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KININ-CONVERTING AMINOPEPTIDASE FROM HUMAN SERUM*

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Abstract—A kinin-converting aminopeptidase from human serum (HuPA) which converts kallidin (lysylbradykinin, LBK) to bradykinin (BK) was purified about 900-fold by a three-step procedure involving ammonium sulfate fractionation, continuous loading Sephadex gel electrophoresis, and gel filtration on Sephadex G-200; the final yield was about 7 per cent. A bioassay was developed in which BK formed in the presence of excess LBK or methionyllysylbradykinin (MLBK), pH 7·8, 37°, was separated on carboxymethylcellulose columns and assayed on the isolated guinea pig ileum. L-Aminoacyl-β-naphthylamidase activities of HuPA measured on L-lysine-, L-arginine- and L-leucylnaphthylamides (Lys-NA, Arg-NA and Leu-NA) were parallel to the kinin-converting activity in all purification steps. The activity of the purest preparation on LBK and MLBK, measured at the approximate enzyme:substrate molar ratio 1:1500, was respectively 267 and 174 nmoles BK/min/mg of protein. Considering the activity on Met-NA as 100, the activities on other naphthylamides were: Leu-NA, 65; Arg-NA, 46; Lys-NA, 29; Asp-NA, 0; Glu-NA, 0. A few tri- and dipeptides, angiotensin II and its amide, polylysine and polyarginine were poorer substrates. The following substances were inhibitors: 1,10-phenanthroline, puromycin, EDTA and 2,3-dimercapto-1-propanol. 1,10-Phenanthroline or EDTA inhibition could be partially reversed by Co^{2+}, Mn^{2+} and Zn^{2+}. The molecular weight was estimated as 95,000 on Sephadex gel filtration. On agarose gel microelectrophoresis, the enzyme migrated as α_1-globulin before the electrophoresis step and had a migration intermediate between α_2- and β-globulin following this step. In this microelectrophoresis, only one band of arylamidase activity was detected on Lys-, Arg- and Leu-NA. The purest preparation was still contaminated with albumin but was free of kininase activity.

THE CONVERSION of kallidin (lysylbradykinin; LBK) to bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) by a human plasma aminopeptidase (HuPA) was simultaneously observed by Webster and Pierce[1] and Erdös *et al.*,[2] and confirmed in horse plasma by Prado *et al.*[3] However, the conversion rate of LBK and methionyllysylbradykinin (MLBK) to bradykinin (BK) by the plasma enzyme has not been measured, probably due to the lack of a quantitative assay and of a purified enzyme preparation. In this paper we report on the development of a purification procedure for the kinin-converting enzyme as well as on its improved bioassay and determined

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the conversion rate of MLBK and LBK to BK. The validity of the assumption made by Berrens[4] that the L-lysyl-β-naphthylamidase of human serum is identical with the kallidin-converting enzyme was tested by following both activities in the purification steps. We have also studied the enzyme action on some peptide substrates, such as a tetradecapeptide containing BK in its sequence, on angiotensin II, and on small peptides. The effect of some inhibitors and ions was also investigated and the molecular weight was estimated to permit a better characterization of the converting enzyme. Partial reports appeared as summaries elsewhere.[5,6]

MATERIALS AND METHODS

Materials

Fresh human pooled serum was obtained by centrifugation from several clotted normal blood samples. Carboxymethyl cellulose, Whatman CM-32, Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden. Bradykinin, lysylbradykinin, methionyllysylbradykinin and 3,4-L-proline-^{14}C-kallidin were obtained through the support of the Radioactive Peptide Synthesis Program of the National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. The radioactive polypeptide was freed from radioactive impurities following carboxymethylcellulose chromatography.[3] Isoleucyl^5-angiotensin-II, bradykinyl-serine, and the tetradecapeptide, Gly-Arg-Met-Lys-Bradykinyl-serine, were products synthesized by the solid phase method by A. C. M. Paiva and M. S. A. Viel in the Department of Biophysics and Physiology of this Medical School. On paper electrophoresis they revealed one main spot and traces of impurities. From the amount of BK liberated from the BK-containing peptides by prolonged incubation with trypsin, their purity was around 90 per cent. Isoleucyl^5-angiotensin-II-amide was the preparation 20'872 in ampules, from Ciba, Basle. Dipeptides and tripeptides were from Cyclo Chemical Corp. and California Corp. for Biochemical Research. Poly-L-arginine-HCl (mol. wt, 55,000) was from Sigma Chemical Co. and poly-L-lysine (mol. wt, 182,000) from Mann Research Laboratories, U.S.A. The aminoacyl-β-naphthylamides of L-lysine, L-arginine and L-leucine were from Cyclo Chemical Co.; those from L-methionine, L-aspartic acid and L-glutamic acid were purchased from Schwarz/Mann, New York; Fast Garnet GBC salt (*O*-amino azotoluene, diazonium salt), practical grade, was a product from Sigma Chemical Co., U.S.A.; puromycin and 2,3-dimercapto-1-propanol (BAL), from Nutritional Biochemicals, and the nonapeptide (BPP) isolated from *B. jararaca* venom by Ferreira *et al.*,[7] possessing probably the following sequence: Pyr-Trp-Pro-Arg-Pro-Gln-Ileu-Pro-Pro,[8] were a gift from C. A. M. Camargo, Department of Pharmacology, Faculty of Medicine, Ribeirão Preto. 1,10-Phenanthroline was obtained from Eastman Kodak Co. and agarose from L'Industrie Biologique Française, S.A., Gennevilliers, France. 1-Dimethylaminonaphthalene-5-sulfonylchloride (DNS) was a product from Calbiochem, U.S.A. Proteins of known molecular weights: horse heart cytochrome *c*, type III, lot 31B-647, mol. wt 12,270, and crystallized ovalbumin, lot 67B-8091, electrophoretic purity approx. 99 per cent, mol. wt 45,000, both were products from Sigma Chemical Co., Missouri, U.S.A. Serum albumin (bovine), twice crystallized, mol. wt 64,500, was obtained from Pentex Biochemicals, Illinois, U.S.A.; γ-globulin (bovine), mol. wt 160,000, was a product from Nutritional Biochemicals Co., U.S.A.

