Purification and Properties of the Thermostable Acid Protease of *Penicillium duponti*†

Shigenori Emi,‡ Dirck V. Myers, and Guillermo A. Iacobucci*

ABSTRACT: An acid protease produced by the thermophilic fungus *Penicillium duponti* K 1014 has been purified by consecutive ion-exchange and gel permeation chromatography, and crystallized from aqueous acetone solution. The purified endopeptidase gave a symmetrical schlieren peak by sedimentation velocity, and was found to be homogeneous upon disc gel electrophoresis at pH 9.5. The enzyme was most active at pH 2.5 against milk casein and showed high thermostability. An isoelectric point of 3.81 was found by isoelectric focusing. A minimum molecular weight of 41 590 was calculated from the amino acid composition, adopting an arginine content of one residue per mole of enzyme. This minimum molecular weight is in good agreement with the value of 41 000 previously found by gel permeation (Hashimoto, H., Iwaasa, T., and Yokotsuka, T. (1973), *Appl. Microbiol. 25,* 578). Besides the thermostability, the purified *P. duponti* protease differs from other well-characterized acid proteases in that it contains carbohydrate, 4.33% expressed as glucose. The enzyme was not affected by *p*-bromophenacyl bromide, but was completely inactivated by α-diazo-*p*-bromoacetophenone, diazoacetyl-DL-norleucine methyl ester, and diazoacetylglycine ethyl ester, in the presence of Cu^{2+}. The complete inactivation of the protease by diazoacetyl-DL-norleucine methyl ester resulted in the specific incorporation of 1 mol of norleucine/mol of enzyme. On the basis of similar behavior of other acid proteases toward this inactivator, the results suggest the presence at the active site of an unusually reactive carboxyl group, involved in the catalytic function. The naturally occurring pepsin inhibitor of *Streptomyces naniwaensis* [Murao, S., and Satoi, S. (1970), *Agric. Biol. Chem. 34,* 1265] inhibited also the protease, at a threefold molar excess with respect to the enzyme.

The best known microbial acid proteases are all produced by mesophilic fungi and are extracellular in nature. Many have been studied in detail, like the ones excreted by *Penicillium janthinellum* (Hofmann and Shaw, 1964), *Aspergillus saitoi* (Ichishima and Yoshida, 1965a), and *Rhizopus chinensis* (Fukumoto et al., 1967), and have been shown to possess enzymatic and molecular properties similar to porcine pepsin. Recent work by Hofmann (Sodek and Hofmann, 1970; Graham et al., 1973) has demonstrated the existence of a strong sequence homology both around the active sites and at the terminal regions of acid proteases.

Lately the active site of pepsin has been probed with the aid of several diazo compounds in the presence of Cu^{2+} (Rajagopalan et al., 1966; Delpierre and Fruton, 1966; Erlanger et al., 1967; Hamilton et al., 1967; Bayliss and Knowles, 1968). It was shown that the inactivation of pepsin results from the esterification by these reagents of the β-carboxyl group of an unique aspartyl residue present at the active site, that plays an essential role in catalysis. Similar results were found for the acid proteases of *P. janthinellum* (Sodek and Hofmann, 1968), *R. chinensis* (Mizobe et al., 1973) and *A. saitoi* (Takahashi and Chang, 1973) using DAN[1] as the inactivator.

Much less information has been published on the acid proteases produced by thermophilic fungi, despite the advantages these enzymes offer for industrial processing. The recent disclosure of the isolation, purification, and some properties of an acid protease from the thermophile *Penicillium duponti* K 1014 (Hashimoto et al., 1973a,b) has prompted us to report the results of an independent study on this enzyme, undertaken as part of a comprehensive evaluation of its use for the industrial production of protein hydrolysates.

This paper deals with an alternative isolation procedure, the characterization of the homogeneous enzyme, and the description of the inactivation by several diazo substrate analogues and the naturally occurring inhibitor S-PI, the *N*-acetyl member of the pepstatin family (Fukumura et al., 1971).

Experimental Section

Materials

The crude protease preparation of *Penicillium duponti* K 1014 was provided by Kikkoman Shoyu Company, Noda, Japan, and consisted of the crude precipitate obtained by adding three volumes of 95% ethanol to one volume of clarified fermented broth, followed by centrifugation and drying in vacuo (Hashimoto et al., 1973b). The dried crude enzyme was a brownish powder, having a specific activity of 618 units/mg of protein. Hammersten quality casein and hemoglobin (standardized for protease assay) were obtained from Nutritional Biochemicals Corporation. Gluten and crystallized and lyophilized egg albumin were purchased from Sigma Chemical Company. Soybean protein isolate (Promine R) was obtained from Central Soya Company, Chicago, Ill. Gelatin and *o*-phenanthroline were products of Eastman Kodak Company. SP-Sephadex C-25, DEAE-Sephadex A-50, and Sephadex G-75 were purchased from Pharmacia Fine Chemicals, Inc. EDTA and mercaptoethanol were products of J. T. Baker Chemical

† From the Corporate R & D Department, The Coca-Cola Company, Atlanta, Georgia 30301. *Received April 15, 1974.*

‡ Visiting Research Scientist. Present address: Enzyme Chemistry Laboratory, Faculty of Science, Osaka City University, Osaka, Japan.

[1] Abbreviations used are: DAN, diazoacetyl-L-norleucine methyl ester; DAG, diazoacetylglycine ethyl ester; S-PI, *Streptomyces naniwaensis* pepsin inhibitor; EDTA, ethylenediaminetetraacetic acid.

