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Some Enzymic Properties of a Protease from *Alternaria tenuissima*

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The purified major protease from the fungus *Alternaria tenuissima* showed an optimum pH range for hydrolysis of casein between pH 7.5 and 10.5 and an optimum temperature at approximately 42°C. It was labile in the purified form above 27°C. Both protease and esterase activity of the enzyme were inhibited by diisopropyl fluorophosphate. The proteolytic activity was also inhibited by some metal ions such as Co^{2+}, Hg^{2+}, and Ag^{+} and by a heat labile factor in human serum. It was not inhibited by soybean trypsin inhibitor, which was used as a substrate by the enzyme. Other substrates of the enzyme were pepsin, hemoglobin, albumin, and various commercial protein-containing products. Di-, tri- and tetrapeptides tested were not hydrolyzed by the enzyme, but poly-L-lysine was hydrolyzed at a relatively low rate at pH 10. *N*-Acetyl-L-tyrosine ethyl ester, *N*-α-benzoyl-L-arginine ethyl ester and *p*-tosyl-L-arginine methyl ester were hydrolyzed by the proteolytic enzyme in pH-stat experiments. The results indicate that the enzyme is an endopeptidase belonging to the group of peptidyl peptide hydrolases (EC 3.4.4 group).

Conditions for submerged production of protease from certain strains of the fungus *Alternaria tenuissima* have been reported earlier.[1,2] It was shown that yields of about 1.5 enzyme units could be obtained per liter of culture fluid from this fungus after 60 h of cultivation under suitable conditions. The major proteolytic enzyme from such fungal cultures has been purified recently with a method including precipitation with ammonium sulfate, adsorption on kieselguhr, precipitation with acetone, and gel filtration.[3] Some of its physical and chemical properties have also been reported.[3] The present paper describes some enzymic properties of that protease.

MATERIALS AND METHODS

Enzyme. The protease was purified from the active culture solution of *A. tenuissima* NRC E-34 [2] as described previously.[3] The final purified fraction was maintained frozen at −20°C in citrate-phosphate buffer pH 6.6 [4] diluted 1:10 as it was obtained from gel

filtration. As reported earlier [3] the specific activity of this preparation was 5.9 units per g of protein.[5] Repeated freezing and thawing of a purified sample was avoided because of losses in activity associated with such treatment. Unless otherwise stated this purified fraction was used. In experiments on action on proteins, however, a less purified fraction, obtained by elution from kieselguhr was used.[3] Its specific activity was 5.2 units per g of protein.

Assay. Protease was estimated as caseolytic activity at pH 9.5 by a modification of Anson's procedure [6] as previously described.[2] Relative activities are given as difference in absorbance at 660 mμ between sample and zero time blank as obtained by this colorimetric method. Mean errors varied between ±1.0 and ±3.6 % at various enzyme concentrations within the absorbance range 0.1 to 0.7. The amount of enzyme used per assay usually corresponded to a difference in absorbance of 0.1 to 0.5. Absolute activities are expressed as enzyme units (EU) or milliunits (mEU), 1 unit being defined as that amount of enzyme which liberated split products equivalent to 1 mequiv./min of tyrosine, under the conditions specified.

For study of the activity on other proteins or protein-containing products, the same assay method was used, but amounts equivalent to 10 mg of substrate protein were in these cases suspended or dissolved without heating in 1 ml glycine-NaOH buffer pH 9.5 for 3 h before incubation with the enzyme. The difference between absorbance of sample and blank was calculated as per cent of the same value for casein proteolysis.

Protein content is given according to the method by Lowry *et al.*[5] with bovine serum albumin (AB Kabi, Stockholm, Sweden) as the standard. After the color reaction, absorbance at 750 mμ was read in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

pH-Stat. The pH-stat experiments were performed in a stirred, 5 ml reaction mixture under nitrogen flow at 30°C. A combination electrode (2026C, Radiometer, Copenhagen, Denmark) was used together with a pH meter (type PHM26, expanded scale) and an automatic titrator (type TTT11b of the same firm). The quantity of alkali added by a magnetic valve was recorded manually at intervals.

