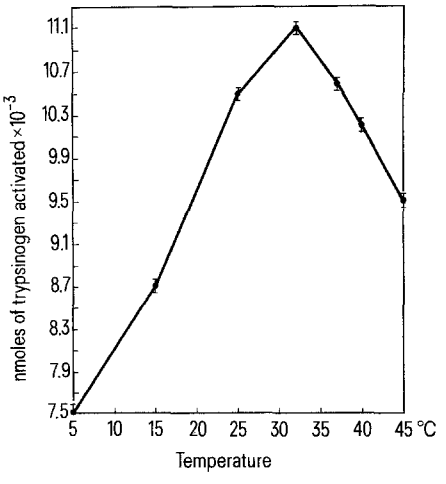


Fig. 2. Effect of temperature on trypsinogen activation. pH of activation 3.5.

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