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PROTEIN CATABOLISM. II. IDENTIFICATION OF NEUTRAL AND ACIDIC PROTEOLYTIC ENZYMES IN *ASPERGILLUS NIGER**

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SUMMARY

Two proteinases of *Aspergillus niger*, one of pH optimum 3.4, the other of pH optimum 7.4, were purified 840- and 740-fold by $(NH_4)_2SO_4$ precipitation and Sephadex G-100, G-150 and G-200 gel chromatography. Both proteinases were assayed using [^{3}H]acetyl hemoglobin as substrate. The purified pH 3.4 proteinase had two bands on sodium dodecylsulfate polyacrylamide gel electrophoresis, while the purified pH 7.4 proteinase gave one band when stained for protein; none of the purified material stained for glycoprotein.

The purified pH 3.4 proteinase had molecular weights of 49 000 and 56 000, pI of 4.1 and 4.5, and a K_m of $1 \cdot 10^{-5}$ M. This acid proteinase showed no cofactor requirements, was inhibited 51% by 0.1 M $HgCl_2$, and was relatively inactive against synthetic substrates of low molecular weight. Analysis of the products of digestion of the [^{3}H]acetyl hemoglobin after 1-h digestion with this enzyme showed that 71% of the product consisted of peptides of molecular weights less than 4000.

The purified pH 7.4 proteinase had a molecular weight of 68 000, pI of 6.2, and a K_m of $5 \cdot 10^{-5}$ M. This neutral proteinase showed no cofactor requirements, was severely inhibited by $HgCl_2$ and diisopropylphosphofluoridate and was also relatively inactive against low molecular weight synthetic substrates. Analysis of the products of digestion with this neutral proteinase showed that 66% of the product consisted of peptides of molecular weights less than 4000.

INTRODUCTION

In comparison to the amount of work performed on protein synthesis, activity and structure little attention has been paid to the subject of protein degradation or catabolism. In particular the subject of proteolytic enzymes has been limited pri-

* This is the second paper in a series entitled "Protein Catabolism". The previous paper is ref. 5.

marily to study of trypsin (EC 3.4.4.4) and certain other mammalian proteases. This lack of attention on proteolytic enzymes is due primarily to the fact that reliable, sensitive assays until recently did not exist, the methods primarily relying on release of Folin–Ciocalteu-positive material from denatured hemoglobin or casein[1,2] or hydrolysis of certain synthetic substrates such as *p*-toluenesulfonyl-L-arginine methyl ester[3] or benzoyl-L-tyrosine ethyl ester[3], which probably do not reflect true *in vivo* enzyme activity. With the advent of a very sensitive assay for proteolytic activity with [^{3}H]acetyl hemoglobin as substrate[4] this laboratory has investigated several aspects of proteolytic activity[5,6]. The present work reports on the substantial purification and the characteristics of two proteolytic enzymes from *Aspergillius niger*, one active at pH 3.4 (cathepsin-like) and another active at pH 7.4.

Various proteolytic enzymes have been described in molds primarily of the *Aspergillus* genus or bacteria. Enzymes identified include an extracellular acid proteinase of *Aspergillus saitoi*[7], an alkaline collagenase from *Aspergillus oryzae*[8], an extracellular alkaline proteinase from *Aspergillus flavus*[9], several neutral proteases from *Aspergillus oryzae*[10], an alkaline protease from *A. oryzae*[11], and an acid protease from *Aspergillus parasiticus*. More extensive biochemical data are available for mammalian proteinases; those described include rabbit, chicken, and human liver[13,14] acid proteinases, and acid and neutral proteinases of rat brain[15]. Finally, recently a neutral proteolytic enzyme has been isolated from *Escherichia coli* and its biochemical properties described[16]. The present paper describes the biochemical characteristics of two proteinases isolated from *Aspergillus niger*.

MATERIALS AND METHODS

Organism

Aspergillus niger was cultured in brain heart infusion (Difco) aerobically. Fungi were purchased from the American Type Tissue Culture collection. The fungi were harvested by centrifugation at 40 000 × *g* and the pellet was used for the experiments described herein; *i.e.* the proteases were isolated from the fungi, even though the culture supernatant contained proteolytic activity indicating release of the enzymes.

