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PROPERTIES OF LEUKOKININOGEN ISOLATED FROM HUMAN NEOPLASTIC ASCITES *

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Abstract—Leukokininogen, the protein precursor of the vasoactive polypeptide leukokinin-H, has been isolated from human ovarian carcinoma ascites. A homogeneous preparation was achieved after a procedure consisting of ammonium sulfate fractionation, gel filtration with Sephadex G-200, DEAE-Sepharose ion exchange chromatography and preparative isoelectric focusing. Leukokininogen has an isoelectric pH of 4.56 and a molecular weight of about 41,000 daltons, determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Kinin-like activity was generated from leukokininogen by incubation of the protein with the leukokininogenase from murine ascites tumor cells and with trypsin. Human plasma kallikrein does not release kinin activity from leukokininogen. The amino acid composition and immunological properties of leukokininogen demonstrate that leukokininogen is distinct from plasma bradykininogens.

Leukokininogen, the protein precursor of the vasoactive polypeptide leukokinin (LK), together with the protein precursor of the nonapeptide bradykinin (BK), bradykininogen, are the principal sources of the vasoactive peptides known as kinins. Kinins have a number of pharmacological actions in common which include their ability to lower blood pressure and increase vascular permeability leading to local edema (see Refs. 1–4). Kinins play a role in many pathological conditions, especially in inflammation [48]. In addition, leukokinin formation has been implicated as a major reason for ascites accumulation in neoplastic disease such as that arising from ovarian carcinoma [5–9] because of its properties of increasing vascular permeability.

There are essentially two major kinin-releasing systems in mammalian physiology. One is the kallikrein–bradykinin-generating system of plasma, in which the plasma protease kallikrein, when activated, can partially hydrolyze the plasma protein known as kininogen or bradykininogen, to release BK. A second kinin-releasing system, the leukokinin system, as described by our laboratory, results in the release of leukokinin from leukokininogen by the action of a cathepsin D-like acid protease [9]. Leukokininogen is a protein which is found primarily in extravascular fluids resulting from inflammation, and in neoplastic ascites [9]. While there are a number of similarities between the general mechanisms of release of both LK and BK, the peptides themselves are chemically and pharmacologically distinct from one another as are the enzymes responsible for their release [7].

Purification of bradykininogen from both human and bovine plasma has revealed that there are several molecular forms differing structurally by their molecular weights and functionally in their susceptibilities to the several known BK-releasing proteases. Much less is known about leukokininogen than about bradykininogen. Leukokininogen is found primarily in pathological fluids such as burn-blister fluid and synovial fluid from rheumatoid arthritis [5]. In addition, leukokininogen is found in ascitic fluid of neoplastic origin such as ascites from mice bearing ascites tumors and human ascites resulting from ovarian carcinoma [5, 6]. Leukokininogen is not usually found in normal human plasmas, or when present, is in much lower concentrations than when it is found in the pathological fluids mentioned above. The structure and amino acid content are unknown.

In an effort to determine the chemical and physical properties of leukokininogen and the relation of it to any of the known form of bradykininogen, the isolation of leukokininogen from human ascites fluid was undertaken. This report describes the isolation, purification and several physico-chemical properties of leukokininogen from human ascites resulting from ovarian carcinoma. Evidence is presented which demonstrates that leukokininogen differs from other known plasma kininogens [high molecular weight (HMW) and low molecular weight (LMW) bradykininogens].

MATERIALS AND METHODS

Chemicals

Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Eastman Kodak Co. (Rochester, NY); ammonium persulfate, *N,N,N',N'*-tetramethylenediamine, riboflavin, and Coomassie Brilliant Blue R-250 from Bio-Rad Laboratories (Richmond, VA); Coomassie Brilliant Blue G-250 from Sreva Co. (Heidelberg); and Amido Black from Allied Chemical Co. (Morristown, NJ). *N*-tosylarginine[^{3}H]methyl ester ([^{3}H]-TAME) was a gift from Dr. Walter Troll, New York University Medical Center, New York NY. [^{3}H]acetylhemoglobin was a gift from Dr. Victor Hatcher, Montefiore Hospital, Bronx, NY. Liquid scintillators, Riafluor and Spectrofluor Butyl BPD, were obtained from New England Nuclear (Boston, MA)

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and Amersham/Searle (Arlington Heights, Il) respectively. Carrier ampholytes, pre-cast thin layers of polyacrylamide containing ampholytes (Ampholine PAG plates) and pre-washed Sephadex G-75 for isoelectric focusing (Ultradex) were obtained from LKB Instruments (Rockville, MD). Sephadex G-200 and DEAE-Sepharose CL-6B, and protein standards for molecular weight determination (aldolase, chymotrypsinogen A, ovalbumin and ribonuclease) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Bovine serum albumin was purchased from Schwarz–Mann Corp. (Orangeburg, NY).

Antisera

Monospecific anti-low molecular weight kininogen was a gift from Dr. Jack Pierce, National Heart and Lung Institute, Bethesda, MD.

