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PURIFICATION AND CHARACTERIZATION OF THE TWO MOLECULAR FORMS OF *ASPERGILLUS ORYZAE* ACID PROTEASE

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Summary

The isolation and partial characterization of the acid proteases A_1 and A_2 (EC 3.4.23.6) from *Aspergillus oryzae* grown on solid bran culture are described. The purified preparations were essentially homogeneous by several criteria including sedimentation analysis and polyacrylamide gel electrophoresis.

The physicochemical properties of the proteases A_1 and A_2 were as follows (in the order: A_1, A_2): molecular weight: 63 000 & 32 000; sedimentation coefficient $s_{20,w}$: 3.93 and 3.16 S; diffusion constant $D_{20,w}$, $5.63 \cdot 10^{-7}$ and $8.61 \cdot 10^{-7}$ cm^2/s, partial specific volume, $\bar{v}$: 0.73 ml/g for both; nitrogen content: 16.30 and 13.42%; $E^{1\%}_{1\,cm}$ at 280 nm: 5.9 and 11.1. The two enzymes had the same pH optima in the acid pH range, and both activated bovine pancreatic trypsinogen. The enzymes were essentially of the same amino acid composition and immunologically cross-reacted with each other.

The protease A_2 contained little or no carbohydrate, whereas the protease A_1 was glycoprotein, containing 49% carbohydrate comprising glucose, mannose, and galactose. These results suggest that the protein portion of acid protease A_1 is the same as that of acid protease A_2.

Introduction

The presence of acid protease (EC 3.4.23.6) with pH optima near that of pepsin has been reported in several *Aspergilli* [1]. The enzyme from *Aspergillus saitoi* has been fully purified and studied extensively by Ichishima and his coworkers [2], but only limited information is available on the protease from *Aspergillus oryzae*. An acid protease with trypsinogen-kinase activity was partially purified from Takadiastase, a crude powder preparation from *A. oryzae*. The enzyme was reported to hydrolyze casein and, in contrast to acid proteases from other *Aspergilli*, a number of small synthetic peptides [3,4]. The enzyme

from *A. oryzae* was also purified and characterized at least partially by several other groups [5—7]. More recently, the acid protease of Polidase-S, a commercial preparation, was highly purified and characterized. This contains 1—2% carbohydrate and exists in two very closely related molecular forms [9].

In our preliminary paper, acid protease of *A. oryzae* was purified to homogeneity and crystallized [10]. However, further study showed that the enzyme exists in two active forms, the larger form containing approximately 50% carbohydrate and the smaller one devoid of detectable carbohydrate.

The present paper describes purification of the two acid proteases from Takadiastase and some physical and chemical properties of the enzymes.

Materials and Methods

Materials. A crude powder preparation (Takadiastase) was a product of Sankyo Co., Ltd., Tokyo. Hammersten milk casein and Z-Glu-Tyr (Z represents benzyloxycarbonyl) were obtained from E. Merck, West Germany. Z-Leu-Arg and Z-Glu-Phe were from Bachem Inc. (Calif., U.S.A.) and Protein Research Foundation, Institute for Protein Research, Osaka University (Osaka, Japan), respectively. Horse heart cytochrome *c* (91% purity) and bovine hemoglobin (enzyme substrate grade) were purchased from Nutritional Biochemical Corp. (U.S.A.), and bovine trypsinogen (once crystallized) from Sigma Chemical Co. (U.S.A.).

Enzyme assays. Proteolytic activity against casein was determined by a modified Anson's method [11]. The reaction mixture (1.5 ml) contained enzyme and 10 mg of milk casein in citric acid/phosphate buffer (26.5 mM citric acid/13.7 mM Na_2HPO_4), pH 3.0. Incubation was conducted at 37°C for 10 min and the reaction was terminated by the addition of 1.5 ml of 10% trichloroacetic acid. After standing for 20 min at 37°C, the resulting precipitate was removed by filtration through a Toyo No. 131 filter paper (Toyo Roshi Company, Japan). 0.5 ml of the filtrate was taken to determine the amount of trichloroacetic acid-soluble products, using Folin-Ciocalteau reagent [12]. One unit of acid protease activity was defined as the amount of enzyme which developed the color equivalent to 1 μmol of tyrosine per min under standard conditions.