Materials

The following synthetic substrates were from Mann Research Laboratories (New York, N.Y.): acetyl-D L-phenyl-alanine-2-naphthyl ester, acetyl-L-tyrosine ethyl ester, benzoyl-DL-arginine-*p*-nitrosnilide, and *N*-benzoyl-DL-arginine-ethyl ester. [^{3}H]Acetic anhydride (spec. act. 400 Ci/mole) was from New England Nuclear. Type I beef blood hemoglobin was from Sigma Chemical Company. Enzyme grade $(NH_4)_2SO_4$ was from Mann Research Laboratories. All other biochemicals and enzymes were purchased from Sigma Chemical Company.

Preparation of substrate

[^{3}H]Acetyl hemoglobin was prepared as described previously[6,17]. [^{3}H]Acetic anhydride (400 Ci/mole) was reacted with the Type I beef blood hemoglobin. The material was purified[6,17] to a specific activity of 162 cpm/pmole based on a molecular weight of 68 000 utilizing the counting procedures described herein. The specific activity of the material was 300 Ci/mole. Routinely in the assay systems described

below 50 μl (136 μg [^{3}H]acetylated hemoglobin; 2 nmoles; 324 000 cpm; 0.6 μCi) of the [^{3}H]acetylated hemoglobin was added per assay.

Enzyme purification

The enzyme purification was accomplished in six steps. 100 g of the pellet described above containing the fungi was homogenized with 500 ml of 0.1% Triton X-100 in a Potter Elvehjem Homogenizer for 30 strokes. This homogenate was then subjected to thirty 15-s homogenization-sonication strokes with a Polytron sonicator. The homogenate was then stirred at 4 °C for 4 h. The homogenate (about 10 g protein) was centrifuged at 70 000 × *g* for 10 min and the supernatant fluid (approx. 1.9 g protein) was made 50% in $(NH_4)_2SO_4$ (w/v). The pellet of this $(NH_4)_2SO_4$ precipitation was exhaustively dialyzed against 0.1% Triton X-100. The resulting material (approx. 640 mg protein) was placed on a Sephadex G-100 column (2.5 cm × 90 cm), equilibrated, and eluted with 0.1% Triton X-100. A flow rate of 80 ml/h was maintained, and samples of 8 ml were collected. Fractions 26 to 50 of this fractionation were dialyzed, lyophilized to 10 ml, and placed on a Sephadex G-150 column prepared and eluted exactly as above. A flow rate of 60 ml/h was maintained and samples of 10 ml were collected. Fractions 19 to 32 and 43 to 60 of this fractionation were dialyzed, lyophilized, and placed on separate Sephadex G-200 columns prepared and eluted as above. Flow rates of 30 ml/h were maintained and fractions of 9 ml were collected. Fractions 40–50 and 50–57 of the respective columns were considered to be the purified pH 7.4 and pH 3.4 proteolytic enzymes in each instance (see Fig. 3). In each instance fractions were chosen to maximize quantity of material as well as specific activity.

Assay of proteolytic activity in 0.1% Triton X-100 extracts of Aspergillus niger as a function of pH

Before complete assay systems (see below) were established, the proteolytic activity of the crude extract of *A. niger* was measured with the Michaelis barbital sodium acetate buffer[18]. The assay included 50 μl of the 0.1% Triton X-100 extract of *A. niger* (100 μg as protein), 50 μl of the [^{3}H]acetylated hemoglobin (2 nmoles), and 100 μl of the buffer at the desired pH. Assays were performed as given below for the complete systems. After these assays were completed, it was decided to perform assays at pH 3.4 (acid proteinase) and pH 7.4 (neutral proteinase).

Assay for pH 3.4 activity

Routinely, 50 to 100 μl of enzyme extract (3 to 150 μg protein, depending on the source of enzyme) was added to 100 μl of a solution of 1.35 M acetic acid and 0.02 M $(NH_4)_2SO_4$, pH 3.4. 50 μl of [^{3}H]acetyl hemoglobin (2 nmoles) was added as the substrate. This mixture was incubated for 1 h at 37 °C in a Dubnoff metabolic shaker. The reaction was terminated by placing the assay tubes in an icewater bath (0–4 °C) and adding 100 μl of 2.5% hemoglobin and 50 μl of 60% trichloroacetic acid. The precipitated material was removed by centrifugation at 5000 × *g* for 5 min and an aliquot of the supernatant fluid was plated on a glass fiber filter; the radioactivity was determined by counting in a liquid scintillation counter[19,20]. Activity is expressed as pmoles of hemoglobin degraded per hour per mg enzyme protein. Suitable blanks consisting of 0.1% Triton X-100 or boiled enzyme (10 min at 100 °C) were added in

place of the enzyme extract and incubated simultaneously. The blank readings never exceeded 5% of the enzyme activity. All experiments were performed in duplicate or triplicate.