Chemicals. Phenol reagent and casein (Hammarsten quality) were obtained from E. Merck AG, Darmstadt, Germany. The other proteins and trypsin inhibitors used were from Nutritional Biochemicals Co., Cleveland, Ohio. The origin of rapeseed oil meal, meat scrap, cottonseed protein, cottonseed flour and skim-milk powder has been given earlier.[7,8] *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) was from The British Drug House Ltd., Poole, U.K. Peptides, *N*-acetyl-L-tyrosine ethyl ester (ATEE) and *p*-tosyl-L-arginine methyl ester hydrochloride (TAME) were from Mann Research Laboratories, Inc., New York, N.Y. Diisopropyl fluorophosphate (DFP) was obtained as an 0.5 M solution in propylene glycol as a gift from Dr. S. Wählby, Institute of Medical Chemistry, Uppsala, Sweden. Buffers were prepared according to Gomory [4] and in some cases with double strength or further diluted with distilled water as stated under Results.

RESULTS

Effect of pH on activity. The purified protease was incubated with casein buffered with equal amounts of double strength buffer.[4] In Fig. 1 the resulting caseolytic activity is plotted against pH in the reaction mixture. At pH 3.6 to 5 casein precipitated on addition of buffer. As shown in the figure, the optimum pH range for hydrolysis of casein by the enzyme under the conditions used was about pH 7.5 to 10.5. At pH 6 and 11 more than 50 % of the maximum activity still remained. The various buffer systems used along the pH scale, citrate-phosphate, veronal and glycine-NaOH, seemed not to affect the activity differently.

Effect of temperature on activity. The caseolytic activity of the purified protease when incubated at various temperatures is given in Fig. 2. As seen, the optimum temperature was about 42°C under the conditions used. At 37°C,

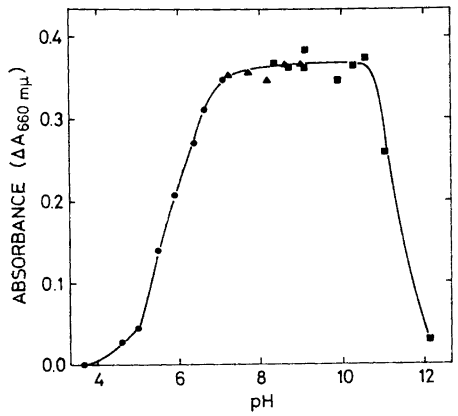

Fig. 1. Effect of pH on hydrolysis of casein by the fungal enzyme. Legend: ●, citrate-phosphate; ▲, veronal; ■, glycine-NaOH buffer.

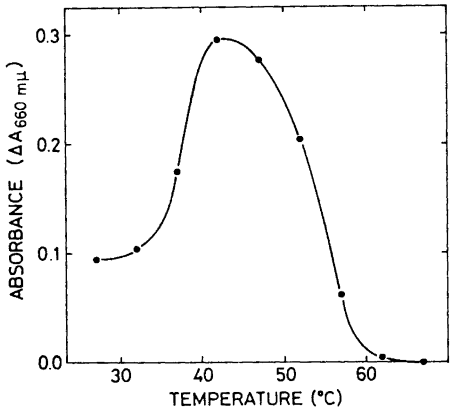

Fig. 2. Effect of incubation temperature on hydrolysis of casein by the fungal enzyme.

the temperature normally used in the assay procedure, 60 % of the maximum absorbance was attained.

Thermal and pH stability. Residual activity was assayed after 15 min preincubation of the purified enzyme in citrate-phosphate buffer at a final pH of 6.9 after dilution 1:150 (Fig. 3). As illustrated in the figure the enzyme was stable up to about 27°C. Above this temperature the activity decreased,

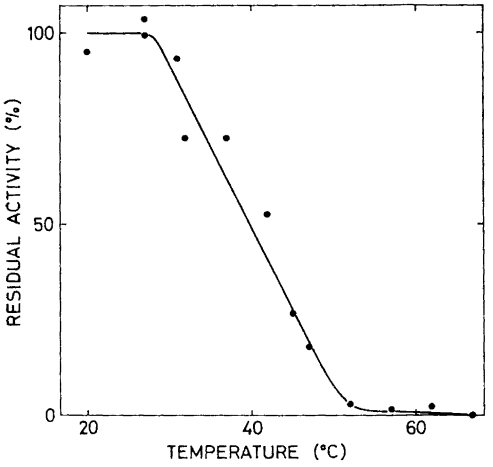

Fig. 3. Heat inactivation of the fungal enzyme by 15 min preincubation at pH 6.9 in citrate-phosphate buffer.