Trypsinogen-kinase activity was determined by the method of Kunitz [13] with the modification given by Hofman and Shaw [14].

Sedimentation analysis. Sedimentation velocity analysis was performed with a Hitachi model UCA-1 ultracentrifuge equipped with the Schlieren cylindrical lens system.

Diffusion constant and partial specific volume. Diffusion constant was measured with a Hitachi Tiselius electrophoresis apparatus model HTB-2A equipped with a Neurath-type diffusion cell at 20°C in 0.1 M sodium acetate buffer, pH 5.0. Patial specific volume was determined either by Schachman's method [15] or by the method of Cohn and Edsall from amino acid composition [16].

Isoelectric focusing. Electrofocusing was carried out on an LKB column (110 ml) containing 1% carrier ampholite "Ampholine" with pH range from 3 to 6. The potential gradient of 700 volts was applied for 65 h at 10°C.

Disc gel electrophoresis. The polyacrylamide gel electrophoresis was performed using 7.5% acrylamide at pH 9.4 [17]. Sodium dodecyl sulfate gel

electrophoresis was carried out as described by Weber and Osborn [18], using gels with a cross-linkage of 10%. Protein bands were stained with 1% Amido black 10B. Carbohydrate was visualized on duplicate gels by periodate-fuchsin stain [19].

Amino acid analysis. Enzyme proteins were hydrolyzed with constant-boiling HCl in sealed, evacuated glass tubes at 110°C for 24, 48 or 72 h. Acid hydrolyzates were run through a Hitachi model KLA-2 automatic amino acid analyzer which was calibrated with D-glucosamine, D-galactosamine and D-mannosamine as well as with amino acids. Cysteine was determined as cysteic acid after performic oxidation [20]. Tryptophan content was determined by the spectrophotometric method of Goodwin and Morton [21].

Carbohydrate analysis. Total carbohydrate content was measured by the phenol-sulfuric acid method [22] and related to glucose. For the identity of each monosaccharide and the determination of carbohydrate composition of acid protease A_1, a sample (10 mg) was hydrolyzed in 1.0 M HCl in a sealed tube for 3 h at 100°C, and for sugar analysis the hydrolyzate was run through a Technicon, autoanalyzer (Technicon, U.S.A.) which was calibrated with D-glucose, D-galactose, D-mannose, D-xylose and L-rhamnose. The identity of each monosaccharide was also established by paper chromatography of the hydrolyzate in the solvent system *n*-butanol/pyridine/water (6 : 4 : 3), and then individual sugars were visualized by $AgNO_3$.

Total hexosamine content of acid proteases was determined by a modified Elson-Morgan's method with D-glucosamine · HCl as a standard [23]. Each hexosamine was identified and determined with the amino acid analyzer, as described above.

Immunochemical analysis. Immunodiffusion tests were performed by the Ouchterlony method [24] with antisera from rabbits that had been given an intramuscular injection of the purified acid protease A_2 in complete Freund's adjuvant.

Protein. Protein concentration was determined from absorbance at 280 nm by the factor $E^{1\%}_{1\,cm} = 11.1$ (See Table III for details).

Results

Purification

All steps described below were carried out at 4—8°C unless otherwise mentioned. Takadiastase powder (490 g) was extracted with 2.2 l of water at room temperature and the insoluble material was removed by centrifugation (Step 1). To the extract (2200 ml) was added 200 ml of 1 M calcium acetate with stirring and the resulting precipitate was removed by centrifugation. The supernatant solution (2260 ml) was exhaustively dialyzed against running tap water and then against 0.1 M sodium acetate buffer, pH 4.0 (Step 2). The dialyzed solution (3200 ml) was passed through a Duolite A-2 column (200—300 mesh, 11 × 80 cm) equilibrated with 0.1 M sodium acetate buffer, pH 4.0. After washing the column with 8 ml of the same buffer, the enzyme was eluted with 0.3 M sodium citrate buffer, pH 4.0. The active fractions (4320 ml) were collected and then dialyzed against running tap water for 48 h (Step 3). The dialyzed solution was brought to 0.1 M sodium acetate buffer, pH 5.0, by

