Properties of a New Alkaline Proteinase from *Aspergillus niger*

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***A. niger* LCF 9 synthesizes a new aspergillopeptidase of potential interest in therapeutics. The properties and operating range of the enzyme were determined. It is a semi-alkaline aspergillopeptidase (EC 3.4.23.4) with one endopeptidase activity. Its pI is 4.10, its molecular weight is 21000 Da and its $A^{1\%}_{1cm}$ at 280 nm is 9.75. It rapidly hydrolyzes casein and hemoglobin. Its optimal pH is 7.8 and optimal temperature is 45 °C. It is thermally labile above 40 °C but can be stabilized by adding calcium ions. It is inhibited by phenylmethylsulfonyl fluoride (PMSF), by ethylenediaminetetraacetic acid (EDTA) and by certain metals ions, *e.g.* copper, manganese and cobalt ions. It has no dipeptidase or tripeptidase activity and its esterase activity is weak. It has a high collagenase activity and is to our knowledge the only aspergillopeptidase that is active towards benzoyl-arginine *p*-nitroanilide (BAPNA).**

Keywords serine proteinase; *Aspergillus niger*; molecular weight; isoelectric point; property

Introduction

Micro-organisms of the genus *Aspergillus* are classically used for the industrial production of proteolytic enzymes. Besides their well-established used in food processing, these enzymes have applications in therapeutics. Those of greatest interest are the carboxylproteinases, which have trypsinogen-kinase activity, and in particular the semi-alkaline proteinases which are used for the cleaning of wounds and which can have marked anti-inflammatory activity.[1] These proteinases can also be used in the production of other materials such as immunizing substances and proteolysates for dermatological preparations (*e.g.* collagen derivatives), and generally as reagents for synthesis.[1]

In previous studies[1,2] a strain from a laboratory collection, *A. niger* LCF 9, was found to synthesize large amounts of proteinases active at weakly alkaline pH. The main enzyme produced was an aspergillopeptidase that was highly active towards benzoyl-arginine *p*-nitroanilide (BAPNA). Optimal fermentation conditions were defined and the process was extended to a pilot scale. To assess possible applications, the properties and operating range of the enzyme were investigated. We report here the results of a study of the activity of this enzyme under various conditions.

Materials and Methods

Apparatus High performance liquid chromatography (HPLC): Waters 510 pumps, Waters U 6K injector, Waters 680 gradient programmer, Waters 490 programmable multi-wavelength spectrophotometer. Merck D_{2000} recorder-integrator. Electrofocusing: Multiphor LKB 2117—301. Analysis: Beckman DB-G spectrophotometer.

Chemicals Reagents were of analytical grade from Merck. Enzyme substrates and inhibitors were purchased from Sigma. Reference proteinases were Pronase grade B (Rapidase), trypsin PTN G-OS (Novo) and trypsin ref. 4035 (Sigma).

Production and Purification of Aspergillopeptidase Semi-alkaline proteolytic enzymes (92.3 IU/ml) were obtained from a culture of *Aspergillus niger* LCF 9 on defatted soy cakes.[2] Two grades of enzyme were prepared; an industrial grade of 80% purity and an analytical grade of 100% purity. The industrial grade enzyme was produced on a pilot scale with a yield of 0.52% from the solid starting material.[3]

Isoelectric Focusing The pI was determined by horizontal analytical electrofocusing on PAG plates (LKB 3.5—9.5). Focusing conditions were 1650 V at 4 °C for 2.5 h. The calibration curve was obtained by electrofocusing of a Pharmacia calibration kit of low pI proteins and solutions of horse myoglobin and trypsinogen.