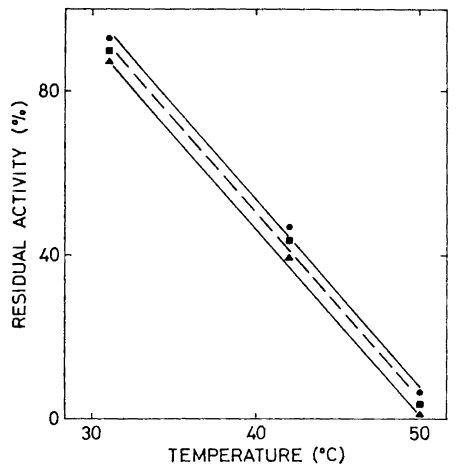

Fig. 4. Effect of pH and temperature on stability of the fungal enzyme. Preincubation in citrate-phosphate buffer at pH 6.0 (●), veronal buffer at pH 8.4 (■), and glycine-NaOH buffer at 9.5 (▲) for 15 min.

and half of the activity had been lost during this incubation at 40°C. Almost no activity remained after preincubation above 50°C at pH 6.9. Incubation at pH 6.0, 8.4, and 9.5 in buffers diluted 1:20 showed a similar picture with only small differences in stability between various pH values (Fig. 4). At pH values higher than 10.0 or lower than 5.5, however, the enzyme was considerably less stable.

Inhibition and activation. The effect of metal ions on the purified protease was studied by preincubation of a dialyzed enzyme sample for 30 min at 21°C in a salt solution before assay. In the solutions pH was adjusted to pH 6 except for $FeSO_4$ (pH 4) and $CuSO_4$ (pH 5). Concentrations during preincubation were 2 mM with respect to the various salts and approximately 0.03 mEU of protease per ml. Most of the salts tested had little or no lasting effect on the residual activity as estimated by the protease assay method. After preincubation with NaCl, $CaCl_2$, $BaCl_2$, $MgSO_4$, $Pb(OOCCH_3)_2$ and $ZnSO_4$ the activities were 95 to 105 % of the control value. Corresponding figures for $K_4Fe(CN)_6$, $FeSO_4$, and $MnSO_4$ were 89 to 91 %, but $CoCl_2$, $HgCl_2$, and $AgNO_3$ inhibited the enzyme considerably: 69, 57, and 54 % residual activity, respectively. Very high absorbance values were found from samples preincubated with $NiSO_4$ and $CuSO_4$, 188 and 230 %. Still higher values were attained at higher concentrations of Cu^{2+} up to 10 mM. However, these values were caused by interference with the assay procedure, and the dry weight of the trichloroacetic-acid precipitable casein remained the same. In addition, a similar increase in absorbance was noted if equivalent amounts of Cu^{2+} or Ni^{2+} were added after the enzyme reaction had been stopped by precipitation with trichloroacetic acid.

Table 1. Effect of various chemicals on the activity of *Alternaria tenuissima* protease.

Addition	Concentration during preincubation	Residual activity (%)
None	—	100
L-Cystein	2 mM	108
Iodoacetic acid	2 mM	103
Ascorbic acid	2 mM	99
Sodium lauryl sulfate	2 mM	98
o-Phenanthroline	2 mM	96
Urea	6 M	94
Ethylenediamine tetraacetate	2 mM	92
N-Ethylmaleimide	0.4 μM	84
p-Chloromercuribenzoate	2 mM	79
Diisopropyl fluorophosphate (1 h)	2 mM	6
Ovalbumin	0.5 mg/ml	115
Bovine serum albumin	0.5 mg/ml	112
Soybean trypsin inhibitor	0.5 mg/ml	115
Limabean trypsin inhibitor	0.5 mg/ml	92
Eggwhite trypsin inhibitor	0.5 mg/ml	75
Human serum	0.5 mg/ml	14
Human serum treated 30 min. 60°C	0.5 mg/ml	98