Enzymes

Kallikrein. Human plasma kallikrein was prepared according to one of the methods described by Henriques *et al.* [10]. One hundred ml of outdated human plasma, obtained from the blood bank of the Columbia Presbyterian Hospital, was shaken with 100-g glass beads, 0.1-mm diameter (A. H. Thomas Co. Philadelphia, PA) for 10 min in a polyethylene tube. After centrifugation, the supernatant fraction was discarded and the beads were washed twice with saline. The beads were allowed to stand at 4° for 2 hr, after which time the adsorbed, activated kallikrein was eluted from the glass beads by washing them with three 25-ml aliquots of 0.1% glycine buffer, pH 6.0, containing 5% sodium chloride. The glycine washes were pooled and dialyzed against 2 litres of 0.01 M Tris buffer, pH 7.5. The kallikrein content was measured by its ability to release kinin from plasma kininogen [11]. In addition, the esterolytic action of kallikrein on the substrate [^{3}H]TAME was determined according to the method of Roffman *et al.* [12].

Trypsin. Twice-crystallized trypsin, in 50% ammonium sulfate, was obtained from Worthington Biochemicals Corp. (Freehold, NJ). The ammonium sulfate was removed by dialysis against 0.001 M cold HCl. The activity of the enzyme was determined by measuring the hydrolysis of [^{3}H]TAME [12].

Leukokinin-forming protease. The acid protease was partially purified from murine L-1210 ascites tumor cells as described previously [13]. The enzyme was tested for its ability to liberate leukokinin from plasma kininogen protein substrate [11]. The enzyme was also tested for its ability to liberate [^{3}H] peptides from [^{3}H] acetylhemoglobin at acid pH, according to the method of Grayzel *et al.* [14], as follows: an incubation mixture was prepared which contained 20μl each of [^{3}H]hemoglobin (0.3 mg/ml, 2 μCi/mg), 0.1 M acetate buffer, pH 4.0, and enzyme solution (25μg protein/ml). After incubation at 37° for 2 hr, 50μl of 3% chilled (4°) unlabeled hemoglobin was added, followed immediately by 100μl of ice-cold 6% trichloroacetic acid (TCA). The TCA-insoluble protein was allowed to precipitate at 4° for 15 min after which time the mixture was centrifuged at 4° at 27,000 *g* for 15 min. One hundred μl of the supernatant fraction was pipetted into a polyethylene liquid scintillation vial (Mini-vial, New England Nuclear, Boston, MA) containing 5 ml of Riafluor liquid scintillant. The radioactivity of the sample was determined in a liquid scintillation counter (1-min count). Control incubation mixtures containing substrate and buffer only (total volume 60μl) were incubated along with those containing enzyme and were treated in a manner similar to the above. In addition, a zero-time incubate was prepared without enzyme and immediately treated with unlabeled hemoglobin and TCA. There was essentially no increase in counts of the supernatant fractions between the zero-time and 2-hr control tubes (i.e. no spontaneous hydrolysis of the substrate).

Collection of ascites

Ascitic fluid was routinely withdrawn by paracentesis from ovarian carcinoma patients at Presbyterian Hospital, New York City, following informed consent, in collaboration with Dr. Henry Frick, II, Columbia University. Fresh fluid, collected in glass bottles, was transferred to polyethylene containers as soon as possible after collection (30–60 min). 8-Hydroxyquinoline (OHQ) was added to a concentration of 0.001 M to stabilize leukokininogen.

Leukokininogen

Leukokininogen in ascites and in fractions obtained during purification was determined by conversion to leukokinin by incubation with the murine leukokinin-forming acid protease at pH 4.0 for 16 hr at 37°. Samples were adjusted first to pH 4.0 and incubated with 100μl of the murine acid protease for 16 hr at 37°. The incubates were diluted with equal parts of water, placed in a boiling water bath for 15 min and centrifuged. The supernatant fractions were adjusted to pH 7.0 and assayed for kinin by measuring the contraction of the isolated estrus rat uterus [15] using synthetic bradykinin as a standard. Leukokininogen is expressed in terms of the amount of leukokinin released after incubation with the leukokinin-forming acid protease at pH 4. One unit leukokininogen is defined as that amount which releases 1 ng leukokinin as determined by bioassay using the rat uterus.

Protein determination

The method of Lowry *et al.* [16] was used to measure protein concentrations of samples, using bovine serum albumin as the standard.

Gel filtration

Gel filtration was carried on a 10 × 85 cm preparative column of Sephadex G-200. The column was equilibrated with 0.1 M ammonium acetate, pH 6.8 7.0, at 4°. Samples of up to 150 ml were applied to the column and were eluted with the equilibration buffer using upward elution. Fractions of 150 ml were collected at a flow rate of 2.5 ml/min. Protein was monitored by measuring the absorbance at 280 nm.