Ammonium sulfate fractionation

To 1 liter of fresh human serum kept at 4°, solid ammonium sulfate was added slowly with stirring up to 0·45 saturation; the precipitate formed in 30 min was separated by 10 min of centrifugation at 22,000 *g* in a refrigerated Sorvall centrifuge. Solid ammonium sulfate was added to the supernatant as before, up to 0·60 saturated; the precipitate formed in 30 min was separated as described above and dissolved in 150 ml of 0·01 M, pH 6·0, Tris-maleate buffer. This solution was dialyzed in the cold room at 4° for 30 hr against the same Tris-maleate buffer and the final volume was 220 ml; 218 ml was concentrated by pressure dialysis (see below) to 45 ml (preparation HuPA-2).

Continuous loading Sephadex gel electrophoresis[9]

The samples were continuously pumped in the electrophoresis chamber (0·25 ml/hr) under a current of 30 mA, 400 V. The elution was continuously made with 0·01 M, pH 6·0, Tris-maleate buffer; 37 fractions of 15 ml volume each were obtained. Up to 1·4 g proteins could be fractionated in one run (about 48 hr total time) with an approximate 30 per cent recovery of enzymatic activity in the peak fractions.

A total volume of 25·5 ml HuPA-2 (in four runs) was purified by this step and concentrated by pressure dialysis to 5·0 ml enzyme solution (HuPA-3).

Sephadex chromatography

A column 55 × 2·1 cm was packed with Sephadex G-200 superfine, washed and equilibrated with 0·025 M, pH 7·8, Tris-HCl buffer containing 0·2 M NaCl. It was initially charged with the enzyme sample (2·5 ml HuPA-3) and eluted with the same buffer in an LBK Ultrorac fraction collector, equipped with a Uvicord II ultraviolet (280 nm) absorptiometer and recorder. The flow was 7·5 ml/hr and the fraction volume was 5 ml.

Kinin standard solutions

Stock solutions (1 mg/ml) of standard BK, LBK and MLBK were made in 1×10^{-3} oxalic acid and kept frozen for periods of about 2 weeks. From these stock solutions, dilute solutions (a few μg/ml) were made daily in boiled 1×10^{-3} M oxalic acid[10] and kept in siliconized tubes in an ice bath during the bioassay. The stock solutions were frequently checked against fresh stocks. In general, our dilute solutions kept well for a working day. It is important to use fresh boiled solutions of oxalic acid.

Kinin-converting assay

In fresh human plasma. An excess of LBK or MLBK (70 nmoles in 0·07 ml oxalic acid, 1×10^{-3} M) was added to a mixture of 0·5 ml plasma without the chelating agent or containing 1×10^{-3} or 3×10^{-3} M 1,10-phenanthroline and Tris-HCl buffer, 0·05 M, 7·8, to make a total volume of 2·0 ml. The mixture was incubated at 37° and at chosen intervals the reaction was stopped by adding it to 4·0 ml ammonium formate buffer, 0·01 M, pH 4·7, in a boiling water bath. After 15 min the coagulated proteins were removed by centrifugation. The clear supernatant was applied to a 0·4 × 15 cm carboxymethyl-cellulose column. After washing the column with 5 ml ammonium formate buffer, 0·04 M, pH 4·7, BK was eluted with 0·09 M, pH 4·7,

ammonium formate buffer, and the nonconverted substrate LBK or MLBK with 0·15 M buffer.[3] Each eluate was assayed on the guinea pig ileum[11] against a standard of the respective peptide. Single and double doses of standard and unknown solutions were given to estimate the unknown activity. Under the conditions described, close to 100 per cent of the added peptides was recovered; this was checked by using radioactive LBK as a tracer in the assay. When BK formed was plotted against time, a straight line was obtained as long as an excess of the substrate was present. In the absence of 1,10-phenanthroline, the initial conversion rate is estimated with less precision; the incubation time was reduced to 2 min to minimize the effect of the well-known plasma kininase activity.[12]

In enzyme preparations. The enzyme aliquot (in less than 0·5 ml) was made up to 2·0 ml with 0·05 M, pH 7·8, Tris-HCl buffer containing the substrate; 1,10-phenanthroline (final concentration 1×10^{-3} M) was added if the enzyme contained kininase. The assay was run as described above for fresh human plasma. The results are expressed as nmoles bradykinin formed at 37°/min/mg of protein.

Rapid semiquantitative kinin-converting assay

This assay is based on the fact that considering the sensitivity of the isolated guinea-pig ileum to BK as 1, its sensitivity to LBK is $1/3 \pm 1/10$ and to MLBK, 1/4 to 1/3.[13] Thus, when LBK or MLBK is completely converted to BK, a 3- to 4-fold increase in the pharmacological activity on the gut is detected; this assay was previously used by Camargo *et al.*[14] The incubates were directly assayed on the ileum without separation of BK in the carboxymethylcellulose column. A semiquantitative estimate of the converting activity was obtained from the incubates between 5 and 15 min, and the presence or absence of kininase in the sample tested may be judged from the activities found in the incubates between 1 and 2 hr of incubation. This assay proved useful to choose enzyme fractions and conditions before the quantitative assay, involving BK separation on carboxymethylcellulose columns, was run. Results for LBK and MLBK values finally given in the tables were obtained with the quantitative method.

