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PURIFICATION AND CHARACTERIZATION OF KININ-FORMING ACID PROTEASE FROM MOUSE FIBROBLASTS L-929*

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Abstract—Purification and further characterization was carried out on a kinin-forming acid protease isolated from a rodent fibroblast cell line L-929 grown in stationary cell culture (N. Back and R. Steger, [7]). The cells, cultured in minimal essential medium containing 10% fetal calf serum and 0.4% lactalbumin, were homogenized, the homogenate dialyzed for 18 hr against 0.01 M phosphate buffer at pH 6.8 in 0.1 M NaCl and 1.0 mM EDTA, and centrifuged at 10000 rpm for 45 min. The supernatant, which digested denatured hemoglobin at pH 4.0, was fractionated first on a G-200 Sephadex column. Kinin-forming activity, compared with that of the supernatant on an isolated perfused rat uterus preparation, was identified in fractions 25–40 when incubated for 24 hr at pH 4.0 with rat plasma kininogen substrate. The active fractions were pooled and purified further on a hydroxypatite column. Treatment of the active fractions with 5 mM cysteine increased the activity 2-fold. Final purification was carried out on a DEAE-A50 Sephadex ion exchange column. The purification factor, compared to the initial supernatant, was 9.4 with a 13.8% yield and a specific activity of 2062.5 ng kinin per mg protein. Dialyzed and centrifuged rat plasma fractionated on a DEAE-A50 Sephadex column initially yielded two apparent kininogen species which resolved into a single major molecular species following passage through a G-100 Sephadex column. The purified enzyme and substrate preparations were used to establish the optimum kinin-forming activity at pH 3.8–4.0. The molecular weights of the enzyme and kininogen were estimated on a G-200 Sephadex column to be 38000–39000 and 115000 respectively. The amount of kinin formed was a function of incubation time and enzyme concentration. The acid protease activity was found localized primarily in the 10000 g supernatant cell fraction. The 500 g cell fraction also exhibited activity.

Kinin-forming protease systems recently have been identified in malignant tissue and fluid. Thus, both alkaline [1–3] and acid proteases [4–7] capable of forming vasopeptide kinins have been found present in the rodent Murphy-Sturm lymphosarcoma [1, 4], malignant cells and ascites tumor fluid [2, 3, 5, 6], and rodent fibroblasts grown in stationary cell culture [7]. The fibroblast acid protease formed kinin when incubated with kininogen substrate from rat plasma and the Murphy-Sturm lymphosarcoma. In the previous report, neither the enzyme nor substrate was purified [7]. In the present study, the mouse fibroblast L-929 cell line was cultured in sufficient quantity to permit the purification and further characterization of the protease. In addition, the rat plasma kininogen substrate was isolated and purified.

MATERIALS AND METHODS

Culture of L-929 cell line. Mouse fibroblast cells L-929 (Grand Island Biological Co., Grand Island, NY) were cultured continuously in Spinner flasks with Minimal Essential Medium (Becton, Dickinson, & Co., Cockesville, MD) containing 10% fetal calf serum and 0.4% lactalbumin. The cell population was maintained at $2 \times 10^5 - 10 \times 10^5$ cells/ml and the pH of the medium automatically controlled between 7.20–7.48 [8]. Cell viability was estimated between 85–98% by the trypan blue exclusion test [9].

Fibroblast acid protease. Initial isolation. L-929 cells in medium (300–500 mg) were centrifuged at 3000 rpm, sonicated for $1\frac{1}{2}$ min 5 times with a Bronwill® Biosonik sonicator (Rochester, NY), and then centrifuged for 15 min at 10000 rpm in a refrigerated centrifuge. The supernatant was dialyzed for 48 hr against a 0.01 M NaH_2PO_4:Na_2HPO_4 buffer, pH 6.8, in 0.1 M NaCl and 1.0 mM EDTA. The dialyzed supernatant had a vol. of 18 ml with a protein concentration of 5.91 mg/ml. Protease activity was measured initially on a denatured hemoglobin substrate (Worthington Labs, Freehold, NJ). Two ml of the supernatant together with 2.0 ml acetate buffer, pH 4.0, were incubated with 4.0 ml of a 2.5% hemoglobin solution for 60 min. Four ml of a 10% trichloracetic acid solution was added, the incubate filtered through a Whatman #1 filter, and the optical density read on a Gilford spectrophotometer at 280 mμ. Control tubes contained equivalent vol. of buffer, supernatant, trichloracetic acid and hemoglobin which were incubated for 60 min and then filtered.

Initial purification. G-200 Sephadex. Initial purification was carried out on 10^9 fibroblast cells that were homogenized for 8 min in a glass cell homogenizer. The homogenate was dialyzed 18 hr against a 0.01 M NaH_2PO_4:NaH_2PO_4 buffer, pH 6.8, in 0.1 M NaCl and 1.0 mM EDTA. After centrifugation

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for 45 min at 12,000 rpm, the 9.0 ml vol. supernatant had a protein concentration of 6.38 mg/ml, which was applied onto a G-200 Sephadex column, 2.5 × 90 cm. Eluent fractions were assayed for kinin-forming activity in the following fashion: 0.5 ml of the fractions was added to 0.3 ml of a 0.56 M acetate buffer at pH 4.0, 0.2 ml citrated rat plasma, 0.1 ml of 1% 1,10-phenanthroline, 0.2 ml soya bean trypsin inhibitor (SBTI, 10 mg/ml), and incubated for 24 hr at 37°. (The SBTI was added to inhibit any possible plasma kallikrein contaminant; SBTI and aprotinin [Trasylol] had been shown previously ineffective against the fibroblast acid protease activity [7]). Following incubation, the mixture was neutralized with 0.3 ml 1.0 M Tris buffer, pH 7.8, and 0.025–0.10 ml vol. bioassayed for smooth muscle stimulating activity (kinin) on the isolated rat uterus perfused with Tyrode's solution in a 10 ml bath at ambient temperature, 20–22° [7]. The heighth of muscle contraction was recorded on a Sanborn polygraph via a transducer, and compared with that obtained with a reference synthetic bradykinin standard (Schwarz/Mann, Orangeburg, NJ). The sp. act. was calculated in terms of ng bradykinin released per mg protein preparation.