Ion-exchange chromatography

Anion exchange chromatography was carried out with a 2.5 × 40 cm column of DEAE-Sepharose CL-6B. The column was equilibrated with 0.1 M ammonium acetate (conductivity = 6 m-mho), pH 6.8, at 4°. The ionic strengths of the equilibration and elution buffers and of the sample and column effluents were measured in terms of the electrical conductivities at 4° using a Radiometer conductivity meter. Samples con-

taining up to 100 mg protein were applied to the column and eluted with a linear gradient of increasing concentration of ammonium acetate. The gradient was formed in a simple two-chamber apparatus, in which the first chamber contained 500 ml of 0.1 M ammonium acetate (C = 6.0 m-mho) and the second contained 500 ml of 0.3 M ammonium acetate (C = 15 m-mho).

Analytical isoelectric focusing

The LKB Multiphor was used for both analytical and preparative isoelectric focusing. Samples were analyzed using LKB Ampholine PAG plates, containing ampholytes with pI values from 3.5 to 9.5. Samples containing 1 mg protein/ml were dipped in small rectangles of filter paper and applied to the surface of the PAG plates. The current was initially started at 200 V, 30 mA, and gradually increased to 1200 V, 10 mA, after 1 hr. This setting was maintained for an additional 30 min, after which current was switched off and the pH determined at the surface of the plate, from anode to cathode, using a micro combination electrode (Microelectrodes, Boston, MA). The plate was then stained in a solution of Coomassie Brilliant Blue R250 (1.115 g in 100 ml of aqueous solution containing 25% methanol and 8% acetic acid, v/v) at 60° for 10 min and destained in the above methanol–acetic acid–water solution.

Preparative isoelectric focusing

Preparative isoelectric focusing was performed in a flat bed of Sephadex G-75 superfine (Ultradex, LKB), in the LKB Multiphor apparatus, according to the manufacturer's instructions. The gel layer was made from 4 g Ultradex suspended in a solution containing 4 ml Ampholine (2.5 ml, pH 4–6, plus 1.5 ml, pH 5–8) and 96 ml water. Approximately 40 mg protein was used for each run, applied either to a trough cut out from the prepared gel layer immediately before current was applied or suspended in the gel slurry before preparation of the gel bed.

Current was maintained such that the power equaled 10 W. The temperature of the gel bed was maintained at 4°, and the focusing was continued for 14 hr. After current was stopped, a "print" was made by laying a piece of filter paper (Whatman No. 1) cut to fit the gel trough on top of the gel surface for 30 sec. The filter paper was then immediately dried in a stream of hot air from a hair dryer and stained in a solution of Coomassie Brilliant Blue G250 (0.2% in 3.5% perchloric acid). The blue bands of stained protein became visible after 20 min and reached a maximum intensity in about 3 hr. Destaining was not necessary to observe the bands since the background color was orange. The print was destained, however, in order to photograph it by washing it in several changes of 3.5% perchloric acid followed by drying as above. Immediately after the print was made from the gel layer, a sectioning grid (LKB) was inserted into the gel bed and the pH of alternate sections was measured directly with a microelectrode as described above. The protein-containing sections were then individually scooped up and transferred to 10 ml syringes containing glass wool plugs. A small amount of water was added to each syringe to allow a small column of gel to settle (1–2 ml of packed gel). The protein was then eluted from each gel with 2 ml of 0.2 M ammonium acetate and the eluates were lyophilized to dryness.

Polyacrylamide gel electrophoresis

Disc gel electrophoresis was performed in 7% gels according to the method of Davis [17]. The gels were stained in a solution of Amido Black (1%) in 7% acetic acid. The gels were destained by diffusion in 7% acetic acid. Alternatively, gels were stained in a solution of Coomassie Blue G-250 (0.2% in 3.5% perchloric acid). No destaining was necessary with the latter staining procedure [18].

Molecular weight determinations

Mobility of proteins in polyacrylamide gels was used to determine the molecular weight according to the method of Weber and Osborn [19]. Samples containing 0.5 mg protein/ml were incubated in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol, for 2 hr at 37°. Electrophoresis was carried out in 10% acrylamide gels in the presence of 0.2% SDS and mercaptoethanol. After completion of electrophoresis, the gels were stained in Coomassie Brilliant Blue R250 and destained by diffusion in a solution consisting of 75 ml acetic acid, 50 ml ethanol and 875 ml water. The mobilities of the standards were calculated and a standard curve was made by plotting the logarithms of the molecular weights of the standards vs their mobilities [19]. The molecular weight of leukokininogen was calculated from this graph.

Amino acid analysis

The amino composition of samples of leukokininogen was analyzed at the Institute for Protein Research, Osaka University, Japan. Protein samples (0.5 to 1.0 mg/ml) were hydrolyzed in 3 ml of constant boiling HCl for 24 hr and the hydrolysates were analyzed as described by Kato *et al.* [20].

