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Aspergillus oryzae Acid Proteinase

PURIFICATION AND PROPERTIES, AND FORMATION OF π-CHYMOTRYPSIN

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An acid proteinase from *Aspergillus oryzae* was isolated from a commercial powder by successive $(NH_4)_2SO_4$ fractionation, acetone precipitation, and ion-exchange chromatography on phosphate- and DEAE-cellulose columns. The purified enzyme was found to be homogeneous by ultracentrifuge-sedimentation analysis ($s_{20,w} = 3.63$ S), but electrofocusing in polyacrylamide gels and electrophoresis at pH3.2 revealed that it consists of two very closely migrating bands. No difference in the amino acid composition and enzymic activities of the two partially separated bands could be detected, and it was concluded that the acid proteinase exists in two molecular forms. The enzyme activates bovine trypsinogen and chymotrypsinogen at pH3.5 (the $k_{cat.}$ and K_m values at 35°C are 11.3 s^{-1}, 0.10mM and 1.14 s^{-1}, 0.18mM respectively). It hydrolyses the Phe-Phe bond of the synthetic pepsin substrates Z-His-Phe-Phe-OEt ($k_{cat.} = 1.65\,s^{-1}$, $K_m = 0.640$mM at pH3.5, 30°C) and Z-Ala-Ala-Phe-Phe-OP_y4P_r ($k_{cat.} = 0.37\,s^{-1}$, $K_m = 0.037$mM at pH2.9, 39°C), where Z represents benzyloxycarbonyl and OP_y4P_r represents 3-(4-pyridyl)-propyl 1-ester. Activation of bovine chymotrypsinogen results from the cleavage of the $Arg_{(15)}$-$Ile_{(16)}$ bond in the zymogen. No other cleavages were observed. The use of *A. oryzae* proteinase provides a simple tool for the production of π-chymotrypsin in good yield and purity.

In recent years acid proteinases from fungi have attracted considerable interest among many enzymologists (see reviews by Sodek & Hofmann, 1970; Matsubara & Feder, 1971; Hofmann, 1975). Partial amino acid sequences of two of them, penicillopepsin from *Penicillium janthinellum* and Rhizopus-pepsin from *Rhizopus chinensis* (Graham *et al.*, 1973; Hofmann, 1974) indicate that these enzymes are homologous to porcine pepsin and chymosin. An active site peptide of an acid proteinase from *Aspergillus awamori* (Lobareva *et al.*, 1972) was shown to be identical with a corresponding peptide of penicillopepsin (Kovaleva *et al.*, 1972).

The acid proteinase from another Aspergillus, *Aspergillus saitoi*, has been studied extensively (see Ichishima, 1970), but only limited information is available on the proteinase from *Aspergillus oryzae*, which has been purified at least partially by several groups (Nakanishi, 1959*a*; Bergkvist, 1963; Robinson *et al.*, 1973). An interesting property of fungal acid proteinases is their ability to activate trypsinogen at pH3–4. First studied by Kunitz (1938) in the growth medium of an unidentified species of *Penicillium* this property has been demonstrated in *Aspergillus oryzae* (Nakanishi, 1959*a*), two other aspergilli and ten species of *Penicillium* (Hofmann, 1963) More recently, this activity was also found in *Rhizopus chinensis* acid proteinase (Graham *et al.*, 1973). in *Endothia parasitica* acid proteinase (Whitaker, 1972) and in the enzymes from *Rhodotorula glutinis* and *Cladosporium* (Morihara & Oka, 1973). These last authors (Morihara & Oka, 1973) show a correlation between the ability to cleave lysyl peptides and trypsinogen activation. An acid proteinase from a protozoon, *Tetrahymena pyriformis*, also activates trypsinogen (J. E. S. Graham & T. Hofmann, unpublished work). Studies on the activation mechanism show that the enzymes cleave the $Lys_{(6)}$-$Ile_{(7)}$ bond (see Sodek & Hofmann, 1970).

The activation of bovine chymotrypsinogen by acid proteinase from *A. saitoi* was studied by Gabeloteau & Desnuelle (1960) and Abita *et al.* (1969). Both groups reported that the activation was much slower than that of trypsinogen; however, unlike in trypsinogen the mechanism of reaction was not elucidated and the activation product was not characterized.

The present report describes a new method for isolation of the *A. oryzae* proteinase, some chemical and kinetic properties, and the mechanism of activation of bovine chymotrypsinogen A by the purified enzyme. It provides also a reliable method for preparation of π-chymotrypsin.