The same preincubation procedure was used to study the effect of various other chemicals listed in Table 1. Concentrations given refer to conditions in the preincubated enzyme solution. As shown in the table, *N*-ethylmaleimide and *p*-chloromercuribenzoate caused some inhibition, whereas higher values than the control were noted for albumin and soybean trypsin inhibitor possibly due to protection of the enzyme. Eggwhite trypsin inhibitor inhibited the enzyme, but this was particularly the case of a heat labile component in human serum. DFP almost completely inhibited the enzyme according to this protease analysis, and no trace of activity could be found in the same inhibited enzyme solution when tested for esterase activity as described below.

Action on peptides. Exopeptidase activity was tested at pH 9.3 in pH-stat experiments with 0.72 mEU of the purified enzyme in a citrate-phosphate buffer (diluted 1:70) with 20 mg of substrate: L-leucyl-L-tyrosine, L-leucyl-L-phenylalanine, glycylglycylglycine, and tetraglycine. No activity could be found on any of these di-, tri-, and tetrapeptides during 2 h of incubation.

Activity on poly-L-lysine was tested in the same way with 10 mg of substrate and 0.52 mEU of the purified enzyme in 0.08 M KCl at pH 10.0. Approximate initial rate of hydrolysis corresponded to 0.15 μeqiv./min of sodium hydroxide added.

Esterase activity. The esterase activity was followed in pH-stat experiments at pH 7.0 with 0.72 mEU of the purified protease per assay and ATEE, BAEE, and TAME as substrates in citrate-phosphate buffer (1:70). As shown in Fig. 5 all these esters were hydrolyzed at a considerable rate. The slow deviation from linearity of the BAEE hydrolysis may be caused by a gradual

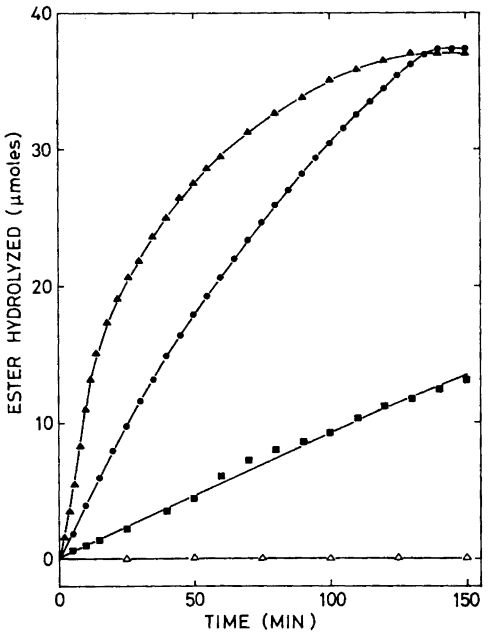

Fig. 5. Hydrolysis of esters in a pH-stat: ATEE (▲), BAEE (●), TAME (■), and ATEE with DFP inhibited enzyme (△). 4 ml 10 mM ester was incubated at 30°C with 0.72 mEU (1 ml) of the fungal protease, pH 7.0. For inhibition the enzyme was preincubated for 1 h at 25°C in 2 mM DFP.

inactivation of the enzyme. Initial rates of hydrolysis were calculated to approximately 1.14, 0.40 and 0.088, μmole/min, respectively. No trace of activity was found on ATEE after preincubation of the enzyme with DFP.

Action on proteins. Action on various proteins were compared with that on casein by using the same incubation and assay method for split products as in the protease assay (Table 2). A partly purified enzyme fraction eluted

Table 2. Action of *Alternaria tenuissima* protease on proteins in relation to that on casein.

Substrate	Activity (%)
Casein	100
Pepsin 3× cryst.	238
Hemoglobin for protease assay	52
Bovine hemoglobin 2× cryst.	39
Soybean trypsin inhibitor 5× cryst.	26
Bovine albumin cryst.	9

from celite was used, which contained 0.05 mEU per assay except for pepsin, where half of that activity was used. In addition to the substrates listed in the table some other compounds were tested: bovine gamma globulin fr. II, ovalbumin 3×cryst., eggwhite trypsin inhibitor, and limabean trypsin inhibitor, but no significant proteolysis could be found even with increased amounts of enzyme (0.17 mEU per 10 mg substrate).