Assay for pH 7.4 activity

Activity at pH 7.4 was analyzed as above except that 100 μl of 0.1 M phosphate buffer, pH 7.4, was substituted as the buffer.

Enzyme characterization

All experiments were performed with the purified enzyme (Sephadex G-200 fractions); enzyme from several purification runs were pooled for the experiments.

Analytic electrophoresis of sodium dodecylsulfate extracts

Extraction and electrophoresis were carried out on the various fractions obtained from the *A. niger* by the method described by Lenard[21].

Following electrophoresis the gels were stained for either protein or glycoproteins as described[22–24].

Protein

Total protein was determined by the method of Lowry *et al.*[25].

Isoelectric focusing

Isoelectric focusing was carried out with the LKB 110-ml electrofocusing apparatus[26].

Proteolytic assays utilizing substrates other than [^{3}H]acetyl hemoglobin

Assays were performed by the method of Anson[1,2] to ensure that true proteolytic and not esterolytic enzymes were being measured with the [^{3}H]acetyl hemoglobin substrate. The values derived by the method described herein in every instance corresponded almost exactly with the Anson method[1,2] although in the latter instance much higher amounts of enzyme (on a mg protein basis) had to be used. Assays utilizing the synthetic peptide substrates were performed as described[16].

RESULTS

Proteolytic activity of crude homogenates of Aspergillus niger as a function of pH

Initial experiments indicated that the crude Triton X-100 homogenate of *Aspergillus niger* had proteolytic activity from pH 2.6 to pH 9.4. Maximal activity was found at pH 3.4 and relatively high activity was found at pH 2.6 to 3.4 and pH 3.4 to pH 5.8. Above pH 5.8 another peak of activity was found at pH 7.4. On the basis of these data, it was decided to attempt to purify and separate the pH 3.4 and pH 7.4 proteinases.

Purification of the pH 3.4 and pH 7.4 proteinases of Aspergillus niger

The data of Table I indicate that following the purification scheme outlined in Materials and Methods the pH 3.4 proteinase was purified 840-fold with a 45% recovery, while the pH 7.4 proteinase was purified 740-fold with a 30% recovery.

TABLE I

PURIFICATION AND ISOLATION OF TWO PROTEOLYTIC ENZYMES OF *Aspergillus niger* BY 0.1% TRITON X-100 EXTRACTION, AMMONIUM SULFATE PRECIPITATION AND COLUMN CHROMATOGRAPHY

Treatment	*pH 3.4 proteinase*					*pH 7.4 proteinase*				
	Total protein (mg)	*Total activity (nmoles Hb/h)*	*% Recovery*	*Specific activity (pmoles Hb/mg protein per h)*	*Purification factor*	*Total protein (mg)*	*Total activity (nmoles Hb/h)*	*% Recovery*	*Specific activity (pmoles Hb/mg protein per h)*	*Purification factor*
0.1% Triton X-100 homogenate	10 000	38.0	100	3.8	1	10 000	23.0	100	2.3	1
20 000 × *g* supernatant	1 880	28.5	75	15.2	4	1 880	18.4	80	9.9	4
50% $(NH_4)_2SO_4$ pellet	640	26.6	70	41.8	11	640	11.5	59	18.0	8
Sephadex G-100 fraction	88	22.8	60	258.4	68	88	9.2	40	104.2	45
Sephadex G-150* fraction	24	19.4	51	820.8	216	9.5	8.3	36	874.0	380
Sephadex G-200* fraction	5.4	17.1	45	3192.0	840	4.0	6.9	30	1725.0	740

* Sephadex fractions refer to different fractions for the G-150 and G-200 column chromatography as explained in the text. See Figs 2 and 3.