RESULTS

Purification of Human LKGN

Ammonium sulfate fractionation (30–70 per cent). Human ascites (1 liter, 51 mg protein/ml) was brought to 30% saturation of ammonium sulfate by addition of

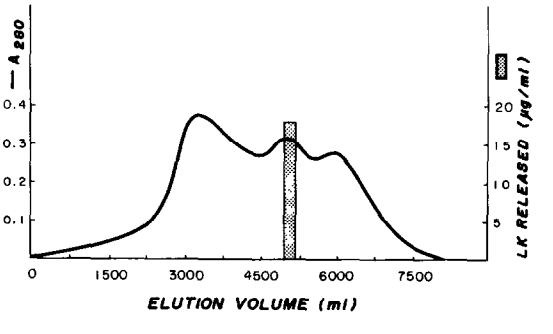

Fig. 1. Gel filtration of the 30–70% SAS fraction (150 ml, 6.0 g protein) on a Sephadex G-200 column (10.0 × 88.0 cm) equilibrated with 0.05 M ammonium acetate (conductivity 7 m-mho), pH 6.8. The column was run with an upward flow of 150 ml/hr and 150-ml fractions were collected.

176 g of solid ammonium sulfate at 20° after centrifugation. The supernatant fraction was adjusted to 70% saturated ammonium sulfate (SAS) by the addition of 273 g ammonium sulfate/liter of 30% SAS. The precipitate formed was collected by centrifugation. LKGN was found to be present only in the 30–70% SAS precipitate. The pellet was suspended in an equal volume of water and dialyzed against 10 vol of 1 mM 8-hydroxyquinoline to prevent peptidase action. The final solution contained 41.0 mg protein/ml.

Sephadex G-200 gel filtration. One hundred and fifty ml (6.2 g protein) of the dissolved 30–70% SAS precipitate was chromatographed on a 10 × 85 cm column

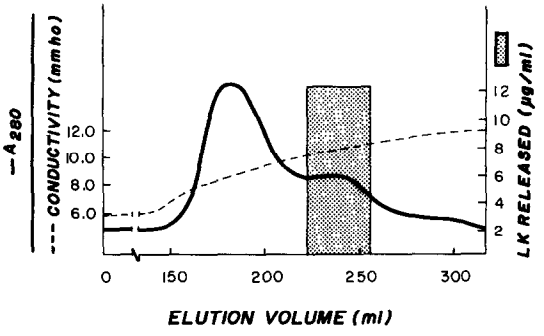

Fig. 2. Anion exchange chromatography of the active fraction from Sephadex G-200 (20 ml. 300 mg protein) on a DEAE-Sepharose CL-6B column (2.5 × 35.0 cm) equilibrated with 0.05 M ammonium acetate (conductivity 6 m-mho) pH 6.8. Gradient elution was performed with a two-chamber apparatus containing 500 ml of 0.05 M ammonium acetate in the first chamber and 500 ml of 0.3 M ammonium acetate (conductivity 20 m-mho) in the second chamber. A flow rate of 20 ml/hr was maintained and 7-ml fractions were collected.

of Sephadex G-200 as described above. Two major protein peaks were resolved and LKGN was present in the low molecular weight peak (Fig. 1).

DEAE-Sepharose chromatography. Twenty ml of the LKGN-containing fraction eluted from the preparative Sephadex G-200 column (26 mg protein) was applied to a 2.5 × 30 cm column of DEAE-Sepharose CL-6B which had been equilibrated with 0.05 M ammonium acetate. An increasing salt gradient was used to elute the protein (see above) (Fig. 2).

Isoelectric focusing. Analytical isoelectric focusing in polyacrylamide indicated that LKGN-containing fractions from DEAE-Sepharose had an isoelectric pH from 4.5 to 5.5 (Fig. 3). The LKGN-containing fractions from DEAE-Sepharose were lyophilized to dryness and suspended in 2 ml water and dialyzed against 100 ml of 1% glycine. The entire sample (34 mg protein) was then subjected to preparative isoelectric focusing in Sephadex G-75 over the pH range from 4 to 6. Two fractions containing LKGN activity were isolated (Fig. 4). The major one had an isoelectric pH of 4.56. A minor fraction at pH 4.98 was recovered but will not be reported on in this paper.

Summary of purification. Table 1 shows the degree of purification along with the recovery of leukokininogen after each purification step. A 347-fold purification of leukokininogen was achieved after isoelectric focusing (as compared to the ascites fluid itself).

