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Some Properties of Acid Protease from the Thermophilic Fungus, *Penicillium duponti* K1014

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A purified acid protease from a true thermophilic fungus, *Penicillium duponti* K1014, was most active at pH 2.5 for milk casein and at pH 3.0 for hemoglobin. The enzyme was stable at a pH range of 2.5 to 6.0 at 30 C for 20 h. The acid protease retained full activity after 1 h at 60 C at a pH range between 3.5 and 5.5. At the most stable pH of 4.5, more than 65% of its activity remained after heat treatment for 1 h at 70 C. These thermal properties show the enzyme as a thermophilic protein. The enzyme activity was strongly inhibited by sodium lauryl sulfate and oxidizing reagents such as potassium permanganate and *N*-bromosuccinimide. No inhibition was caused by chelating reagents, potato inhibitor, and those reagents which convert sulfhydryl groups to mercaptides. Reducing reagents showed an activating effect. The enzyme showed the trypsinogen-activating property at an acidic pH range; optimal trypsinogen activation was obtained at a pH of approximately 3.0. The isoelectric point of the enzyme was estimated to be pH 3.89 by disk electrofocusing. By using gel filtration, an approximate value of 41,000 was estimated for the molecular weight.

A number of extracellular acid protease from various strains of *Penicillia* (7, 27) and other mesophilic fungi (18, 24, 26) have been purified. Hofmann and Shaw (7) reported the purification and characterization of a trypsinogen-activating proteolytic enzyme, peptidase A, of *Penicillium janthinellum*. The *P. janthinellum* peptidase A is called penicillopepsin (22) because of similarities to pepsin in many of its properties (14, 21, 22). Milk-clotting acid proteases from thermophilic species of *Mucor*, *Mucor pusillus* (1, 10) and *Mucor miehei* (16, 17), have been purified and characterized. However, the properties of acid protease from other thermophilic fungi have not been described in detail; only a few reports (4, 5) have been published at this time.

We reported (4) that a true thermophilic fungus, *Penicillium duponti* K1014, produces a thermostable acid protease; the enzyme has been isolated (5). The dual properties of acid and thermal stability are useful not only for industrial purposes but also for attempts to obtain more information on the structure and function of proteases. In this study (presented in part at the Annual Meeting of the Agricultural Chemical Society of Japan, 1 April 1970, at Fukuoka, Japan), a purified protease from *P. duponti* was examined to obtain information on properties of the enzyme.

MATERIALS AND METHODS

Purification of acid protease. *P. duponti* K1014 was grown in submerged culture, and the acid protease was isolated and purified as described in a previous report (5). The purified enzyme was used throughout this work.

Assay of protease activity. Protease activity was assayed as described in detail previously (4). When necessary, the casein-275-nm method (3) was used. A reaction mixture containing 1 ml of enzyme solution and 5 ml of 0.6% Hammarsten milk casein in 0.05 M acetate buffer (pH 2.5) was incubated at 30 C for 10 min. The enzyme reaction was stopped by adding 5 ml of 0.11 M trichloroacetic acid containing 0.22 M acetic acid and 0.33 M sodium acetate. The terminated reaction mixture was allowed to stand for 30 min at 30 C and then was filtered. The extinction values at 275 nm were read against a blank containing the reagents.

Hydrolysis of various proteins. Five percent of each protein substrate was boiled for 5 min in 0.05 M lactic acid. After cooling, the protein suspension was adjusted to pH 3.8 by the addition of diluted HCl.

The reaction mixture (pH 3.8), which contained 1 ml of a 0.0267% enzyme solution in buffer and 5 ml of a 5.0% substrate, was incubated at 60 C. The extent of hydrolysis was measured by the increase of non-precipitated Kjeldahl nitrogen in 0.05 M trichloroacetic acid and was expressed as a fraction of total nitrogen present in the sample. Protein samples used are as follows: Hammarsten milk casein (Merck & Co., Inc.), hemoglobin (Koso Chemical Co., Ltd.), soybean casein and egg albumin (Wako Pure Chemical Ind., Ltd.). Total nitrogen was estimated by Kjeldahl's micro- or macromethod, and formol nitrogen was estimated by the formal titration procedure of Sorensen (6).