Experimental

Materials

Polidase-S, a crude powder preparation from *A. oryzae*, was purchased from Schwartz and Mann (Orangeburg, N.Y., U.S.A.). Phosphate-cellulose (P-cellulose) (medium fibrous powder P-11) and DEAE-cellulose DE-23 were obtained from Whatman Biochemical Ltd. (Maidstone, Kent, U.K.). Bovine trypsinogen (once-crystallized), α-chymotrypsin (five times crystallized), δ-chymotrypsin (salt-free; freeze-dried) and di-isopropyl phosphorofluoridate-treated carboxypeptidase B were products of Worthington Co. (Freehold, N.J., U.S.A.). Bovine chymotrypsinogen A (seven times crystallized), containing only 0.02% of active enzyme, was a gift from Dr. K. Kurachi (University of Washington, Seattle, Wash., U.S.A.). Crystalline porcine trypsin was purchased from Novo Industri A/S (Copenhagen, Denmark). Tos-Arg-OMe,* Ac-Trp-OEt, Ac-Tyr-OEt and Bz-Tyr-OEt were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and Ampholine was from LKB (Stockholm, Sweden). *N*-Acetyl-L-phenylalanyl-L-3,5-di-iodotyrosine and Z-His-Phe-Phe-OEt were products of E. Merck (Darmstadt, Germany). Z-Ala-Ala-Phe-Phe-OP$_r$4P$_r$ was a gift from Dr. Z. Bohak (Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel). *p*-Nitrophenyl-*p'*-(ω-dimethylsulphonioacetamido)benzoate bromide, a specific active-site titrant of chymotrypsin (Wang & Shaw, 1972), was a gift from Dr. E. Shaw. Di-isopropyl phosphorofluoridate was purchased from Pierce Chemical Co. (Rockford, Ill., U.S.A.) and diluted with dry propan-2-ol.

Enzyme assays

The trypsinogen-activating activity of *A. oryzae* proteinase was assayed by the casein method (Kunitz, 1938) as described by Sodek & Hofmann (1970). One unit of enzymic activity was defined as the amount of enzyme required for activation of 1 μmol of trypsinogen/min, under the experimental conditions. The specific activity was calculated per E_{280} unit. A protein solution giving an absorbance of 1.00 at 280nm through a 1cm path-length cell was defined as possessing one E_{280} unit.

Trypsin and chymotrypsin activities were also determined titrimetrically. The assay was carried out in a Radiometer Titrator in 0.1M-KCl–0.005M-Tris–0.05M-$CaCl_2$ buffer, pH8.0, by using Tos-Arg-OMe (0.01M), as trypsin substrate or Ac-Trp-OEt (0.005M) and Ac-Tyr-OEt (0.01M) as chymotrypsin substrates, at 25°C.

The spectrophotometric assay of chymotrypsin by using Bz-Tyr-OEt (0.0005M) was carried out at 25°C in a Gilford 2400 spectrophotometer as described by Walsh & Wilcox (1970). One unit of activity was equal to the hydrolysis of 1 μmol of substrate/min.

Active-site titration of chymotrypsin

Active-site titrations were carried out with the specific active-site titrant *p*-nitrophenyl-*p'*-(ω-dimethylsulphonioacetamido)benzoate bromide (Wang & Shaw, 1972).

Activation of chymotrypsinogen

To 1ml of a solution of 1mM-HCl containing 1.0mg of chymotrypsinogen was added 1ml of 0.2M-Tris–HCl buffer, pH8.0, containing 0.1mg of porcine trypsin. Porcine rather than bovine trypsin was used because its activity toward Ac-Tyr-OEt and Bz-Tyr-OEt is negligibly low. The solution was incubated for 2h at 2°C and then assayed for chymotrypsin activity.

Purification of A. oryzae proteinase

All steps were carried out at 4°C.

(a) *Preparation of an acetone precipitate.* Polidase-S (27g) was slowly stirred with 270ml of 60%-satd. $(NH_4)_2SO_4$ (390g added to 1 litre of water) for 30min. The precipitate was separated by centrifugation (10min at 16000*g*) and the clear supernatant was adjusted to 100% saturation by the slow addition of crystalline $(NH_4)_2SO_4$. After a further 15min of stirring the precipitate was collected by centrifugation (10min at 16000*g*), dissolved in 100ml of water and dialysed against 2×6 litres of water. For every 100ml of the contents of the dialysis bag 1ml of 1% (w/v) NaCl and 200ml of cold acetone were added. The precipitate formed was separated by centrifugation (10min, 16000*g*), dissolved in water, dialysed against water and freeze-dried.

(b) *Fractionation on P-cellulose column.* P-cellulose was prepared by successive washing with 0.5M-NaOH, water, 0.5M-HCl, water and the starting buffer, 0.026M-sodium formate buffer, pH3.8 (the molarity is that of the anionic component except where mentioned otherwise). The acetone precipitate (2.4g) was dissolved in 40ml of the starting buffer and applied to the P-cellulose column (4.2cm×30cm), previously equilibrated with the starting buffer. Elution was performed with the starting buffer (1600ml), followed by 0.26M-sodium formate buffer, pH3.8 (700ml). The flow rate was adjusted to 90ml/h and 9ml fractions were collected. The protein peak, eluted with 0.26M-sodium formate buffer (all tubes with E_{280} above 0.4), was pooled and dialysed overnight, against 2×6 litres of 0.05M-sodium acetate buffer, pH4.5.