Homogeneity of leukokininogen

When the major LKGN fraction recovered from preparative isoelective focusing was analyzed by disc polyacrylamide gel electrophoresis in 7% gels (100 μg protein/gel), only one protein band was observed (Fig. 5). The broad lower band in the gel on the right is due to

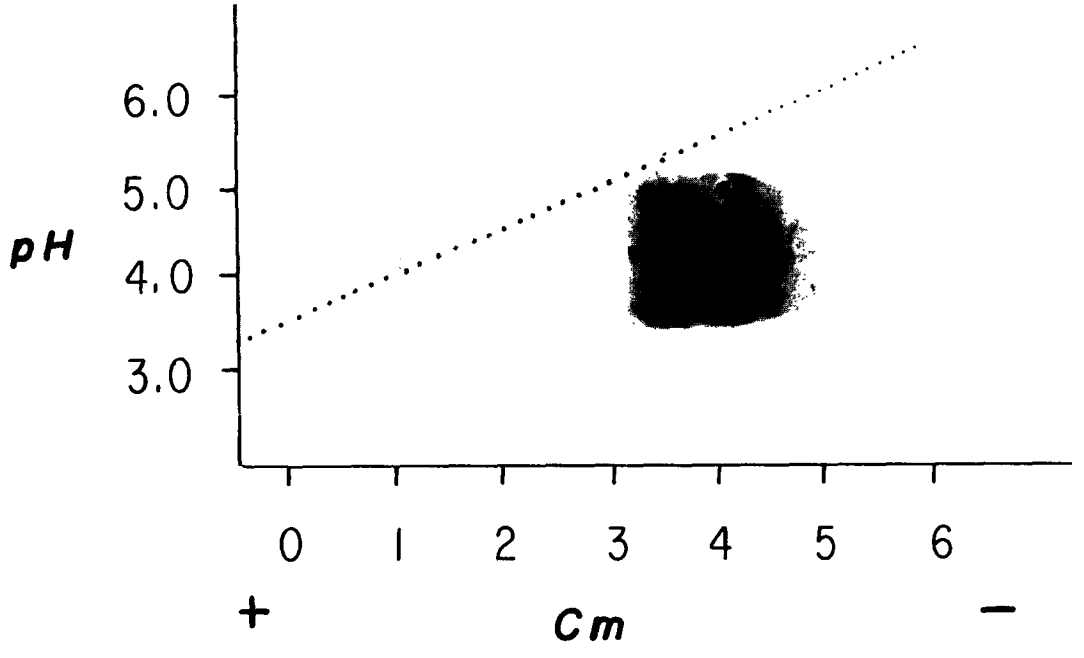

Fig. 3. Isoelectric focusing of the active fraction from DEAE-Sepharose, performed in polyacrylamide gel slab containing ampholytes over the range from pH 3.5 to 9.5 (LKB Ampholine PAG plates). Protein was stained with Coomassie Brilliant Blue R-250.

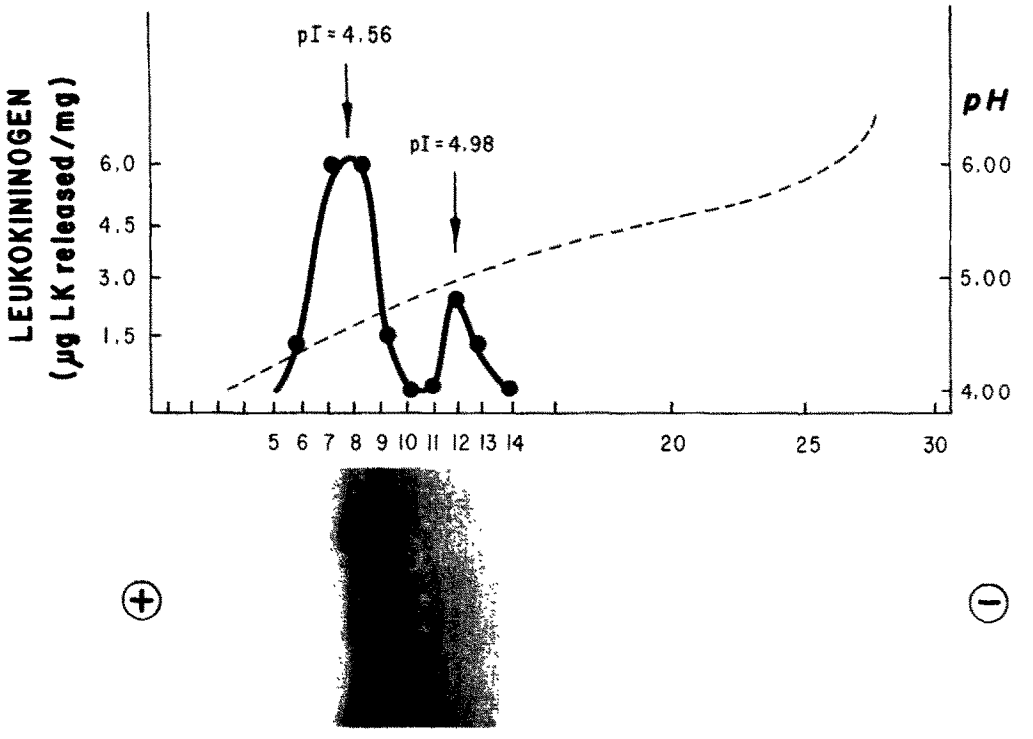

Fig. 4. Preparative isoelectric focusing of the active fraction form DEAE-Sepharose, performed in a flat bed of Sephadex G-75 superfine containing ampholytes from pH 4 to 6. A print was made from the gel surface after completion of the run using Whatman No. 1 filter paper. Protein was stained with Coomassie Brilliant Blue G-250 in 3.5% performic acid.