Trypsinogen activation. Trypsinogenkinase activity was assayed according to slightly modified method of Hofmann and Shaw (7). Reaction mixture containing 1.0 ml of 0.1 M acetate buffer, 0.5 ml of 10^{-5} M trypsinogen (containing 40% $MgSO_4$; Miles-Seravac Ltd.) in 0.0025 M HCl and 0.5 ml of enzyme solution was incubated at 30 C for 10 min. Except where specified, enzyme reactions were carried out at pH 3.0. After exactly 10 min, the quantity of trypsin formed was assayed at 30 C according to the casein–275-nm method. To 5 ml of 0.6% Hamarsten milk casein in 1.0 M tris(hydroxymethyl)aminomethane buffer (pH 8.0), 0.5 ml of the reaction mixture and 0.5 ml of 1.0 M phosphate buffer (pH 8.0) were added; after 10 min the undigested casein was precipitated by adding 5 ml of 12% trichloroacetic acid. After 30 min of standing at 30 C, the precipitate was filtered through Toyo Roshi no. 5 C (9 cm) filter paper. The extinction values at 275 nm were read against a blank containing the reagents.

Estimation of isoelectric point. Isoelectric focusing on polyacrylamide gel was carried out by using carrier ampholite (LKB-produkter A.B.) in a pH range of 3 to 10 (13). The protein mixed into the gel was run at the constant voltage of 80 V for 180 min at 5 C. After electrofocusing, the gel was divided into 2-mm slices which were individually extracted with 2 ml of water. The enzyme activity and the pH of each fraction were measured.

Estimation of molecular weight by gel filtration. The molecular weight of the enzyme was estimated by gel filtration (25). Each protein sample (20 mg) in 1 ml of 0.1 M acetate buffer (pH 6.0) was applied to a column (2.5 by 115 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer at 5 C. Elution was performed with the same buffer at a flow rate of 15 ml/h; 3-ml fractions were collected. The void volume of the column was measured with blue dextran. Marker proteins used as standards were as follows: trypsin (mol wt = 23,300; Nutritional Biochemicals Co. [N.B.C.]), pepsin (mol wt = 35,400; N.B.C.), serum albumin (mol wt = 67,000, contained dimer; N.B.C.), and ovo-albumin (mol wt = 45,000; E. Merck AG).

RESULTS

Effect of pH on the enzyme. The effect of pH on acid protease activity is shown (Fig. 1). The optimal point for pH was 2.5 for milk casein and at 3.0 for hemoglobin. The enzyme is stable for 20 h at 30 C between pH 2.5 and 6.0 (Fig. 2). More than 95% of original activity remained after treatment in the pH range of 3.5 to 5.5 for 1 h at 60 C. Beyond this pH range there was a rapid drop in activity. It was also found that the enzyme was most stable at a pH of approximately 4.5. After heat treatment at pH 4.5 for 1 h at 70 C, the acid protease retained about 65% of its original activity. Complete inactivation was observed at pH 4.5 for 10 min at 80 C (data not shown). These results are in close agreement with the data obtained from a crude enzyme preparation (4).

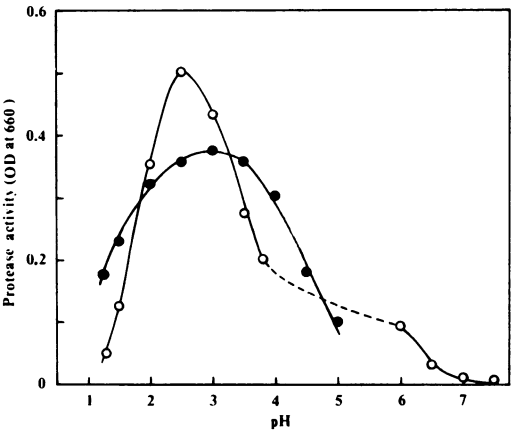

FIG. 1. *Effect of pH on acid protease activity. Symbols:* ○, *milk casein;* ●, *hemoglobin.*

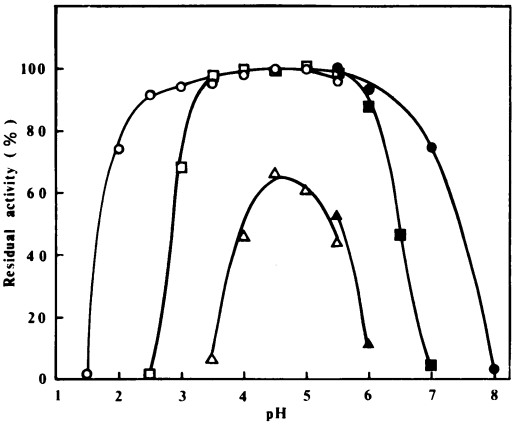

FIG. 2. *Effect of pH on acid protease stability. Symbols:* ○(●), *at 30 C for 20 h;* □(■), *at 60 C for 1 h;* △(▲), *at 70 C for 1 h; open symbols, incubated in acetate buffer; dark symbols, incubated in phosphate buffer.*

Effect of temperature on the enzyme. The enzyme solution was incubated for 30 min at different temperatures with hemoglobins of pH 2.5, 3.6, and 4.6, respectively. The temperature optima for the acid protease as well as its thermal stabilities were dependent on pH values. The enzyme displayed an optimal temperature of 55 C at pH 2.5, of 70 C at pH 3.6, and of 75 C at pH 4.6 (Fig. 3).