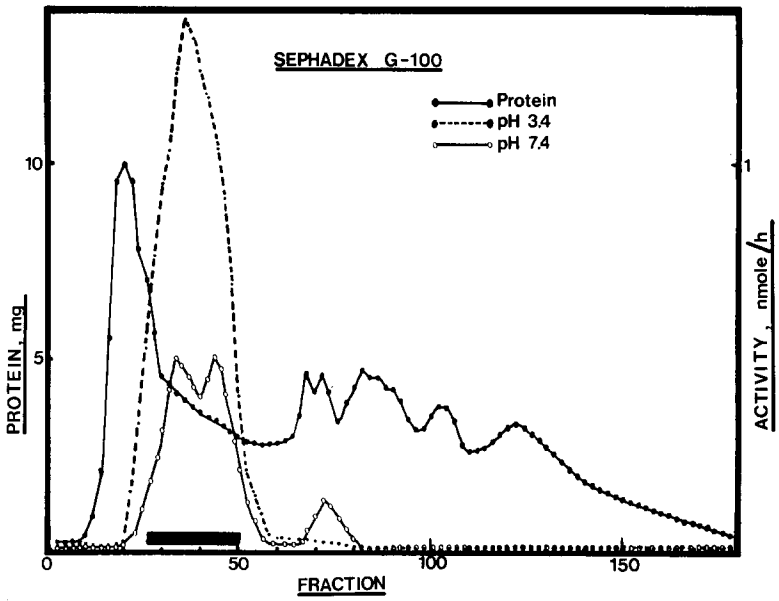

Fig. 1. Gel chromatography of proteases of *Aspergillus niger* on Sephadex G-100. The dialyzed 50% $(NH_4)_2SO_4$ pellet was placed on a Sephadex G-100 column. Details of the fractionation are described under Materials and Methods. The heavy line indicates the fraction placed onto Sephadex G-150 (Fig. 2).

Major purification (4- to 5-fold in each instance) occurred with the 20 000 × *g* centrifugation and gel chromatography on Sephadex G-100 (Fig. 1). On Sephadex G-100 the pH 3.4 proteinase eluted in one rather sharp peak while the pH 7.4 proteinase eluted in 1 volume (Fractions 25 to 55) containing two peaks and in another volume (Fractions 68 to 80) containing one minor peak. On Sephadex G-150 gel chromatography (Fig. 2) the two proteinases were essentially separated, each eluted in a rather broad peak. Taking material from these peaks and placing them on separate Sephadex G-200 columns (Fig. 3) resulted in further purification and virtual separation of the two enzymes. The purification scheme was repeated 4 times with similar results.

Characteristics of the assay for the two Aspergillus niger proteinases

Experiments indicated that both the proteinases were dependent on non-heat denatured enzyme, time of incubation, and incubation temperature for activity. The pH 7.4 enzyme had 17% the activity of trypsin against the hemoglobin substrate on a mg protein basis and was inhibited 16% by a 10-fold excess of soybean trypsin inhibitor; the pH 3.4 proteinase was not inhibited by the soybean trypsin inhibitor.

Activity of the two Aspergillus niger proteases as function of temperature of assay

The pH 3.4 proteinase had high activity from 30 to 60 °C with maximal activity at 50 °C; there was no activity above 70 °C or below 10 °C. The pH 7.4 proteinase had high activity between 30 and 50 °C with maximal activity at 45 °C; little activity occurred above 70 or below 15 °C.

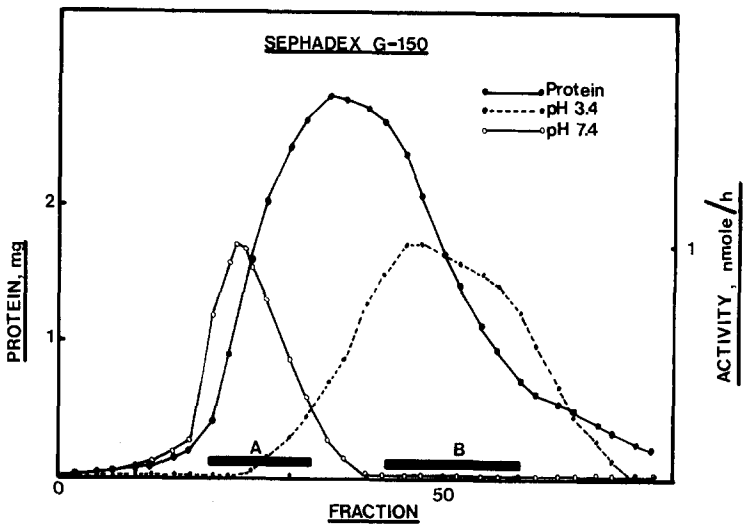

Fig. 2. Gel chromatography of proteases of *Aspergillus niger* on Sephadex G-150. The material from the Sephadex G-100 fractionation (Fig. 1) was fractionated on Sephadex G-150. Details of the fractionation are described under Materials and Methods. The heavy lines indicate the fractions placed onto Sephadex G-200 (Fig. 3).