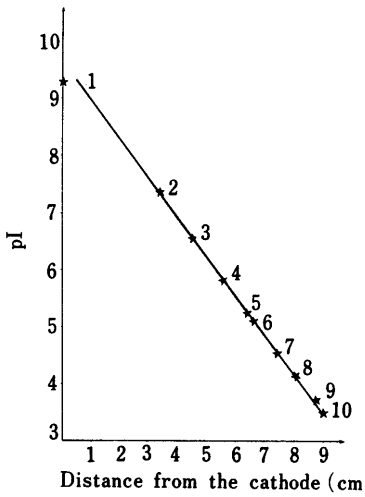

Fig. 1. Determination of the pI of *Aspergillus niger* LCF 9 Proteinase by Analytical Electrofocusing

(1) Trypsinogen (pI 9.3), (2) horse myoglobin (pI 7.35), (3) human carbonic anhydrase (pI 6.55), (4) bovine carbonic anhydrase (pI 5.85), (5) lactoglobulin (pI 5), (6) *A. niger* LCF 9 proteinase, (7) soybean trypsin inhibitor (pI 4.55), (8) glucose oxydase (pI 4.1), (9) methyl red (pI 3.75), (10) amyloglucosidase (pI 3.5).

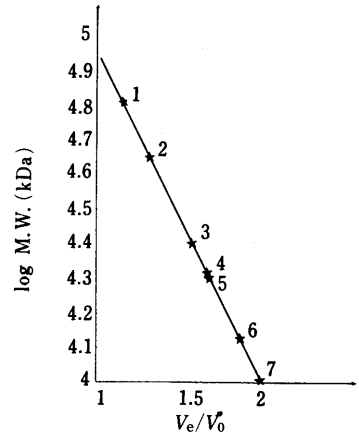

Fig. 2. Determination of the Molecular Weight of *Aspergillus niger* LCF 9 Proteinase by HPSEC.

Conditions: 0.78 × 30 cm Protein-Pack I_{125} Waters column, isocratic elution with Na_2HPO_4 buffer (pH 7) containing 0.32 M NaCl–96% EtOH (80 : 20, v/v), flow rate, 1 ml/min; detection at 280 nm; sensitivity, 0.005 aufs.

(1) Bovine albumin (M.W. 66000), (2) egg ovalbumin (M.W. 45000), (3) α-chymotrypsinogen (M.W. 25000), (4) *A. niger* LCF 9 proteinase, (5) Soybean trypsin inhibitor (M.W. 20100), (6) ribonuclease A (M.W. 13700), (7) tripeptide (M.W. < 1000).

Molecular Weight Molecular weight was determined by high performance size exclusion chromatography (HPSEC) by the method of Hefti.[4] A Waters 0.78 × 30 cm Protein-Pack I_{125} column was used. The eluant was a mixture (80:20, v/v) of 0.08 M Na_2HPO_4 buffer (pH 7.0) containing 0.32 M NaCl and 96% ethanol. The flow rate was 1 ml/min and detection was at 280 nm with a sensitivity of 0.005 aufs (absorbance unit full scale). The calibration curve was obtained by injecting successively 50 μg of each standard. The retention time corresponding to the dead volume of the column was determined by injecting ferritin (M. W. 470000 Da). Maximum duration of analysis was determined by injecting a tripeptide, glycyl-glycyl-proline.

Assays of Enzyme Activity Proteolytic Activity: A. On Casein Substrate: Caseinolytic activity was determined by incubating 1 ml of enzyme solution with 1 ml of 1% Hammarsten casein preincubated at 40 °C. After 10 min of incubation at 40 °C, the reaction was stopped by adding 3 ml of 5% trichloroacetic acid. After 5 min, the unhydrolyzed casein was removed by centrifugation at 3000 rpm for 10 min. Then 4 ml of 0.5 M Na_2CO_3 and 1 ml of Folin-Ciocalteu reagent were added to 1 ml of the supernatant. The mixture was left for 15 min at 20 °C and then the absorbance was measured at 660 nm. One unit of the enzyme activity was defined as the amount of the enzyme that released 1 μmol of tyrosine per min under the standard conditions.

B. On Hemoglobin as Substrate: Activity on hemoglobin was determined by the method of Anson.[5]

Esterase Activity was determined with benzoyl-arginine ethyl ester (BAEE) using the method of the French Pharmacopoea[6] and with benzoyl-benzoyl tyrosine ethyl ester (BTEE) using the same method. Measurements were made at 253 nm for BAEE and at 256 nm for BTEE.