Aminoacyl-β-naphthylamidase (arylamidase) assay

The hydrolysis of the β-aminoacylamides used was measured essentially by the method of Goldbarg and Rutenburg.[15] A typical assay mixture contained 200 nmoles substrate and 0·01 to 0·05 ml of enzyme solution and the volume made up to 1·5 ml with 0·9% NaCl; to this mixture 1·5 ml Tris-maleate buffer, 0·01 M, pH 6·0, was added. After a 30-min incubation period at 37°, the reaction was interrupted by adding 1 ml of freshly prepared Garnet reagent (0·1% Fast Garnet GBC salt in 0·2 M, pH 4·2, acetate buffer containing 10%, v/v, of Tween 20), and the red color was read at 525 nm. A standard curve was prepared simultaneously with β-naphthylamine. Results were expressed in nmoles hydrolyzed/min/mg of protein. Effect of pH was measured at every 0·5 unit in the pH range 5 to 8·5. A pH optimum at 6·0 to 6·5 was found for the hydrolysis of Lys-NA. But at pH 7·5 to 8·0, about 80 per cent of the enzyme activity persisted.

Aminopeptidase activity

On polyamino acids. Polyarginine or polylysine (25 nmoles) was incubated with 110 μg protein of HuPA-4 or with 10 μg trypsin for 72 hr in 0·05 M, pH 7·5, Tris-HCl

buffer at 37°. Aliquots were chromatographed on Whatman 3 MM paper developed with *N*-butanol–acetic acid–water–pyridine (30:6:24:20, v/v) and sprayed with ninhydrin. Controls of lysine, arginine, the polyamino acids and the enzymes alone were also run.

On LBK, MLBK and Gly-Arg-Met-Lys-Bradykinyl-Ser. The incubation mixtures at 37° had 86 μg HuPA-3 and 84 nmoles LBK or 76 nmoles MLBK or 250 nmoles of the tetradecapeptide in a total volume of 200 μl completed with 0·05 M, ph 7·5 Tris-HCl buffffer. Aliquots of 20–30 μl were removed between 1 and 2 hr and dansylated.[16,17] Thin-layer chromatograms on plates coated with Silica gel H (20 $\times$ 20 $\times$ 0·025 cm) were developed for about 150 min with 2-methylpropanol–methylacetate–concentrated ammonium hydroxide (90:70:40, v/v), dried at 60° and analyzed under u.v. light. Dansyl derivatives of standard amino acids and peptides were also run.

Rates of hydrolysis of LBK and MLBK were determined as described under kinin-converting assay. One assay is shown in Fig. 1 for preparation HuPA-3.

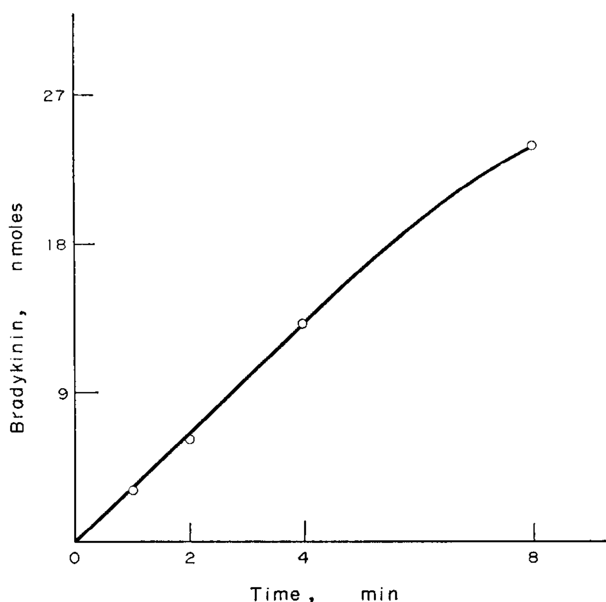

Fig. 1. Conversion of LBK to BK. The incubation mixture (200 μl) at 37° contained 86 μg HuPA-3 and 70 nmoles LBK in 0·05 M, pH 7·8, Tris-HCl buffer. BK formed at the indicated times was separated on carboxymethylcellulose microcolumns and assayed on the isolated guinea-pig ileum.

On angiotensin II and angiotensin-II-amide. To 20 μg (17 nmoles) of isoleucyl^5-angiotensin II or its amide in 1 ml of 0·05 M, pH 7·8, Tris-HCl buffer was added 44 μg HuPA-4 protein (100 μl). The mixture was incubated at 37° and 0·1-ml aliquots (2 μg peptides) were removed at 0, 1, 2, and 16 hr, added to 1·9 ml of boiling Tyrode's solution and boiled for 5 min more. Residual activities were measured on the isolated guinea-pig ileum[18] suspended in atropinized (1 μg/ml) Tyrode's solution.

On small peptides. One μmole of five dipeptides (Gly-Gly; Ala-Gly; Lys-Val; Arg-Val and Leu-Gly) and two tripeptides (Gly-Gly-Gly and Lys-Gly-Gly) in 2 ml of 0·05 M, pH 7·5, phosphate buffer were incubated at 37° with 22 μg protein of the

purified preparation HuPA-4. Aliquots were removed between 0 and 240 min and the color developed with ninhydrin by the method of Matheson and Tattrie.[19] Standard curves obtained from the unhydrolyzed peptides plus known proportions of the corresponding amino acids formed by hydrolysis were used for comparison.

Protein determination

Protein was determined by the method of Lowry *et al.*[20]

Pressure dialysis

Dialysis in Visking tubing (The Scientific Instrument Centre Ltd., London, England) under a pressure of 1·25 kg/cm^2, at 4°, was made against 0·01 M, pH 6·0, Tris-maleate buffer to concentrate dilute enzyme fractions obtained in three steps of the preparation scheme. Reductions of 5–100 times in volume were obtained without loss of proteins and enzymatic activities.

