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MECHANISM OF DIGESTION OF BRADYKININ AND LYSYLBRADYKININ (KALLIDIN) IN HUMAN SERUM

ROLE OF CARBOXYPEPTIDASE, ANGIOTENSIN-CONVERTING ENZYME AND DETERMINATION OF FINAL DEGRADATION PRODUCTS*

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Abstract—Degradation of bradykinin and lysylbradykinin was studied in plasma and serum, and the results were compared to those seen with mixtures of carboxypeptidase N and angiotensin-converting enzyme (ACE), the two recognized kininases in blood. Angiotensin-converting enzyme was an effective kininase in mixtures with carboxypeptidase N at physiologic concentration and digested bradykinin to the dipeptides Phe- Arg and Ser-Pro plus the pentapeptide Arg-Pro-Pro-Gly-Phe. Carboxypeptidase N slowly removed the C-terminal Arg from bradykinin to yield des-Arg^9-bradykinin (DBK); the latter was digested by ACE to yield the aforementioned pentapeptide and the tripeptide Ser-Pro-Phe. In serum, however, the C-terminal Arg was removed from bradykinin about five times faster than was accounted for by the activity of carboxypeptidase N. The primary substrate of ACE in serum, therefore, was des-Arg^9-bradykinin and not bradykinin. The products of this reaction, pentapeptide and tripeptide, were unstable in serum and were cleaved by enzymes that have not yet been characterized. One product, free phenylalanine, was used to monitor these reactions by HPLC. Our studies indicate that the final products of bradykinin degradation were the tripeptide Arg-Pro-Pro, one mole each of Ser, Pro, Gly, and Arg, and two moles of phenylalanine. Since the serum level of carboxypeptidase N did not account for the rapid kinin degradation seen, other carboxypeptidases may have been operative, perhaps released as a result of blood clotting, or a serum cofactor may augment carboxypeptidase N activity.

The half-lives of bradykinin and lysylbradykinin in biologic fluids such as plasma are brief due to rapid degradation by kininases. Thus, assessment of the kinin system in human disease has proven to be particularly difficult. In the present study, we determined the pathway(s) of kinin degradation in serum and hope to then develop methods for quantitative assessment of the degradation products.

Two plasma kininases, designated kininases I and II, have been described [1, 2], and these proteins are identical to carboxypeptidase N and (CPN) and angiotensin-converting enzyme (ACE) [3] respectively. Using purified enzymes and synthetic substrates, we confirmed earlier observations that carboxypeptidase N removes the C-terminal arginine from bradykinin to form des-Arg^9-bradykinin, and that angiotensin-converting enzyme sequentially removes the dipeptides Phe-Arg and Ser-Pro to form a transient heptapeptide and then the pentapeptide Arg-Pro-Pro-Gly-Phe [4, 5]. We also have demonstrated that des-Arg^9-bradykinin (the product of carboxypeptidase N cleavage) can be further degraded by angiotensin-converting enzyme to yield Arg-Pro-Pro-Gly-Phe plus Ser-Pro-Phe [6].

Since bradykinin and lysylbradykinin are acted upon by both kininases *in vivo*, we next set out to follow kinin degradation by a mixture of these enzymes at physiologic concentrations. We then assessed kinin degradation in whole serum, so as to identify the final degradation products, and, where possible, to determine the enzyme responsible for each successive cleavage.

MATERIALS AND METHODS

Materials. Bradykinin (BK), des-Arg^9-bradykinin (DBK), Lys-bradykinin (LBK), angiotensin I (AI), angiotensin II (AII) and hippuryl-His-Leu (HHL) were obtained from Sigma (St. Louis, MO); captopril (SQ 14225) was from Squibb (Squibb, NJ). Highly purified rabbit pulmonary angiotensin-converting enzyme (EC 3.4.15.1) having a specific activity of 0.1 units/μg protein (1 unit releases 1 μmol of hippuric acid from hippuryl-His-Leu per min at 37°) was provided by Dr. Richard L. Soffer, Cornell Medical Center, New York, NY. Highly purified human plasma carboxypeptidase N (CPN; EC 3.4.12.7) having a specific activity of 0.14 units/μg protein (1 unit removes 1 μmol of Lys from benz-Ala-Lys per min at 37°) was a gift from Dr. Ervin G. Erdos, University of Texas, Dallas, TX. Bovine pancreatic carboxypeptidase A (EC 3.4.12.2) and di-isopropyl fluorophosphate (DFP)-treated bovine pancreatic