Table 1. Summary of purification of leukokininogen

Fractionation step	Volume (ml)	Total protein (mg)	Total leukokininogen* (LK units)	Yield (%)	Sp. act. (LK units/mg protein)	Purification (fold)
1. Ascites fluid	1,000	51,000	81,000	100	1.6	—
2. 30–70% SAS	500	20,500	131,200†	131	6.4	4
3. Sephadex G-200	1,000	1,300	18,200	13.9†	14.0	9
4. DEAE-Sepharose	35	94	11,730	8.9‡	125.0	78
5. Preparative isoelectric focusing	13	16	8,791	6.7†	555.0	347

* Expressed in terms of ng leukokinin (LK) formed after incubation with acid protease, pH 4.0, 37° for 16 hr/LK was determined by bioassay on the estrus rat uterus, using synthetic bradykinin as the standard. One LK unit is equivalent to 1 ng BK in this assay.

† See text.

‡ Compared to step 2.

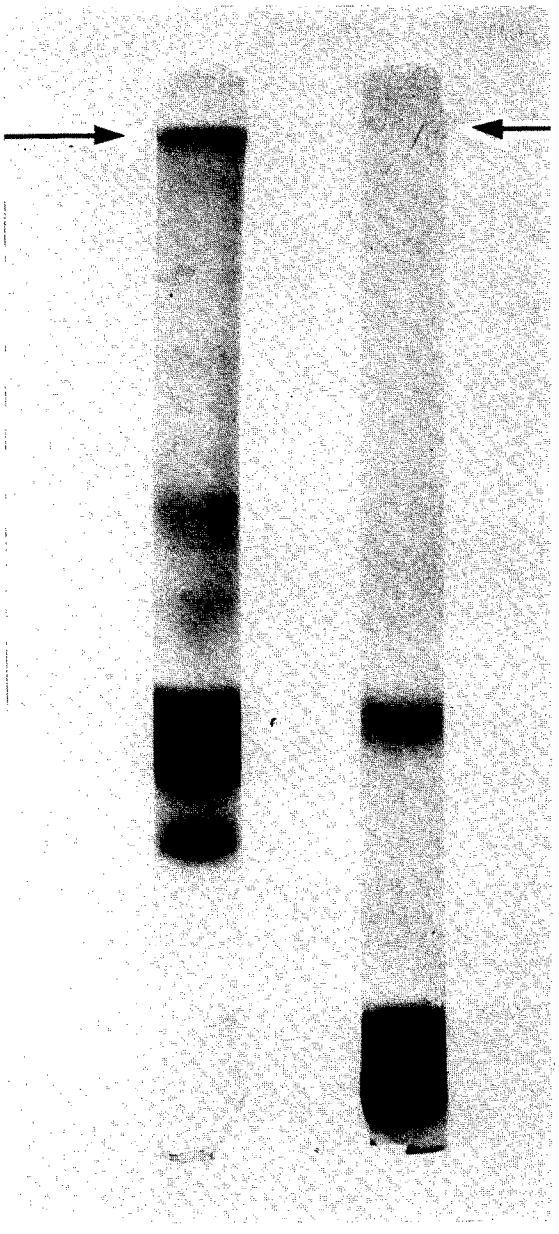

Fig. 5. Disc polyacrylamide gel electrophoresis of leukokininogen isolated by preparative isoelectric focusing. Electrophoresis was performed in 7% gels, pH 8.9. The gels were stained in 0.1% Amido Black in 7% acetic acid and destained by diffusion in 7% acetic acid. The gel on the left shows the protein composition of the active material from DEAE-Sepharose. The pattern seen in the gel on the right is that of fraction 7 from the preparative isoelectric focusing step (Fig. 4). The broad band at the bottom of this gel is due to the staining of ampholytes present in the sample. The origin of protein migration is indicated by the arrows.

the staining of ampholytes in the sample. After denaturation and reduction in the presence of SDS and 2-mercaptoethanol, for 2 hr at 37°, electrophoresis in 10% polyacrylamide gels containing 1% SDS again resolved only one band of protein (Fig. 6).