Electrophoresis

The electrophoretic migration was determined by two methods: microelectrophoresis on: (1) agarose gel,[21] and (2) poly-acrylamide-gel disc.[22] In the first method, the agarose gel was prepared on single (2·5 × 7·5 cm) or double (5 × 7·5 cm) microscope slides and 10–100 μg protein was applied to cuts in the cathode side of the gel. A 40–50 mA current, 150–200 V, was applied for about 50 min. After a 20-min fixation step in a solution of ethanol–acetic acid–water (5:1:4, v/v), the slides were covered with filter paper moistened with the same solution and dried under a current of hot air. The remaining film on the slide was stained for 10 min in a 0·1% amido black solution in the fixation fluid and rinsed with the fixation solution to remove the background coloration. In order to locate the enzyme activity, the gel was previously cut into two longitudinal sections following the electrophoretic run; one was used for fixation and staining as described, and the other was cut into several transversal segments, 0·2–0·5 cm long, which were immersed in buffer and assayed for arylamidase activity. It is also possible to freeze the segments overnight, thaw them, and with the fluid separated by centrifugation and which is close to 100% of the gel weight,[23] run the usual arylamidase assay.

The polyacrylamide gel disc electrophoresis was only used to observe the electrophoretic pattern of the purified preparation; 20–100 μg protein in 200 μl sucrose solution was added to the gel top (tubes of 0·7 × 10 cm containing 7·5% polyacrylamide gel). The buffer was 0·05 M, pH 8·6, Tris-glycine and the run was made for 2 hr at 250 V, 4 mA per tube. The staining was done with 0·05% amido black in 7% acetic acid for 40 min and the rinsing with several washings of 7% acetic acid.

Molecular weight estimation

A Sephadex G-200 column (2·1 × 55 cm) was calibrated with the following four proteins of known molecular weights: cytochrome *c* (mol. wt 12,270); ovalbumin (mol. wt 45,000); bovine serum albumin (mol. wt 64,500) and bovine γ-globulin (mol. wt 160,000). When the protein mixture (mg amounts) was eluted with 0·01 M, pH 7·1, phosphate buffer containing 0·1 M NaCl, protein peaks (readings at 280 nm)

were identified by microelectrophoresis of the eluates on agarose gel against the respective protein controls. The enzyme preparation was added to the protein mixture in μg amounts and its elution volume determined from the arylamidase activity. The molecular weight of the arylamidase was estimated by the procedure of Andrews.[24]

RESULTS

Purification. The purification steps and a summary of the results appear in Table 1. Overall purification of the kinin-converting activity, estimated by the method involving BK separation, was 867-fold for LBK conversion, with a 6·5 per cent recovery; and 1208-fold for MLBK, with a 9·1 per cent yield of active material. The kinin-converting specific activities in steps 1 and 2 (Table 1, in parentheses) were obtained

TABLE 1. PURIFICATION OF KININ-CONVERTING AMINOPEPTIDASE FROM HUMAN SERUM*

Steps	Substrate	Specific activity (nmoles hydrolyzed/ min/mg protein)	Purification	Recovery (% of total activity)
HuPA-1				
Human serum	Lys-NA	0·094	1	100
	Arg-NA	0·174	1	100
	Leu-NA	0·275	1	100
	LBK	0·308 (0·056)†	1	100
	MLBK	0·144 (0·028)†	1	100
HuPA-2	Lys-NA	0·62	6·6	50
0·45–0·60 Ammonium	Arg-NA	0·97	5·6	43
sulfate precipitate	Leu-NA	1·52	5·5	42
and pressure	LBK	(0·255)†	4·6‡	35‡
dialysis	MLBK	(0·220)†	7·8‡	59‡
HuPA-3	Lys-NA	13·1	139	12·5
Continuous	Arg-NA	24·3	164	12·5
sephadex	Leu-NA	32·0	116	10·5
electrophoresis	LBK	37·7	122	11·0
	MLBK	30·1	209	19·0
HuPA-4	Lys-NA	93	989	7·4
Sephadex	Arg-NA	170	977	7·4
chromatography	Leu-NA	240	873	6·6
	LBK	267	867	6·5
	MLBK	174	1208	9·1

* Lys-NA, Arg-NA and Leu-NA are the β-naphthylamides; LBK, kallidin (lysylbradykinin); MLBK, methionyllysylbradykinin.

† Values in parentheses were obtained in the presence of 1×10^{-3} M 1,10-phenanthroline.

‡ Calculated considering as 100 the activity obtained in HuPA-1 in the presence of 1×10^{-3} M 1,10-phenanthroline.

in the presence of 1×10^{-3} M 1,10-phenanthroline; it will be shown that this chelating agent inhibits the kinin-converting enzyme, but the values obtained with the same concentration of inhibitor in steps 1 and 2 were used to calculate the purification factor. The specific activities in step 1 obtained in the absence of 1,10-phenanthroline are approximate values measured in a short incubation period (Fig. 2) to minimize the action of plasma kininase. In step 2 this could not be done because the kininase activity of this preparation was about 7-fold higher than in serum. Although step 3 gave a somewhat smaller yield (about 30 per cent) than the other steps (35 to 60 per

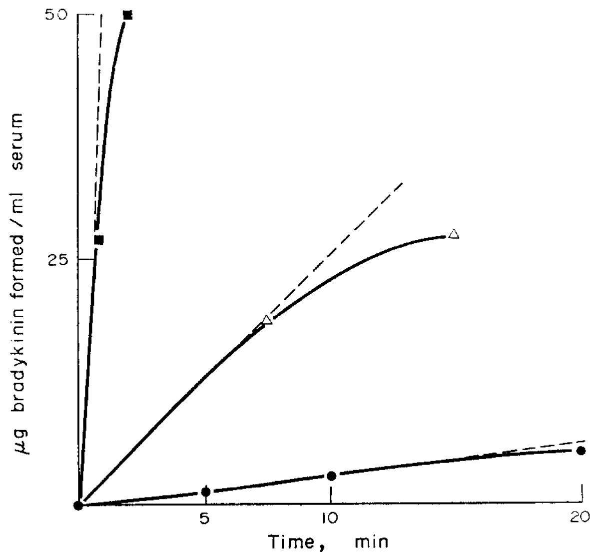

FIG. 2. Action of 1,10-phenanthroline on the converting activity of human serum. The kinin-converting activity of fresh human serum was measured as described in Methods either in the absence (■) or in the presence of 1×10^{-3} M (△) or 3×10^{-3} M (●) 1,10-phenanthroline in the incubation mixture.