adding 1 M sodium acetate buffer, pH 5.0, and was then applied to a DEAE-Sephadex A-50 column (3.5 × 40 cm) equilibtated with 0.1 M sodium acetate buffer, pH 5.0. The column was first washed with 500 ml of the same buffer. Next a linear gradient elution was performed with 1300 ml of 0.1 M sodium acetate buffer, pH 5.0, in the mixing chamber and 1300 ml of 0.1 M sodium acetate buffer, pH 4.0, containing 0.4 M NaCl in the reservoir. The active fractions (565 ml) were adjusted to pH 4.0 with HCl and exhaustively dialyzed against 0.01 M sodium acetate buffer, pH 4.0 (Step 4). The dialyzed solution (632 ml) was applied to a column of CM-Sephadex C-50 (3 × 40 cm) equilibrated with 0.01 M sodium acetate buffer, pH 4.0. The column was then washed with 220 ml of the same buffer. Under these conditions, part of the enzyme was recovered in the effluent (Peak 1, 815 ml), which was designated as acid protease A_1, and the remainder was adsorbed on the column, designated as acid protease A_2 (Fig. 1). Acid protease A_2 was then eluted as shown in Fig. 1. The active fractions (Peak 2, 165 ml) were collected (Step 5).

The effluent of Step 5 (815 ml) was dialyzed against 0.1 M sodium acetate buffer, pH 6.0, and then applied to a column of DEAE-Sephadex A-50 column (3 × 30 cm) equilibrated with the same buffer. The column was washed with 300 ml of the same buffer. A gradient elution was performed with 700 ml of the aforementioned buffer and 700 ml of 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl. To the active fractions collected (273 ml) were added 2 volumes of cold acetone at −20° C. The resultant precipitate was collected by centrifugation and was then dissolved in 15 ml of 0.1 M sodium acetate buffer, pH 5.0 (Step 6). The enzyme solution of Step 6 was subjected to Sephadex G-100 gel filtration as described in Fig. 2. The enzyme was recovered in the first protein peak, as a symmetrical peak which had constant specific activity throughout the active fractions. In Table I results of the purification procedure

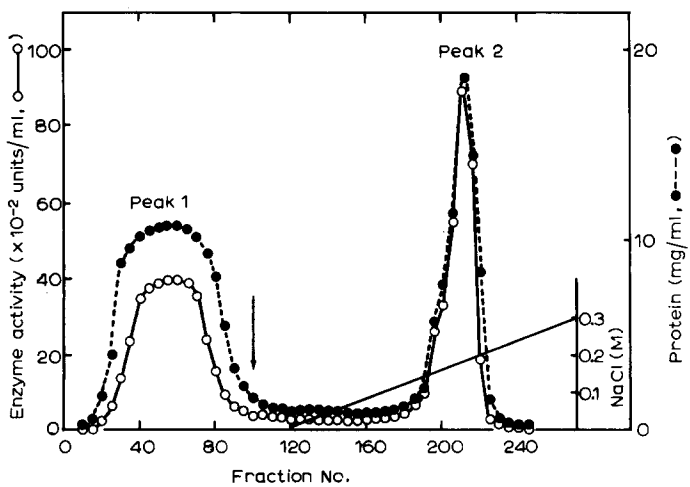

Fig. 1. CM-Sephadex chromatography of *A. oryzae* acid proteases. Elution of acid protease A_2 started as indicated by the arrow, was performed by a linear gradient elution with 800 ml of 0.01 M acetate buffer, pH 4.0, in the mixing chamber and 800 ml of 0.3 M sodium acetate buffer, pH 4.5, in the reservoir. Fractions of 10 ml were collected at a flow rate of 80 ml per h. See text for details.

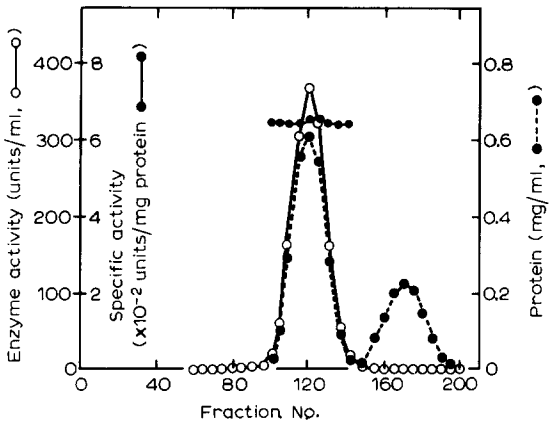

Fig. 2. Sephadex G-100 gel filtration of *A. oryzae* acid protease A_1. 3 ml of the enzyme from Step 6 (15 ml) was applied to a column (3 × 170 cm) which was equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The column was developed with the same buffer at a flow rate of 8 ml per h. Fractions of 5 ml were collected.