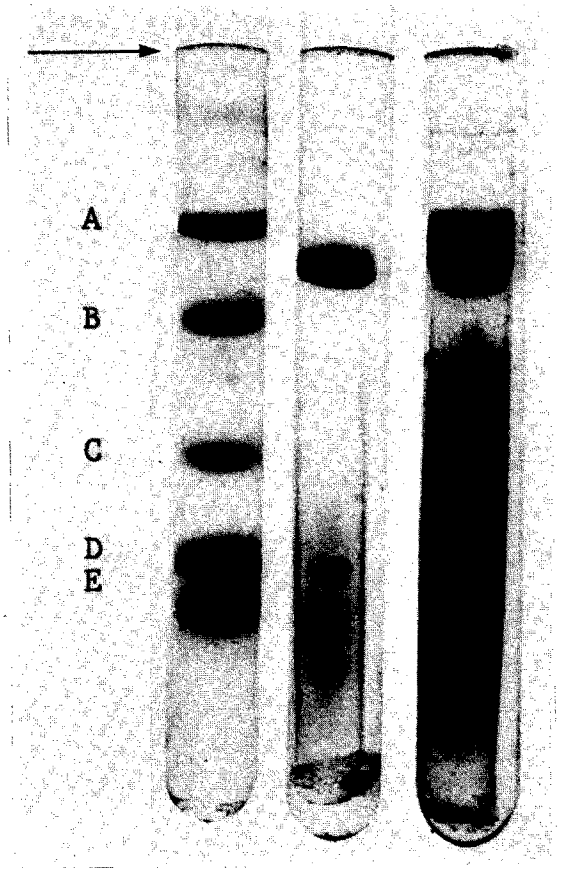

Fig. 6. Polyacrylamide gel electrophoresis of leukokininogen and several protein standards. The left-hand gel shows the migration of the standards: (A) bovine serum albumin (mol. wt 67,000), (B) ovalbumin (mol. wt 43,000); (C) chymotrypsinogen A (mol. wt 25,000), (D) myoglobin (mol. wt 17,200); and (E) cytochrome *C* (mol. wt 11,700). The middle and right-hand gels show leukokininogen in the absence and presence of SDS respectively. The origin of migration is designated by the arrow.

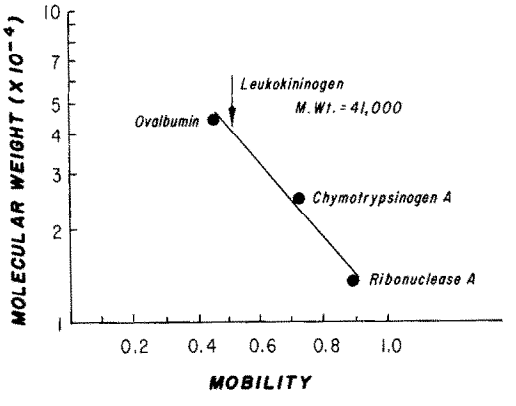

Fig. 7. Molecular weight determination of leukokininogen in SDS polyacrylamide gels. Standard proteins are indicated by the solid circles. The mobility of the leukokininogen (LKGN) is shown by the arrow. The gel concentrations was 10% acrylamide. All proteins were denatured in the presence of 0.5% each of SDS and 2-mercaptoethanol for 2 hr at 37°.

Molecular weight determination

LKGN was found to have a mobility corresponding to a protein with a molecular weight of 41,000, as determined by the method of Weber and Osborn [19] (Fig. 7). A sample of LKGN was analyzed by Dr. Kato, Protein Research Institute, Osaka, Japan, and similar results were obtained. In these studies LKGN had a mobility between that of ovalbumin and bovine serum albumin, corresponding to an apparent molecular weight of about 50,000 (Fig. 6).

Amino acid composition of LKGN

Table 2 shows the amino acid composition of bovine LMW kininogen (low molecular weight bradykininogen) along with that of human LKNG. It is clear that LKGN has an amino acid composition which differs significantly from that of LMW kininogens.

Table 2. Comparisons of the amino acid composition of human leukokininogen and bovine LMW kininogen

Amino acid	Leukokininogen * (residues/mole)	Bovine LMW kininogen † (Kato *et al.* [20]) (residues/mole)
Asp	38	37
Thr	20	24
Ser	18	28
Glu	57	46
Pro	20	26
Gly	13	16
Ala	42	26
½ Cys	11	16
Val	28	30
Met	5	5
Ile	4	15
Leu	41	23
Tyr	13	13
Phe	20	14
Try		4
Lys	38	9
His	14	26
Arg	14	11

* Determined according to the procedure of Kato *et al.* [20].

† Low molecular weight bradykininogen.

Release of kinin from LKGN by trypsin and plasma kallikrein

Pure LKGN (200 units) was incubated with trypsin (15 TAME units) in 0.05 M Tris buffer, pH 7.5, containing 0.2 $CaCl_2$, for 2 hr at 37°. The mixture was then heated on a boiling water bath for 15 min and an aliquot assayed for kinin on the isolated estrus rat uterus. A total of 100 BK equivalents was generated in the incubation mixture (Table 3).

Table 3. Kinin release from leukokininogen by trypsin and plasma kallikrein

	Trypsin (ng BK Equiv./mg protein)	Plasma kallikrein (ng BK Equiv./mg protein)
Leukokininogen (fraction 4, from DEAE-Sepharose)	600	88
Leukokininogen (fraction 5, after isoelectric focusing)	100	0

A parallel experiment was also carried out with an equivalent amount of LKGN as above using human plasma kallikrein (15 TAME units, the same TAME esterase activity as that of trypsin). In contrast to trypsin, kallikrein did not release kinin activity from LKGN. However, both trypsin and plasma kallikrein did liberate kinin from a partially purified preparation of leukokininogen, obtained after DEAE-Sepharose chromatography (fraction 4), which would indicate the presence of bradykininogen in the partially purified fraction of leukokininogen.