cent), the purification in this step (25–30 times) was better than in the other steps (5–7 times). On the whole, the purification factors and yields obtained for the three naphthylamidase activities tested followed closely those obtained with the kinin-converting assays; the only discrepant total purification factor of 1208 for the MLBK-converting activity instead of the general value of about 870–990 arylamidase purification factor is probably explained by errors introduced by the bioassays involved. The purest preparation was free of kininase activity; 11 μg of this preparation incubated for 2 hr at 37° with 30 μg BK in Tris-HCl buffer, 0·05 M, pH 7·8, did not affect the peptide activity on the ileum.

Electrophoretic behavior. The electrophoretic migration on agarose gel of the arylamidase activity in some purification steps is illustrated in Fig. 3. In fresh human serum and following step 2, the enzyme activity migrated as α_1-globulin; after the continuous loading Sephadex gel electrophoresis and the Sephadex column gel filtration, it migrated as an α_2- or even as a β-globulin. We have no explanation yet for this change in migration rate, but it was verified that pressure dialysis alone did not alter the electrophoretic migration. In all microelectrophoresis runs made on agarose gel, only one band of active material toward lysyl-, arginyl- and leucyl-β-naphthylamides was found. In terms of enzyme activity, the recoveries in these microelectrophoresis runs were close to 100 per cent.

Polyacrylamide-gel disc and agarose gel electrophoresis of HuPA-4 revealed an enzyme preparation still contaminated with albumin.

Molecular weight. Results in Fig. 4 show that a molecular weight of 95,000 was extrapolated for human serum arylamidase. Two estimations were made: in the first experiment a mixture containing 500 μg of each protein of known molecular weight and 44 μg of HuPA-4 was used, and in the second filtration the control proteins were

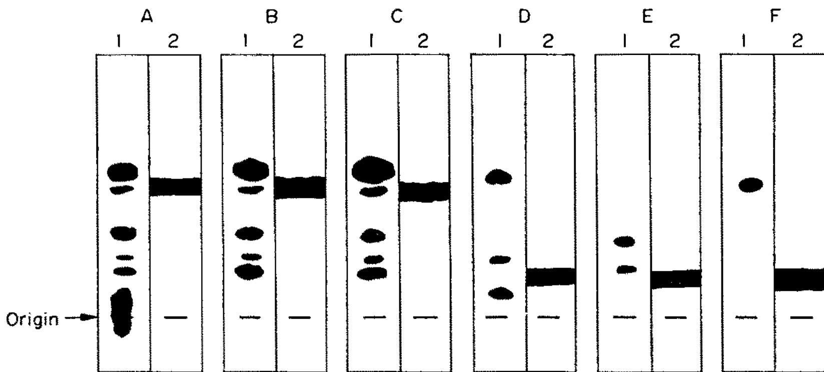

FIG. 3. Scheme of electrophoretic behavior on agarose gel microelectrophoresis of the purification steps of arylamidase activity. Aliquots (2–5 μl) were applied at the origin in two cuts of each slidd The electrophoretic run, protein revelation (side 1) and zymograms (side 2) were made as describee. in Methods. (A) human serum (HuPA-1); (B) and (C) 0·45 to 0·60 ammonium sulphate precipitate before (B) and after (C) pressure dialysis; (D) after continuous loading electrophoresis (HuPA-3); (E) human serum purified directly on Sephadex-gel filtration-electrophoresis; (F) HuPA-4. Arylamidase activity behaved as α_1-globulin in A, B and C; and as α_2- or β-globulin in D, E and F. HuPA-4 is still contaminated with albumin.

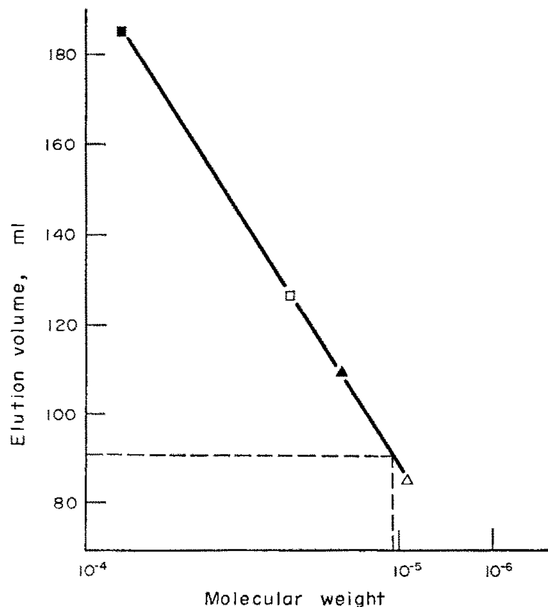

FIG. 4. Estimation of molecular weight of human arylamidase (Lys-NA) by Sephadex G-200 gel filtration. ■, Cytochrome C; □, ovalbumin; ▲, serum albumin; △, gamma globulin. A mixture of these four proteins (500 μg or 10 mg of each) and HuPA-4 (44 μg) was applied to a 2·1 × 55 cm Sephadex column and eluted (2-ml fractions; flow 10 ml/hr) with 0·01 M, pH 7·1, phosphate buffer.

increased to 10 mg each. The two estimations gave identical results. This estimated molecular weight was used to calculate the relative enzyme:substrate molar ratios given in Table 2.