Effect of metal ions on the enzyme. No significant inactivation or inhibition of enzyme activity was observed with the following chemicals at concentrations of 10^{-2} M or 10^{-3} M: KCl, NaCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $FeSO_4$, $NiSO_4$, $CdCl_2$, $AlCl_3$, etc. $CuSO_4$ and $HgCl_2$ inhibited enzyme activity by 30 and 60%, respectively, at a concentration of 10^{-2} M; little or no inhibition was observed at a concentration of 10^{-3} M.

Effect of various reagents on the enzyme activity. Table 1 shows the results of experiments undertaken in an attempt to obtain some information about the active site of the acid protease. The enzyme activity was not inhibited by metal chelating reagents, reducing reagents, and sulfhydryl reagents, whereas strong inhibition was observed by such oxidizing reagents as *N*-bromosuccinimide, potassium ferricyanide, and potassium permanganate. Sodium lauryl sulfate caused severe inactivation at 10^{-3} M and pH 2.5.

Action of the enzyme on various proteins at an elevated temperature. Hydrolysis of the protein substrates by the purified acid protease was carried out at 60 C at a substrate-enzyme weight ratio of 100:0.107. The enzyme readily hydrolyzed hemoglobin, soybean casein, milk casein, and egg albumin (Fig. 4). Therefore, it demonstrated little structural specificity toward different substrates. The ratio of formol nitrogen to total nitrogen in 0.05 M trichloroacetic acid-soluble clear hydrolysate was almost constant during the hydrolysis of each protein.

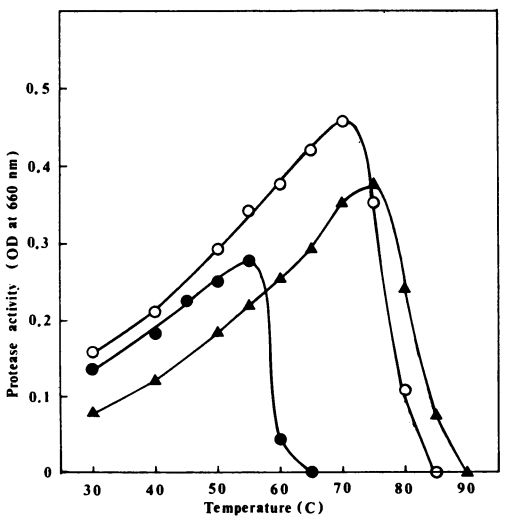

FIG. 3. *Effect of temperature on acid protease activity. Symbols: ●, at pH 2.5 for 30 min; ○, at pH 3.6 for 30 min; ▲, at pH 4.6 for 30 min.*

TABLE 1. *Effect of various reagents on acid protease activity*[a]

Reagents	Concn (M)	Activity (%)
None		100
L-Cysteine-hydrochloride	10^{-4}	136
Mercaptoethanol	10^{-4}	131
Potato inhibitor[b]		111
N-Ethylmaleimide	10^{-3}	108
Ethylenediaminetetraacetate	10^{-3}	101
o-Phenanthroline	10^{-3}	99
Sodium citrate	10^{-3}	98
Iodoacetic acid	10^{-3}	98
Sodium oxalate	10^{-3}	97
Semicarbazid-hydrochloride	10^{-3}	95
Sodium thioglycolate	10^{-3}	86
p-Chloromercuribenzoate	10^{-3}	81
Potassium ferricyanide	10^{-3}	29
N-Bromosuccinimide	10^{-3}	19
Potassium permanganate.	10^{-3}	9
Sodium lauryl sulfate	10^{-3}	2

[a] Enzyme and reagents were preincubated in 0.05 M acetate buffer (pH 2.5) for 30 min at 30 C, and then the remaining activity was assayed.

[b] Crude extract (0.1 ml) was used.

Trypsinogen activation with the enzyme. Figure 5 shows the effect of pH on trypsinogen activation. Optimal trypsinogen activation was obtained at a pH of about 3.0.