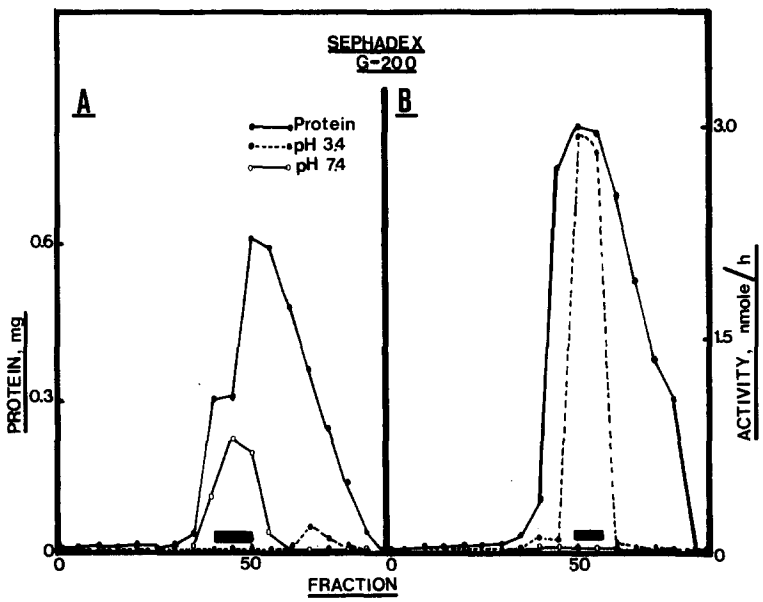

Fig. 3. Gel chromatography of the proteases of *Aspergillus niger* on Sephadex G-200. The material labeled A and B from the Sephadex G-150 fractionation (Fig. 2) was fractionated on separate Sephadex G-200 columns as described in Materials and Methods. The heavy lines indicate the fractions considered to be the purified pH 7.4 (A) and pH 3.4 (B) proteinases, respectively.

Activity of the two Aspergillus niger proteases as function of pH of assay

The pH 3.4 enzyme had maximal activity at pH 3.4 with no activity above pH 5; at pH 1.5, 33% of the activity still was present. The pH 7.4 enzyme had maximal activity at pH 7.4 and rather high activity within *plus* or *minus* 2 pH units of pH 7.4. No activity was found with this enzyme above pH 10.5 or below pH 3.5. The data demonstrate that the pH 3.4 enzyme was free of pH 7.4 enzyme contamination and *vice versa*.

Effects of divalent cations and group specific reagents on the two proteolytic enzymes of Aspergillus niger

The results given in Table II demonstrate that the pH 3.4 protease did not require a divalent cation for activity and was not inhibited too severely by the

TABLE II

INFLUENCE OF DIVALENT CATIONS AND GROUP SPECIFIC REAGENTS ON THE TWO PROTEASES OF *Aspergillus niger**

Addition	*Concentration (mM)*	*Relative activity (%)*	
		pH 3.4 protease	*pH 7.4 protease*
None	—	100	100
$CoCl_2$	0.1	60	100
$HgCl_2$	0.1	49	2
$PbCl_2$	0.1	81	106
$MnCl_2$	0.1	70	104
$MgCl_2$	0.1	104	96
$CaCl_2$	0.1	80	101
$CdCl_2$	0.1	84	100
$CuCl_2$	0.1	95	106
$FeCl_2$	0.1	102	100
$ZnCl_2$	0.1	84	100
EDTA	0.1	100	104
DFP	0.1	89	6
Cysteine	1	86	89
Mercaptoethanol	1	89	96
Sodium *p*-chloromercuribenzoate	1	92	89
2,4'-Dibromoacetophenone**	0.1	98	92
2-Bromo-2-phenyl acetophenone**	0.1	96	96
Iodoacetate	0.1	96	94

* The purified enzymes were incubated with the indicated compounds at the final concentration designated for 10 min at 25 °C, pH 7.0, before the substrate was added. Data are given as relative activity; means from five independent determinations.

** Dissolved in dimethylsulfoxide which was diluted to 0.8% (v/v) in the assays.

divalent cations or group specific agents studied. $HgCl_2$ and to a lesser extent $CoCl_2$ and $MnCl_2$ inhibited the pH 3.4 proteinase of *A. niger*. The pH 7.6 protease of *A. niger* was not dependent on a divalent cation for activity but was inhibited severely and selectively by 0.1 mM $HgCl_2$ and 0.1 mM diisopropylphosphofluoridate.