Arylamidase activity. Table 2 gives the hydrolysis rates of HuPA-4 on six β-amino-acylnaphthylamides. With the exception of Asp- and Glu-NA, which were not hydrolyzed, the others were split with different rates. The K_m value was determined for Lys-NA by the method of double-reciprocal plots and found to be $3 \cdot 33 \times 10^{-5}$ M.

TABLE 2. RATES OF HYDROLYSIS OF SEVERAL SUBSTRATES BY PURIFIED HUMAN SERUM KININ-CONVERTING ENZYME (HuPA-4)

Substrates	Enzyme:Substrate molar ratio*	Activity (nmoles/min/mg protein)
Met-NA	1:2,000	371
Leu-NA	1:2,000	240
Arg-NA	1:2,000	170
Lys-NA	1:2,000	93
Asp-NA	1:2,000	0
Glu-NA	1:2,000	0
Arg-Val	1:4,100	75
Lys-Val	1:4,100	48
Ala-Gly	1:4,100	31
Leu-Gly	1:4,100	27
Gly-Gly	1:4,100	11
Gly-Gly-Gly	1:4,100	24
Lys-Gly-Gly	1:4,100	24
LBK	1:1,500	267
MLBK	1:1,500	174
Tetradecapeptide	1:5,400	Present; not measured
Angiotensin II	1:37	1·3
Angiotensin-II-amide	1:37	1·6
Polylysine	1:22	Present; not determined
Polyarginine	1:22	Present; not determined

* Calculated considering the molecular weight as 95,000 and the enzyme preparation as pure.

Aminopeptidase activity. The hydrolysis of the five dipeptides and two tripeptides used was followed and the hydrolysis rates are given in Table 2.

Incubates of HuPA-3 with LBK, MLBK and the tetradecapeptide Gly-Arg-Met-Lys-Bradykinyl-Ser were dansylated. Thin-layer chromatography of the dansyl derivatives showed that lysine was formed from LBK; methionine and lysine from MLBK; and glycine, arginine, methionine, lysine and bradykinyl-serine from the tetradecapeptide. Rates of hydrolysis of LBK and MLBK by preparation HuPA-4, measured from the amount of BK separated on carboxymethylcellulose columns (see Methods), are given in Table 2.

When isoleucyl^5-angiotensin-II and its amide were incubated with HuPA-4 at pH 7·8 (enzyme:substrate molar ratio 1:250) for 2 hr, their activities on the isolated guinea-pig ileum were not affected. It was necessary to increase the E:S ratio to 1:37 to observe slow inactivation. The hydrolysis rates are also given in Table 2.

On polylysine and polyarginine, the action of HuPA-4 was detected by paper chromatography of the incubates, using trypsin as control. Trypsin digestion liberated

intermediary peptides and the amino acids, lysine and arginine, as previously observed.[25] In 72 hr, HuPA-4 formed the free amino acids only, and left some undigested polyamino acid at the origin. Hydrolysis rates were not measured.

Inhibitors. When human serum was pre-incubated for 15 min with 1,10-phenanthroline, its kinin-converting activity both on LBK and MLBK was inhibited, as may be seen in Table 3. This effect was also measured on the arylamidase activity of human serum (Table 3) and gave identical inhibitions. Figure 2 illustrates the phenanthroline effect on kinin conversion by human serum.

Results obtained by pre-incubating preparation HuPA-4 for 15 min with puromycin, L-methionine, BAL and BPP are also shown in Table 3. It may be observed that both the arylamidase and kinin-converting activities were similarly affected and, when

TABLE 3. EFFECT OF SOME SUBSTANCES ON THE ARYLAMIDASE AND KININ-CONVERTING ACTIVITIES OF HUMAN SERUM OR HuPA-4*

Substance	Concn (M)	Inhibition (%)		
		Arylamidase activity†	Kinin-converting activity	
			LBK	MLBK
1,10-Phenanthroline	1×10^{-3}	ND‡	82	81
	3×10^{-3}	93	98	ND
Puromycin	4×10^{-5}	32	35	38
	8×10^{-5}	40	44	46
	$1{\cdot}6 \times 10^{-4}$	57	67	59
L-Methionine	4×10^{-3}	9	ND	ND
	8×10^{-3}	30	Partial§	Partial§
	$1{\cdot}6 \times 10^{-2}$	55	Partial§	Partial§
BAL	4×10^{-4}	35	ND	ND
	2×10^{-3}‖	ND	100	ND
BPP	4×10^{-5}	0	ND	ND
	2×10^{-4}	0	0	0

* Results with 1,10-phenanthroline were obtained with serum; the others, with preparation HuPA-4.
† Measured on Lys-NA.
‡ These assays were not done.
§ Semiquantitative assays.
‖ This BAL concentration interfered with the color development.

measurements were made, in a parallel way. Using one enzyme concentration and two concentrations of Lys-NA (0·4 and 0·8 mM), the effect of three concentrations of puromycin (0·04, 0·08 and 0·16 mM) on the arylamidase activity was measured. The inhibition was of the competitive type and the K_i value for puromycin was calculated by Dixon's method[26] and found to be 7×10^{-5} M. Methionine was less effective; its K_i, also calculated by Dixon's method,[26] was 1×10^{-3} M. The bradykinin-potentiating nonapeptide from *B. jararaca* venom (BPP), had no effect on the arylamidase and kinin-converting activities; during the bioassay, however, its typical potentiating action on BK was evident.