Hydroxylapatite column. The 17 ml vol. of the G-200 Sephadex-purified preparation (3.8 mg/ml) then was applied onto a hydroxylapatite column, 2 × 30 cm. The initial equilibrating buffer was 0.01 M phosphate buffer, pH 6.8, in 0.1 M NaCl and 1.0 mM EDTA. After elution of the first peak, a linear gradient of 150 ml of the initial buffer to 150 ml of 0.12 M phosphate buffer in 1.0 M NaCl and 1.0 mM EDTA was used. All the fractions were assayed for kinin-forming activity as described above, and sp. act. calculated.

Cysteine activation. The active fractions from the hydroxylapatite column were pooled, the protein concentration determined to be 0.65 mg/ml (14 ml), and then dialyzed for 18 hr against 500 ml 0.01 M KH_2PO_4:K_2HPO_4 buffer at pH 6.8 in 0.1 M KCl containing 5 mM cysteine. Aliquots of 0.5 ml of dialysate were incubated with 0.2 ml rat plasma kininogen substrate, 0.3 ml acetate buffer, 0.1 ml 1,10-phenanthroline, and 0.1 ml aprotinin (50000 units/ml). After neutralization with 0.3 ml Tris buffer, 0.025–0.1 ml vol. were bioassayed for kinin-forming activity, and sp. act. calculated.

DEAE-A50 ion exchange column. The cysteine-activated protease preparation (11 ml with a protein concentration of 0.73 mg/ml) was applied onto a DEAE-A50 ion exchange column, 1 × 20 cm. The column was equilibrated with 0.01 M KH_2PO_4:K_2HPO_4 buffer, pH 6.8, in 0.1 M KCl and 2.5 mM mercaptoethanol. The initial eluting buffer consisted of 50 ml of the equilibrating buffer. A linear gradient of 150 ml of the initial buffer to 150 ml of 0.01 M KH_2PO_4:K_2HPO_4 in 0.1 M KCl and 2.5 mM mercaptoethanol was used. The fractions were assayed for kinin forming activity, and the sp. act. calculated.

Rat plasma kininogen purification. DEAE-A50 column. Blood was drawn from the abdominal aorta of anesthetized Holtzman female rats, anticoagulated with 3.8% sodium citrate, and plasma collected following centrifugation. Twenty-five ml of the rat plasma was dialyzed for 10 hr at 4° against a 0.01 M sodium phosphate buffer, pH 6.8 in 0.1 M NaCl and 1.0 mM EDTA. The dialysate was centrifuged at 3000 rpm for 30 min, and the supernatant filtered through a Whatman #1 filter paper. The filtrate (20 ml with a protein concentration of 39 mg/ml), was applied onto a DEAE-A50 column, 2.5 × 30 cm. The flow rate was 20 ml/hr. The column was developed with 240 ml of the starting buffer followed by a linear gradient of 95 ml 0.1 M NaCl to 95 ml 0.5 M NaCl in the 0.01 M phosphate buffer, pH 6.8. A vol. of 190 ml phosphate buffer in 0.5 M NaCl was added followed by a linear gradient of 75 ml 0.8 M NaCl to 75 ml 1.2 M NaCl in the phosphate buffer, pH 6.8. The fractions were assayed for kininogen by incubation of 0.5 ml aliquots for 18 hr at 37° with 0.1 ml (1.2 mg/ml) purified acid protease, neutralized with 0.2 ml 1.0 M Tris buffer, pH 7.8, and kinin formed estimated on the isolated perfused rat uterus as described above. Kininogen was calculated in terms of ng kinin formed per mg kininogen protein.

G-100 Sephadex column. 4 ml of kininogen I peak (1.64 mg/ml) and 3 ml of kininogen peak II (7.75 mg/ml) were applied onto a G-100 Sephadex column, 2.5 × 90 cm, equilibrated with 0.01 M KH_2PO_4:K_2HPO_4 buffer in 0.1 M KCl and 2.5 mM mercaptoethanol. Aliquots of the eluted fractions were assayed for kininogen on the rat uterus, and the sp. act. calculated.

Disc gel electrophoresis. The extent of homogeneity of both the purified enzyme and kininogen preparations was determined by disc gel electrophoresis [10]. The gel solution was prepared by mixing 20 ml of 0.12 M Tris-glycine, pH 8.6 with 0.1 ml 100% *N,N,N'-N'*-tetraethylethylene diamine, 0.15 ml 20% ammonium persulfate, and 47 ml distilled water. Then 15 ml of a filtered 20% cyanogum 41 solution was added and the solutions pipetted into glass tubes (0.6 cm × 12 cm) mounted on a vertical disc-gel electrophoresis apparatus. The gel was allowed to solidify within 10 min, and a drop of distilled water added onto the top of the gel surface to prevent concavity formation. The upper and lower chambers of the electrophoresis apparatus were filled with 0.03 M Tris-glycine, pH 8.6. A vol. of 50 μl of the enzyme or substrate test samples obtained during the various steps of purification was added to the gel tubes. The DEAE-A50-purified enzyme sample (0.1 ml) was treated for 15 min with 10 mg cysteine and refrigerated overnight before disc gel electrophoresis. The electrophoresis was carried out for 1½ hr with a current of 2.5 ma per tube and a voltage of 280 V in the entire system (LKB power supply). At the end of the electrophoresis, the gel from each tube was removed and immersed into 12.5% trichloracetic acid for 15 min. The gel then was transferred to a 25 ml glass tube filled with 10 mg% coomassie brilliant blue in 12.5% trichloracetic acid. After 1½ hr, the gel was washed overnight with 5% acetic acid, and the protein bands in each of the tubes assayed for respective activities. Duplicate tubes were run for correlation of protein band with activity.