Company. Twice crystallized swine pepsin was obtained from Worthington (lot no. 2M-2LB) and used without further purification. *p*-Bromophenacyl bromide was purchased from Aldrich Chemical Co., and *p*-bromobenzoyl chloride from Eastman Organic Chemicals. Glycylglycine ethyl ester hydrochloride and glycyl-DL-norleucine were products of Cyclo Chemical Corp. and Schwarz/Mann Research Inc., respectively. S-PI was the generous gift of Professor S. Murao of the University of Osaka Prefecture, Japan. Deionized water was used in all the experiments and was prepared with an ion-exchanger Research Model II from Illinois Water Treatment Co.

Methods

Proteolytic Activity Assays. (a) *P. duponti* Protease. Substrate was prepared by suspending 1.2 g of casein in 50 ml of 0.05 M HCl. The suspension was dissolved by stirring on a boiling water bath. The solution was then adjusted to pH 2.5 with 0.05 M sodium citrate, made up to 100 ml with 0.05 M sodium citrate–HCl buffer (pH 2.5), and stored at 4 °C. The proteolytic activity was assayed by adding 1 ml of the enzyme solution to 5 ml of the substrate at 30 °C. After 10-min incubation at the same temperature, the reaction was stopped by the addition of 5 ml of the Cl_3CCOOH reagent of Hagihara et al. (1958). The precipitated protein was removed by filtration through Whatman filter paper no. 42 after standing at 30 °C for 30 min. The optical density of the filtrate was read at 275 nm against a blank, using a Zeiss PMQ-II spectrophotometer. The blank was prepared by first mixing 5 ml of substrate solution with 5 ml of the Cl_3CCOOH reagent, followed by 1 ml of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme able to liberate in 1 min the Cl_3CCOOH-soluble 275 nm absorbance equivalent to 1 μg of tyrosine, under the above conditions. The specific activity of the enzyme was defined as the number of proteolytic units per mg of protein. (b) Pepsin. Peptic activity was measured by the method suggested by Ryle (1970) using hemoglobin as a substrate, dissolved in 0.05 M sodium citrate–HCl buffer (pH 1.8), at 37 °C; an $E_{1\text{ cm, }280\text{ nm}}$ (1%) 14.7 (Perlmann, 1959) was used to determine pepsin concentration, adopting a molecular weight of 35 000 (Bovey and Yanari, 1960).

Determination of Protein Concentration. The value $E_{1\text{ cm, }280\text{ nm}}$(1%) 11.45, previously obtained on a sample of dry, crystalline enzyme, was used.

Desalting of Enzyme Solutions. Desalting of enzyme solutions was done by gel filtration on a Sephadex G-25 column (2.5 × 100 cm) equilibrated with the appropriate buffer. The volume of enzyme solution charged was always less than one-tenth of the column's bed volume.

Ion-Exchange Chromatography. The ion-exchange dextrans were always conditioned by consecutive washings with 0.5 M HCl, water, 0.5 M NaOH, and water until neutral. The regenerated dextran, either SP-Sephadex C-25 or DEAE-Sephadex A-50, was equilibrated prior to use with the proper buffer. After deaeration in vacuo, the equilibrated dextran was packed, and buffer was passed overnight through the column at 4 °C. Once the enzyme solution was charged, the column was washed with two bed volumes of buffer. The adsorbed enzyme was eluted with a NaCl linear gradient generated in the same buffer by a LKB Ultrograd 11300, and the flow rate of each column was maintained constant with a peristaltic pump LKB ReCyChrom 4912 A.

Ultracentrifugal and Electrophoretic Analysis. Sedimentation velocity studies were carried out at 60 000 rpm and 20 °C with a Beckman preparative ultracentrifuge, Model L2-65B, equipped with schlieren optics, with 1.25% enzyme in 0.1 M acetate buffer (pH 4.5). The homogeneity of the enzyme was also examined by polyacrylamide gel disc electrophoresis at pH 9.5 (Ornstein, 1964; Davis, 1964), using a Canalco apparatus. The protein, 0.1 mg, was charged in 40% w/v sucrose solution with a spacer gel on the top of a 0.5 × 5 cm column of 7.5% polyacrylamide gel. The separation was run with a current of 2 mA/column, at 4 °C. The gels were stained with 1% Amido Schwarz in 7% acetic acid for 20 min, followed by destaining in 7% acetic acid using a Canalco gel destainer.

The isoelectric point of the enzyme was measured with a LKB electrofocusing apparatus, containing a 1% solution of carrier ampholytes in the pH range 3.0–6.0; 2 ml of 0.5% enzyme solution in water was charged in a LBK 8101 column (containing a 0 to 50% w/v sucrose gradient), and the electrofocusing was run at 4 °C by applying 300 V for the first 2 h, followed by 500 V during the following 48 h. By this time a steady current of 1.8 mA was obtained. Fractions of 2.5 ml were collected, and 280-nm absorbance, proteolytic activity, and pH were measured on each fraction.

Determination of the Extent of Protein Hydrolysis by the Ninhydrin Method. To 8 ml of 1.25% protein solution in 0.05 M acetate buffer (pH 3.2) was added 2 ml of enzyme solution (2 mg of crystalline enzyme, or equivalent on activity basis), and the mixture was incubated at 37 °C under toluene. Appropriate aliquots (1 ml) of the reaction mixture were periodically removed, and to them was added 1 ml of 2% dodecyl sulfate to terminate the reaction as well as to solubilize the aliquots totally. The liberation of α-amino groups was measured by the ninhydrin method (Moore and Stein, 1954). The degree of peptide bond cleavage was calculated by comparing the ninhydrin value of the test samples to a reference value given by the equivalent amount of protein hydrolyzed with 6 M HCl, at 110 °C for 24 h.