Effect of ions. A solution of 0·22 mg HuPA-4 in 2·0 ml water was dialyzed at 4° against 5×10^{-3} M EDTA (3×1000 ml in 24 hr) and then against glass-distilled water (3×1000 ml in 24 hr). Aliquots of 50 μl of this dialyzed solution and control

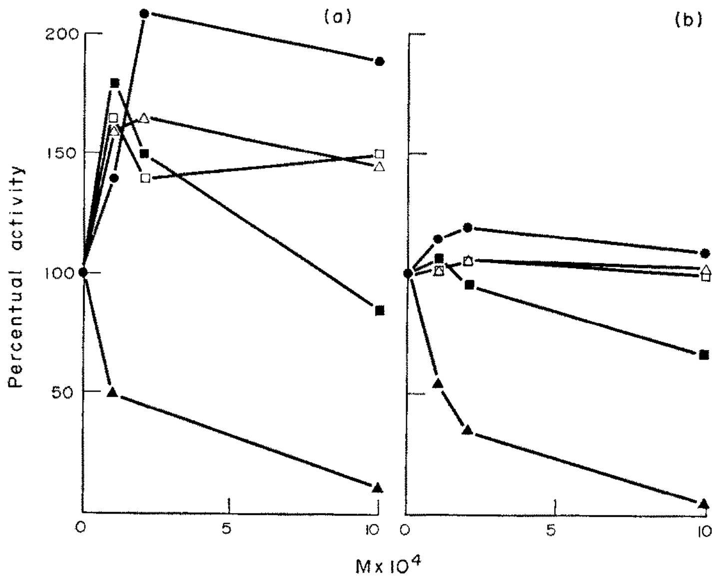

FIG. 5. Effect of ions on arylamidase activity of HuPA-4. Two identical solutions of preparation HuPA-4 were used: one of them (a) was dialyzed against EDTA, 5×10^{-3} M, and glass-distilled water; the other (b) was not dialyzed. They were pre-incubated for 16 hr with 1×10^{-4} M, 2×10^{-4} M and 1×10^{-3} M salt solutions of $CO(No_3)_2$, ●; $MnSO_4$, □; $MgCl_2$, △; $ZnSO_4$, ■; and $CuSO_4$, ▲. Although their activities before pre-incubation with the salts were different (see text), they were taken as 100 and the ion effect is shown as percentual modifications of the respective pre-incubation activities.

aliquots of nondialyzed enzyme were used for assaying arylamidase activity on Lys-NA as described in Methods, but the incubation time was 6 hr. Dialysis alone reduced to 34 per cent the activity of the enzyme. Aliquots of these two enzyme solutions were then pre-incubated with $CO(NO_3)_2$ (R.P. Carlo Erba, Italy); $MnSO_4$ (Pro analysi,

TABLE 4. INHIBITION OF ARYLAMIDASE ACTIVITY BY 1,10-PHENANTHROLINE* AND REVERSAL BY ADDITION OF IONS†

Aliquot	Treatment	Ion (M)	Activity (%)
1	None	0	100
2	None	0	100
3	1,10-Phenanthroline	0	9
4	1,10-Phenanthroline	0	9
5	1,10-Phenanthroline	0	15
6	1,10-Phenanthroline	5×10^{-3} $Co(NO_3)_2$	89
7	1,10-Phenanthroline	5×10^{-3} $ZnSO_4$	38
8	1,10-Phenanthroline	$2{\cdot}5 \times 10^{-3}$ $MnSO_4$	33
9	1,10-Phenanthroline	5×10^{-3} $MnSO_4$	48
10	1,10-Phenanthroline	$2{\cdot}5 \times 10^{-3}$ $MgCl_2$	6
11	1,10-Phenanthroline	5×10^{-3} $MgCl_2$	8

* The enzyme preparation (Sp. act., 800) was inhibited by 1 hr of incubation with 1×10^{-3} M 1,10-phenanthroline.

† Six aliquots of 1,10-phenanthroline-inhibited enzyme were incubated for 16 hr with the ions shown.

E. Merck, Darmstadt); $MgCl_2$ (Baker Analyzed, São Paulo); $ZnSO_4$ (Pro analysi, E. Merck, Darmstadt) and $CuSO_4$ (Baker Analyzed, São Paulo) at three concentrations for 16 hr, and their arylamidase activities determined. Results are shown in Fig. 5. Co^{2+}, Mg^{2+} and Mn^{2+} stimulated less than 20 per cent the activity of the nondialyzed enzyme and more strongly that of the dialyzed arylamidase. Zn^{2+} stimulated at the lower concentrations and inhibited about 20 per cent at the highest concentration. Cu^{2+} was inhibitory in the three concentrations.

One additional experiment was made with a preparation with specific activity 800. Eleven identical aliquots were used. Two were controls and the others were incubated for 1 hr at 37° with 1×10^{-3} M, 1,10-phenanthroline; the arylamidase activity of Lys-NA dropped to an average of 11 per cent (Table 4). Co^{2+}, Zn^{2+} and Mn^{2+} were able to restore partially the activity of the 1,10-phenanthroline-inhibited enzyme; Mg^{2+} had no effect.

DISCUSSION

The kinin-converting activity of human serum which converts LBK or MLBK to BK was purified by a three-step procedure which involved ammonium sulfate fractionation, continuous Sephadex gel electrophoresis[9] and Sephadex column chromatography. The parallelism which was found between the kinin-converting activity and the arylamidase activity on Lys-, Arg- and Leu-NA in the purification steps is compatible with the idea that one enzyme is responsible for all these actions and gives support to Berren's[4] assumption mentioned at the beginning of the present paper. The enzyme migration is intermediate between α_2- and β-globulin on agarose gel following the Sephadex gel electrophoresis, has a molecular weight of about 95,000, was purified about 900-fold by the procedure developed, and behaved like a metallopeptidase. Among the ions tested, Co^{2+} was the most effective to restore the activity of the enzyme previously inhibited by dialysis against EDTA or by addition of 1×10^{-3} M 1,10-phenanthroline. This chelating agent was shown to produce a parallel inhibition of both activities, kinin-converting and arylamidase, an observation which also favors the identity of the two activities. Puromycin, L-methionine and BAL were also inhibitors, but BPP, the nonapeptide isolated from *B. jararaca* venom which potentiates BK,[7] had no effect on either activity.