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carboxypeptidase B (CPB; EC 3.4.12.3) were bought from Sigma as well as from Worthington (Malvern, PA). Phe-Arg, Ser-Pro, Gly-Phe, and the pentapeptide Arg-Pro-Pro-Gly-Phe were purchased from Serva Biochemicals (Garden City, NY); the tripeptide Ser-Pro-Phe was prepared in our laboratory as described below. HPLC-grade acetonitrile, EDTA, and all inorganic salts used were purchased from Fisher (Springfield, NJ); trifluoroacetic acid was from Pierce (Rockford, IL); CFA-25 membrane cones were from Amicon (Lexington, MA); and heparin was from Lyphomed Inc., Melrose Park, IL.

Preparation of "physiologic buffer". Blood (30 ml) of a normal subject was drawn into a glass tube and was clotted at room temperature for 30 min. The tube was spun for 15 min at 2000 rpm in a J-6B Beckman Centrifuge set at 4°. About 15 ml serum thus obtained was filtered through CFA-25 membrane cones by spinning for 8 hr at 2600 rpm to yield 7.8 ml of clear aqueous fluid termed physiologic buffer. The pH of the fluid obtained was 8.55 which was then adjusted to 7.4 with 16 μl of 6% H_3PO_4. Bradykinin was stable in this buffer for 24 hr at 37°.

Preparation of Ser-Pro-Phe. Bradykinin (500 μl, 10 μg/μl) was incubated with 3 μl of carboxypeptidase-B DFP (2.2 μg/μl) at 37° for 30 min in the present of 60 μl of Tris–HCl buffer (0.5 M; pH 7.5). HPLC analysis of 1 μl of the reaction mixture demonstrated the complete conversion of the BK into des-Arg^9-bradykinin. After addition of 20 μl of 3 M NaCl, the resultant des-Arg^9-bradykinin was next digested with 35 μl of the angiotensin-converting enzyme (350 ng/μl) at 37° for 1 hr. The analysis of 1 μl of the reaction mixture showed complete degradation of the DBK into the tripepetide Ser-Pro-Phe plus tetrapeptide Arg-Pro-Pro-Gly and free Phe (the latter two products are the result of degradation of the pentapeptide Arg-Pro-Pro-Gly-Phe by the CPB [4, 5] already present in the reaction mixture). Ser-Pro-Phe was separated from the other products by HPLC and lyophilized to complete dryness to give 1.37 mg (88% yield) of the pure peptide. The identity of the material was confirmed by amino acid analysis and gave a single peak on HPLC.

Preparation of serum and plasmas. Human blood (20 ml) was withdrawn by venipuncture from a healthy male into a glass tube and was clotted for 30 min at room temperature. The tube was centrifuged for 15 min at 2000 rpm in a J-6B Beckman model at 4° to give approximately 10 ml of serum. In addition, 10 ml of blood from the same donor was drawn into syringes containing either 100 μl of 4% sodium citrate or 100 μl of heparin (1000 units/ml). These blood samples were also placed in glass tubes for 30 min at room temperature and were centrifuged exactly in the same way as described above to give citrated plasma and heparinized plasma (approx. 5 ml of each). Serum and plasmas were used either directly or stored at −22° for future use.

Digestion in serum of plasma. Ten microliters of BK, LBK and DBK, containing 1 μg/μl of the substrate, were added to 490 μl of plasma or to serum to yield a final concentration of 20 ng/μl. At timed intervals, 50-μl aliquots were added to 500 μl of 3.3 N HCl to precipitate protein, and the mixture was heated at 37° for 10 min. After spinning at 13,700 rpm for 3 min in a 235B Fisher Centrifuge, the supernatant fraction was mixed with 150 μl of 10 N NaOH to neutralize HCl and complete the process of precipitation. The resultant turbid solution was again centrifuged for 3 min and directly loaded onto the HPLC column for analysis. In experiments to follow the degradation of pentapeptide (Arg-Pro-Pro-Gly-Phe) or tripeptide (Ser-Pro-Phe), we utilized a starting solution of 10 μg/μl and otherwise followed the above protocol. This was necessary to distinguish more clearly their degradation products from substances present in serum.