Isoelectric point of the pH 3.4 protease of Aspergillus niger

The pH 3.4 enzyme showed two peak isoelectric points of pH 4.1 and 4.5.

Isoelectric point of the pH 7.4 proteinase of Aspergillus niger

The pI of the pH 7.4 optimum enzyme was 6.2 and the purified enzyme was quite homogeneous when subjected to isoelectric focusing.

Molecular weight of the pH 3.4 proteinase and the pH 7.4 proteinase of Aspergillus niger

Utilizing migration in sodium dodecylsulfate polyacrylamide gel electrophoresis as a determinant of molecular weight and homogeneity of the purified enzymes, the pH 3.4 protease was found to have two bands with molecular weights of 49 000 and 56 000 and the pH 7.3 protease one band with a molecular weight of 68 000 (Fig. 4). Neither of the purified preparations of proteinase stained with the periodic acid–Schiff reaction, indicating that neither is of glycoprotein nature.

Lineweaver-Burk plot of the purified pH 3.4 and pH 7.4 optimum proteinases of Aspergillus niger

Linear double reciprocal plots were obtained with each of the purified protein-

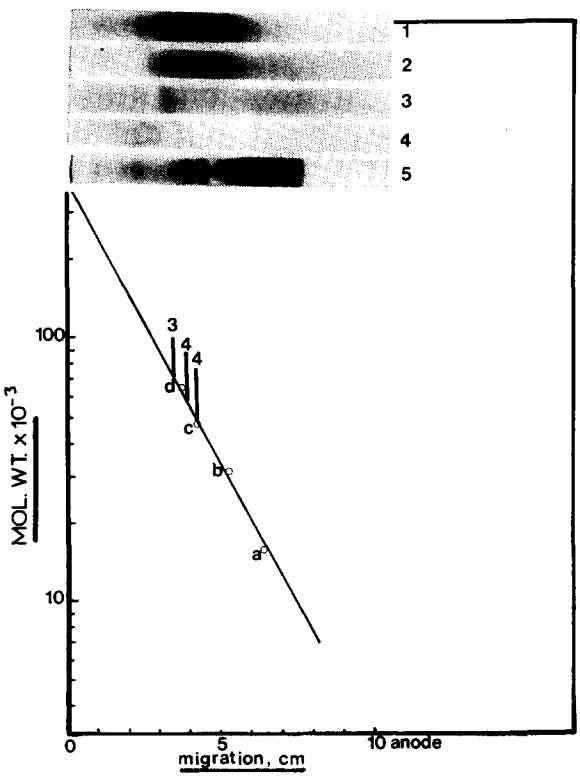

Fig. 4. Polyacrylamide gel electrophoresis in sodium dodecylsulfate of the various fractions of *Aspergillus niger* and hemoglobin standards and a plot of migration *vs* molecular weight. Electrophoresis was performed as given in Materials and Methods. The gels are (1) 50% $(NH_4)_2SO_4$ pellet (Table 1); (2) Fractions 26 to 50 of the Sephadex G-100 column (Fig. 2); (3) The purified Sephadex G-200, pH 7.4 proteinase (Fig. 4); (4) The purified Sephadex G-200, pH 3.4 proteinase (Fig. 4); (5) hemoglobin standard. All gels were stained with Coomassie Brilliant Blue; there was no staining of the purified proteinases with the periodic acid–Schiff reagents. Standards on the molecular weight plot are (a) hemoglobin monomer (17 000) (b) hemoglobin dimer (34 000), (c) hemoglobin trimer (51 000), and (d) hemoglobin tetramer (68 000).

