Biochemical Pharmacology, Vol. 32, No. 13, pp. 2071–2077, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

RODENT KININ-FORMING ENZYME SYSTEMS—II

PURIFICATION AND CHARACTERIZATION OF AN ACID PROTEASE FROM MURPHY–STURM LYMPHOSARCOMA*

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(*Received* 17 *October* 1982; *accepted* 16 *December* 1982)

Abstract—An acid protease from the rat Murphy–Sturm lymphosarcoma (MSLS) tumor was purified 640-fold by extraction of the tumor tissue, acid precipitation with glacial acetic acid, ammonium sulfate precipitation, DEAE-Sephadex A-50 batch adsorption, QAE-Sephadex A-50 column chromatography, Sephadex G-200 gel filtration, and CM-32 cellulose chromatogaphy. The protease hydrolyzed bovine hemoglobin and formed vasopeptide kinins when incubated with purified rat plasma kininogen. Two protease fractions obtained by Sephadex G-200 gel filtration had identical molecular weights of 39,500–41,000 and were similar in other physico-chemical and kinetic characteristics. The purified enzyme showed three major isozymic forms (α, β and γ) with isoelectric points (pI) of 5.2, 5.5 and 5.8, respectively, and nearly identical amino acid compositions. The enzyme had a high moles percent of both aspartic and glutamic acids. The carbohydrate moiety of the enzyme contained 2 moles of *N*-acetylglucosamine and 8 moles of mannose per mole of enzyme. The pH optimum for the digestion of bovine hemoglobin was approximately 3.0 with a sharp decline of activity on either side of the pH optimum. The protease activity was very stable above pH 3.4. The K_m values for the purified enzyme fractions A and B were 31.17 and 31.19 μM, respectively, and the corresponding V_{max} values were 6.17 and 5.5 μM tyrosine per mg per min at 37° and pH 3.0. The enzyme was inhibited strongly by pepstatin ($K_i = 31 \times 10^{-9}$ M and $\alpha = 0.1$). The acid protease released kinin from purified rat plasma kininogen at an initial rapid rate which plateaued at 460 ng bradykinin equivalents/mg substrate after a 2-hr incubation at 37°.

Acid proteases have been reported in a wide variety of mammalian cells and tissues [1] as well as in human gastric carcinoma tissue [2], renal carcinoma [3], Jensen sarcoma [4], and tumor interstitial fluid [5]. Acid proteases capable of forming vasopeptide kinins have been found in the rodent Murphy–Sturm lymphosarcoma [6, 7], mouse fibroblast cell line [8, 9], malignant cells and ascites tumor fluid [10–14]. In a continuing effort to explore the possible role of proteases in cellular control mechanisms, the purification and characterization of the earlier reported acid protease from the Murphy–Sturm lymphosarcoma [7] were completed and are reported in this study.

MATERIALS AND METHODS

The analytical materials used are described in the preceding paper [15]. Polyacrylamide electrophoresis and amino acid and sugar analysis procedures also have been described [15].

Molecular weight determinations

Molecular weight was determined by gel filtration on a Sephadex G-200 column (1.5 × 95 cm) equilibrated and eluted with 0.05 M Na_2HPO_4/0.2 M NaCl, pH 6.8. The column was calibrated with the following proteins: catalase, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and soybean trypsin inhibitor. The void volume (V_o) and the total column volume (V_t) were determined, respectively, by running blue dextran and methyl red through the column. The partition coefficient (K_{av}) values of each standard protein and acid protease were calculated from the formula:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e represents the elution volume of each protein. The molecular weights of subunits were determined by sodium dodecylisulfate (SDS)-polyacrylamide gel electrophoresis with or without treatment with 2-mercaptoethanol as described previously [15].