Estimation of isoelectric point. Figure 6 shows the isoelectric focusing pattern of the acid protease. Acid protease activity was assayed by the casein-275-nm method at pH 2.5. The trypsin which was formed at pH 3.0 was also determined by the same method but was based on casein hydrolysis at pH 8.0. The isoelectric point of the acid protease was estimated to be pH 3.89 ± 0.05. Trypsinogen activation is due, not to contaminating enzyme, but to the acid protease (Fig. 6).

Estimation of molecular weight. Figure 7 shows the relationship on a Sephadex G-100 column between the elution volume and void volume ratio and the logarithmic values of the molecular weights of standard proteins and the acid protease. The molecular weight of the acid protease was estimated to be approximately 41,000 by this method.

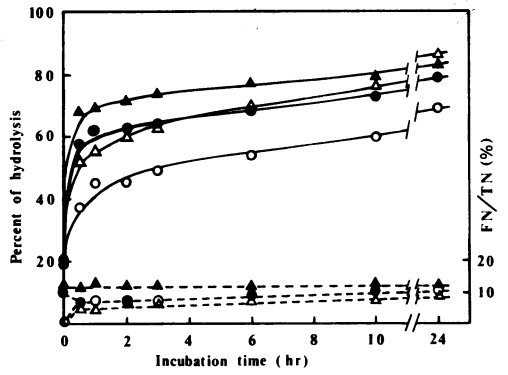

FIG. 4. *Hydrolysis of various proteins at 60 C at pH 3.8 by P. duponti acid protease. Symbols: O, egg albumin; ●, soybean casein; Δ, milk casein; ▲, hemoglobin; solid lines, percent hydrolysis; dashed lines, formol nitrogen (FN) per solubilized nitrogen (TN).*

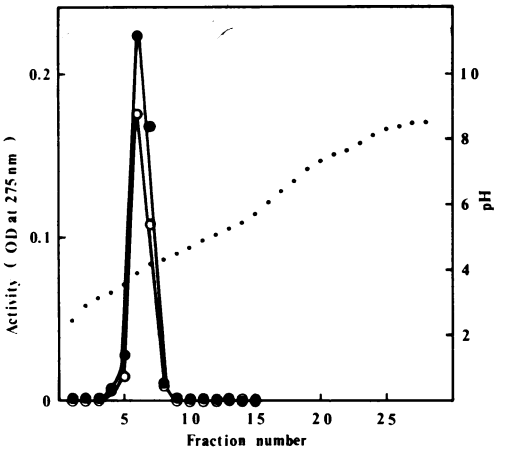

FIG. 6. *Isoelectric focusing pattern of P. duponti acid protease. Symbols: O, acid protease activity; ●, trypsin activity formed at pH 3.0; dashed line, pH.*

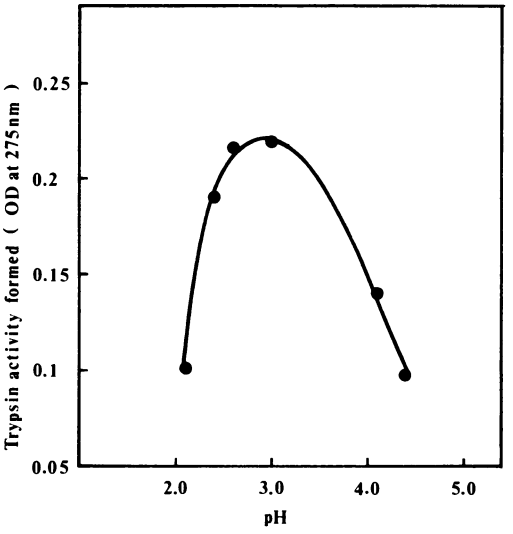

FIG. 5. *Effect of pH on trypsinogen activation by P. duponti acid protease. Trypsin formed at the acidic pH range by P. duponti acid protease was assayed by the casein-275-nm method at pH 8.0.*

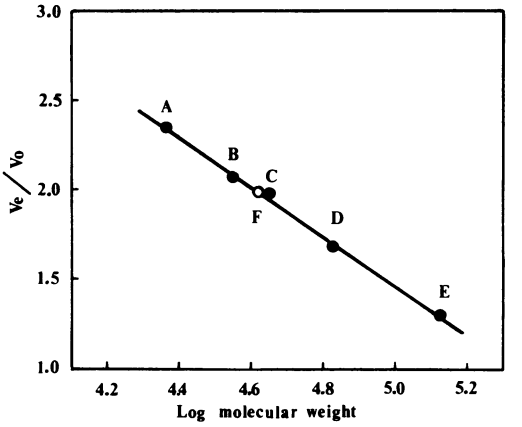

FIG. 7. *Relationship between elution volume (Ve) and molecular weight of proteins on a Sephadex G-100 column. Void volume, Vo. Symbols: A, trypsin; B, pepsin; C, ovalbumin; D, serum albumin; E, serum albumin dimer; F, P. duponti acid protease.*