Leucine aminopeptidase (LAP) activity was determined with leucyl-naphthylamide using the method of Goldbarg and Rutenburg[7] and with leucinamide using the method of Appel.[8]

Amino acid arylamidase activity was determined with various *p*-nitroanilide substrates using the method of Fritz *et al.*[9]

Dipeptidase and tripeptidase activity was measured by the formol titration method of Appel.[10]

Carboxypeptidase activity was measured toward various carbobenzoxypeptides by incubating 0.5 ml of 1 mg/ml enzyme solution with 1 ml of substrate for 30 min at 40 °C. The reaction was stopped by placing the tubes in a bath at −15 °C and the lysate was assayed by the ninhydrin method.[11] The results are expressed in μmol of released glycine per mg of enzyme per min.

Collagenase activity was determined by the method of Appel, with glycyl-propyl-glycyl-glycyl-propyl-alanine.[12]

Determination of Hydrolysis Products Released acids were separated by thin layer chromatography (TLC) on Kieselguhr F_{1500} silica gel plates with *n*-BuOH–CH_3COOH–H_2O (3:1:1, v/v/v) and $CHCl_3$–MeOH–CH_3COOH (95:5:3, v/v/v) as developing solvents and visualized by spraying the plates with ninhydrin–collidine and heating in an oven at 110 °C for 5 min. Benzyloxycarbonylated radicals were eliminated before visualization by spraying with 40% hydrobromic acid.

The hydrolysis products were characterized by reverse-phase TLC on C_{18} gel plates with CH_3Cl–CH_3COOH–H_2O (85:5:10, v/v/v) containing 0.1 M HCl. The TLC behavior of reaction products was compared with that of control samples.

Determination of Inhibitors Inhibitors were identified by the method of Fritz *et al.*[9] with BAPNA.

Results

Physicochemical properties were determined with analytical-grade (100% purity) enzyme.

Absorption Coefficient: The absorption coefficient was determined by UV spectroscopy of analytical-grade enzyme in 0.05 M Tris–HCl buffer (pH 7.5). The $A_{1\,cm}^{1\%}$ at 280 nm was 9.75.

Isoelectric Point: Isoelectric focusing showed that the pI value of the enzyme was 4.1.

Molecular Weight: The aspergillopeptidase was eluted in 8.87 min, which corresponds to a molecular weight of 21100 Da. SDS-electrophoresis gave a value of 21000 Da.

Enzyme Activity Since recent tests, to be published shortly, have shown that the industrial-grade enzyme is of practical interest, all the measurements of enzyme activity were made using the industrial-grade material.

Activity towards Naturally Occurring Substrates: The enzyme is a true endopeptidase that rapidly hydrolyses casein and hemoglobin. Its specific activities towards these

Table I. Activity towards BAPNA, Comparison with Various Commercially Available Enzymes

Enzyme	Final concentration (μg)	Duration of incubation (min)	OD/min
Aspergillopeptidase[a]	10	3	0.005
		5	0.005
		10	0.004
	50	3	0.043
		5	0.042
		10	0.043
	100	3	0.088
		5	0.090
Sigma trypsin	10	3	0.045
		5	0.045
	50	3	0.226
Novo trypsin	10	3	0.028
		5	0.029
	50	3	0.145
Pronase grade B	50	3	0.008
		5	0.009
	100	3	0.021
		5	0.021
	500	3	0.086
		5	0.085

a) Industrial quality.