Amino Acid Analysis. A Beckman amino acid analyzer, Model 120-C, fitted with a single column system Durrum DC-1A and a Beckman Model 125 integrator, was used. The lyophilized enzyme preparation was hydrolyzed in duplicate with 3 M HCl and 6 M HCl, at 110 °C, for 24, 48, and 72 h, in vacuo, and the results for each acid concentration were computed separately. The values for threonine and serine were obtained by extrapolation to zero hydrolysis time and amide-N was estimated from the NH_3 value obtained by extrapolation to zero time. The maximum values (72 h) were adopted for valine and isoleucine, while for the other amino acids, values were averaged from the 24-, 48-, and 72-h hydrolysates.

Methionine and half-cystine were determined after performic acid oxidation, as recommended by Moore (1963). The tryptophan content was secured by a method perfected in our laboratory (Wilkinson et al., 1975), using ion-exchange chromatography after alkaline hydrolysis in the presence of 5-methyltryptophan as internal standard.

Ultraviolet Absorption Spectra. The uv spectra of the enzyme were measured in 0.1 M acetate buffer (pH 4.5) and 0.1 M NaOH solutions, using a Beckman recording spectrophotometer, Model DK-2.

Preparation of Inactivators. α-Diazo-*p*-bromoacetophenone was prepared from *p*-bromobenzoyl chloride by the method of Erlanger et al. (1967). DAG was prepared by diazotization of glycylglycine ethyl ester hydrochloride (Riehm and Scheraga, 1965). DAN was prepared from glycyl-DL-norleucine in 48% yield, mp 50–51 °C, by the meth-

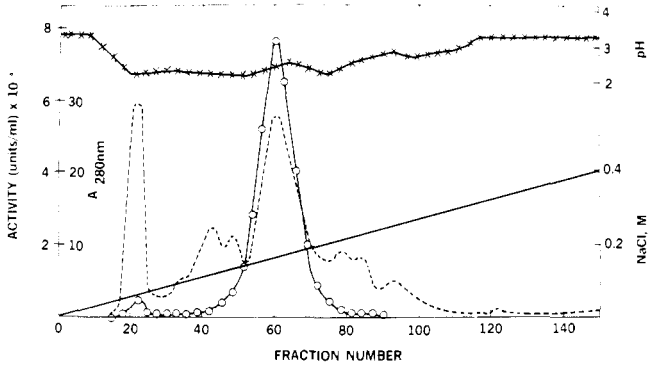

FIGURE 1: Chromatography of *Penicillium duponti* acid protease on a SP-Sephadex C-25 column (2.5 × 100 cm). 1500 ml of enzyme solution ($A_{280\ nm}$, 14.8; protease, 18 300 units/ml) was charged. The flow rate was 50 ml/h; 20-ml fractions were collected. Other details are described in Methods and Results sections. (O) Protease activity; (- - -) $A_{280\ nm}$; (x) pH; (—) NaCl concentration.

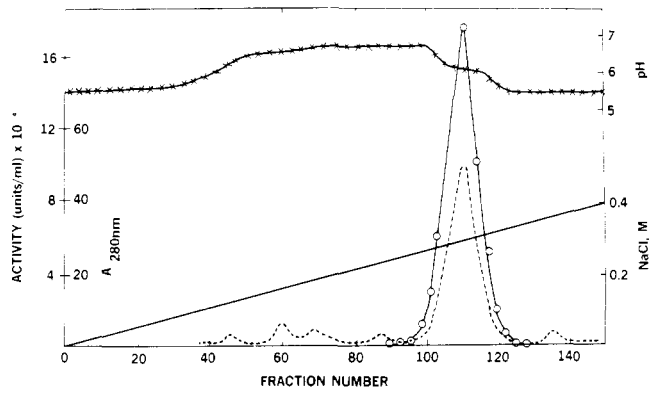

FIGURE 2: Chromatography of *Penicillium duponti* acid protease on a DEAE-Sephadex A-50 column (2.5 × 100 cm). 400 ml of enzyme solution ($A_{280\ nm}$, 28.5; protease, 85 000 units/ml) was applied. The flow rate was 50 ml/h; 20-ml fractions were collected. (O) Protease activity; (- - -) $A_{280\ nm}$; (x) pH; (—) NaCl concentration.

od of Rajagopalan et al. (1966) who reported a melting point of 49–50 °C.

Inactivation of P. duponti Protease with DAN: Norleucine Incorporation. To the protease (100 mg) dissolved in 125 ml of deionized water, were added 17 ml 0.1 M sodium acetate buffer (pH 5.5) and 8.3 ml of 0.024 M cupric acetate. After 10-min incubation at 14 °C, 17 ml of methanol containing 25 μmol of DAN was added. As a control for the nonspecific incorporation of norleucine, the enzyme was treated with DAN in the absence of Cu^{2+} under the same conditions. After incubation for 60 min at 14 °C, EDTA was added to a final concentration of 0.01 M to inhibit further reaction. For both reactions, the product was concentrated to 10 ml in an Amicon 401 ultrafiltration cell fitted with PM-10 Diaflo membrane, and the retentate was permeated through a 2.5 × 100 cm column of Sephadex G-25, using water as eluent. Fractions containing the treated enzyme as evidenced by 280-nm absorbance were pooled, dialyzed against deionized water overnight at 4 °C, and recovered by freeze-drying. The norleucine incorporation was quantified by amino acid analysis, on samples hydrolyzed in triplicate at 110 °C in 6 M HCl, for 24 h in vacuo. The incorporation of norleucine was expressed in molar terms, on the basis of an arginine content of one residue per mole of enzyme.