(c) *Fractionation on DEAE-cellulose.* DEAE-cellulose prepared similarly to P-cellulose was equilibrated with 0.05M-sodium acetate buffer, pH4.5 (starting buffer) and packed into a column

* Abbreviations: Tos, toluene-*p*-sulphonyl; Ac, acetyl; Bz, benzoyl; Z, benzyloxycarbonyl; -OP$_r$4P$_r$, 3-(4-pyridyl)-propyl 1-ester.

(3.2cm × 30cm). The dialysed fraction obtained in the previous step was applied to the column and the elution was carried out with the starting buffer (540ml) followed by stepwise increases in buffer concentration as follows: 0.125M (360ml), 0.22M (540ml) and 0.40M (720ml). The flow rate was adjusted to 100ml/h and 9ml fractions were collected. The active fraction that eluted with 0.4M-sodium acetate buffer (all tubes with E_{280} above 0.4) was combined, dialysed against water and freeze-dried. This fraction, which was used for further studies, was designated *A. oryzae* proteinase.

Electrophoresis on cellulose acetate membranes

Electrophoresis was performed in a Beckman Microzone Electrophoresis system model R-100 in 0.08M-collidine–acetate buffer, pH7.0, 0.1M-pyridine–acetate buffer, pH5, or 0.1M-pyridine–formate buffer, pH3.2 (the molarity is that of the cationic component). Samples (0.25–0.75μl) of protein solution were applied to the membrane and the electrophoresis was performed for 60min, at 200V. The membranes were stained with Ponceau-S as described in the Beckman Method Manual (Model R-1: Microzone Electrophoresis System Rm-TB-010; 1967).

Electrofocusing in polyacrylamide gels

The electrofocusing was performed as described by Wrigley (1968) in tubes (0.5cm × 12cm) in the pH ranges 3–5 and 3–10 with a final concentration of polyacrylamide of 4.6%. The protein (50–150μg) was applied in 10% (w/v) sucrose solution, and electrofocusing was carried on for 6h at 350V and an initial current of 1mA per tube. The protein was fixed with 12% (w/v) trichloroacetic acid and stained with 1% Coomasie Blue in 10% trichloroacetic acid. The gels were destained with water–methanol–acetic acid (13:5:2, by vol.). At least one gel per run was electrofocused without protein. The pH gradient in these gels was checked by measuring the pH of slices of the gel soaked in water.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Protein samples were denatured by adding them to a boiling solution of 1% sodium dodecyl sulphate in 0.01M-sodium phosphate buffer, pH7.0, containing 1%-mercaptoethanol. After 10min at that temperature the samples were cooled and applied to gels by the general procedure of Weber *et al.* (1972).

Ultracentrifuge analysis

(a) *Determination of the sedimentation coefficient.* A sedimentation-velocity analysis was carried out in a Spinco model E ultracentrifuge at 56000rev./min, 20°C, with 0.8% *A. oryzae* proteinase in 0.1M-NaCl–0.01M-sodium acetate buffer, pH5.0.

(b) *Determination of the molecular weight.* The high-speed equilibrium method of Yphantis (1964) was used. The ultracentrifuge was operated at 32000rev./min or 36000rev./min at 20°C, until equilibrium was reached (24h). Three concentrations of protein (0.26, 0.4 and 0.53mg/ml) in 0.1M-NaCl–0.01M-sodium acetate buffer, pH5.0, were run in a six-place cell, after overnight dialysis against the same buffer. Photographs of the interference fringes were measured with a Nikon comparator.

Amino acid analysis

The freeze-dried protein (about 3mg) dissolved in about 3ml of 5.7M-HCl was hydrolysed in 0.5ml batches under vacuum for 24, 48, 72 and 96h at 107°C. Norleucine and α-aminoguanidinopropionic acid were added as standards. For determination of half-cystine as cysteic acid 20μl of dimethyl sulphoxide was added before hydrolysis (Wold, 1969).

Tryptophan was determined after 24h hydrolysis in the presence of 4% thioglycollic acid (Matsubara & Sasaki, 1969). Cysteic acid was also determined after performic acid oxidation (Hirs, 1967). Glucosamine was determined on the 15cm of a Spinco 120B amino acid analyser. All other analyses were carried out on a Spinco 121C instrument.

Detection of the N-terminal amino acid

The *N*-terminal amino acid was determined by Beckman sequencer model 890, by the method of Edman & Begg (1967) as modified by Hermodson *et al.* (1972).

Determination of kinetic parameters with different substrates

(*a*) *Trypsinogen and chymotrypsinogen.* A 1ml portion of bovine trypsinogen (0.17–1.70mg/ml) or chymotrypsinogen (0.39–3.9mg/ml) in 0.1M-sodium formate buffer, pH3.5, was incubated for 3min at 35°C with 50μl of *A. oryzae* proteinase containing 1.5 and 15μg of protein respectively. It was found in preliminary experiments that under these conditions the activation even at low substrate concentrations is within the initial velocity limits. The amount of trypsin or chymotrypsin formed was determined immediately by esterolytic assays at pH8.0 with Tos-Arg-OMe and Ac-Trp-OEt; this also stopped the activation reaction.