Table II. Activity towards Synthetic Peptides

Substrate	Specific activity (μmol/min·mg)
Arylamidase activity	
Leucyl-naphthylamide	—
Leucinamide	—
Leucine-*p*-nitroanilide	—
Glutamyl-*p*-nitroanilide	—
Proline-*p*-nitroanilide	—
N-α-Benzoyl-arginine *p*-nitroanilide	0.257
Esterase activity	
Benzoyl-arginine ethyl ester	0.342
Benzoyl-tyrosine ethyl ester	0.338
Carboxypeptidase activity	
Z-glutamyl-tyrosine	—
Z-prolyl-leucine	—
Z-glycyl-phenylalanine	—
Z-glycyl-glycine	—
Proteinase activity	
Z-prolyl-leucyl-glycine	—
Z-glycyl↓prolyl-leucine	0.00035
Z-glycyl↓glycyl-proline	0.1298
Z-glycyl↓leucyl-proline	0.0012
Z-glycyl↓prolyl-glycyl-leucyl-proline	0.0024
Z-leucyl-lysyl-prolyl-glycine	—
Z-glycyl↓prolyl-glycyl↓glycyl↓prolyl-alanine	0.1976
Dipeptidase and tripeptidase activity	
Glycyl-proline	—
Prolyl-glycine	—
Prolyl-leucine	—
Glycyl-prolyl-alanine	—
Leucyl-glycyl-proline	—

substrates were 0.913 and 0.190 IU/mg, respectively at pH 7.8 and 40 °C. Its caseinolytic activity was marked, being equal to that of Pronase grade B, twice that of Novo trypsin and 1.3 times that of Sigma trypsin. Its activity towards hemoglobin was about three times that of Novo trypsin but it is only 15% of that of Proteinase II of *A. oryzae* which is one of the most active enzymes towards casein.

Activity towards Synthetic Substrates: The aspergillopeptidase had neither carboxypeptidase nor aminopeptidase activities. It was inactive towards dipeptides and tripeptides and its esterase activity was very low. However, it was very active towards BAPNA, a specific trypsin and plasmin substrate. Its activity towards this substrate was 20% of that of Sigma trypsin and 30% of that of Novo trypsin. Hence the peptide bond involving the carboxyl group of arginine is susceptible to be one of the scissile bonds by the enzyme, in contrast to other aspergillopeptidases which have a chymotrypsin-type specificity.[1] Three carbobenzoxytripeptides were hydrolyzed. No free amino acids could be detected and so the points of attack of the enzyme must be those given in Table II.

The Y–Gly–X bond is hydrolyzed very rapidly when Y is glycine and much more slowly when Y is proline. This property suggests the likelihood of a collagenase type activity. This was confirmed by determining the activity of the aspergillopeptidase towards a synthetic substrate classically used for assay of clostridiopeptidase, Gly–Pro–Gly–Gly–Pro–Ala, which was hydrolyzed very rapidly. Reverse phase TLC of the hydrolysate showed two tripeptides and traces of glycine. The point of attack of the enzyme is therefore the same as that of clostridiopeptidases A, namely the Gly–Gly linkage. As the enzyme used for these trials was the industrial grade material, it is likely that the very slight iminopeptidase activity observed was due to a contaminant and that the aspergillopeptidase from *A. niger*, like aspergillopeptidase C from *A. oryzae*,[13] is inactive towards the bonds involving the imine end of proline or hydroxyproline. This was confirmed by trials with analytical quality enzyme.

Optimal Conditions for Action: Optimal conditions for action were determined by measuring caseinolytic activity under various conditions. Activity was maximal in 0.05 M Tris–HCl buffer (pH 7.8) with an enzyme–substrate ratio of 1/40 and at an incubation temperature of 45 °C. Under these conditions the activity was linear over the range of incubation times between 10 and 45 min.

Influence of pH on Activity and Stability: The aspergillopeptidase was active over a wide range of pH (5—8.5). Its range of efficiency, defined by Reet[14] as the range over which the enzyme retains 80% of its activity, was pH 7.2 to 8.5.

To determine the influence of pH on the stability of the enzyme, two series of tests were run, one at 4 °C and the other at 40 °C.[15] The enzyme was incubated with the relevant buffer for 24 h at 4 °C and for 45 min at 40 °C. After incubation, the solutions were diluted tenfold with 0.05 M Tris–HCl (pH 7.8) and the activity determined as above. At 4 °C the enzyme in solution was stable for 24 h between pH 5 and 7.5, but at 40 °C it was rather unstable even at the pH of optimum stability. Freeze-dried enzyme stored at −18 °C had lost no activity after eight months.