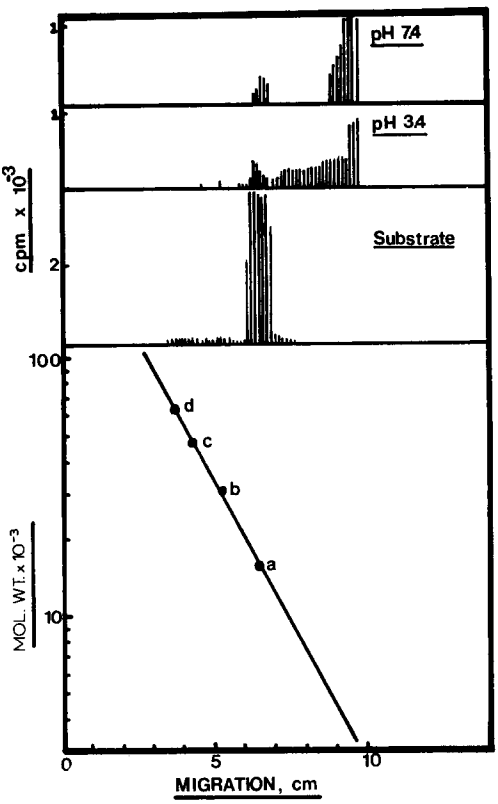

Fig. 5. Polyacrylamide gel electrophoresis of the [^{3}H]acetyl hemoglobin substrate and the products after digestion with the pH 3.4 and pH 7.4 proteinases of *Aspergillus niger*. Assays were performed as given in Materials and Methods with the complete systems and after 1 h incubation polyacrylamide gel electrophoresis was performed. For the standards of the molecular weight *vs* migration graph see Fig. 4. Substrate from the equivalent of 0.7 normal reactions and reaction mixtures from 0.7 assays were placed on the polyacrylamide gels. For the pH 3.4 enzyme 71% of the product was of molecular weight less than 4000. For the pH 7.4 proteinase 66% of the product was of molecular weight less than 4000.

ases. From the graphs a K_m of $1 \cdot 10^{-5}$ M is obtained for the pH 3.4 proteinase and a K_m of $5 \cdot 10^{-5}$ M is obtained for the pH 7.4 proteinase of *A. niger*.

Nature of product after digestion of the [^{3}H]acetyl hemoglobin substrate by the pH 3.4 and pH 7.4 proteinases of Aspergillus niger

The data of Fig. 5 illustrate that the substrate on polyacrylamide gel electrophoresis under the conditions described in Materials and Methods is essentially all of molecular weight 17 000. This is due to the sodium dodecylsulfate and boiling procedure prior to sodium dodecylsulfate gel electrophoresis and the substrate the enzyme actually digests is primarily of molecular weight 68 000 with relatively small amounts of material of molecular weight 51 000, 34 000, and 17 000. The data in Fig. 5 show that after 1-h digestion with the purified pH 7.4 proteinase 66% of the material is of molecular weight less than 4000 by this method. Similarly after digestion with the pH 3.4 enzyme, 71% is of molecular weight less than 4000. It is of interest to

note that the pH 7.4 product contains peptides of approx. 17 000 daltons and material of approx. 4500 daltons with no intermediate molecular weight peptide, while the pH 3.4 digest contains essentially a continuum of molecular weight peptides from approx. 17 000 to 4000.

Activity of the purified pH 3.4 and pH 7.4 proteinases of Aspergillus niger on low molecular weight substrates

The two proteinases were very inactive toward low molecular weight substrates on a molar basis compared with the usual [^{3}H]acetyl hemoglobin substrate. The pH 3.4 proteinase was active only with benzoyl-DL-arginine-*p*-nitroanilide, while the pH 7.4 proteinase was active only with acetyl-DL-phenylalanine-2-naphthyl ester and *N*-benzoyl-DL-arginine-ethyl ester. Neither enzyme showed activity with acetyl-L-tyrosine ethyl ester.

DISCUSSION

The results of this paper demonstrate that *Aspergillus niger* contains two proteases, one active at pH 3.4 and another active at pH 7.4. Following the nomenclature of this field the former enzyme might be termed an acid protease or "cathepsin-like" enzyme and the latter a neutral protease.

In Table III are summarized some recent work on acid, alkaline, and neutral proteinases of fungal, bacterial and mammalian sources. Except for the acid proteinase (as described, ref. 12) of *Aspergillus parasiticus*, which has a pH optimum of 6.5, the acid proteinases tabulated from diverse sources have remarkably similar optimal pH and range of pH for activity. Similarly the neutral proteinases, by definition, also have very similar optimal pH even though they are derived from diverse sources.

The data of Table III indicate a similarity of molecular weight for the acid proteinases reported, ranging from 30 000 to 60 000. Also the isoelectric points for the three acid proteinases for which data exist are within 3 pH units of each other. The data for the neutral and alkaline proteinases do not offer such homogeneity with respect to molecular weight and pI, the molecular weight ranging from 17 800 to over 100 000 and the pI ranging from 4.6 to 8.3.