The amount of activated enzymes was calculated by assuming $k_{cat.}$ of 147s^{-1} for trypsin and 46.5s^{-1} for chymotrypsin (Cunningham, 1965).

The molar concentrations of the zymogens were calculated respectively from their specific extinctions ($E_{280}^{1\%}$) of 13.9 and molecular weights of 23700 for trypsinogen and of 20.0 and 25700 for chymotrypsinogen.

The $k_{cat.}$ and K_m values for the activation of the zymogens by *A. oryzae* proteinase as well as for its

action on synthetic substrates were calculated from double-reciprocal Lineweaver–Burk plots.

(*b*) *Z-His-Phe-Phe-OEt.* The activity on this substrate was followed by measuring the decrease in absorbance at 225nm owing to hydrolysis of the peptide bond. The reaction was carried out at 30°C in a Gilford 2400 spectrophotometer with an expanded scale (0.1 $E=100\%$). Substrate solution (0.5mM) was prepared by dissolving 6.1mg of the substrate in 70μl of formic acid, diluting to 20ml with water and adjusting the pH to 3.4 with 2M-NaOH. Further dilution of the substrate was made with the buffer prepared in a similar manner. The reaction was carried out in a cuvette which contained 2ml of the substrate (0.031–0.5mM) and 40μl of the enzyme (1mg/ml). The extent of hydrolysis for the peptide-bond cleavage was calculated assuming $\Delta\varepsilon_{225}=161.4$. This value was obtained experimentally by following the complete hydrolysis of glycylglycine. The only products of the enzymic reaction were identified as Z-His-Phe and Phe-OEt by t.l.c. as described by Inouye *et al.* (1966).

(*c*) *Z-Ala-Ala-Phe-Phe-OP*$_y$*4P*$_r$. The activity on this substrate was followed by the automated ninhydrin assay of Hollands & Fruton (1968), by using a Technicon autoanalyser. The substrate solution was prepared by dissolving 57.9mg of the substrate in 0.6ml of dimethylformamide and diluting to 60ml with 0.1M-HCl–KCl buffer, pH2.97. The reaction was carried out at 39°C with substrate concentrations varying from 0.235 to 0.7mM. The enzyme concentration was 7.3μg/ml. The reaction rates were calculated from the absorbance changes. The correlation between the absorbance and molarities was obtained by calibrating the system with 0.1mM solutions of leucine. It should be noted that the ninhydrin yield colour of Phe-OEt is 98% of that of leucine (Moore & Stein, 1948).

Activation of chymotrypsinogen by A. oryzae proteinase

Samples (1ml) of bovine chymotrypsinogen (1mg/ml) in 0.1M-sodium formate buffers (pH2.50–4.72) were incubated at 25° with 50μl of *A. oryzae* proteinase (75μg). Samples were removed from the activation mixtures at different times and assayed immediately for chymotrypsin activity with Ac-Tyr-OEt as a substrate.

Preparation of A. oryzae proteinase-activated chymotrypsin

Chymotrypsinogen (60mg) and the *A. oryzae* proteinase (5mg) were dissolved in 20ml of 0.05M-sodium formate buffer, pH3.5, and incubated at 0°C. This temperature was chosen because chymotrypsin remained fully active in the presence of the *A. oryzae* proteinase for at least 24h. After 4h of activation over 90% of the zymogen was activated. The pH was raised to 4.5 with 1M-NaOH and the activation mixture was applied to a DEAE-cellulose column (0.9cm×20cm) previously equilibrated with 0.05M-sodium acetate buffer, pH4.5. The column was developed with the same buffer at 60ml/h. All the chymotrypsin was recovered in the 'break-through', fraction whereas *A. oryzae* proteinase was wholly retained on the column and was eluted only by increasing the buffer concentration to 0.4M. The fractions which contained the chymotrypsin activity (24ml) were pooled, desalted on a Sephadex G-25 column (2.6cm×35cm) equilibrated with 1mM-HCl, freeze-dried. The yield was 52mg. All the chromatographic procedures were carried out at 4°C.

Digestion of A. oryzae proteinase-activated chymotrypsin by carboxypeptidase B

A. oryzae proteinase-activated chymotrypsin (10mg) was dissolved in 2ml of 1mM-HCl. Samples were removed for determination of the molar concentration and active-site titration. To the rest of the material 5μl of 1.0M-di-isopropyl phosphorofluoridate and 0.12ml of 1M-Tris–HCl buffer, pH8.5, were added. After 10min no chymotryptic activity was left. Carboxypeptidase B (10μl, containing 35μg) was added and the incubation was continued at 23°C. Samples were removed after 0, 0.5, 1.0, 3.0, 5.0 and 24h, mixed with 0.2M-sodium citrate buffer, pH2.2, and analysed for their amino acid content.