Results

Purification and Crystallization of the Enzyme. The crude *Penicillium duponti* enzyme preparation (200 g) was suspended in 3 l. of 0.01 M acetate buffer (pH 4.5) and, after the suspension was stirred at 4 °C for 4 h, the insoluble material was removed by centrifugation. To the clear supernatant, ammonium sulfate was added to 50% saturation, and the suspension was kept at 4 °C for 4 h. The resulting precipitate was removed by centrifugation and discarded. At this stage the recovery of proteolytic activity in the supernatant was 99%, while 18% of the 280-nm absorbance was removed with the insolubles.

Ammonium sulfate was added to the supernatant up to 80% saturation, and the suspension was kept overnight at 4 °C. The precipitate was collected by centrifugation, dissolved in 700 ml of 0.02 M acetate buffer (pH 3.7), and desalted on a Sephadex G-25 column. The desalted enzyme (2.2 l.) was decolorized batchwise with 100 g (dry weight) of SP-Sephadex C-25 previously equilibrated with 0.02 M acetate buffer (pH 3.7). The suspension was stirred for 1 h at 25 °C and the dextran was removed by filtration and washed twice with a small volume of buffer. The decolorized filtrate and the washings were combined, and the pH was lowered to 3.2 with 0.05 M HCl. The enzyme solution (3 l.) was then applied on a column of SP-Sephadex C-25 equilibrated with 0.02 M acetate buffer (pH 3.2). The proteolytic activity was completely adsorbed by the dextran at this pH and, after washing the column with the same buffer, the activity was eluted with a NaCl gradient (0–0.4 M). The results are shown in Figure 1, indicating the presence of two proteases eluted at NaCl concentrations of 0.04–0.06 and 0.12–0.20 M, containing 2 and 80% of the applied activity, respectively. At this stage, the specific activity of the major protease was increased approximately twofold.

The pooled fraction containing the major protease (980 ml) was adjusted to pH 4.5 with 1 M NaOH, ammonium sulfate was added to 80% saturation, and the suspension was kept overnight at 4 °C. The precipitate was collected by centrifugation, dissolved in 130 ml of 0.02 M acetate buffer (pH 5.5), and desalted. The enzyme solution (400 ml) was adsorbed on a column of DEAE-Sephadex A-50, which had been equilibrated with 0.02 M acetate buffer (pH 5.5). After washing the column with the same buffer, the adsorbed protease was eluted with a NaCl gradient (0–0.4 M). The results of the chromatography are illustrated in Figure 2, showing that the active peak was eluted at 0.3 M NaCl. The active fractions were combined (400 ml), the pH was adjusted to 4.5 with 1 M HCl, and ammonium sulfate was added to 80% saturation. The precipitated enzyme was dissolved in 80 ml of 0.01 M acetate buffer (pH 4.5), and desalted. The enzyme (300 ml) was purified further by rechromatography on DEAE-Sephadex A-50, using a NaCl gradient (0–0.4 M) in 0.01 M acetate buffer (pH 4.5). The active fractions were combined (420 ml), and ammonium sulfate was added to 80% saturation. The precipitated enzyme was collected by centrifugation, dissolved in 60 ml of 0.01 M acetate buffer (pH 4.5), and subjected to gel filtration on a 5 × 100 cm column of Sephadex G-75 equilibrated with the same acetate buffer (pH 4.5). The permeation resulted in a symmetrical protease peak having a constant specific activity of 4700 units/mg of protein. The fractions with equal specific activity were combined, ammonium sulfate was added to 80% saturation, and the suspension was kept overnight at 4 °C. The resulting precipitate was collected by centrifugation, dissolved in the least possible volume of 0.02 M sodium acetate–HCl buffer (pH 3.5),

Table I: Summary of Purification Procedure of *Penicillium duponti* Acid Protease.

Step	pH	Total Activity (units × 10^{-5})	Specific Activity (units/mg of Protein)	Activity Recovery (%)
First extract	4.5	680	618	100
$(NH_4)_2SO_4$ (80% satn.) precipitate	3.7	598	1050	87.9
Decolorized with SP-Sephadex C-25 (pH 3.7)	3.8	558	1420	82.1
Eluate from SP-Sephadex C-25 (pH 3.2)	2.4	381	2730	56.0
Eluate from DEAE-Sephadex A-50 (pH 5.5)	6.2	301	4140	44.3
Eluate from DEAE-Sephadex A-50 (pH 4.5)	4.5	253	4390	37.2
Eluate from Sephadex G-75	4.5	218	4670	32.1
Crystallized		167	4810	24.6
Recrystallized		151	4810	22.2
Lyophilized		150	4810	21.6

and then dialyzed against the same buffer for 2 days at 4 °C, with frequent changes of dialyzing buffer. Cold acetone (−10 °C) was added dropwise to the dialyzed enzyme solution at 4 °C until a slight cloudiness appeared. The acetone concentration was gradually increased over a 5-day period to a final value of 37%. At this time about 90% of the enzyme activity had crystallized out in plaquettes. The crystals were collected by centrifugation at 4 °C, dissolved in a minimum amount of 0.02 M sodium acetate–HCl buffer (pH 3.5) to give a 10% enzyme solution, and recrystallized by adding acetone as before. A photomicrograph of the recrystallized enzyme is shown in Figure 3. The crystalline enzyme was finally lyophilized without any loss of activity.

The purification procedure is summarized in Table I. The activity recovered in the crystalline enzyme was 22% of the total present in the crude preparation, with an eightfold increase in specific activity. The yield of pure enzyme obtained from 200 g of crude preparation was 3.3 g.

The enzymatic and chemical properties of this pure preparation were established in the series of experiments that follow.

Homogeneity. Figure 3 shows the enzyme sediments as a single symmetrical peak. Homogeneity was also confirmed by polyacrylamide disc gel electrophoresis, where a single band was observed.