of acid protease A_1 are summarized (Step 7). To the active eluate of Step 5 (165 ml) were added 330 ml of cold acetone (−20° C) with stirring. The resultant precipitate was collected by centrifugation and dissolved in 25 ml of cold water. Cold acetone was added drop by drop to the solution until the solution became slightly cloudy. After standing at 3—4° C for 4 h, the crystals that were formed were collected by centrifugation. Recrystallization of the enzyme gave no further increase in its specific activity (Step 8). The results of purification procedure are summarized in Table I.

The specific activity of the purified A_2 preparation (Step 8) was slightly higher than that of A_1 (Step 6). The two preparations were used in the studies described below.

Criteria of homogeneity

The purified preparations of the acid proteases A_1 and A_2 gave one symmetrical peak on sedimentation analysis, and migrated as a single band on disc

TABLE I

PURIFICATION OF *A. ORYZAE* ACID PROTEASES A_1 AND A_2

Step	Fraction	Total activity (units · 10^{-3})	Yield (%)	Specific activity (units/mg protein)
1	Crude extract	11 500	100	43.1
2	Calcium acetate	10 960	98	48.7
3	Duolite A-2	8 720	78	307
4	DEAE-Sephadex A-50	4 610	41.4	397
5	CM-Sephadex C-50, A_1	1 310	11.9	267
	A_2	940	8.4	457
6	DEAE-Sephadex A-50 (A_1)	732	6.6	365
7	Sephadex G-100 (A_1)	418	3.8	660
8	First crystallization (A_2)	391	3.5	815
	Second crystallization (A_2)	299	2.6	809

gel electrophoresis, although acid protease A_1 gave a slightly broad band. When acid protease A_1 was subjected to isoelectric focusing, two activity peaks were obtained (Fig. 3). These peaks were coincident with two protein peaks and had constant specific activity throughout the active fractions. The isoelectric point was 3.15 for peak A_{1a} and 3.50 for peak A_{1b}, respectively. On the other hand, protease A_2 gave a single protein peak with enzyme activity which showed an isoelectric point of 3.9 (Fig. 3).

The purified proteases A_1 and A_2 released no detectable amino acid residues upon prolonged incubation at pH 3 and 30° C from synthetic dipeptides tested (Z-Leu-Arg, Z-Glu-Tyr and Z-Glu-Phe), indicating no contamination with carboxypeptidase-like enzymes.

Optimum pH

The effect of pH on the activity of the acid proteases with several protein substrates was determined in McIlvaine's citric acid/phosphate buffer [25]. As shown in Fig. 4, the optimum pH with horse heart cytochrome *c* as substrate was 3.0 for both enzymes, while it was around 4.2 with urea-denatured bovine hemoglobin for both enzymes. The enzymes hydrolyzed casein at pH 3.0 and 5.8, but the hydrolytic activity was higher at the former than at the latter pH for both enzymes. Measurements of the caseinolytic activity at pH values between these two values gave no reliable results because of insolubility of the substrate.