Table 1. *Purification of A. oryzae acid proteinase*

The DEAE-cellulose eluate was designated as the *A. oryzae* proteinase

Fraction	Volume (ml)	E_{280}	Total protein (E_{280} units)	Specific activity (munits/E_{280} units)	Total activity (munits)	Yield (%)
Polidase-S solution	266	132.00	35000	0.106	3720	100
Acetone precipitate	40	95.00	3800	0.27	1045	28
P-cellulose eluate	200	2.50	500	1.34	670	18
DEAE-cellulose eluate	105	1.22	128	2.54	326	9

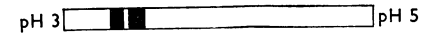

Fig. 1. *Isoelectric focusing of A. oryzae proteinase in* 4.6% *polyacrylamide gels*

The pH gradient was 3–5 (from left to right); 50μg of protein was applied per tube and the isoelectric focusing was performed for 6h at 350V with maximal current of 1mA per tube.

Table 2. *Amino acid composition of A. oryzae proteinase*

The number of residues per molecule was the average (±S.D.) from four analyses after 24, 48, 72 and 96h of hydrolysis based on mol.wt. 39400, except for the values of threonine and serine which were extrapolated to zero time of hydrolysis. Half-cystine was measured as cysteic acid after oxidation, and tryptophan after hydrolysis in the presence of 4% thioglycollic acid.

Amino acid	Number of residues per molecule	Nearest integral
Lysine	21.6±0.8	22
Histidine	5.5±0.2	5–6
Arginine	1.6±0.1	1–2
Aspartic acid	44.6±0.6	45
Threonine	24.5	24–25
Serine	30.9	31
Glutamic acid	34.5±0.5	34–35
Proline	15.6±0.6	13
Glycine	43.2±0.1	43
Alanine	32.2±0.2	32
Half-cystine	2.1	2
Valine	28.7±0.3	29
Methionine	1.1±0.1	1
Isoleucine	16.4±0.3	16
Leucine	23.9±0.6	24
Tyrosine	15.6±0.3	16
Phenylalanine	19.7±0.3	20
Tryptophan	6.3	6
Glucosamine	1.4±0.1	1–2
Other hexoses (assumed)	3	2–4
Total		347–351

Results

Purification and homogeneity of A. oryzae proteinase

The purification procedure is summarized in Table 1. As shown 27g of Polidase-S yielded about 100mg of purified enzyme and the yield of activity was approx. 10%.

It should be noted that the first step of purification resulted in rather poor yield of activity, although the presented procedure is optimal. This is most probably because the acid proteinase is precipitated in a rather wide range of $(NH_4)_2SO_4$ or acetone concentrations.

The specific extinction coefficient of *A. oryzae* proteinase was calculated from the residue weight of the constituent amino acid residues after amino acid analysis with norleucine and α-aminoguanidinopropionic acid as standards (which were added to a solution of enzyme of known E_{280}) and was found to be $E^{1\%}_{1cm} = 16.0$ at 280nm.

The homogeneity of the *A. oryzae* proteinase was tested by electrophoresis, isoelectric focusing in polyacrylamide gels and ultracentrifugation. Only one band was found in electrophoresis at pH5 and 7; however, electrophoresis at pH3.2 and isoelectric focusing (see Fig. 1) revealed that the material is not homogeneous but consists of two very closely migrating bands. On the other hand, homogeneity in size was indicated by sedimentation analysis. A single symmetrical peak was obtained in a sedimentation-velocity experiment and linear Yphantis (1964) plots were obtained in sedimentation-equilibrium experiments at 32000 and 36000rev./min. Attempts to separate the two bands obtained in gel electrophoresis were made by using preparative isoelectric focusing in sucrose gradients (Haglund, 1971) at pH ranges of 3–6 and 3.8–4.9 and resulted only in a partial separation in the latter gradient. However, since the amino acid composition and specific activities of the partially separated peaks (pI values 3.9 and 4.1) were identical it was concluded that the *A. oryzae* proteinase exists most probably in two forms that differ very little from each other.

Molecular weight and amino acid composition

Since sedimentation analysis resulted in one symmetrical peak with $s_{20,w} = 3.63$S (concentration = 0.8%) the molecular weight was calculated from sedimentation equilibrium by the meniscus-depletion method of Yphantis (1964). A partial specific volume $v = 0.726$ was calculated from the amino acid composition. The average value from three runs made at 36000rev./min was 39400±350 daltons. At 32000rev./min a slightly higher value (40700±500) was obtained, but since at both speeds no concentration-dependence was found this difference is probably due to a small amount of aggregate.

The amino acid composition of *A. oryzae* proteinase is summarized in Table 2. The results were calculated by assuming a molecular weight of 39400. One molecule consists therefore of 363–367 amino acids and contains 1–2 molecules of glucosamine. If one assumes two hexose residues per one glucosamine unit (Graham *et al.*, 1973) one molecule of *A. oryzae* proteinase contains three to six sugar molecules, to give a carbohydrate content of 1–2%. The value of arginine (1.6 residues/molecule) is relatively far from the integral values of one or two. So far no explanation for this result can be proposed.