Molecular weight approximations of acid protease and rat plasma kininogen. The purified preparations of the acid protease and kininogen were subjected to Sephadex G-200 gel filtration for molecular weight approximations. The columns, 1 × 57 cm, were cali-

brated with the following markers of known mol. wt: aldolase (25 mg), ovalbumin (5 mg), chymotrypsinogen A (4 mg), and ribonuclease (4 mg) (Pharmacia, Upsala, Sweden). The calibration curves were constructed from multiple runs of individual markers and runs performed with mixtures of standards with wide molecular weight ranges. The void volume (*Vo*) was determined with blue dextran, and the total column volume (V_t) calculated on the basis of the formula:

$$V_t = (\tfrac{1}{2}d)^2(\pi)(\text{column height}) = 45.4\text{ ml}$$

The elution volume (*Ve*) was determined in the following manner: the *Ve* values of the standards (aldolase, ovalbumin, chymotrypsinogen A, ribonuclease) were determined by applying each standard onto the Sephadex column and pooling the eluent from the first tube to the maximum peak consecutively. The peak maxima were recorded on a UV Scan III UV Monitor with a 260 mμ filter (Buchler, Fort Lee, NJ). The V_e values of both the DEAE-A50 purified protease (0.80 mg) and kininogen II (0.88 mg) were determined by also pooling the eluent from the first tube to the maximum peak. The peak maxima were determined by the appropriate bioassay technique described previously. The partition coefficient (K_{av}) values of each standard and test agent were calculated from the formula:

$$K_{av} = (Ve\text{-}Vo)/(V_t\text{-}Vo)$$

where V_t = total column volume; *Vo* = void volume; *Ve* = elution volume of standard or test agent.

pH profile of acid protease. The pH profile for the kinin-forming activity of the purified acid protease on the kininogen substrate was determined using the following pH solutions: pH 1.0, 0.05 M KH_2PO_4:HCl; pH 1.5, 0.05 M $KHPO_4$ buffer; pH 2.0 and pH 2.5, glycine: HCl; pH 2.8, citric acid: NaOH; pH 3.0 and pH 3.3, citric acid: sodium citrate; pH 3.6, pH 3.8, pH 4.0, pH 4.2, formic acid: sodium formate; pH 4.5, pH 4.8, pH 5.0, acetic acid: sodium acetate; pH 5.5, citric acid: sodium citrate; pH 6.0, histidine buffer; pH 7.0, imidazole buffer; pH 8.0, Tris buffer; pH 9.0, histidine buffer; pH 10.0, glycine buffer; pH 11.0 glycocoll buffer; pH 12.0, phosphate buffer. The incubation consisted of 0.2 ml of rat plasma kininogen peak II (1.2 mg/ml), 0.2 ml acid protease (0.6 mg/ml), and 0.3 ml of the respective buffer. The mixture was incubated for 15 hr at 37°, boiled for 15 min, neutralized with 0.2 ml 1.0 M Tris buffer, pH 7.8, and kinin assayed as described previously.

Kinin release with respect to time and enzyme concentration. A fixed concentration of 0.075 mg DEAE-Sephadex purified plasma kininogen was incubated with DEAE-Sephadex purified acid protease in concentrations of 0.019 mg, 0.038 mg, 0.075 mg, and 0.120 mg for $\frac{1}{2}$, 1, 2, 4, and 8 hr respectively. The incubation vol. was 0.3 ml of 0.56 M acetate buffer, pH 4.0. At the end of each incubation time, the mixture was boiled for 3 min and 0.1 ml of 1 M Tris buffer, pH 7.8 was added. The kinin formed was bioassayed on the isolated perfused rat uterus.

Enzyme localization. Cell fractionation was carried out to determine the localization of the acid protease activity in various cell fractions prepared by centrifugation. Fibroblast cells (10^9) were homogenized three times for 2 min each time, and dialyzed for 22 hr against 0.01 M phosphate buffer, pH 6.8 in 0.1 M NaCl and 1 mM EDTA. The dialysate was centrifuged at 1500 *g* for 15 min and the precipitate collected as the 1500 g fraction. The supernatant then was centrifuged at 10000 *g* for 20 min yielding the 10000 g soluble supernatant fraction and the 10000 g precipitate fraction. For assay, the respective fractions were reconstituted with the initial phosphate buffer to the following vol.: 1500 g fraction, 23 ml; 10000 g supernatant fraction, 21 ml; 10000 g precipitate fraction, 21 ml. Each fraction was assayed for kinin-forming activity by incubating 2.5 ml of each fraction with 1.0 ml rat plasma, 1.5 ml acetate buffer (pH 4.0), 0.5 ml, 1,10-phenanthroline (10 mg/ml), and 0.5 ml aprotinin (50,000 units/ml) for $1\frac{1}{2}$, 2, 4, 5 3/4, 9,19, and 48 hr. Aliquots of 1.2 ml of the incubation mixture were withdrawn at each time interval, 0.3 ml of 1 M Tris buffer, pH 7.8, added, and the mixture boiled for 15 min. Microliter vol. were withdrawn and assayed for estimation of kinin on the isolated perfused rat uterus. Appropriate control tubes were prepared containing all of the above components and an additional 2.5 ml of acetate buffer in place of the cell fraction.

RESULTS

Initial acid protease assay. The L-929 cell dialysate digested denatured hemoglobin when incubated together at pH 4.0 for 60 min. Tubes containing the cell-sonicated dialysate and hemoglobin gave an extinction (E) reading at 280 mμ of 1.097 while control tubes gave a reading of 0.914. Further evidence of an acid protease activity was obtained when the dialysate, incubated with rat plasma at pH 4.0, generated smooth muscle stimulating activity (kinin) as measured on the isolated perfused rat uterus. This smooth muscle stimulating activity was abolished when the dialysate-substrate mixture was incubated with the known kininase carboxypeptidase B (Worthington Labs., Freehold, NJ).