Activity and Stability of the Enzyme. A series of conventional studies on enzymatic hydrolysis was conducted for several protein substrates. The pH optimum found for casein was 2.5, while the optima for hemoglobin and egg albumin were displaced upwards, between 3.0 and 3.5.

The enzyme was stable between pH 2.5 and 6.5 after 24-h incubation at 30 °C. For 1-h incubation at 60 °C, the original activity was retained over a narrower pH range, between 3.5 and 5.5. When the enzymatic reaction was carried out on casein for 10 min at various temperatures, the optimal temperature at pH 2.5 was 60 °C; whereas at pH 3.7 it was 75 °C. The thermal stability of the enzyme was examined by incubation at pH 2.5 and 4.5 at various temperatures. The former pH was found optimal for the hydrolysis of casein at 30 °C and the latter was judged the most stable pH for the enzyme. The enzyme retained its original activity at pH 4.5 at temperatures below 65°C following a 15-min incubation, and below 60 °C for a 60-min incubation. In contrast, no remaining activity was found when the enzyme was incubated at pH 2.5, at 65 °C after 15 min, or at 60 °C after 60 min.

Extent of Hydrolysis on Various Proteins. The degree of peptide bond cleavage was established for casein, hemoglobin, egg albumin, soybean protein, gluten, and gelatin, by using the pure crystalline enzyme as well as the crude preparation. Hemoglobin and egg albumin were the best substrates, with about 30% of the peptide bonds split after 48-h hydrolysis, at 37 °C and pH 3.0. In all cases, the crude enzyme preparation hydrolyzed proteins to a larger extent than the pure endopeptidase, suggesting the presence in the former of additional proteolytic enzymes. When both preparations were tested against carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalamine, two known substrates for acid carboxypeptidases (Shaw, 1964; Ichishima, 1972), the crude protease was able to hydrolyze both synthetic substrates, while the crystalline endopeptidase did not.[2] Thus, only the crude enzyme possesses carboxypeptidase activity.

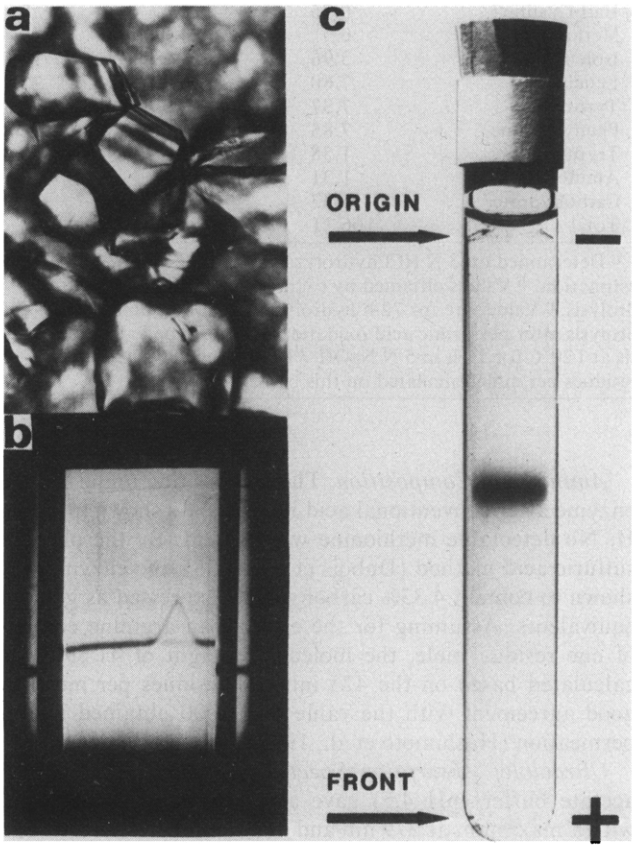

FIGURE 3: Homogeneity of the crystalline preparation of *P. duponti* acid protease. (a) Photomicrograph (×200). (b) Ultracentrifugal pattern. Direction of sedimentation from right to left. (c) Polyacrylamide disc electrophoresis. See Methods for details.

[2] Unpublished data.

Table II: Amino Acid Composition of *P. duponti* Protease

Amino Acid	g of Residue/ 100 g of Protease	Residues/mol of Protease	
		Calcd	Integral
Lysine	4.03	12.4	12
Histidine	0.84	2.4	2
Arginine[a]	0.39	1.0	1
Aspartic acid	12.88	44.4	44
Threonine[b]	7.03	27.6	28
Serine[b]	10.70	48.7	49
Glutamic acid	13.52	41.5	42
Proline	4.41	18.0	18
Glycine	6.61	46.0	46
Alanine	5.11	28.5	29
Valine[c]	5.84	23.4	23
Half-Cystine[d]	0.75	2.9	3
Methionine[d]	0	0	0
Isoleucine[c]	3.96	13.9	14
Leucine	7.60	26.6	27
Tyrosine	7.37	17.9	18
Phenylalanine	7.85	21.1	21
Tryptophan[e]	1.58	3.4	3
Amide-NH_2	1.31	32.4	32
Carbohydrate[f]	4.33	10.7	11
Total	106.11		423

[a] Determined on 3 N HCl hydrolyzates to allow a more accurate estimation. [b] Values obtained by extrapolation to zero time of hydrolysis. [c] Values are for 72-h hydrolysates. [d] Determined by hydrolysis after performic acid oxidation. [e] Determined after hydrolysis at 120°C for 18 h in 5 N NaOH. [f] Determined as glucose, and residues per mole calculated on this basis.