Kinetic properties

Some kinetic properties are given in Table 3. The enzyme hydrolyses Z-His-Phe-Phe-OEt by cleaving

Table 3. *Kinetic parameters of A. oryzae proteinase*

The experiment with Z-Ala-Ala-Phe-Phe-OP_y4P_r was performed in pH2.97 since the substrate is insufficiently soluble at higher pH.

Substrate	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)	Temp. (°C)	pH
Z-His-Phe-Phe-OEt	0.064	1.65	30.6	30	3.5
Z-Ala-Ala-Phe-Phe-OP_y4P_r	0.037	0.35	9.5	39	2.97
Bovine trypsinogen	0.10	13.0	113.0	35	3.5
Bovine chymotrypsinogen A	0.18	1.14	6.3	35	3.5

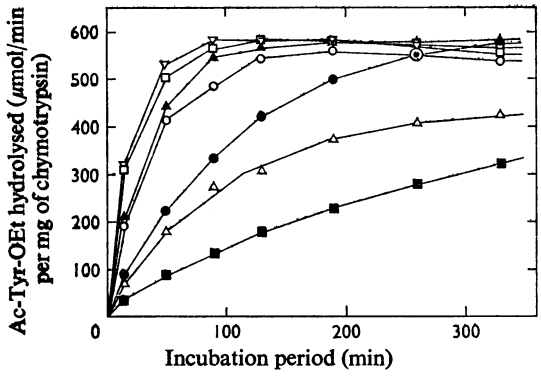

Fig. 2. *Effect of pH on activation of chymotrypsinogen by A. oryzae proteinase*

Samples (1 ml) of bovine chymotrypsinogen (1 mg) in 0.1 M-sodium formate buffers (pH2.50, △; pH2.85, ○; pH3.10, □; pH3.6, ▽; pH3.95, ▲; pH4.35, ●; pH4.72, ■) were incubated with 50 μl of *A. oryzae* (75 μg) at 25°C. Samples were removed from the activation mixture and immediately assayed for chymotrypsin activity by using Ac-Tyr-OEt as substrate.

the Phe-Phe bond, and Z-Ala-Ala-Phe-Phe-OP_y4P_r, presumably also at the Phe-Phe bond. The kinetic constants are similar to those observed with other acid proteinases, both mammalian and fungal, acting on similar substrates (Voynick & Fruton, 1971). Like other acid proteinases *A. oryzae* proteinase acts only very slowly on small peptides. Thus preliminary experiments indicate that acetylphenylalanyl-3,5-diiodotyrosine is cleaved at a rate less than 1% of that of Z-His-Phe-Phe-OEt.

The rate of activation of bovine trypsinogen is greater than that of chymotrypsinogen by a factor of about 10, whereas the K_m values are comparable. Although the activation of chymotrypsinogen by acid proteinases has been observed previously (Gabeloteau & Desnuelle, 1960) no detailed study of the mechanism has been carried out. The action of *A. oryzae* proteinase on chymotrypsinogen was therefore investigated in detail.

(*a*) *Time-course and pH-dependence.* The time-course of activation of chymotrypsinogen at different pH values is summarized in Fig. 2. The maximal rate of activation was at pH3.2 and 3.6, but activation at pH2.85, 3.95 and 4.35 gave the same specific activity after full activation.

This specific activity was almost the same as that obtained by trypsin activation under conditions which lead to formation of δ- and π-chymotrypsin (Miller *et al.*, 1971) and higher than that of commercially available α- and δ-chymotrypsins (Table 4). The ratio of the activity of α-chymotrypsin with Bz-Tyr-OEt to that with Ac-Tyr-OEt is considerably lower than that for δ-chymotrypsin and offers a good criterion for distinguishing between the two forms (Table 4). The *A. oryzae* proteinase-activated zymogen had the same ratio as that of chymotrypsin activated by trypsin under conditions which favour the formation of π- and δ-chymotrypsin. Fig. 2 shows that the chymotrypsin formed by *A. oryzae* proteinase is stable over at least twice the time required for full activation.

(*b*) *Isolation and characterization of the activation product.* The isolation procedure is described in the Experimental section. Electrophoresis of the isolated chymotrypsin in polyacrylamide gels containing sodium dodecyl sulphate and mercaptoethanol showed a single band of molecular weight around 25000. Active-site titration revealed 0.87 mol of active site/mol of enzyme. *N*-Terminal analysis yielded only two sequences: the major one, Ile-Val-Asn-Gly-Glu-Ala, corresponds to the known sequence of chymotrypsin beginning at isoleucine-16 and the minor one, ?-Gly-Val-Pro-Ala, corresponds to the sequence of the activation peptide (Dayhoff, 1972), starting at cysteine-1.