Initial purification on G-200 Sephadex. The elution profile of the fibroblast acid protease through G-200 Sephadex is shown in Fig. 1. Kinin-forming activity was present in fractions 25–29 (low activity) and 33 42. The pooled 25–42 fractions had a yield of approximately 100 per cent based on the amount of kinin formed per ml eluant. The sp. act. of the supernatant dialysate was 220.5 ng kinin/mg protein whereas the 25–41 fraction pool had a sp. act. of 520.7 ng kinin/mg protein. The purification was increased over the dialysate by a factor of 2.4.

Hydroxylapatite chromatography. Kinin-forming activity was found in fractions 49–61 when the G-200 Sephadex purified fractions were chromatographed through hydroxylapatite, Fig. 2. The pooled fractions, when concentrated to 20 ml with an Amicon UM #2 membrane and dialyzed against a potassium phosphate-KCl-cysteine buffer system, had a sp. act of 1064.8 ng kinin/mg protein. These steps increased the purification almost 5-fold with a 34.9 per cent yield, Table 1.

DEAE-A50 Sephadex anion exchange. Fig. 3 represents the ion exchange profile of the acid protease when passed through a DEAE-A50 column. The

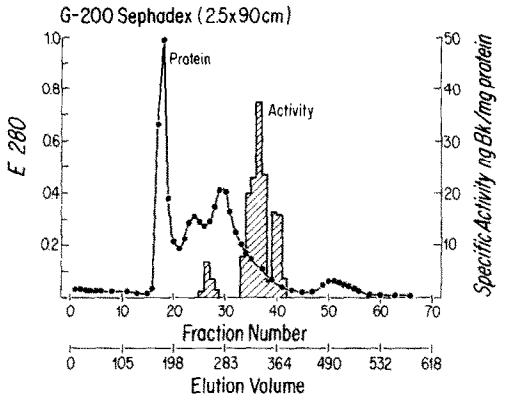

Fig. 1. Fractionation on G-200 Sephadex (2.5 × 90 cm) of fibroblast L-929 (10^9 cells) cell homogenate dialysate. Cells were homogenized for 8 min, dialyzed 18 hr against a 0.01 M NaH_2PO_4:Na_2HPO_4 buffer, pH 6.8, in 0.1 M NaCl and 1 mM EDTA, and centrifuged at 12000 rpm for 45 min. A 9 ml vol. containing 6.38 mg protein/ml was applied. Eluent fractions were monitored for protein concentration (E of 280), and 0.5 ml aliquots measured for kinin forming activity on rat plasma kininogen in 0.56 M acetate buffer, pH 4.0, containing 1, 10-phenanthroline and soya bean trypsin inhibitor (SBTI).

enzyme eluted from the column first without the need to apply a salt gradient. The final sp. act. was 2062.5 ng kinin/mg protein with a 13.8 per cent yield. Compared to the activity of the supernatant dialysate, the final purification was almost 10-fold, Table 1.

Purification of rat plasma kininogen. Two substrate peaks for the purified acid protease were obtained when 25 ml of rat plasma was fractionated on a DEAE-A50 ion exchange column, Fig. 4. Fractions 25–45, which constituted peak I, when concentrated to 8.0 ml, had a protein concentration of 1.64 mg/ml and a sp. act. of 22.9 ng kinin/mg protein, Table 2. A second kininogen peak II was found in fractions 55–63 and, when concentrated to 8.7 ml, had a protein concentration of 7.75 mg/ml. The sp. act. of peak II was 77.4 ng kinin/mg protein with a yield of 18.6 per cent and an almost 3-fold purification compared to the original rat plasma.

G-100 fractionation. Four mls of peak I and 3 mls of peak II kininogen fractions, when applied onto a G-100 Sephadex column, yielded a major peak in fractions 14–20, and a smaller peak in fractions 21–23, Fig. 5. Fractions 15–17, representing the peak activity fractions, were pooled and had a protein concentration of 0.36 mg/ml. The sp. act. of this fraction pool was 112.2 ng kinin/mg protein and the purification factor was 3.9, Table 2.

Disc gel electrophoresis. Apparent homogenous acid protease was obtained as indicated by the disc gel patterns, Fig. 6a. Step-wise purification was achieved yielding a single band with protease activity. Disc gel electrophoretic patterns for rat plasma and kininogen purified in a subsequent study by similar techniques indicate a major kininogen protein band and major protein band of impurity, Fig. 6b.

Molecular weight determinations. The elution vol. (*Ve*) of the standards and test materials were: aldolase, 24.7 ml; ovalbumin, 31.7 ml; chymotrypsinogen A, 36.3 ml; ribonuclease, 38.9 ml; acid protease, 33.0 ml; peak II kininogen, 26.2 ml. From the G-200 gel filtration data, the mol. wt of the acid protease was estimated at 38000–39000 and the peak II kininogen mol. wt estimated at 115000, Fig. 7. The partition coefficient of each component is shown on the ordinate and the mol. wt shown on the abscissa.