Mixed enzyme digestion. Incubation of BK, with a mixture of ACE plus CPN or ACE plus CPB at 37, occurred in a total volume of 500 μl that contained 100 mM NaCl buffered at pH 7.4 with 3 mM sodium phosphate to approximate the concentration of these salts in human plasma. The final concentration of

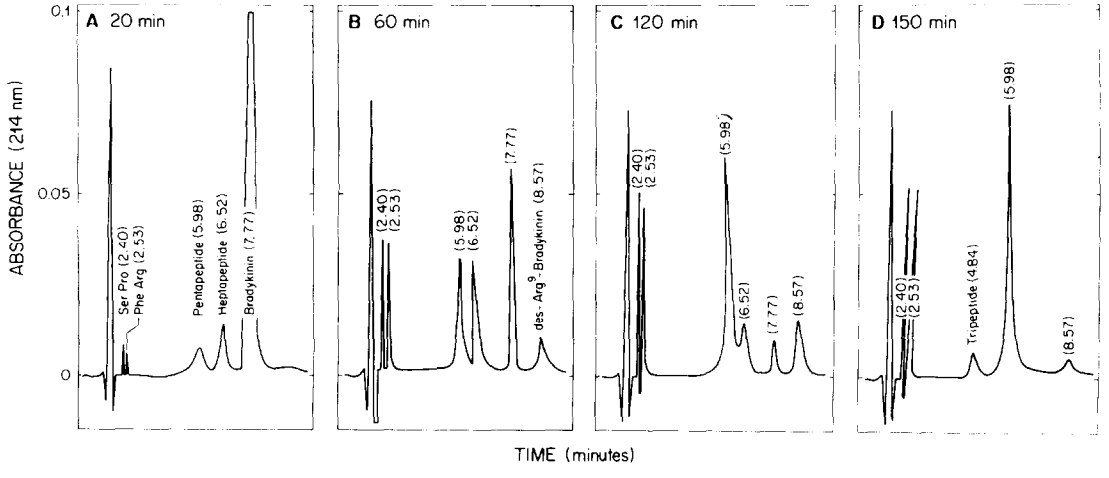

Fig. 1. Elution pattern of bradykinin degradation by a mixture of ACE and CPN as assessed by HPLC. Four time points are shown in panels A–D at 20, 60, 120 and 150 min respectively. Each peak is identified at its initial appearance; retention times are given in parentheses.

the substrate was 0.02 μg/μl (20 μM), while those of ACE, CPN and CPB were 0.4 ng/μl (2.85 nM), 0.04 μg/μl (0.14 μM) and 0.03 ng/μl (0.88 nM) respectively. The concentrations of ACE and CPN were selected to equal that in plasma [7, 8]; the substrate concentrations were chosen so as to yield clear peaks by HPLC. At timed intervals, 50-μl aliquots were quenched with 250 μl of 15 mM HCl, pH 2, and directly loaded onto an HPLC column for analysis.

High performance liquid chromatography. HPLC analyses were performed with a Waters Associate system containing a set of 6000 A pumps, Data Module 730, System controller 720, Automated Injector (Wisp) 710-B, and Extended Wavelength Module 440 Absorbance Detector. Peptide products were applied to a μ-Bondapack C-18 reverse phase column (3.9 mm i.d. × 30 cm) of 10 μm particle size (Waters). The separations were carried out with different linear gradients and isocratic systems of 0.065% (v/v) acetonitrile: trifluoroacetic acid (solvent B) and 0.1% (v/v) trifluoroacetic acid in deionized water (solvent A). For the analysis of BK, LBK, and DBK, and their degradation products, a linear gradient of increasing concentrations of solvent B from 10 to 50% in a span of 10 min was applied with a flow rate of 1.5 ml/min at ambient temperature. The products detected at 214 nm were identified by co-elution with peptide standards, and each peak was confirmed by amino acid analysis. Substrate degradation was calculated by comparing the integrated peak area to the peak area of a known amount of authentic standard.