DISCUSSION

In order to investigate the nature of the leukokininogen molecule, the isolation and purification of leukokininogen from human ascites resulting from ovarian carcinoma were undertaken. The high levels of leukokininogen in these fluids facilitated its purification.

An unexpected result was obtained during the ammonium sulfate fractionation. The total amount of leukokininogen in the 30–70% SAS pellet exceeded that in whole ascites (Table 1). It seems probable, therefore, that during the ammonium sulfate fractionation, an inhibitor of leukokinin formation was removed.

The final purification step, preparative isoelectric focusing, resulted in the isolation of two protein fractions, each of which released a uterus-contracting substance after incubation with the murine leukokinin-forming acid protease. The protein fraction yielding the higher amount of kinin-like activity was further characterized as leukokininogen. It consists of a single polypeptide chain with a molecular weight of 41,000 daltons and has an isoelectric pH of 4.55 to 4.56. The homogeneity and lack of subunits in the preparation were demonstrated by the resolution of only one protein band when analyzed by disc polyacrylamide gel electrophoresis both in the presence and absence of sodium dodecyl sulfate (Figs. 5 and 6). The other protein fraction containing releasable kinin activity will be

Table 4. Physicochemical properties of bradykininogens and leukokininogen

Kininogen	Mol. wt	Isoelectric pH
Leukokininogen (human ascites)	41,000	4.5–4.9
HMW Kininogen * (human)	200,000† 70,000†	4.5–4.7‡
LMW Kininogen (human)	50–60,000†‡	4.9‡
HMW Kininogen (bovine)	76,000§	4.5§
LMW Kininogen (bovine)	48,000§	3.3§

* Refers to bradykininogen.

† Ref. 21.

‡ Refs. 22, 23.

§ Ref. 24.

characterized in future studies in order to determine if a heterogeneity exists for leukokininogen as it does for the bradykininogens.

Based on our current evidence, leukokininogen and the HMW and LMW bradykininogens appear to be different proteins. Leukokininogen is clearly different from HMW kininogen on the basis of its molecular weight and isoelectric pH and by the failure of plasma kallikrein to liberate kinin from leukokininogen. While it is similar to LMW kininogen in terms of the physical parameters measured (Table 4), the amino acid composition of leukokininogen is different from that of human or bovine LMW kininogens (Table 2) and human LMW bradykininogen (Dr. H. Kato, personal communication).

An additional similarity between leukokininogen and LMW kininogen was that trypsin was found to release a rat-uterus contracting peptide from leukokininogen as it does from LMW kininogen. Whether this peptide is an analogue of leukokinin or a biologically active peptide such as bradykinin is currently under study, although the latter possibility seems remote because of the significant differences in the composition of leukokininogen and LWM kininogen.

In studies carried out by Dr. H. Kato of the Protein Institute, Japan, leukokininogen was found not to react with either monospecific anti-human or anti-bovine antisera (personal communication). In our laboratory, using anti-human LMW kininogen antisera, generously supplied to us by Dr. Jack Pierce, National Heart Lung Institute, Bethesda, leukokininogen was unreactive in double diffusion studies at reasonable concentrations (11μg/well). At high concentrations (33μg/well) a precipitin reaction was formed (data not shown). The possible contamination of leukokininogen with LMW kininogen or the presence of antibodies against leukokininogen in the antisera cannot be entirely ruled out, although the purity of leukokininogen was confirmed by Dr. Kato (personal communication).

From all of these data we conclude that, while leukokininogen may possibly have certain antigenic determinants in common with LMW kininogen, leukokininogen represents a unique protein, distinct from any of the bradykininogens so far characterized. This conclusion is consistent with previous data demonstrating differences in amino acid composition of leukokinin and bradykinin, and the mechanisms of their release [9, 25–27]. The observations that both leukokininogen and the leukokinin-forming acid protease are present in neoplastic ascites have led us to postulate that leukokinin formation in neoplastic ascites may result in increased permeability of the vasculature of the peritoneum, thus enhancing ascites accumulation [7]. This theory is supported by the demonstration that pepstatin, an inhibitor of leukokinin formation *in vitro*, dramatically inhibits ascites formation in mice bearing certain ascites tumors [6–9]. While it is presumed that, since the neoplastic cells isolated from the murine ascites are rich in the leukokinin-forming protease, these cells are the source of the enzyme in ascites, the source of leukokininogen in ascites is not clear at this time. Plasma does not normally contain significant levels of leukokininogen but may contain a precursor protein, pro-leukokininogen, which is presumably converted to leukokininogen by an aprotinin-sensitive protease [9]. Studies are now being directed toward the determination of the mechanism responsible for the conversion of a pro-leukokininogen molecule into leukokininogen. The isolation and characterization of human leukokininogen should enhance our understanding of alternate pathways of kinin generation, such as the leukokinin-generating system.

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