Influence of Temperature on Activity and Stability: Two types of test were carried out at pH 5.2, the pH value of optimum stability, and at pH 7.8 the pH value of optimum activity. At each pH, the influence of calcium on stability

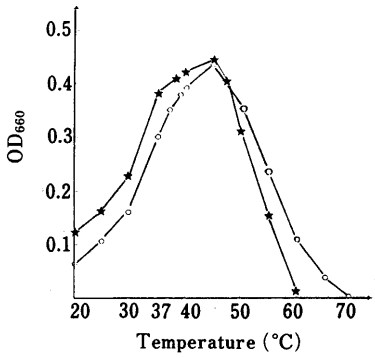

Fig. 4. Influence of Temperature on the Activity of *Aspergillus niger* LCF 9 Proteinase

The enzyme (1 ml, 250 μg/ml) without preincubation (★) or preincubated for 10 min at the relevant temperature (○) was mixed with 1 ml of 1% Hammarsten casein solution. The substrate was dissolved in 50 mM Tris–HCl buffer, pH 7.8. After incubation for 10 min, the proteinase activities were assayed.

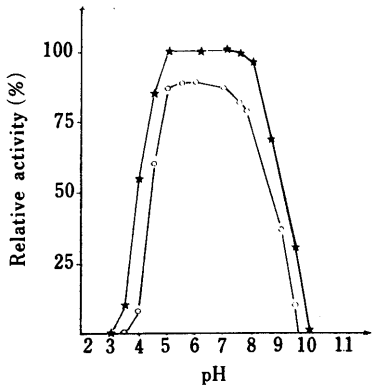

Fig. 3. Influence of pH on Stability of *Aspergillus niger* LCF 9 Proteinase

The proteinase (1 ml, 50 μg/ml) was mixed with the relevant buffer for 24 h at 4 °C (★) or 45 min at 40 °C (○). After incubation, the solutions were diluted tenfold with 0.05 M Tris–HCl (pH 7.8) and their activities were assayed at 40 °C. Buffers (0.5 M) used were sodium acetate, HCl buffer (pH 3—5), bis-Tris–HCl buffer (pH 5.5—7), Tris–HCl buffer (pH 7.5—9) and glycine, NaOH Sorensen buffer (pH 9.5—11).

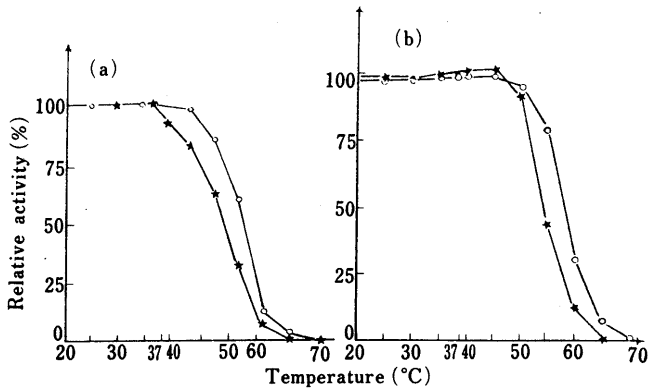

Fig. 5. Influence of Temperature on the Stability of *Aspergillus niger* LCF 9 Proteinase

The enzyme (1 ml, 250 μl/ml) was preincubated for 15 min at various temperature without $CaCl_2$ (★) or with $CaCl_2$ (2 mM) (○) and mixed with 1% Hammarsten casein preincubated for 10 min at the same temperature. After incubation for 15 min, at the relevant temperature, the remaining activities were assayed at 40 °C. (a) Optimum pH of activity (50 mM Tris–HCl buffer, pH 7.8). (b) Optimum pH of stability (50 mM bis-Tris–HCl buffer, pH 5.2).