Isoelectric focusing

Analytical isoelectric focusing was carried out in 7% polyacrylamide gel rods containing 1% carrier ampholites, pH 3–10. Proteins were focused at a constant voltage of 300 V for 6 hr at 4°. Protein bands were fixed for 1 hr with a solution of 3.5% sulfosalicylic acid (w/v), 12% trichloroacetic acid in 30% (v/v) methanol and were stained overnight in 0.1% Coomassie Brilliant Blue R-250 in the fixing solution. Preparative isoelectric focusing was performed in an LKB 8101 column with pH 4–8 carrier ampholites at 4° according to the method of Vesterberg and Svensson [16].

* Supported by U.S. Public Health Service Grant HE-11492, National Heart Institute.
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Enzyme assay

During the purification steps of the Murphy–Sturm lymphosarcoma (MSLS) acid protease, the enzyme activity was assayed routinely by a slight modification of the method of Anson [17]. Activity was measured at pH 4.0 in incubation mixtures (1.0 ml) containing 0.5 ml of 0.1 M acetate buffer, pH 4.0, 0.25 ml of 4% hemoglobin solution, and 0.25 ml of enzyme or distilled water (for control). After a 30-min incubation period at 37°, the reaction was stopped by the addition of 1.0 ml of 6% ice-cold trichloroacetic acid solution. The precipitate was allowed to stand for 10 min at 0° and centrifuged, and the absorbance of the supernatant fraction was measured at 280 nm. For kinetic determinations the enzyme assay was carried out by the same procedure except that 0.1 M sodium citrate-phosphate buffer, pH 3.0, was used. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmole tyrosine/min. The specific activity of the enzyme is expressed in units/mg protein.

For the study of the effect of inhibitors, the assay was carried out by the procedure described above except that each inhibitor was dissolved in the buffer prior to adjustment to pH 3.0.

Determination of pH optimum for activity and stability

The pH dependence of activity was determined by the above assay procedure using 0.1 M glycine buffers in the pH range of 2.6 to 3.7, and 0.1 M citrate-phosphate buffers of pH values between 2.8 and 7.0. For the determination of optimum pH for stability, 100 μl of the purified enzyme solution was incubated with 100 μl of the buffers of differing pH at 37° for 30 min. Aliquots were assayed for acid protease activity at pH 3.0 as described above.

Antiserum preparation and immunological assays

Antiserum against the purified acid protease was raised in a male white New Zealand rabbit (3–4 kg) by multiple subcutaneous injections of 100 μg of enzyme emulsified with complete Freund's adjuvant (1:1). The animal was given a booster shot twice at 1-month intervals with 50 μg of protease emulsified with incomplete Freund's adjuvant and was bled by heart puncture after the second booster injection. The antiserum was obtained by incubating the blood at 37° for 1 hr and then overnight at 4° after which the serum was separated by centrifugation at 2000 *g* for 30 min. Agar double-diffusion analyses were performed by the technique of Ouchterlony [18].

Tumor tissue transplantation and collection

Approximately 500-mg fragments of Murphy–Sturm lymphosarcoma (MSLS) tissue were transplanted subcutaneously into male Sprague–Dawley rats ranging in weight from 250–300 g. The animals were maintained on Purina food pellets and water *ad lib.* and housed in air-conditioned, humidity-controlled facilities. Ten days after tumor transplant, the tumors were excised, freed from necrotic tissue, and stored at −70° until needed.

Purification of acid protease

All operations were performed at 4°, and the protease activity was determined during all preparative steps according to the assay procedure described above. The centrifugations were performed in a refrigerated Sorvall RC-2B centrifuge.

Step 1: Tumor extraction. Tumor tissue (575 g) was minced and homogenized in a Vortex blender for 2 min with an equal volume of 0.02 M sodium phosphate buffer, pH 6.8. The homogenate was centrifuged at 12,000 *g* for 30 min and filtered through cheesecloth. The pellet was resuspended, re-homogenized, and centrifuged as above. The two supernatant fractions were combined and processed as described below.