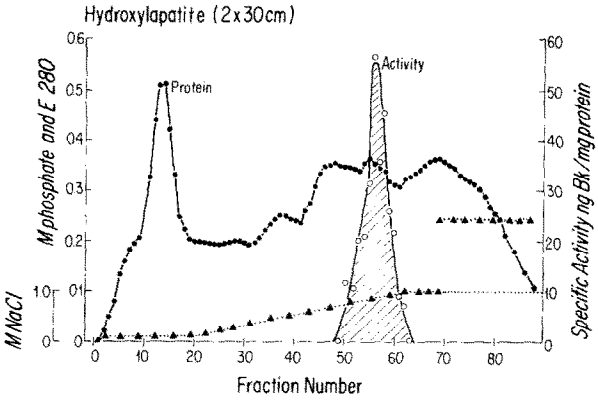

Fig. 2. Chromatography on hydroxylapatite column (2 × 30 cm) of G-200 Sephadex-purified acid protease (17 ml, 3.8 mg protein/ml; sp. act. of 520.7 ng kinin/mg protein). Initial equilibrating buffer was 0.01 M phosphate buffer, pH 6.8, in 0.1 M NaCl and 1 mM EDTA. After appearance of first protein peak, a linear gradient was applied of 150 ml 0.01 M phosphate buffer in 0.1 M NaCl, 1 mM EDTA to 150 ml 0.12 M phosphate buffer in 1.0 M NaCl, 1 mM EDTA. Protein was monitored continuously and eluent fractions assayed for kinin-forming activity as indicated in Fig. 1.

Table 1. Summary of purification of acid protease from L 929 fibroblast

Procedures	Vol (ml)	Protein (mg/ml)	Kinin-forming activity* (ngBK/ml)	Specific activity ngBK/mg protein	Yield (%)	Purification factor
L929 cell (10^9) Supernatant dialysate	17.0	8.8	1940.0	220.5	100	1
G-200 Sephadex	17.0	3.8	1978.7	520.7	102	2.4
Hydroxylapatite (After dialysis vs cysteine)	13.0	0.73	727.0	1064.8	34.9	4.8
DEAE-A50 Sephadex	11.0	0.20	412.5	2062.5	13.8	9.4

* Activity in terms of kinin formed following 24 hr incubation of 0.5 ml fraction with 0.2 ml rat plasma

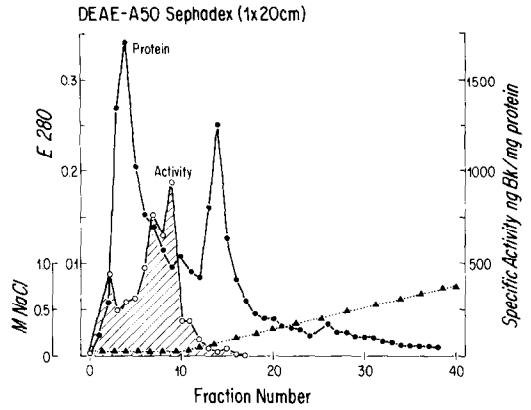

Fig. 3. Fractionation of cysteine-activated, hydroxylapatite-purified acid protease (11.0 ml, 0.73 mg protein/ml, sp. act., 1064.8 ng kinin/mg protein) through DEAE-A50 ion exchange column (1 × 20 cm). The column was equilibrated with 0.01 M KH_2PO_4:K_2HPO_4 buffer, pH 6.8, in 2.5 mM mercaptoethanol. Initial buffer was 50 ml of equilibrating buffer followed by 150 ml of equilibrating buffer to 150 ml of a 0.01 M KH_2PO_4:K_2HPO_4, 1.0 M KCl, 2.5 mM mercaptoethanol linear gradient. Bioassay conditions were as described in Fig. 1. Sp. act. of the eluent was 2062.5 ng kinin/mg protein.

pH profile of the acid protease. The acid protease formed kinin within a relatively narrow pH range, Fig. 8. Optimal activity was at pH 4.0. At that pH, 137 ng kinin/mg substrate/mg enzyme were generated following 15 hr incubation at 37°.

Time course of kinin release. The time course of kinin release was directly proportional to the enzyme concentration at a fixed substrate concentration, Fig. 9. With increasing concentrations of enzyme, increasing amounts of kinin were formed. Kinin release appeared to increase nonlinearly with respect to time at higher enzyme concentrations.

Subcellular localization of acid protease. The major amount of kinin-forming activity was contained in the 10000 g supernatant fraction of the cell, Fig. 10. This fraction contained 61.5 per cent of the activity calculated after 48 hr of incubation. The 1500 g cell fraction contained 38.5 per cent of activity whereas the 10000 g precipitate fraction was devoid of activity.

DISCUSSION

Data herein presented do extend previously published findings regarding the kinin-forming acid protease identified in mouse fibroblasts [7]. This acid protease was shown to utilize hemoglobin as a substrate similar to other acid proteases reported in the rat Murphy-Sturm lymphosarcoma [4], human gastric carcinoma [11], renal carcinoma [12], Jensen sarcoma [13], and tumor interstitial fluid [14]. A 10-fold purification of the fibroblast acid protease was achieved using column chromatographic technique. Undoubtedly, the purification factor would have been considerably higher had the activity in the crude cell homogenate been adopted as the reference activity rather than the activity of the dialyzed supernatant of the cell homogenate. The purification data would