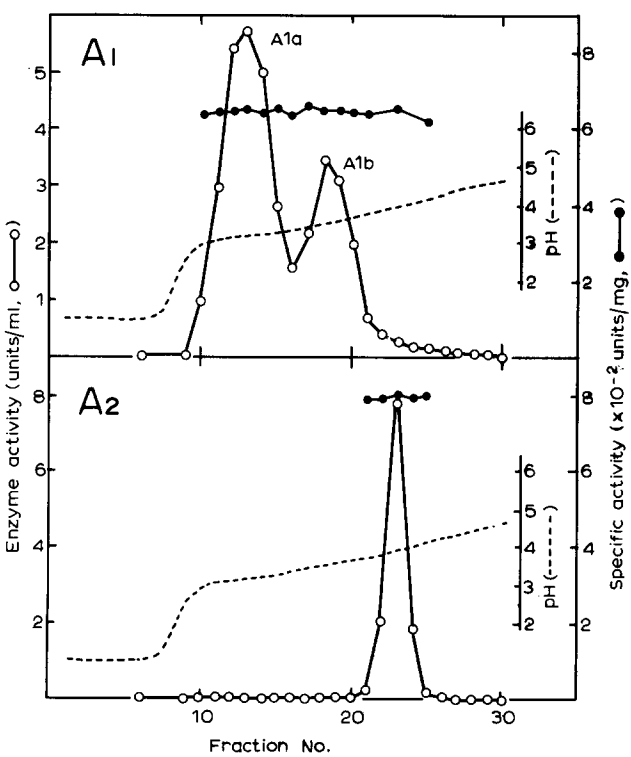

Fig. 3. Electrofocusing profiles of *A. oryzae* acid proteases. Experiments were carried out as described in Materials and Methods. A_1, acid protease A_1; A_2, acid protease A_2.

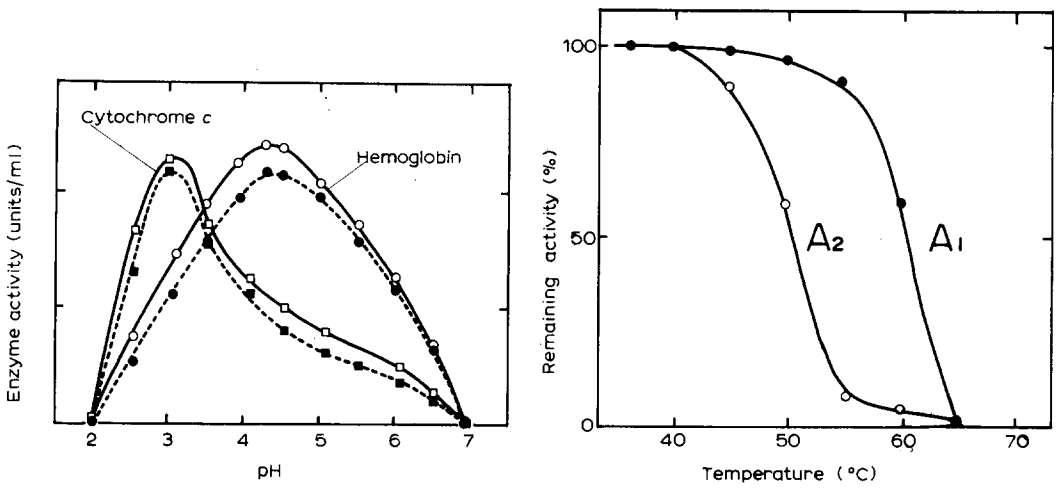

Fig. 4. Optimum pH of *A. oryzae* acid proteases. Enzyme activity was determined as described in Materials and Methods except that pH values of McIlvaine's citric acid/phosphate buffer were varied as indicated and that urea-denatured bovine hemoglobin or horse heart cytochrome *c* was used as substrate.

Fig. 5. Heat stability of *A. oryzae* acid proteases. Purified enzymes in 5 mM sodium acetate buffer, pH 5.0, were heated for 10 min at various temperatures and the remaining activities were determined. ———●———, acid protease A_1; ———○———, acid protease A_2.

Inhibitors

The two proteases were not inhibited by *p*-chloromercuribenzoate at 0.17 mM and monoiodoacetate at 1.7 mM. No inhibition was observed with ethylenediaminetetraacetate, *o*-phenanthroline, diisopropylfluorophosphate, *N*-bromosuccinimide and ϵ-aminocaproate at 1.7 mM. Metals tested, including $HgCl_2$, NaF, $FeCl_3 \cdot 6\ H_2O$, $FeCl_2 \cdot 4\ H_2O$, $CoCl_2$, $MgCl_2$, $CaCl_2$ and $ZnSO_4$, showed no significant stimulatory nor inhibitory effect at 1.7 mM. The enzymes were not inhibited by soybean trypsin inhibitor at a concentration of 0.017%. As reported with some of the fungal acid proteases [5,6,26], the two acid proteases were completely inactivated upon preincubation with 5.1 mM sodium lauryl sulfonate at 37°C for 10 min.