Step 2: Acid precipitation. The supernatant fraction was adjusted to pH 4.2 with glacial acetic acid. The resulting precipitate was removed by centrifugation at 12,000 *g* for 30 min. The supernatant fraction is referred to as the "acid supernatant".

Step 3: Ammonium sulfate precipitation. The acid supernatant was brought to 60% saturation by stepwise addition of solid ammonium sulfate. After 1 hr the precipitate was collected by centrifugation at 12,000 *g* for 30 min, dissolved in cold water, and dialyzed against 0.05 M Tris–HCl buffer, pH 8.0. The precipitate formed on dialysis was removed by centrifugation at 14,000 *g* for 30 min.

Step 4: DEAE-Sephadex batch absorption. The enzyme solution (350 ml) from the above step was swirled for 1 hr with an equal volume of DEAE-Sephadex preequilibrated with 0.05 M Tris–HCl buffer, pH 8.0, and then suction filtered. The filter cake was resuspended with 2 × 500 ml equilibrating buffer for 1 hr each time and suction filtered. Finally, the enzyme was eluted from the DEAE-Sephadex with 3 × 350 ml of 0.05 M Tris–HCl/0.5 M NaCl, pH 8.0. The combined filtrate was concentrated by passage through an Amicon PM-10 membrane and dialyzed against 0.05 M Tris–HCl buffer, pH 8.0.

Step 5: QAE-Sephadex chromatography. A 2147 mg sample of partially purified enzyme from step 4 was applied to a column (2.5 × 100 cm) of QAE-Sephadex in 0.05 M Tris–HCl buffer, pH 8.0, and eluted with a stepwise gradient of NaCl in this buffer. Fractions were collected at a flow rate of 40 ml/hr and assayed for protease activity. Enzyme-containing fractions were pooled and concentrated by ultrafiltration.

Step 6: Sephadex G-200 chromatography. The enzyme concentrate (15 ml) from the QAE-Sephadex column was applied to a Sephadex G-200 (fine) column and eluted with 0.02 M Na_2HPO_4/0.2 M NaCl, pH 6.8. Fractions (5 ml) were collected at a flow rate of 12 ml/hr. The acid protease fractions were pooled into two fractions based on their respective elution profile, concentrated by ultrafiltration, and dialyzed against 0.05 M $C_2H_3NaO_2$, pH 5.0. Each fraction (A and B) was purified further separately.

Step 7: CM-32 cellulose chromatography. Fractions A and B were applied separately on CM-cellulose columns (1.5 × 30 cm) equilibrated previously with 0.05 M $C_2H_3NaO_2$, pH 5.0. A linear NaCl gradient was applied after elution with a volume of buffer equivalent to three times the volume of the column.

Enzyme-containing fractions were pooled and concentrated.

Step 8: Sephadex G-200 chromatography. As the final step, the enzyme concentrate of fraction A was applied to a Sephadex G-200 (2.5 × 80 cm) column and eluted with 0.05 M Na_2HPO_4/0.2 M NaCl, pH 6.8. The fractions showing protease activity were pooled and concentrated through an Amicon PM-10 membrane.

RESULTS

Purification of the MSLS acid protease

The purification of the acid protease is summarized in Table 1. As noted, acidification (step 2) increased activity 2.4-fold with a concomitant removal of 63% of the total protein. Ammonium sulfate (0–60%) precipitated more than 75% of the total acid protease (step 3). During dialysis of this fraction against 0.05 M Tris–HCl buffer, pH 8.0, almost 70% of the proteins precipitated, resulting in a 25% loss of protease activity. The acid protease bound to the DEAE-Sephadex in 0.05 M Tris–HCl buffer, pH 8.0 (step 4), and eluted with increasing sodium chloride concentration. The enzyme eluted from the QAE-Sephadex column at 0.1 M NaCl concentration (step 5), resulting in a 56.5-fold purification compared to the crude extract. The major portion of the activity eluted from the Sephadex G-200 column in the molecular weight range of 40,000 to 45,000 and was preceded by a shoulder that eluted with the bulk of the protein applied (step 6). Fraction A (tubes 33–59) and Fraction B (tubes 60–77) showed identical elution profiles on CM-cellulose chromatography (step 7). Fraction B showed a single band on polyacrylamide gel electrophoresis whereas Fraction A showed some additional slower moving bands which were removed by Sephadex G-200 gel filtration (step 8). The final purification factor for Fraction B was 640 and for Fraction A 467.