TABLE III. Effect of Chemicals and Divalent Metal Ions on BAPNA Activity

Compound	Final concentration (mM)	Relative activity (%)
None		100
$CaCl_2$[a]	5	104
	10	106
	15	107
$CuSO_4$[a]	5	14.6
$MgSO_4$[a]	5	100
	10	95
$ZnSO_4$[a]	5	102
	10	112
	15	100
$ZnCl_2$[a]	5	104
	10	112
	15	107
$CoSO_4$[a]	5	51.2
	10	31.7
$MnSO_4$[a]	5	51.2
$HgSO_4$[a]	5	29.2
EDTA[b]	1	27
PMSF in propanol 2[b]	0.1	1
Propanol 2[b]		95

Conditions: enzyme concentration, 500 μg; BAPNA concentration, 0.77 mM; incubation temperature, 35 °C (a) or 40 °C (b) reading at 405 nm against air. Buffer and solutions of inhibitor were preincubated. The solutions of enzyme and inhibitor were mixed and preincubated for 30 min (a) or 5 min (b).

was studied by preincubating the enzyme for 15 min at each temperature with and without 0.2 M $CaCl_2$. The results of the two series are shown in Figs. 4 and 5. At the pH of optimum stability, the enzyme was unstable up to 45 °C and became totally inactive beyond 65 °C. At the pH of optimum activity, inactivation occurred more rapidly, presumably as a result of autolytic effects. The presence of calcium stabilized the enzyme and protected it against thermal denaturation.

Influence of Metal Ions on Activity: The enzyme was preincubated at 35 °C for 30 min with triethanolamine buffer without $CaCl_2$ containing 1.5, 10 and 15 mM of various ions ($CaCl_2$, $CuSO_4$, $MgSO_4$, $ZnCl_2$, $CoSO_4$, $MuSO_4$ and $HgCl_2$). The residual activity towards BAPNA was then measured. Copper, manganese and cobalt had an inhibiting effect. Zinc and magnesium had no effect. Phosphate had a slight inhibiting effect (20%) as is frequently the case for aspergillopeptidases.

Influence of a Serine Protease Inhibitor and of Ethylenediamine Tetraacetic Acid (EDTA): The aspergilloproteinases active over this pH range are either serine proteinases or metal proteinases. A specific serine inhibitor, phenylmethanesulfonyl fluoride (PMSF), and a metal ion chelating agent, EDTA, both strongly inhibited the enzyme. PMSF was used in isopropanol solution, being unstable in water. Hence the enzyme is a serine proteinase (EC 3.4.23.4). The rapid inactivation of the enzyme by EDTA may be due to the removal of calcium ions which provide protection against thermal denaturation.[1]

Discussion

We characterized a new aspergillopeptidase from *A. niger*. This enzyme is active over the pH range of 5—8.5, its optimum pH for activity is pH 7.8 and its optimum temperature for activity is 45 °C. It is a true endopeptidase and differs from other aspergillopeptidases active over this pH range by its trypsin specificity. Its molecular weight is 21100 Da, its maximal stability is in the range of pH 5.5—7.5, is thermally inactivated at above 40 °C and its sensitivity to inhibitors shows it to be a serine proteinase. These results coincide with those obtained for the semi-alkaline serine proteinases from *Aspergillus melleus* and *Aspergillus oryzae*.[16-18]

Semi-alkaline serine proteinases from *Aspergillus* constitute a subgroup of enzymes with useful therapeutic properties. The best known is the aspergillopeptidase from *A. melleus*,[19] which is used for its anti-inflammatory properties. The aspergillopeptidase from *A. niger* may find use in two areas. Firstly, its arginyl carboxyamidase activity enables it to cleave the 4–5 bond of bradykinin and thereby inactivate it, suggesting it should have an anti-inflammatory activity. The results of trials to assess this activity will be reported shortly. Secondly, this aspergillopeptidase is very active towards substrates with a Gly–X–Y sequence. Such a sequence occurs 338 times in collagen I. Collagen derivatives used in therapeutics are highly pure polypeptides of well-defined molecular weights. These cannot be obtained by chemical hydrolysis, and so the use of an enzyme would be advantageous. Trials are being carried out on various commercially available native collagens and the initial results are promising.

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