Proteolytic enzymes can be classified in one of four groups: (a) serine proteinases, (b) acid proteinases, (c) thiol proteinases, and (d) metal activated proteinases[27]. As demonstrated in Table II, the thiol specific reagents, iodoacetate, *p*-mercuribenzoate, mercaptoethanol, and cysteine failed to substantially inhibit either of the proteinases; however, $HgCl_2$ was inhibitory to both enzymes, especially the pH 7.6 proteinase. This inhibition by $HgCl_2$ is insufficient evidence to class the enzymes as thiol proteinases[27] and certainly as shown in Table III neither of the enzymes can be considered a metal activated enzyme. The specific inhibitors of pepsin 2,4'-dibromoacetophenone and 2-bromo-2-phenylacetophenone[28,29] also had no effect on the proteinases of *A. niger*. The pH 7.6 proteinase was severely inhibited by diisopropylphosphofluoridate, which limits its classification to the "serine proteinase group." The pH 3.4 proteinase fits the requirements of the "acid proteinase group" and has many properties similar to cathepsin D (EC 3.4.4.23) (ref. 30).

Finally, the present work should be placed into context with that performed by Japanese workers on proteases of *Aspergillus*. The initial work of Ichishima and

TABLE III

COMPARISON OF VARIOUS PARAMETERS OF ISOLATED PROTEINASES

Dash (—) indicates data not given in original paper.

Ref.	*Enzyme*	*Source*	*Mol. wt*	K_m* *(M)*	*pH* optimum (range)*	*Purification fold*	*Isoelectric point*	*Inhibitors***
7, 31	Acid proteinase	*Aspergillus saitoi*	30 000–40 000	$1.3 \cdot 10^{-5}$	4.5 (2.5–5.0)	—	3.65	—
12	Acid proteinase	*A. parasiticus*	—	—	6.5 —	—	—	EDTA
13, 14	Acid proteinase	Rabbit liver	50 000–52 000		3.2 (2.0–6.0)	1000	5.5–6.5	—
6	Acid proteinase	Human erythrocyte plasma membrane	33 000–54 000	$1 \cdot 10^{-5}$	3.4 (2.0–5.0)	182	—	—
15	Acid proteinase	Rat brain	60 000	$7.6 \cdot 10^{-3}$	3.8 (2.0–5.0)	110	—	—
Present work	Acid proteinase	*A. niger*	49 000, 56 000	$1 \cdot 10^{-5}$	3.4 (1.5–4.5)	840	4.1, 4.5	
8	Alkaline collagenase	*A. oryzae*	20 000	—	9.0 (6.0–10.5)	40	—	DFP
9	Alkaline proteinase	*A. flavus*	17 800–22 000	— —	8.0 (6.0–10.0)	120	—	
10	Neutral proteinase	*A. oryzae*	—	—	7.6 —	—	4.6, 5.9, 6.0, 8.3	—
11	Alkaline proteinase	*A. oryzae*	—	—	—	60	7.6	—
15	Neutral proteinase	Rat brain	>100 000	$2.7 \cdot 10^{-2}$	7.6	18	—	Heavy metals, PCMB***
16	Neutral proteinase	*E. coli*	43 000	—	7.5	1000		DFP
Present work	Neutral proteinase	*A. niger*	68 000	$5 \cdot 10^{-5}$	7.4 (6.0–9.0)	740	6.2	DFP, Hg^{2+}

* Values reported for hemoglobin as substrate where possible.

** Compounds identified as given major inhibition.

*** *p*-Chloromercuribenzoate.

Yoshida[7] on acid protease of *A. saitoi* is considered in Table III. Subsequent work performed on this protease is summarized by Ichishima[31] and additional characteristics of the enzyme are given. Aside from the fact that the *A. satoi* acid proteinase[31] is isolated from extracellular secretions of the mold, it is quite similar in properties and characteristics to the *A. niger* acid proteinase described herein. Other culture products of genus *Aspergillus* contain acid proteases[32–34]. The isolation and characteristics of alkaline proteinases from *Aspergillus* genus extracellular secretions is reviewed by Nakagawa[35]; alkaline proteases have been characterized from *A. sojae*[36], *A. sydowi*[37,38] and *A. fumigatus*[39]. These alkaline proteinases ("serine proteinases") are characterized by mol. wt of 18–25 000, optimum pH of 9–10, and inhibition by diisopropylphosphofluoridate and *N*-bromosuccinate. Therefore these alkaline proteinases differ in mol. wt and optimum pH from the neutral proteinase described herein from *A. niger*.

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