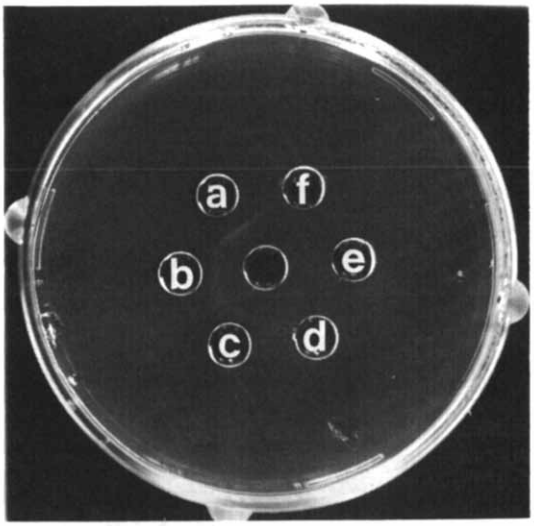

Fig. 1. Radial immunodiffusion of MSLS tumor acid protease (4–5 μg, center well) and different dilutions of antiserum prepared to MSLS tumor acid protease, pool b. A volume of 10 μl of antiserum was applied to each pool on a 1% agarose gel in barbital acetate buffer, pH 8.2. Dilutions of the antiserum were (a) 1:1, (b) 1:2, (c) 1:4, (d) 1:8, (e) 1:16, and (f) 1:32.

Properties of the purified enzymes—Immunologic homogeneity

The homogeneity of the purified acid protease Fractions A and B each was established by polyacrylamide disc gel electrophoresis, SDS-polyacrylamide disc gel electrophoresis, and double-diffusion Ouchterlony analysis (Fig. 1).

Table 1. Purification of acid protease from MSLS tumor

Step	Treatment	Protein (mg)	Activity* (units)	Specific† activity (× 10^3)	Purification factor	Yield (%)
1.	Crude extract	39,383	377.4	9.6	1.0	100.0
2.	Acid supernatant	13,938	315.3	22.5	2.4	83.6
3.	60% $(NH_4)_2SO_4$ precipitate	8,891	237.3	26.7	2.8	62.9
4.	DEAE-Sephadex A-50 batch adsorption	2,147	144.2	67.1	7.0	38.2
5.	QAE-Sephadex A-50 chromatography	185	105.7	542.4	59.8	28.1
6.	Sephadex G-200 gel filtration					
	Fraction A	103	31.1	303.3	31.7	8.2
	Fraction B	43	61.0	1,417.6	80.1	16.2
7.	CM-32 cellulose chromatography					
	Fraction A	9.5	18.4	1,942.9	203	4.9
	Fraction B	6.2	38.0	6,123.8	640	10.6
8.	Sephadex G-200 gel filtration					
	Fraction A	2.3	10.4	4,466.7	467	2.8

* A unit of enzyme is defined as the amount of enzyme that releases 1 μmole of tyrosine/min under the assay conditions employed.

† Units per mg protein.