DISCUSSION

The purified acid protease from a true thermophilic fungus, *P. duponti* K1014, was most active at pH 2.5 for milk casein and at pH 3.0 for hemoglobin. These results were similar to those of most fungal acid proteases (8).

Koaze (12) reported that a new proteolytic enzyme, tentatively named acid protease A which was secreted by *Aspergillus niger* var. *macrosporus*, was most active at 70 C at pH 2.6. However, an acid protease which is similar to the thermostable neutral (2) and alkaline (15) proteases in the thermal stability has not been reported so far. An acid protease from a thermophilic fungus, *M. pusillus*, was irreversibly destroyed at 65 C for 15 min, showing a 90% loss of activity (23). The results in the present work demonstrated that an acid protease of *P. duponti* retained more than 65% of its original activity after treatment for 1 h at 70 C; the reaction rate at pH 4.6 increased steadily from 30 C to about 75 C and then dropped sharply at 80 C due to rapid enzyme inactivation at this temperature. These temperature characteristics suggest that the enzyme may be the most thermostable of fungal acid proteases which

have been isolated thus far. These data agree with the thermal properties obtained with a crude enzyme preparation (4).

The enzyme activity was not inhibited by metal chelating reagents (Table 1). Neither significant activation nor inhibition of the enzyme was observed with any metal ions at 10^{-3} M. The results indicate that no metal ion is essential for proteolytic action, although it is possible that an ion essential to activity was very tightly bound to the enzyme and thus was not released by chemical treatment. Potassium permanganate, potassium ferricyanide, and *N*-bromosuccinimide exhibited strong inhibition. These results resemble those obtained on the acid proteinases of *Aspergillus saitoi* (9) and *Paecilomyces varioti* (19). Sodek and Hofmann (22) reported that penicillopepsin, like pepsin, has an aspartic acid residue at the active site. The inhibitory effect of iodoacetate, *N*-ethylmaleimide, and *p*-chloromercurobenzoate on the acid protease of *P. duponti* was moderate, and reducing reagents (cysteine and mercaptoethanol) activated the enzyme. These observations may indicate that sulfhydryl groups are not essential to proteolytic activity. The present enzyme, like other fungal acid proteases (8), was almost completely inhibited by sodium lauryl sulfate, which is a pepsin inhibitor. Thus, fungal acid proteases differ from the extracellular acid protease of *Rhodotorula glutinis* (11) in their susceptibility to sodium lauryl sulfate.

The present acid protease was capable of hydrolyzing proteins at high temperature, but the degree of hydrolysis of egg albumin was less than milk casein, hemoglobin, and soybean casein. These results allow for a direct comparison of enzyme activity upon different substrates. Shinano and Fukushima (20) reported that an acid protease from lotus seed (*Nelumbo nucifera* Gaertn) was active on casein and hemoglobin, but hardly hydrolyzed egg albumin. From these results, the acid protease of *P. duponti* may be useful for the hydrolysis of various proteins. Furthermore, an advantage of hydrolyzing proteins at high temperature and acidic conditions is the low probability of putrefaction during incubation.

The acid protease from *P. duponti* K1014 was capable of converting trypsinogen into trypsin at an acidic pH range. This trypsinogen-activating property is similar to that of enzymes from *P. janthinellum* (7) and *A. saitoi* (9), and differs from that of pepsin (14). An acid protease from thermophilic fungus, *M. pusillus*, lacks this ability (10). The mechanism of activation of bovine trypsinogen by the mold enzymes is known to be identical with that of autocatalytic activation. It involves the specific cleavage of the Lys-Ile bond with the liberation of the hexapeptide valyl (aspartyl)$_4$ lysine.

The only other well characterized acid protease from thermophilic fungus is that from *M. miehei* (16) which has a molecular weight of 38,200 and an isoelectric point of 4.2. The *P. duponti* acid protease had a molecular weight of 41,000 and an isoelectric point of 3.89. It thus appears that the *Mucor* and *Penicillium* acid proteases are similar in these properties.

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