Amino Acid Composition. The composition found for the enzyme after conventional acid hydrolysis is shown in Table II. No detectable methionine was present. By the phenol–sulfuric acid method (Dubois et al., 1956), the enzyme was shown to contain 4.33% carbohydrate, expressed as glucose equivalents. Assuming for the enzyme an arginine content of one residue/mole, the molecular weight of 41 590 was calculated based on the 423 integral residues per mole, in good agreement with the value of 41 000 obtained by gel permeation (Hashimoto et al., 1973a).

Ultraviolet Absorption Spectra. The enzyme in 0.1 M acetate buffer (pH 4.5) gave a typical protein spectrum, with a maximum at 279 nm and a minimum at 250 nm. The specific extinction coefficient at 280 nm, $E_{1\text{ cm, }280\text{ nm}}(1\%)$ 11.45, has been used in this work for the calculation of protein concentrations. The calculation of the number of tyrosine and tryptophan residues from the spectrum of the enzyme in 0.1 M NaOH by the method of Goodwin and Morton (1946) gave values of 17.0 and 3.3 mol, respectively, adopting a molecular weight of 41 590 for the enzyme. These values are in good agreement with those obtained by amino acid analysis.

Determination of the Isoelectric Point. The isoelectric point of the enzyme was determined with an isoelectric focusing apparatus as described in Methods. A single symmetrical peak of coinciding absorbance and proteolytic activity was formed at pH 3.81. Over 95% of both the 280-nm absorbance and the activity applied on the column was recovered in the peak.

Effect of p-Bromophenacyl Bromide. This inactivator is known to react with the β-carboxyl group of an aspartyl residue in pepsin (Erlanger et al., 1965, 1966; Gross and Morell, 1966). Pepsin treated with a 10% molar excess of the reagent lost 72% of the original activity against hemoglobin after a 6-h incubation at pH 2.8 and 37 °C. This partial inactivation was in agreement with previous observations (Erlanger et al., 1965). On the other hand, *P. duponti* protease was not inactivated by *p*-bromophenacyl bromide under the same conditions. Furthermore, inactivation did not occur upon incubation of the enzyme (0.016 μmol) for 24 h with 0.1 μmol of *p*-bromophenacyl bromide in 1 ml of 0.1 M citrate–phosphate buffer at pH 2.8, 3.5, and 4.5.

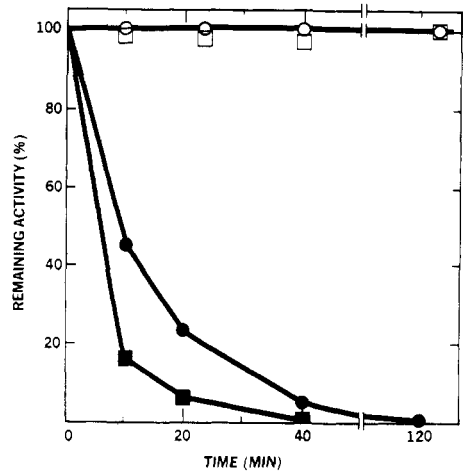

FIGURE 4: Effect of α-diazo-*p*-bromoacetophenone on the activity of *Penicillium duponti* acid protease and pepsin. Enzyme (0.28 μmol) was dissolved in 3.4 ml of 0.006 M acetate buffer (pH 5.0). To this solution was added 0.75 ml of 0.01 M $CuSO_4$, and after 10-min incubation at 27 °C, 0.25 ml of ethanol containing 1.07 μmol of the inhibitor was added. The reaction mixture was kept at 27 °C and appropriate aliquots were periodically removed to determine the remaining activity. *Penicillium duponti* acid protease: (○) in the absence of Cu^{2+}; (●) in the presence of Cu^{2+}. Pepsin: (□) in the absence of Cu^{2+}; (■) in the presence of Cu^{2+}.

Effect of α-Diazo-p-bromoacetophenone. This inactivator is the diazo analogue of *p*-bromophenacyl bromide and has been shown to cause the total loss of the protease activity of pepsin when reacted in the presence of Cu^{2+} (Erlanger et al., 1967). Figure 4 indicates that α-diazo-*p*-bromoacetophenone inactivated totally the *P. duponti* protease in the presence of Cu^{2+}, but was without effect in the absence of Cu^{2+}. The rate of inactivation was about half of that observed for pepsin under similar conditions. Cupric ion alone, at the same concentration, had no effect on the activity of both enzymes.

Effect of DAN and DAG. As shown in Figure 5, DAN inactivated the *P. duponti* protease with rates dependent on the Cu^{2+} concentration. In the presence of a tenfold molar excess of DAN and an 80-fold molar excess of Cu^{2+} with respect to the enzyme, inactivation was complete after 15 min, at 14 °C and pH 5.5. Cupric ions are essential for the inactivation with this diazo compound, as was found before for pepsin (Lundblad and Stein, 1969). The rates of inactivation of *P. duponti* protease by DAG were comparable to those of DAN under similar conditions.

Inactivation of P. duponti Protease with DAN: Norleucine Incorporation. The enzyme was treated with DAN in the presence and absence of Cu^{2+}, in order to correlate norleucine incorporation with enzymatic inactivation. The molar ratio of enzyme:DAN:Cu^{2+} in this experiment was 1:10:80. After incubation at 14 °C for 1 h, the protease lost activity completely in the presence of Cu^{2+}, but remained fully active in the control without copper. The results indicated that 1.20 norleucine residues/mole of enzyme was incorporated in the presence of Cu^{2+}, whereas only 0.28 resi-