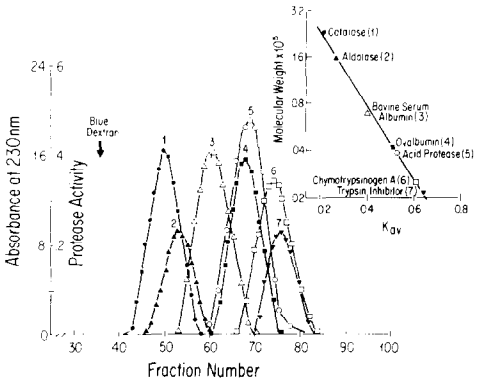

Fig. 2. Estimation of the molecular weight of MSLS tumor acid protease by gel filtration. Acid protease was applied to a column (1.5 × 95 cm) of Sephadex G-200 equilibrated with 0.05 M Na_2HPO_4/0.2 M NaCl, pH 6.8. Fractions (2 ml) were collected at a flow rate of 10 ml/hr. The column was calibrated previously with different protein markers: (1) catalase (232,000), (2) aldolase (158,000), (3) bovine serum albumin (68,000), (4) ovalbumin (43,000), (6) chymotrypsinogen A (25,000) and (7) soybean trypsin inhibitor (21,500). Elution profile of acid protease (○—○) and protein markers on Sephadex G-200 is shown. Inset shows the plots of molecular weight and K_{av}.

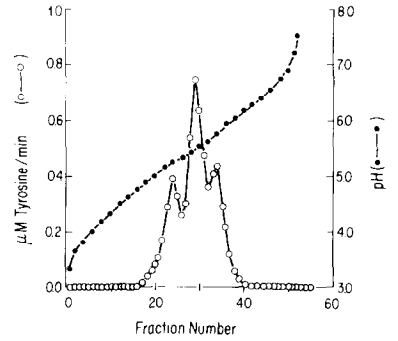

Fig. 4. Column isoelectric focusing of MSLS tumor acid protease. The enzyme solution was subjected to column electrophoresis at 50° with sucrose density gradient containing 2%, pH 4–8, carrier ampholites in an LKB 8101 column. Fractions of 2 ml were collected after isoelectric focusing for 60 hr at a constant voltage (600 V). Each fraction was assayed for enzyme activity and pH.

Molecular weight and subunit structure

The molecular weight of both Fractions A and B, estimated from their chromatographic mobilities on a calibrated Sephadex G-200 column, was 39,500 (Fig. 2). The molecular weight of 41,000 was estimated from polyacrylamide gel electrophoresis in 0.1% SDS (Fig. 3). SDS-gel electrophoresis of Fraction A and B acid proteases in the presence of β-mercaptoethanol revealed a major protein band of 41,000 molecular weight and two fainter bands in the molecular weight range of 27,000 and 12,000. It is assumed that both acid proteases are made up predominantly of a single chain 41,000 molecular weight protein and that the smaller molecular weight species arise from the proteolytic degradation of the enzyme.

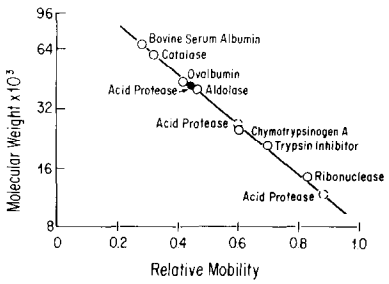

Fig. 3. Estimation of the molecular weight of MSLS tumor acid protease subunits. SDS-polyacrylamide disc gel electrophoresis of standard proteins and enzyme in 10% acrylamide and 0.1% sodium dodecyl sulfate was carried out as described in the preceding paper [15]. Electrophoretic mobilities were determined relative to that of bromophenol blue.

Isoelectric focusing

Analytical isoelectric focusing of the purified acid protease Fractions A and B revealed three protein staining bands having isoelectric points (pI) of approximately 5.2, 5.6 and 6.1 respectively. The isoelectric points were estimated from a calibration plot generated by running, simultaneously, markers from a Pharmacia Fine Chemical pI Calibration Kit. The three isozymes were isolated by preparative isoelectric focusing and were designated as α, β, and γ with isoelectric points of 5.2, 5.5 and 5.8 respectively (Fig. 4).