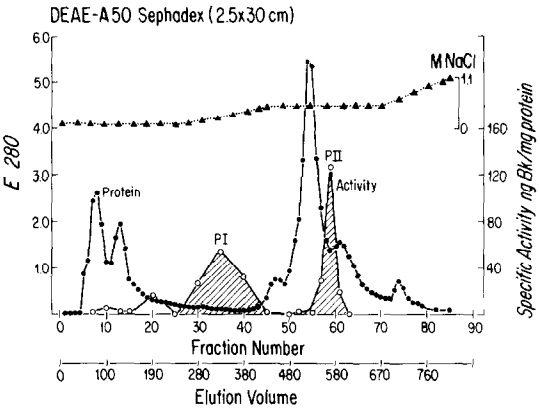

Fig. 4. Fractionation of rat plasma through DEAE-A50 Sephadex column (2.5 × 30 cm) for isolation of rat plasma kininogen. 25 ml of rat plasma was dialyzed for 10 hr against 0.01 M sodium phosphate buffer, pH 6.8, in 0.1 M NaCl and 1 mM EDTA. The dialysate was centrifuged at 3000 rpm for 30 min, the supernatant filtered through a #1 Whatman paper, and 20 ml of filtrate (39 mg/ml) applied. The flow rate was 20 ml/hr. The column was developed with 240 ml of the starting buffer followed by 95 ml of 0.1 M NaCl to 95 ml 0.5 M NaCl linear gradient in 0.01 M phosphate buffer, pH 6.8. An eluting vol. of 190 ml of 0.5 M NaCl in 0.01 M phosphate buffer then was followed by 70 ml of 0.8 M NaCl to 75 ml 1.2 M NaCl linear gradient in 0.01 M phosphate buffer. Fractions (0.5 ml) were assayed for kininogen by incubation for 18 hr with G.200 Sephadex-purified acid protease (1.2 mg/ml) in 0.56 M acetate buffer (pH 4.0) and SBTI. After neutralization with 1.0 M Tris buffer, pH 7.8, microliter aliquots were bioassayed for kinin on the isolated perfused rat uterus against a reference bradykinin (BK) standard. Activity of both peak I and peak II is expressed in terms of ng Bk/mg protein eluate.

Table 2. Summary of purification of rat plasma kininogen

Procedures	Vol (ml)	Protein (mg/ml)	Activity ngBK/ml	Specific activity ngBK/ mg Protein	Yield %	Purification factor
Rat Plasma	25.0	39.0	1124	28.8	100	1
DEAE Sephadex						
Peak I	8.0	1.64	37.6	22.9	1.06	0.8
Peak II	8.7	7.75	600.0	77.4	18.6	2.7
G-100 Sephadex	30	0.36	40.3	112.2	4.3	3.9

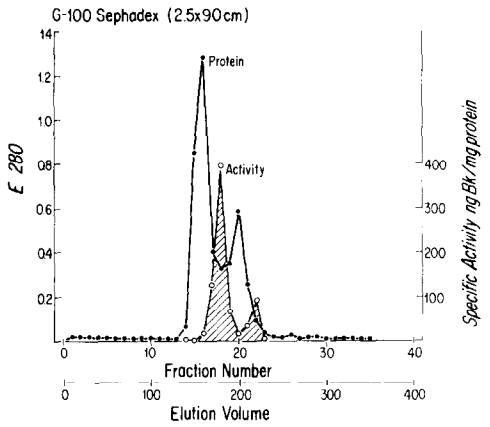

Fig. 5. Fractionation of DEAE-A50 Sephadex-purified kininogen through G-100 Sephadex column (2.5 × 90 cm). 4 ml of peak I kininogen (1.64 mg/ml) plus 3 ml of peak II kininogen (7.75 mg/ml) was applied onto the column equilibrated with 0.01 M KH_2PO_4:K_2HPO_4 buffer, pH 6.8 in 0.1 M KCl and 2.5 mM mercaptoethanol. Kininogen content in the eluants was assayed and activity expressed as described in Fig. 4.

appear comparable, however, to the 6-fold purification reported of cathepsin A and D from extracts of chicken breast muscle [15]. At a pH optimum of 4.0, the purified enzyme released significant quantities of kinin from a rat plasma kininogen substrate in a time- and enzyme concentration-dependent manner.

Disc gel electrophoretic patterns indicated the apparent homogeneity of the enzyme and substrate reagents. Whether or not the acid protease is homogenous needs other purity-test studies. G-200 gel filtration technique revealed the protease to have an approximate mol. wt of 38000–39000 as estimated by bioassay analysis. A leukocyte cathepsin D proteinase was reported to have a mol. wt of 52000 ± 3000 [16] whereas rat liver lysosomal cathepsin B_1 and B_2 had mol. wt of 24000 and 50000 respectively [17]. The estimated mol. wt of the fibroblast acid protease does agree reasonably well with the 37000 for spleen cathepsin D [18] and 36000 for chicken muscle cathepsin D [19].

The acid protease was found to be activated by 5 mM cysteine suggesting the possible involvement of a sulfhydryl group in the enzyme for enzymatic activity. The enhanced activity also may relate to interaction of cysteine with the kininogen substrate. Similar observations were reported with a kinin-forming