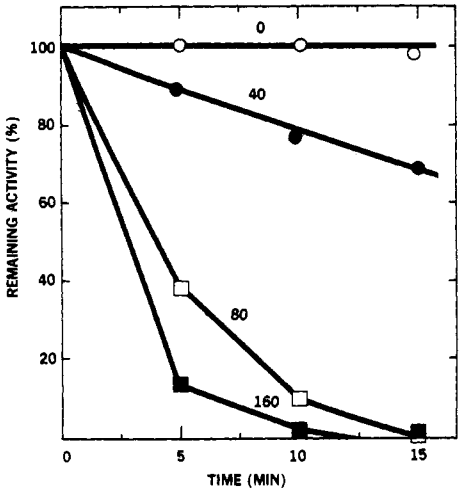

FIGURE 5: Effect of the concentration of Cu^{2+} on the inactivation of *Penicillium duponti* acid protease by DAN. Enzyme (0.015 μmol) was dissolved in 0.75 ml of deionized water. To this solution were added 0.1 ml of 0.1 M acetate buffer (pH 5.5) and 0.05 ml of cupric acetate solution. After 10-min incubation at 14 °C, 0.1 ml of the diazo compound was added. The reaction mixture was kept at 14 °C, and at intervals aliquots of 0.1 ml were removed for enzyme activity assay. The molar ratio Cu^{2+}:enzyme in each experiment is given on the figure.

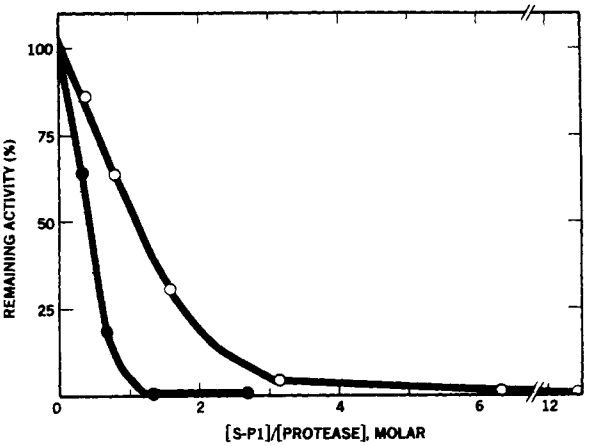

FIGURE 6: Effect of S-PI on the activity of *P. duponti* acid protease (○) and pepsin (●). Inhibition performed as described by Satoi and Murao (1971) using 0.1 M sodium citrate-HCl buffer (pH 1.8 for pepsin, pH 2.5 for *P. duponti* protease). Protease concentrations: pepsin, 14.5 μM; *P. duponti* protease, 12.3 μM. Remaining activity assayed as described in the Experimental Section.

due/mole was in the control. If the last value is considered nonspecific, then the actual norleucine incorporated at the active site was 0.92 residue/mole.

Effect of S-PI. S-PI is a specific pepsin inhibitor produced by *Streptomyces naniwaensis* (Murao and Satoi, 1970), closely related to the pepstatins excreted by *Streptomyces testaceus* and *Streptomyces argenteolus* var. *toyonakensis* (Umezawa et al., 1970). These inhibitors are derivatives of a common pentapeptide, having different acyl substituents at the N-terminus. S-PI is the acetyl derivative (Fukumura et al., 1971), whereas pepstatin A has an isovaleryl substituent. Both inhibitors strongly bind to pepsin to form 1:1 stoichiometric complexes (Satoi and Murao, 1971; Kunimoto et al., 1972). As shown in Figure 6, the complete inhibition of *P. duponti* protease required a threefold molar excess of S-PI, while pepsin was totally inhibited at equimolar concentrations. However, the *P. duponti* protease appears to be more sensitive to S-PI than the other proteases examined by Satoi and Murao (1971).

Discussion

The acid protease from the thermophile *Penicillium duponti* K 1014 has been purified and crystallized, and the pure enzyme was found homogeneous by ultracentrifugal and electrophoretic analysis. The enzyme was most active at pH 2.5 against casein, and at pH 3.0–3.5 against hemoglobin. At pH 4.5 in the absence of substrate, the enzyme retained full activity when kept for 1 h at 60 °C, illustrating its exceptional thermostability.

The amino acid composition of the protease was thoroughly determined by the conventional 6 M HCl hydrolysis, as well as under milder conditions using 3 M HCl. The latter was needed to measure accurately the arginine content, upon which the minimum molecular weight was calculated. The arginine values obtained from the 3 M HCl hydrolysates were higher and had a smaller deviation than those from the 6 M acid. The minimum molecular weight of 41 590 found for the *P. duponti* protease assuming one arginine residue per mole of enzyme, was in good agreement with the value of 41 000 obtained by gel permeation, whereas the molecular weights of other acid proteases are known to be lower, within the range of 30 000–35 000.

The enzyme contained no methionine, as found also for the acid proteases of *Penicillium janthinellum* (Hofmann and Shaw, 1964) and *Aspergillus saitoi* (Ichishima and Yoshida, 1965b). The amino acid composition of *P. duponti* protease, however, differed from that of *P. janthinellum,* because of the lack of arginine and half-cystine in the latter; it resembled better the *A. saitoi* protease in the lack of methionine, the low content of arginine, histidine, and half-cystine, and the high content of lysine.

P. duponti protease was found to contain 4.33% carbohydrate expressed as glucose, a value that remained constant upon precipitation and washing with 10% Cl_3CCOOH. Although several enzymes are known to be glycoproteins (Spiro, 1973), the existence of constitutive carbohydrates in acid proteases seems to be quite rare. To the best of our knowledge, the extracellular acid protease of *Mucor miehei* (Rickert and Elliott, 1973) is the only previously recorded case. The possibility that the carbohydrate may play a role in determining the thermostability of *P. duponti* protease deserves further investigation.

The experiments with diazo inactivators have shown that *P. duponti* protease resembles the other known fungal acid proteases on being (a) totally inactivated by α-diazo-*p*-bromoacetophenone, DAN, and DAG, in the presence of Cu^{2+}; (b) unlike pepsin, completely inert toward the action of *p*-bromophenacyl bromide.