Table 2. Amino acid* and carbohydrate† composition of MSLS acid protease isozymes

Amino acid	Intact	Isozymes α	β	γ
Lysine	6.1	6.3	6.3	6.2
Histidine	1.8	1.9	1.6	2.0
Arginine	2.8	2.8	2.5	3.2
Aspartic acid	11.6	11.8	12.1	11.8
Threonine	7.1	7.0	6.9	7.4
Serine	7.6	8.2	8.3	8.9
Glutamic acid	10.9	11.7	10.4	11.1
Proline	5.7	4.6	5.0	5.2
Glycine	9.0	9.1	9.6	9.3
Alanine	13.9	13.6	13.8	14.0
Half cystine	1.2	1.4	0.9	1.0
Valine	6.6	5.8	5.9	6.6
Methionine	0.2	0.1	0.2	0.2
Isoleucine	3.5	3.5	3.9	3.4
Leucine	8.0	7.9	7.2	7.8
Tyrosine	2.9	2.6	2.8	2.7
Phenylalanine	3.4	3.7	3.4	2.9
Tryptophan	ND‡	ND	ND	ND
N-Acetylglucosamine	1.1	ND	ND	ND
Mannose	4.0	ND	ND	ND

* Moles percent.
† Grams per 100 g protein.
‡ Not determined.

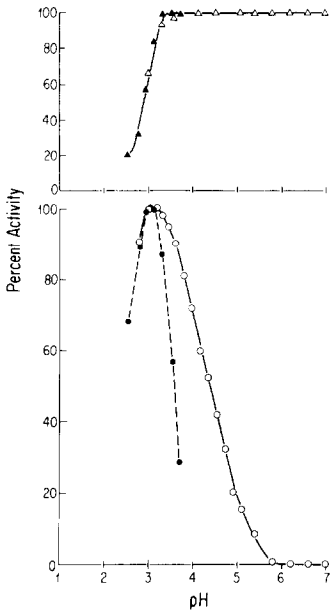

Fig. 5. Effect of pH on the stability and activity of MSLS tumor acid protease. A 100-μl sample of purified enzyme was preincubated for 30 min at 37° with 100 μl of the 0.1 M glycine buffers of pH 2.6 to 3.7 (▲) and 0.1 M citrate-phosphate buffers of pH 2.8 to 7.0 (△). The enzyme assay was performed in 0.1 M citrate-phosphate buffer, pH 3.0. The pH dependence of activity was determined under usual assay conditions in 0.1 M glycine buffers of 2.6 to 3.7 (●) and 0.1 M citrate-phosphate buffers of pH 2.8 to 7.0 (○).

Amino acid and carbohydrates composition

The amino acid compositions of the acid protease isozymes, summarized in Table 2, were very similar to each other and consistent with the composition reported for unfractionated enzyme. The enzyme showed a high moles percent of both aspartic and glutamic acids. The acid protease was identified as a glycoprotein containing *N*-acetylglucosamine and mannose in a molar ratio of 1 : 4. Based on molecular weight of 40,000, the carbohydrate moiety was found to contain 2 moles of *N*-acetylglucosamine, and 8 moles of mannose per mole of enzyme.

Optimum pH for activity and stability

The pH optimum for digestion of bovine hemoglobin for both Fraction A and Fraction B acid proteases was 3.0 (Fig. 5, bottom curve). A sharp decline in the activities of both enzymes was observed on either side of the pH optimum.

For pH stability studies, an untreated enzyme sample was used as a control and was assumed to have 100% activity. Activities of both fractions were very stable in the pH range 3.4 to 7.0 and were unstable at pH below 3.4 (Fig. 5, top curve).

Effect of substrate concentration on enzyme catalysis

The effect of substrate concentration on the velocity of the enzyme reaction was investigated using various concentrations of bovine hemoglobin. A plot of observed velocity against substrate concentration (Fig. 6, inset) produced a hyperbolic curve. The apparent Michaelis constants (K_m) and maximum velocities (V_{max}) for both Fraction A and B acid proteases were computed by the least squares method from the Lineweaver–Burk plots ([19]; data shown only for Fraction B in Fig. 6). The K_m values were 31.17 and 31.19 μM with enzyme Fractions A and B respectively. The corresponding V_{max} values were 6.17 and 5.5 μM tyrosine per mg per min at 37° and pH 3.0.