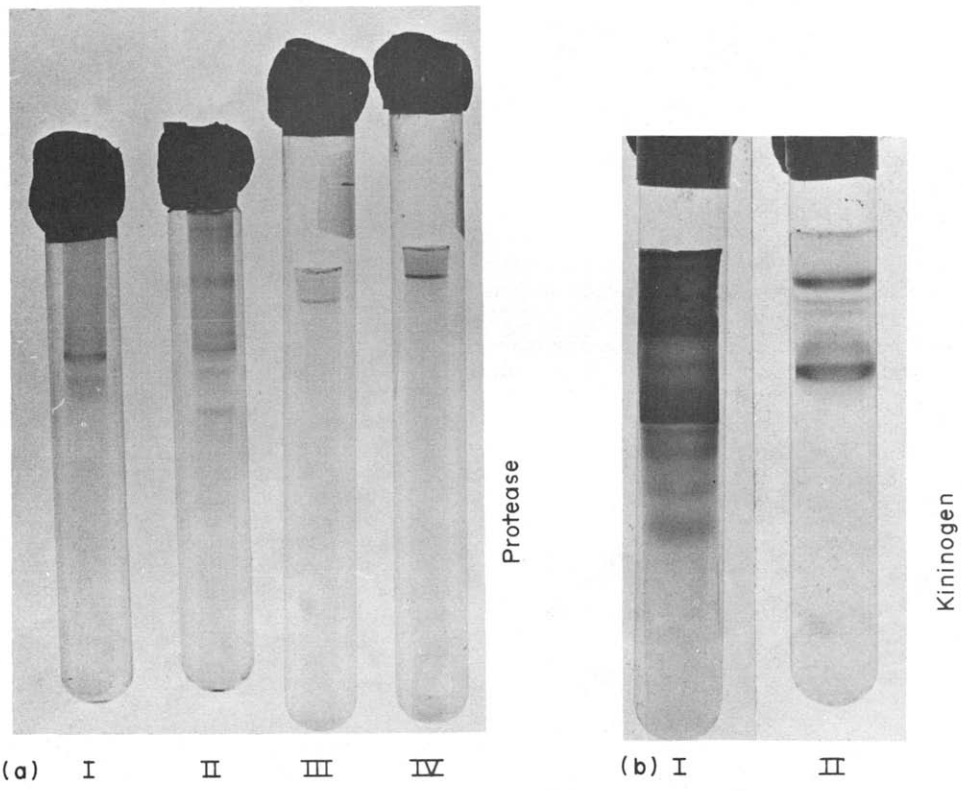

Fig. 6.(a) Disc gel patterns of acid protease during purification. A vol. 50 μl of the following enzyme preparations was added to the gel tubes: I = L-929 supernatant dialysate; II = G-200 Sephadex purified; III = DEAE-A50 Sephadex purified in presence of cysteine; IV = DEAE-A50 Sephadex purified in presence of 2-mercaptoethanol. The electrophoresis was carried out for 1½ hr with a current of 2.5 ma per tube and a voltage of 280 V in the entire system. (b) Disc gel patterns of rat plasma kininogen. A 50 μl vol. of the following substrate preparations was added to the gel tubes: I = rat plasma; II = Sephadex-purified fraction. The electrophoresis was carried out for 1½ hr with a current of 2.5 ma per tube and a voltage of 280 V in the entire system.

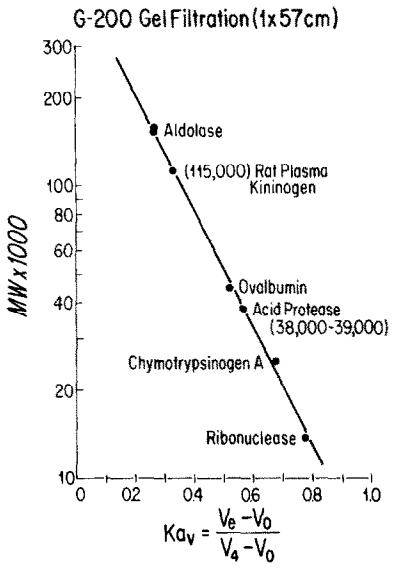

Fig. 7. Gel filtration of acid protease and kininogen through G-200 Sephadex column (1 × 57 cm) for estimation of mol. wt. The column was equilibrated with 0.01 M NaH_2PO_4 : Na_2HPO_4 buffer, pH 6.8, in 0.1 M NaCl, and 1 mM EDTA. Markers included aldolase (25 mg), ovalbumin (4 mg), chymotrypsinogen A (4 mg) and ribonuclease (4 mg). All marker proteins were dissolved in 0.5 ml of the starting buffer.

acid protease isolated and purified from the rat Murphy-Sturm lymphosarcoma [20]. An acid protease from rabbit lysosomes also was shown to be activated by cysteine [21].

A leukokinin-forming acid protease has been reported in rabbit polymorphonuclear (PMN) leukocytes [22]. While proteases from human polymorphonuclear leukocytes ordinarily are active at neutral pH [23], the similarity between the PMN leukocyte protease and the fibroblast protease has been alluded to previously [7]. Similar in pH optima and effect of inhibitors, their respective sites of cellular localization and substrate specificities may be different. The subcellular distribution of both proteases awaits more definitive study. While differential centrifugation, according to Poole [24], may solve the problem of damage of the subcellular organelles, these studies do show that the fibroblast protease existed both in the nuclear and cytoplasmic fractions. There are reports indicating that, in tumor homogenates, the total lysosomal enzyme activities present in the soluble (cytosol) fraction frequently are higher than those observed in normal cells [24]. Strain L cells also were reported to release their cytoplasmic enzymes [25]. Thus, cathepsin D was found to be released in both protein-free and protein-containing media [25].

The fibroblast acid protease hydrolyzed the same kininogen substrate utilized by trypsin and plasma kallikrein. The acid protease from the Murphy-Sturm lymphosarcoma formed kinin from tumor kininogen as well as 80°-heated rat plasma substrate [4]. A single kininogen was isolated from the rat plasma with a mol. wt of 115000, similar to the high mol. wt human kininogen following passage through a guanidine-Sepharose-4B column [26]. There are reports, however, indicating that high mol. wt bovine and human kininogen have a mol. wt ranging from 60000 to 80000 [27, 28]. In our initial study, two kininogen peaks were obtained from the passage of the rat plasma through a DEAE-A50 Sephadex column (Fig. 4). Hamberg [29] also found two

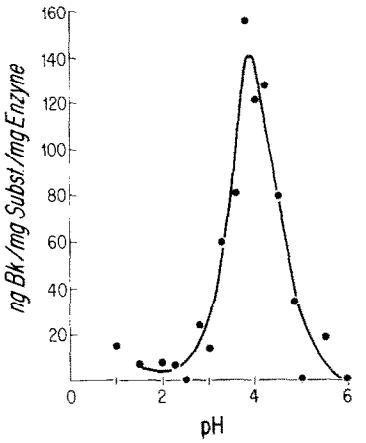

Fig. 8. pH profile of the kinin-forming activity of the acid protease on rat plasma kininogen. The following pH points were studied and solutions were used: pH 1.0, 0.05 M KH_2PO_4 : HCl; pH 1.5, 0.05 M KH_2PO_4; pH 2.0, 2.3, 2.5, glycine: HCl; pH 2.8, citric acid: NaOH, pH 3.0, 3.3, citric acid: sodium citrate; pH 3.6, 3.8, 4.0, 4.2, formic acid: sodium formate; pH 4.5, 4.8, 5.0, acetic acid: sodium acetate; pH 5.5, citric acid: sodium citrate; pH 6.0, histidine buffer; pH 7.0, imidazole buffer; pH 8.0, Tris buffer; pH 9.0, histidine buffer; pH 10.0, glycine; pH 11.0, glycocoll buffer; pH 12.0 phosphate buffer.