Several commercial protein-containing products were hydrolyzed by the same procedure (Table 3). Before incubation, the fish meal, rapeseed oil meal, and meat scrap were pulverized to pass a 250-μ sieve. The protein of most of these products is largely denaturated, and high blanks indicated that some of them contained large amounts of free amino acids and peptides

Table 3. Action of *Alternaria tenuissima* protease on protein-containing products in relation to that on casein.

Protein-containing product	Activity (%)
Casein	100
Skim-milk powder	77
Standardized milk (3 % fat)	73
Skim milk	72
Farmyard milk	49
Cottonseed flour	55
Fishmeal	36
Rapeseed oil meal	30
Cottonseed protein	19
Gelatin (Difco)	16
Meat scrap	12

before the test. This was particularly true in the case of rapeseed oil meal and fishmeal.

Proteolysis of pepsin, bovine albumin and casein was further studied in pH-stat experiments at pH 9.5 and 30°C with 0.13 mEU of the same enzyme preparation as above per assay in glycine-NaOH buffer diluted 1:100. As

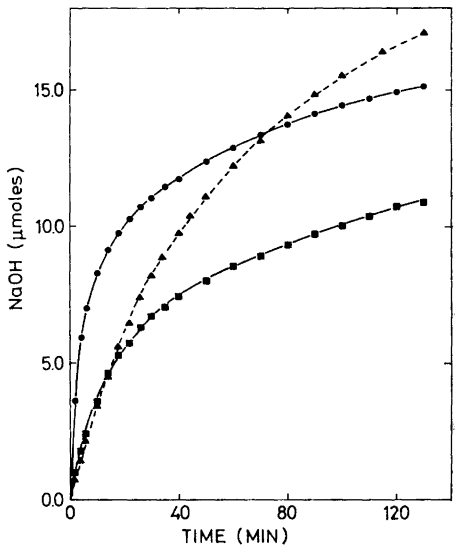

Fig. 6. Hydrolysis of casein (▲), pepsin (●), and serum albumin (■) by the fungal protease in a pH-stat. 20 mg substrate was incubated with 0.13 mEU of protease in 5 ml glycine-NaOH buffer pH 9.5 at 30°C.

shown in Fig. 6 pepsin was hydrolyzed with the highest initial rate, which corresponded to approximately 2.5 μequiv./min of sodium hydroxide added, whereas the corresponding figures for serum albumin and casein were 0.6 and 0.5, respectively.

DISCUSSION

Like some other fungal proteases, for example those from *Gliocladium roseum* [9] and from *Penicillium cyaneo-fulvum*,[10] the major protease purified from culture solutions of *A. tenuissima* showed a wide pH optimum with a considerable activity on casein from pH 6 to 11. At pH-values higher than 10.0 or at temperatures above 30°C the enzyme was, however, relatively labile.

The enzyme appeared not to be dependent on metal ions for activity. Thus it was not significantly inactivated by metal-chelating agents such as EDTA or *o*-phenanthroline and not activated by the metal ions tested. Ni^{2+} and Cu^{2+} caused increased colorimeter readings apparently due to the reactions of these ions with the split products.[5] In cases where increased sensitivity of the assay method is wished, reaction with small amounts of copper may be useful.[11] The increase in color by 5 mM Cu^{2+} in the incubated casein-enzyme solution was 2.5- to 3-fold for several microbial proteases at an absorbance of about 0.1 (without Cu^{2+}). In some cases, for example after preincubation

with $HgCl_2$, the inhibition may have been more or less reversed during the assay procedure.

The strong inactivation by DFP indicates that the enzyme belongs to the group of serine enzymes, but it was also slightly affected by the sulfhydryl inhibitor reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide but not by iodoacetic acid. The factor in human serum, wich was another strong inhibitor of the enzyme, was heat-labile like the α_1 trypsin inhibitor of blood serum.

The experiments with hydrolysis of proteins and lack of activity towards di-, tri-, and tetrapeptides tested indicate that the enzyme is an endopeptidase belonging to the group of peptidyl peptide hydrolases (EC 3.4.4 group).

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