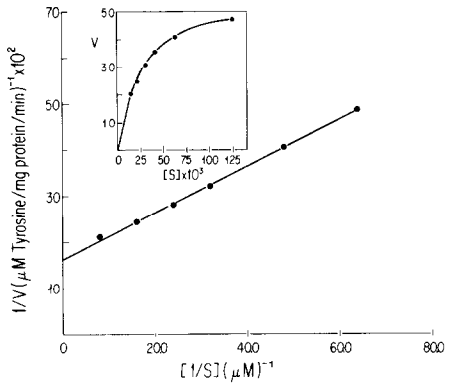

Fig. 6. Lineweaver–Burk plot for the hydrolysis of hemoglobin and MSLS tumor acid protease (0.1 mM). The effect of substrate concentration on the enzyme catalysis was studied by using 12.5 to 125 μM solutions of hemoglobin at 37° in 100 mM citrate-phosphate buffer, pH 3.0. The molar values of substrate and enzyme were calculated assuming molecular weights of 64,000 and 40,000 for hemoglobin and enzyme respectively. The initial velocity (V) versus (S) plot is shown in inset.

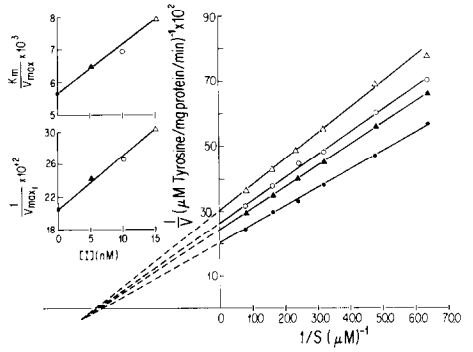

Fig. 7. Inhibition of MSLS tumor acid protease by pepstatin. For each of the three inhibitor concentrations, 5 μM (▲), 10 μM (○), and 15 μM (△), the substrate concentration was varied from 12.5 to 125 μM, and enzyme concentration was 0.13 nM. The assay mixture was incubated at 37° for 30 min. Replots of inhibitor concentration (I) versus K_m/V and $1/V_{max}$ (left inset) were used to determine inhibition constant, K_i, and α respectively.

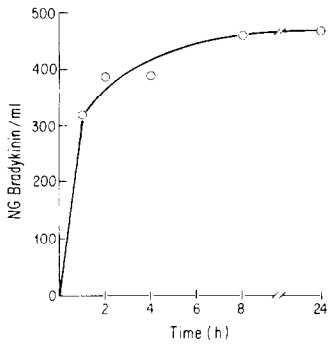

Fig. 8. Time course of kinin release from rat plasma kininogen. Purified rat plasma kininogen (1 mg/ml) was incubated at pH 3.0 with 10 mU acid protease. Aliquots were withdrawn at different time intervals, and reaction was stopped by mixing with 0.1 M Tris buffer, pH 8.5. Kinin formed was assayed and calculated by bioassay on rat uterus [21].

Effects of activators and inhibitors

Of the various inhibitors tested (pepstatin, leupeptin, soybean trypsin inhibitor, EDTA and dithiothreitol), only pepstatin was found to be a potent inhibitor of the MSLS acid protease, causing 50% inhibition at 7×10^{-9} M. Lineweaver–Burk plots revealed a mixed type inhibition (Fig. 7). Secondary plots of inhibitor concentration (I) versus slope (K_m/V_{max}) and intercepts ($1/V_{max}$) of Lineweaver–Burk plots (Fig. 7, left inset) were used to determine inhibition constant, K_i, and α respectively [20]. The values thus obtained were 31×10^{-9} M and 0.1 for K_i and α respectively.