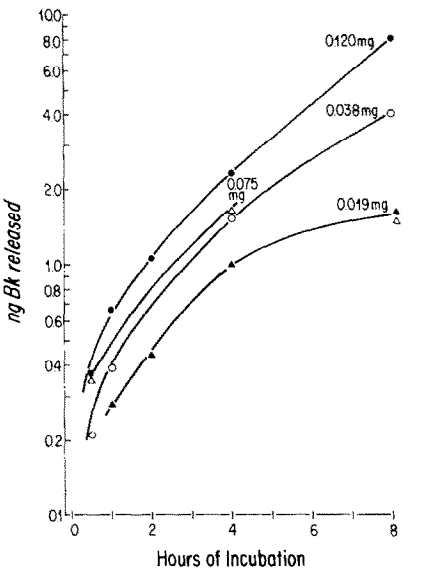

Fig. 9. Time course of kinin release with respect to acid protease enzyme concentration. DEAE-A50 Sephadex-purified acid protease at concentrations of 0.019 mg (▲——▲), 0.038 mg (O——O), 0.075 mg (△——△), and 0.120 mg (●——●) were incubated with 0.075 mg DEAE-A50 Sephadex-purified rat kininogen at pH 4.0 for 0.5, 1, 2, 4, and 8 hr. Kinin formed was assayed and calculated as described in Fig. 1.

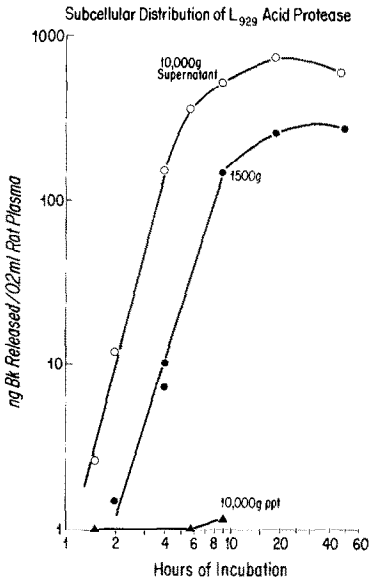

Fig. 10. Cell fraction localization of the acid protease activity. 10^9 fibroblasts were homogenized three times for 2 min each time and dialyzed 22 hr against 0.01 M phosphate buffer, pH 6.8, in 0.1 M NaCl and 1 mM EDTA. The preparation was centrifuged for 15 min at 1500 *g* to obtain the 1500 g precipitate fraction. Centrifugation at 10000 *g* for 20 min yielded a 10000 g precipitate fraction and 10000 g supernatant fraction. Assay for kinin-forming activity was carried out on rat plasma kininogen substrate at pH 4.0 following incubation for 1.5, 2, 4, 5.75, 9, 19, and 48 hr. Estimates of kinin formed were made by bioassay technique.

kininogen species after fractionation of human plasma on DEAE-Sephadex using a salt gradient of 0.06 M to 0.30 M. However, by using steeper gradients only one kininogen species was isolated [29]. In our chromatography study, after mixing the two kininogen peaks obtained from the DEAE-A50 Sephadex and passing this pool through a G-100 Sephadex column equilibrated with phosphate buffer and 2-mercaptoethanol, only one major kininogen peak and a very small secondary peak were obtained (Fig. 5). The results suggest that, although two kininogen species may exist in rat plasma as suggested by Jacobsen [30], there is the possibility that in fact only one species is present. The minor component may represent either an aggregate, as suggested by Hamberg [29], or may be due to minor heterogeneity of the kininogens [31].

Interstial fluid of solid tumor transplants were shown to be rich in acid proteases and other peptidases [14] suggesting that tumor cells grow in a medium which provides a rapid external supply of small peptides and amino acids. While tumor cells were found devoid of significant peptidase activity, cathepsin and amino-peptidase activities were found, in decreasing order of activity, in macrophages, mast cells, and fibroblasts localized between the growing edges and necrotic tumor transplants [32]. A fibroblast network was found in the Murphy-Sturm lymphosarcoma from which an acid protease was isolated [1, 4]. Fibroblast proliferation was noted at the transplant site during the initial growth phase following tumor transplantation [33]. While the functional role of fibroblasts and associated protease activity in neoplasia is uncertain, evidence has been summarized in a recent symposium suggesting some involvement in cell transformation, tumor morphology, growth, and invasiveness [34]. Free and total cathepsin levels were higher in the growing regions of the regressing rodent Jensen sarcoma than in non-regressing tumors [13], and cathepsin-like enzymes have been suggested associated with the invasiveness of human gynecologic tumors [35]. Acid proteases originating from fibroblasts within neoplastic tissue may form vasoactive mediators that may cause chemotaxis, vasculogenesis, and alterations of tumor blood flow. Tumor cell chemotaxis was produced by the intradermal injection of a neutral protease found recently in the ascites hepatoma AH109A (36). Algire, *et al.* [37] reported that L-929 fibroblasts grown in cell culture, when transplanted into skin-fold transparent chambers established in mice, caused considerable vasculogenesis as noted by direct microscopic examination.

Two kinin peptides have been isolated and purified from incubates of rat plasma kininogen and fibroblast acid protease (unpublished data). That an appropriate tissue pH environment may exist for such protease action is suggested by reports that the pH of negatively charged cell surfaces is significantly lower than that of the bulk phase [24]. This excess of hydrogen ions in the immediate vicinity of predominantly negatively charged cell surfaces may be capable of lowering the local pH by as much as 2 units. The characterization of the pharmacologic profile of the fibroblast kinins now in progress may contribute toward elucidating the role of proteases in the neoplastic process.

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