This peptide appeared as a minor chain because it is relatively soluble in the sequencer solvents and was washed out of the cup as indicated by the appearance of arginine in the aqueous phase [arginine is estimated by a spot test, which reacts also with arginine peptides as described by Hermodson *et al.* (1972)], and the low yield of the phenylthiohydantoin-amino acids. The absence of other sequences indicated that *A. oryzae* proteinase had only cleaved

Table 4. *Activities of A. oryzae proteinase-activated chymotrypsin, trypsin-activated chymotrypsin, α- and δ-chymotrypsin on Bz-Tyr-OEt and Ac-Tyr-OEt*

The activation with *A. oryzae* proteinase was carried out at 25°C with *A. oryzae* proteinase chymotrypsinogen ratio of 1:13.3 (w/w) and the activation with porcine trypsin was carried out at 0°C with a trypsin chymotrypsinogen ratio of 1:10 (w/w). The activity is expressed in μmol of substrate hydrolysed/min per mg of enzyme.

Enzyme	Activity on: (1) Bz-Tyr-OEt	(2) Ac-Tyr-OEt	Ratio of (1)/(2)
A. oryzae proteinase-activated chymotrypsin at pH3.1 for 190min	95	580	0.164
A. oryzae proteinase-activated chymotrypsin at pH3.6 for 190min	94	575	0.164
Trypsin-activated at pH8.0 for 120min	101	602	0.168
α-Chymotrypsin	54	502	0.108
δ-Chymotrypsin	83	535	0.157

Table 5. *Release of arginine from A. oryzae proteinase-activated chymotrypsin by digestion with carboxypeptidase B*

Activated chymotrypsin (10mg) was rapidly inactivated by excess of di-isopropyl phosphorofluoridate and digested at pH8.5 with carboxypeptidase B (35μg). Samples were removed during the time-course of the activation and assayed for amino acid content. The concentration of the active enzyme was estimated by active-site titration before inactivation by di-isopropyl phosphorofluoridate.

Time of incubation (h)	Arginine (mol/mol of active enzyme)
0	0.03
0.5	0.69
1.0	0.74
3.0	0.79
5.0	0.82
24.0	0.93

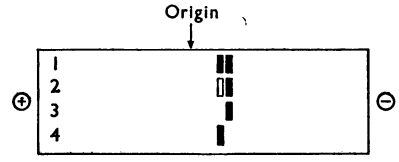

Fig. 3. *Cellulose acetate electrophoresis of A. oryzae proteinase-activated chymotrypsin*

The electrophoresis was performed at room temperature in 0.08M-collidine–acetate buffer, pH7.0, for 30min, at a potential of 400V (the cathode on the right side). Samples (0.5μl) containing 10μg of the following proteins were applied: (1) *A. oryzae* proteinase-activated chymotrypsin; (2) *A. oryzae* proteinase-activated chymotrypsin pre-treated with di-isopropyl phosphorofluoridate; (3) chymotrypsinogen; (4) δ-chymotrypsin. Colour intensity: ■, strong; □, weak.

the $Arg_{(15)}$-$Ile_{(16)}$ bond giving π-chymotrypsin, although the possibility existed that another cleavage in the activation peptide (residues 1–15) had occurred leading to δ-chymotrypsin or a similar form of the enzyme. In order to test this, *A. oryzae* proteinase-activated chymotrypsin was rapidly inactivated with a large excess of di-isopropyl phosphorofluoridate and digested with carboxypeptidase B. Table 5 shows that up to 0.9mol of arginine/mol of active enzyme was released and it was concluded that the activation peptide had remained at least 90% intact. This conclusion is warranted because any cleavage within the activation peptide would have released a small peptide containing arginine-15. This would have been removed by the passage of the preparation through Sephadex G-25. Surprisingly electrophoresis on cellulose acetate of the *A. oryzae* proteinase-activated chymotrypsin at pH7.0 (see Fig. 3) revealed the presence of two approximately equal bands, one of them with the mobility of chymotrypsinogen, which is equal to that of π-chymotrypsin at this pH and a second one corresponding to δ-chymotrypsin which has one positive charge less owing to removal of the dipeptide $Ser_{(14)}$-$Arg_{(15)}$. In the enzyme which was rapidly inactivated with di-isopropyl phosphorofluoridate the band corresponding to δ-chymotrypsin was very weak, however, and since the conversion of π-chymotrypsin into δ-chymotrypsin is extremely fast (Bettelheim & Neurath, 1955) we consider that the appearance of the band corresponding to δ-chymotrypsin results from partial $\pi \rightarrow \delta$ conversion during the electrophoresis at pH7.0. This was further substantiated by the observation that when the *A. oryzae* proteinase-activated chymotrypsin was dissolved in 0.05M-Tris–HCl buffer, pH8.0, a progressive disappearance of the faster band and simultaneous appearance of the slower band was observed. Unfortunately electrophoresis at pH3–4, where the conversion would not occur, did not resolve the bands. It may therefore be concluded that the activation of chymotrypsinogen by *A. oryzae*

proteinase is due to the split of the $Arg_{(15)}$-$Ile_{(16)}$ bond, and the main if not the sole product is π-chymotrypsin.