Activation of trypsinogen

The two acid proteases were found to activate bovine pancreatic trypsinogen at pH 3.4, as demonstrated with some fungal acid proteases [1]. The enzymes A_1 and A_2 activated 13.6 and 10.6 nmol of trypsinogen per unit of enzyme at 36°C for 10 min, respectively, under the conditions described by Hofman and Shaw [14].

Stability

The purified A_1 and A_2 preparations were stable for at least two years when stored at −20°C in 50 mM sodium acetate buffer, pH 5.0. However, the homogeneous A_2 preparations occasionally showed one major protein band and additional three faintly stained minor bands after several months of storage under those conditions. When heated at 55°C for 10 min in 50 mM sodium acetate buffer, pH 5.0, acid protease A_1 lost approximately 10% of its original activity

in contrast to almost complete loss of activity by A_2 (Fig. 5). Accordingly, it is obvious that acid protease A_1 is much more stable than A_2.

Molecular weight

The apparent sedimentation constant was determined at various enzyme concentrations, and the extrapolation to zero concentration yielded a value of 3.93 S for acid protease A_1 and 3.16 S for A_2, respectively. The diffusion constant $D_{20,w}$ of proteases A_1 and A_2 was $5.71 \cdot 10^{-7}$ and $8.61 \cdot 10^{-7}$ cm^2/s, respectively. The partial specific volume was determined to be 0.73 ml/g for both enzymes. From these data, the molecular weight was calculated to be 63 000 for acid protease A_1 and 32 000 for A_2 according to Svedberg's equation.

Nitrogen and carbohydrate

Nitrogen analyses gave a value of 6.30%, an unusually low nitrogen content, for acid protease A_1 and 13.42% for A_2. The carbohydrate content was 49% for protease A_1 while less than 0.3% for A_2. On the basis of these data, the molecular weight of the protein moiety of protease A_1 was considered to be 32 000 which corresponds to that of protease A_2. Carbohydrate analysis of the acid hydrolyzate of acid protease A_1 revealed that the enzyme contained glucose, galactose, and mannose. The number of residues of these hexoses per molecule of the protease were found to be 56—58 for glucose, 96—101 for galactose and 22—24 for mannose, respectively. These three hexoses were also detected and

TABLE II

AMINO ACID COMPOSITION OF *A. ORYZAE* ACID PROREASE A_1 AND A_2

Amino acid	Molar ratio *		Residues per molecule ***	
	A_1	A_2	A_1	A_2
Lysine	0.560	0.579	15	15
Histidine	0.126	0.138	3	4
Arginine	0.067	0.064	2	1
Aspartic acid	1.425	1.413	39	36
Threonine **	0.872	0.907	21	23
Serine **	1.004	1.048	26	27
Glutamic acid	1.010	1.031	27	27
Proline	0.537	0.552	15	17
Glycine	1.257	1.310	33	35
Alanine	1.000	1.000	27	26
Half-cystine	0.117	0.101	3	3
Valine	0.845	0.869	23	24
Methionine	0.032	0.034	1	1
Isoleucine	0.472	0.497	13	14
Leucine	0.710	0.722	19	19
Tyrosine	0.455	0.485	14	12
Phenylalanine	0.563	0.572	15	15
Tryptophan	0.173	0.189	5	5
		Total	301	304

* Average values of two 24-h hydrolyzates for A_1 and of two 24-, two 48- and two 72-h hydrolyzates for A_2.

** Values extrapolated to zero time.

*** A molecular weight of 32 000 for acid protease A_2 and for the protein portion of A_1 was used.

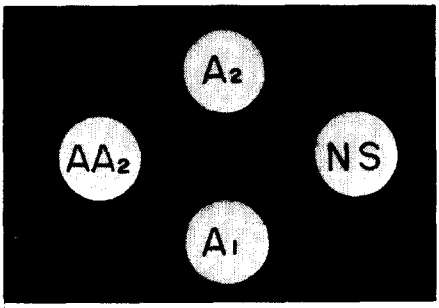

Fig. 6. Double diffusion analysis of *A. oryzae* acid proteases. A_1, acid protease A_1 (8 μg); A_2, acid protease A_2 (5 μg); AA_2, rabbit antiserum against acid protease A_2; NS, normal rabbit serum.

identified by paper chromatography of the acid hydrolyzate of acid protease A_1.