Time course of kinin release

The time course of kinin released from rat plasma kininogen is shown in Fig. 8. The initial rate of release was rapid and plateaued at 460 ng bradykinin equivalents/mg substrate. The amount of kinin released was comparable to that released by trypsin in the same 2-hr time period (data not shown).

DISCUSSION

On the basis of mounting evidence linking proteases to the control of normal and malignant cell growth [22], the purification and characterization of an acid protease from rodent Murphy–Sturm lymphosarcoma (MSLS) were undertaken. The purification scheme outlined above resulted in a 640-fold purification yielding a homogeneous preparation as confirmed by disc gel electrophoresis and by Ouchterlony immunodiffusion techniques. During the various purification steps, DEAE-Sephadex batch adsorption was found to provide better resolution of the proteins in the subsequent steps. Chromatography on Sephadex G-200 yielded a major protease peak preceded by a shoulder. These two enzyme pool fractions were purified separately to determine if the two peaks were due to different proteases as had been observed in rat thoracic duct lymphocytes and rat lymphoid tissues [23]. Both peaks, however, showed identical elution profiles on a CM-cellulose column. Further, since both protease fractions showed a single band with identical relative mobility on polyacrylamide gel electrophoresis in the absence and presence of SDS and, also, showed immunological crossreactivity against antiserum raised against Fraction B, it can be presumed that a single acid protease exists which has a tendency to aggregate.

The molecular weight of 39,500 estimated by Sephadex G-200 gel filtration and of 41,000 by polyacrylamide gel electrophoresis is within the range of values reported for cathepsin D-like proteases from other tissue sources [24–27]. The polyacrylamide gel electrophoresis under denaturing conditions revealed that the acid protease comprised a major band of molecular weight 41,000 with two fainter bands of molecular weights 27,000 and 12,000. A comparative study of brain cathepsin D showed that enzymes from bovine, human and guinea pig differed from rat brain cathepsin D in having prominent bands with molecular weights of 31,000 and 13,000 [24].

The purified enzyme showed three major isozymic forms. The amino acid compositions of the three isozymes were nearly identical and followed a pattern similar to that reported for acid protease purified from other tissue sources [25, 28]. The enzyme is a glycoprotein consisting of only mannose and *N*-acetylglucosamine as the carbohydrate moieties. Based on a molecular weight of 41,000, the MSLS acid protease appears to have 8–9 mannose and 2 *N*-acetylglucosamine residues. The carbohydrate composition suggests that the enzyme contains a high mannose-type asparagine-linked carbohydrate chain. The enzyme resembles a cathepsin D-like protease from human gastric mucosa [29] in its carbohydrate composition, but it appears to differ from rat spleen cathepsin D, which also contains galactose and fucose [25].

The following evidence strongly supports the conclusion that the MSLS purified acid protease is a cathepsin D-like enzyme. First, the protease has a molecular weight of 41,000, as against the 90,000 reported for rat spleen cathepsin E [30]. The enzyme was not inhibited by *p*-chloromercuribenzoate, soybean trypsin inhibitor, EDTA, 1,10-phenanthroline, or leupeptin, but it was inhibited very strongly by pepstatin, confirming the carboxyl peptidase nature of the enzyme [31]. The inhibitory action of pepstatin on MSLS acid protease activity had been reported previously by our laboratory [32]. Fifty percent inhibition obtained at a 7×10^{-9} M pepstatin concentration is similar to that observed with cathepsin D-like acid protease from human gastric mucosa [29] and Rhesus monkey lung [33]. Inhibition with pepstatin was not of a pure competitive type but rather a mixed type inhibition. A pH optimum of 3.0 also is in agreement with that expected for a carboxyl peptidase [31].

The purified enzyme hydrolyzed purified rat plasma kininogen to release a smooth muscle stimulating vasopeptide differing from bradykinin in size. The vasopeptide has been isolated and purified. Detailed characterization of the peptide will be published separately.

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