Discussion

Although there has been an increasing interest in the structure and function of fungal acid proteinases in recent years most of the enzymes studied can only be prepared in quantities needed for structural studies if large-scale fermentation units are available. The purification of *A. oryzae* proteinase, however, starts from material which is readily available commercially and thus makes this enzyme more generally accessible. *A. oryzae* proteinase is clearly related to other fungal acid proteinases such as penicillopepsin and aspergillopeptidase A, as the present study shows. Their molecular weights are similar, they hydrolyse small peptides only poorly, if at all, and appear to require an extended peptide chain for optimal activity. This property is also observed with pepsin (Fruton, 1970) and chymosin (Voynick & Fruton, 1971), enzymes which are homologous to the fungal enzymes (Hofmann, 1975). Comparative studies of the mammalian and fungal enzymes may well become valuable in the elucidation of their mechanism of action, which is as yet poorly understood (Hofmann, 1975).

In addition the fungal enzymes have been useful in the study of trypsinogen activation (Nakanishi, 1959*b*; Gabeloteau & Desnuelle, 1960; Sodek & Hofmann, 1970; Robinson *et al.*, 1973). The purification method described here leads to an enzyme preparation which appears enzymically homogeneous, although after electrophoresis two bands are observed.

The difference in the electrophoretic properties could result from deamidation of one or more amido groups, during the purification procedure. We tried to test this possibility by preincubating *A. oryzae* proteinase in 0.1 M-sodium acetate buffer, pH4.5, for 5h at 37°C before electrophoresis. No change in the electrophoretic pattern was observed after this treatment, hence this possibility is not very likely although it cannot be excluded.

A limited autolytic cleavage cannot account for the difference because no free *N*-terminal groups could be detected in a sequenator analysis of the two proteins.

The presence of glucosamine in the hydrolysate of the enzymes shows the presence of a sugar moiety which probably has sialic acid in a terminal position. A difference in sialic acid content could very well account for the electrophoretic difference. Sialic acid is often found as part of proteinbound carbohydrate chains (Spiro, 1970) but small differences in amino acid sequence, evidence for genetic variants, cannot be ruled out.

It should be noted that microbial acid proteinases from *Rhizopus chinensis* (Graham *et al.*, 1973) and *Rhizopus oligosporus* (Wang & Hesseltine, 1970) also exist in two or more multiple forms, although in the case of *Rhizopus chinensis* the difference in the isoelectric points is somewhat larger (5.2 and 6.0) and the two isoenzymes were successfully separated (Graham *et al.*, 1973).

Although the molecular weight and the enzymic properties suggest that *A. oryzae* proteinase is closely related to other acid proteinases there is as yet no good evidence for homology. Sequence studies could readily provide such information, but unfortunately an attempt to determine the *N*-terminal sequence in a sequenator failed. It appears that the *N*-terminus is blocked.

The maximum rate of the trypsinogen activation by *A. oryzae* proteinase ($k_{cat.} = 11.3 s^{-1}$) is almost identical with that of penicillopepsin at the same pH and temperature ($k_{cat.} = 11 s^{-1}$; Hofmann & Shaw, 1964), but there is a greater than 10-fold difference in the K_m (100 μM for *A. oryzae* proteinase and 7.6 μM for penicillopepsin). Whitaker (1972) measured the same parameters for an enzyme from the mould *Endothia parasitica* at 30°C and pH4.0 and found $k_{cat.} = 49\ s^{-1}$ and $K_m = 83\ \mu$M. Morihara & Oka (1973) found large variations in the rate of trypsinogen activation during a study of the relationship between trypsinogen-activating ability of seven acid proteinases and their specificity towards lysine peptides. An enzyme from *Mucor mihei* did not activate trypsinogen; it also failed to hydrolyse lysine peptides.

A. oryzae proteinase readily activates chymotrypsinogen, although the rate is only one-tenth of that of trypsinogen activation. The Michaelis constant, however, is similar. Only a little work has been reported in the literature of chymotrypsinogen activation by fungal acid proteinases. Penicillopepsin (Hofmann & Shaw, 1964) and *Endothia* acid proteinase (Whitaker, 1972) failed to activate this zymogen. Activation was observed by Gabeloteau & Desnuelle (1960) by an enzyme from *A. saitoi*. The rate of activation, however, was much lower than that of *A. oryzae* proteinase, only 75% of the maximal potential activity was reached and the product formed was unstable, probably owing to concurrent partial digestion by the active enzyme (or other contaminating peptidases). Our study, in contrast, shows that the π-chymotrypsin formed by *A. oryzae* proteinase is not further cleaved and remains stable over an extended period. The specific activity of π-chymotrypsin produced by *A. oryzae* proteinase (580 μmol of Ac-Tyr-OEt/min per mg) is very close to that reported by Wilcox (1970) of 600 μmol of Ac-Tyr-OEt/min per mg. Whereas previously π-chymotrypsin could only be obtained by rapid tryptic activation in the presence of β-phenylpropionate (Bettelheim & Neurath, 1955), which limited the $\pi \rightarrow \delta$ conversion

but did not completely prevent it, the use of *A. oryzae* proteinase as described here now offers a method for the production of a stable π-chymotrypsin in good yield and purity. If it is stored after freeze-drying from a solution at pH3–4 it should be stable for prolonged periods.

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