Total hexosamine content was less than 0.1% for both of acid proteases A_1 and A_2, that is, less than 0.3 residue of total hexosamine per molecule for both enzymes. When a sample (2 mg) of each acid protease was hydrolyzed in 6 M HCl for 24 h at 110°C and assayed for glucosamine, galactosamine, and mannosamine by amino acid analyzer, none of these hexosamines was detectable in either enzyme. The carbohydrate moiety of acid protease A_1 fractionated together with protein through a variety of procedures including column chromatography on Duolite A-2 and DEAE-Sephadex, gel filtration through Sephadex G-100, and disc gel electrophoresis. In addition, protease A_1 remained soluble in several protein precipitants such as saturated $(NH_4)_2SO_4$ and trichloroacetic acid, while A_2 was easily precipitated by these agents.

Amino acid composition

The results of amino acid analyses are shown in Table II. The values are expressed as number of residues per molecule, assuming a molecular weight of 63 000 (49% carbohydrate) for acid protease A_1 and 32 000 for A_2. The results indicate that both enzymes contain 301—304 amino acid residues per molecule. No essential differences were observed between the two enzymes.

Immunodiffusion

The immunodiffusion studies showed a strong reaction between acid proteases A_1 and A_2 and rabbit antiserum against A_2 (Fig. 6). The continuity between the precipitin bands of the two enzymes suggested a fairly high degree of similarity between them.

Discussion

The two acid proteases A_1 and A_2 of *A. oryzae* have been purified to homogeneity from Takadiastase, a commerical preparation. The enzymes have pH optima in the acid pH range with several protein substrates. They have no hydrolytic activity towards several synthetic dipeptides which were reported to be hydrolyzed by fungal acid carboxypeptidate [27]. They activate pancreatic trypsinogen in the acid pH range. Those properties are also observed

TABLE III

SUMMARY OF PHYSICAL AND CHEMICAL PROPERTIES OF *A. ORYZAE* ACID PROTEASE A_1 AND A_2

Properties	A_1	A_2
$E^{1\%}_{1\,cm}$ at 280nm	5.9	11.1
Elementary analysis (%):		
N	16.3	13.4
C	44.2	48.3
H	6.7	7.1
Carbohydrate content (%)	49	0.3
Isoelectric points	3.15, 3.50	3.90
Sedimentation constant, $S_{20,w}$ (S)	3.93	3.16
Diffusion constant, $D_{20,w}$ (cm^2/s)	$5.65 \cdot 10^{-7}$	$8.61 \cdot 10^{-7}$
Partial specific volume, ν (ml/g)	0.73	0.73
Molecular weight	63 000	32 000

with acid proteases from other fungi including the genera *Aspergillus*, *Penicillium* and *Phizopus* [1].

No differences could be observed in the amino acid composition and enzymatic activities of the two *A. oryzae* acid proteases. In addition, acid protease A_1, a glycoprotein, reacts clearly with rabbit antiserum against protease A_2 devoid of detectable carbohydrate, suggesting that the amino acid sequence of acid protease A_2 is the same as that of the protein portion of A_1.

The physical and chemical properties of the two acid proteases A_1 and A_2 are summarized in Table III. Acid protease A_1 is a glycoprotein which contains as much as about 50% carbohydrate, consisting of glucose, galactose and mannose, while A_2 is almost devoid of carbohydrate. The difference in carbohydrate content could very well account for differences between the two enzymes in isoelectric point as well as molecular weight. It should be noted that *Aspergillus sojae* acid protease is also a glycoprotein which contains glucose, galactose and mannose but does not contain hexosamines, although its carbohydrate content is somewhat lower (12.5%) [28].

The acid protease of *A. oryzae* was recently isolated and characterized by Davidson et al. [9]. The enzyme can be precipitated with $(NH_4)_2SO_4$ and contains only a small quantity of carbohydrate (1—2%). In addition, the isoelectric point of the enzyme is around 4. These findings indicate that the enzyme reported by Davidson et al. corresponds to acid protease A_2 described in this paper, although molecular weight of their enzyme (39 000) is larger than that of acid protease A_2 (32 000).

In view of the precipitability with $(NH_4)_2SO_4$, the acid protease A_2 seems to correspond to the trypsinogen-kinase which was isolated from Takadiastase by Nakanishi [3].

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