From a study on the rates of hydrolysis of several peptide substrates, it was observed that the purified enzyme attacks LBK 1·5 times faster than MLBK, when BK was the end product measured. It was shown that methionine and lysine, and not the dipeptide, are removed from MLBK to form BK and it is probable that they are removed sequentially, as was shown to be the case for rabbit brain kinin-converting enzyme;[14] the sequential removal of methionine and lysine would explain the slower formation of BK from MLBK. Tripeptides and dipeptides studied were attacked less efficiently. The hydrolysis rates of the kinin-containing tetradecapeptide, polylysine and polyarginine were not measured; however, glycine, arginine, methionine and lysine were liberated from Gly-Arg-Met-Lys-Bradykinyl-Ser within 1–2 hr of incubation, at an E:S ratio of 1:5400, while polyarginine and polylysine had to be incubated for 72 hr and at an E:S ratio of 1:22 to liberate amino acids. This seems to suggest that the tetradecapeptide is a better substrate than the polyamino acids and may approach LBK and MLBK in its sensitivity. This important point requires further experiments.

The aminoacyl-β-naphthylamides were also hydrolyzed with very different rates; Met-NA, which seems to be a better substrate than LBK, was the best substrate among the naphthylamides; the acidic ones were not attacked. It seems that aspartic acid is not easily removed from the polypeptide substrates because in angiotensin-II (Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe), aspartic acid was hard to split. If aspartic acid were easily removed, inactivation of the peptides would have occurred[27] much faster than observed. Blocking of the free γ-carboxyl group of aspartic acid in angiotensin-II-amide did not alter its resistance, showing that the γ-carboxyl group is not the only hindrance. It is true that at the end of 16 hr the angiotensins were finally inactivated, but it was necessary to increase the E:S ratio from 1:250 to 1:37.

Among the arylamidases, the Co^{2+}-activated and puromycin-sensitive arylamidase of human plasma[28] may be close to HuPA; the order in which it cleaves Met-, Leu-, Arg- and Lys-NA is identical in both enzymes. The rat brain arylamidases described by Marks *et al.*[29] have a relative specific activity towards Leu-, Lys-, Arg- and Met-NA, which differs from that observed in the enzyme that we are studying. Camargo *et al.*[14] described a kinin-converting enzyme from the supernatant fraction of homogenates of frozen rabbit brain. This enzyme has similarities with the serum enzyme here reported; they found a K_i for puromycin ($3{\cdot}5 \times 10^{-5}$ M) similar to that found for the serum aminopeptidase (7×10^{-5} M). But, although their enzyme was only partially purified, the relative hydrolysis rates of Lys-, Arg- and Leu-NA were different for the serum enzyme. The rates of hydrolysis of LBK and MLBK cannot be compared with our results because one does not know the molar enzyme concentration that they used in their experiments. The bovine brain arylamidase, purified 205-fold by Brecher and Suskiw,[30] was completely inhibited by Co^{2+} and Zn^{2+}, while mercaptoethanol was an activating agent and its estimated molecular weight was 70,000. Suskiw and Brecher[31] have also verified that the 600-fold purified bovine brain arylamidase does not hydrolyze dipeptides and tripeptides. We think, in conclusion, that the brain arylamidases probably differ from the aminopeptidase here studied.

Hopsu-Havu *et al.*[32,33] and Mäkinen and Hopsu-Havu[34] purified from rat liver an aminopeptidase B specific for arginine and lysine residues of peptides and naphthylamides. They observed that it was activated by chloride ions and was inhibited by chelating agents, but they found no activating metals. Hopsu-Havu *et al.*[35] claimed that the enzyme splits lysine from LBK, but the LBK-converting activity was not measured. The possibility that the human serum kinin-converting enzyme might be identical with aminopeptidase B but contaminated by an arylamidase active on neutral amino acids seems to be ruled out by the observation here reported that only one band of enzyme active towards lysyl-arginyl- and leucyl-β-naphthylamides was found on agarose gel microelectrophoresis of the purest preparation. The activity ratio Leu-NA/Arg-NA and Leu-NA/Lys-NA in the purification steps of the plasma enzyme remained practically constant; this is taken as further evidence that we are dealing with a single enzyme which splits basic and neutral aminoacyl-β-naphthylamides. The arylamidase purified 2650-fold from human liver by Smith *et al.*[36] is active on neutral aminoacylnaphthylamides and inactive on Glu-NA; the activity of EDTA-inactivated enzyme was also restored by Co^{2+} and Zn^{2+}, while Mg^{2+} was less effective. Its activity on basic naphthylamides was not reported. Experiments are under way to obtain information on the possibility of using human liver as a source of a kinin-converting enzyme.

It has been observed by Webster and Pierce[1] that by adding kallidin to human plasma a part of the peptide was converted into bradykinin in 1·5 min at 24°. Later it was shown that horse plasma was able to transform 18 per cent of kallidin to bradykinin in 5 min in the presence of 3×10^{-3} M 1,10-phenanthroline.[3] We know now that the horse plasma enzyme must have been close to 100 per cent inhibited and consequently, in plasma without any inhibitor, the conversion would be larger. In the present paper, we measured the approximate initial conversion rate of human plasma without inhibitor and found that it amounted to about 28 μg BK formed/ml of plasma/min (0·308 nmoles/min/mg protein) in the presence of excess LBK. We believe that in our previous paper[3] the kinin-converting aminopeptidase activity left after 5 min of pre-incubation with 3×10^{-3} M phenanthroline was sufficient to convert 18 per cent of kallidin to bradykinin.

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