The inactivation with DAN has been studied in detail on pepsin (Rajagopalan et al., 1966) and on the acid proteases of *P. janthinellum* (Sodek and Hofmann, 1968), *R. chinensis* (Mizobe et al., 1973), as well as those of *Aspergillus saitoi* and *Trametes sanguinea* (Takahashi and Chang, 1973). In all cases, the inactivation resulted in the covalent incorporation of 1 mol of norleucine/mol of enzyme. The reaction of *P. duponti* protease with DAN was quantitatively studied under conditions of complete inactivation and resulted also in the specific incorporation of 1 mol of norleucine/mol of enzyme. Although the isolation of the peptide containing the DAN-reacted residue was not attempted, the similarity in behavior with other acid proteases strongly suggests that an aspartyl residue occurs at the active site and is essential for catalysis.

The pepsin inhibitor S-PI was found to inhibit the protease, in a manner not yet understood. Inactivation studies using both DAN and S-PI would clarify the mechanism of S-PI inhibition and provide more information on the active site.

Despite the many similarities with pepsin and the pepsin-like fungal acid proteases, the acid protease of *P. duponti* remains unique in possessing an exceptional thermal stability.

Acknowledgments

We are grateful to Mrs. M. Wilkinson for her valuable assistance in the amino acid analysis. Our special thanks to Professor Sawao Murao, University of Osaka Prefecture, Sakai, Japan, for a sample of the pepsin inhibitor from *Streptomyces naniwaensis*. We would like also to express our appreciation to Dr. T. Yokotsuka, Kikkoman Shoyu Co., Noda, Japan, for the generous gift of crude enzyme preparation of *P. duponti* K 1014.

References

Bayliss, R. S., and Knowles, J. R. (1968), *Chem. Commun.*, 196.

Bovey, F. A., and Yanari, S. S. (1960), *Enzymes, 3rd Ed. 4*, 63.

Davis, B. L. (1964), *Ann. N.Y. Acad. Sci. 121*, 404.

Delpierre, G. R., and Fruton, J. S. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56*, 1817.

Dubois, M., Gilles, K. A., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., and Cooper, A. G. (1965), *J. Biol. Chem. 240*, PC 3447.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., and Cooper, A. G. (1966), *Biochem. Biophys. Res. Commun. 23*, 243.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., and Cooper, A. G. (1967), *Biochem. Biophys. Res. Commun. 28*, 203.

Fukumoto, J., Tsuru, D., and Yamamoto, T. (1967), *Agric. Biol. Chem. 31*, 710.

Fukumura, M., Satoi, S., Kuwana, N., and Murao, S. (1971), *Agric. Biol. Chem. 35*, 1310.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Graham, J. E. S., Sodek, J., and Hofmann, T. (1973), *Can. J. Biochem.* 51, 789.

Gross, E., and Morell, J. L. (1966), *J. Biol. Chem. 241*, 3638.

Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K. (1958), *J. Biochem. (Tokyo) 45*, 185.

Hamilton, G. A., Spona, J., and Crowell, L. A. (1967), *Biochem. Biophys. Res. Commun. 26*, 193.

Hashimoto, H., Iwaasa, T., and Yokotsuka, T. (1973a), *Appl. Microbiol. 25*, 578.

Hashimoto, H., Kaneko, Y., Iwaasa, T., and Yokotsuka, T. (1973b), *Appl. Microbiol. 25*, 584.

Hofmann, T., and Shaw, R. (1964), *Biochim. Biophys. Acta 92*, 543.

Ichishima, E. (1972), *Biochim. Biophys. Acta 258* 274.

Ichishima, E., and Yoshida, F. (1965a), *Biochim. Biophys. Acta 99*, 360.

Ichishima, E., and Yoshida, F. (1965b), *Biochim. Biophys. Acta 110*, 155.

Kunimoto, S., Aoyagi, T., Morishima, H., Takeuchi, T., and Umezawa, H. (1972), *J. Antibiot. 25*, 251.

Lundblad, R. L., and Stein, W. H. (1969), *J. Biol. Chem. 244*, 154.

Mizobe, F., Takahashi, K., and Ando, T. (1973), *J. Biochem. (Tokyo) 73*, 61.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 905.

Murao, S., and Satoi, S. (1970), *Agric. Biol. Chem. 34*, 1265.

Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121*, 321.

Perlmann, G. E. (1959), *Proc. Natl. Acad. Sci. U.S.A. 45*, 915.

Rajagopalan, T. G., Stein, W. H., and Moore, S. (1966), *J. Biol. Chem. 241*, 4295.

Rickert, W. S., and Elliott, J. R. (1973), *Can. J. Biochem. 51*, 1638.

Riehm, J. P., and Scheraga, H. A. (1965), *Biochemistry 4*, 772.

Ryle, A. P. (1970), *Methods Enzymol. 19*, 316.

Satoi, S., and Murao, S. (1971), *Agric. Biol. Chem. 35*, 1482.

Shaw, R. (1964), *Biochim. Biophys. Acta 92*, 558.

Sodek, J., and Hofmann, T. (1968), *J. Biol. Chem. 243*, 450.

Sodek, J., and Hofmann, T. (1970), *Can. J. Biochem. 48*, 1014.

Spiro, R. G. (1973), *Adv. Protein Chem. 27*, 349.

Takahashi, K., and Chang, W. J. (1973), *J. Biochem. (Tokyo) 73*, 675.

Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, H., and Takeuchi, T. (1970), *J. Antibiot. 23*, 259.

Wilkinson, M., Iacobucci, G. A., and Myers, D. V. (1975), *Anal. Biochem.* (in press).