Amino acid analysis. Amino acid analysis was performed by Dr. Marshal Elzinga, Brookhaven National Laboratories, Upton, NY as follows. The peak obtained by HPLC was lyophilized and exposed to 6 N HCl overnight at 110°. The sample was dried under nitrogen, dissolved in 0.2 M sodium citrate buffer, pH 2.0, and loaded into the column. Separation and identification were performed utilizing the methods and instrumentation described by Hewick *et al.* [9].

RESULTS

Since bradykinin is degraded by a mixture of kininases, we first incubated bradykinin with a mixture of ACE and CPN at physiologic concentrations (0.4 μg/μl and 40 ng/ml respectively) in phosphate-buffered saline (PBS) and monitored the digestion by HPLC (Fig. 1). Bradykinin eluted at 7.77 min. At 20 min (Fig. 1A) four additional peaks were seen and identified as heptapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro), pentapeptide (Arg-Pro-Pro-Gly-Phe), and the dipeptides Ser-Pro and Phe-Arg. These are consistent with the digestion of bradykinin by ACE. Des-Arg^9-bradykinin (DBK), the product of bradykinin digestion by CPN, was not observed at this stage. As the digestion progressed, these products accumulated further and by 60 min only a small quantity of DBK was seen (Fig. 1B). By 120 min the peaks of bradykinin and heptapeptide were diminished while those of Ser-Pro, Phe-Arg, and pentapeptide increased, indicating further digestion by ACE; the DBK peak increased but only minimally.

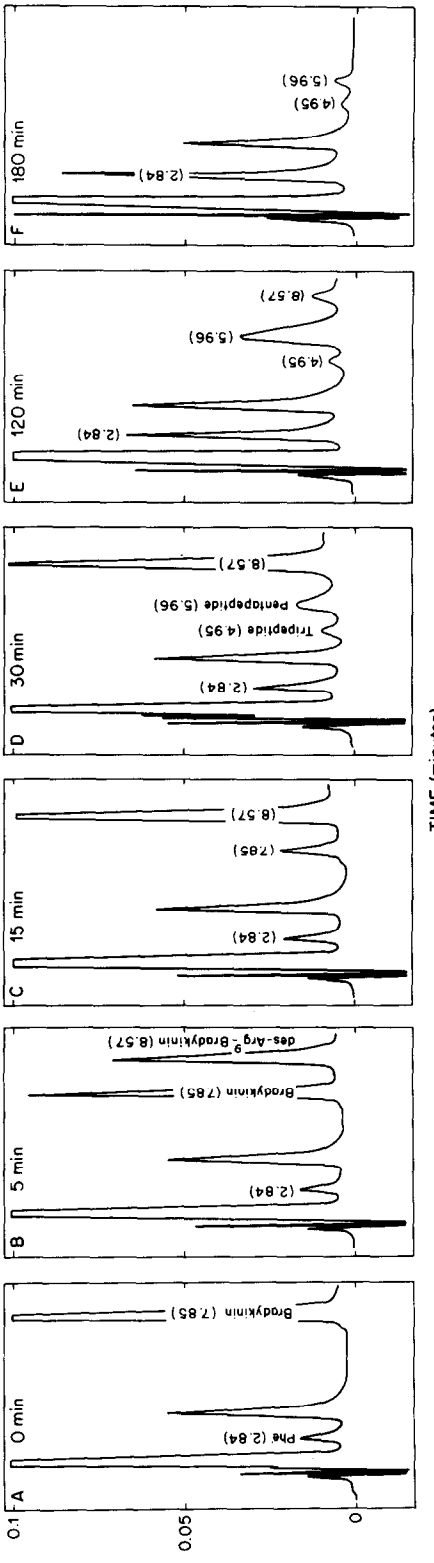

Fig. 2. Elution pattern of the degradation of bradykinin in human serum. Time points at 0, 5, 15, 30, 120 and 180 min are shown in panels A–F. Each peak is identified at its initial appearance, and retention times are shown in parentheses.

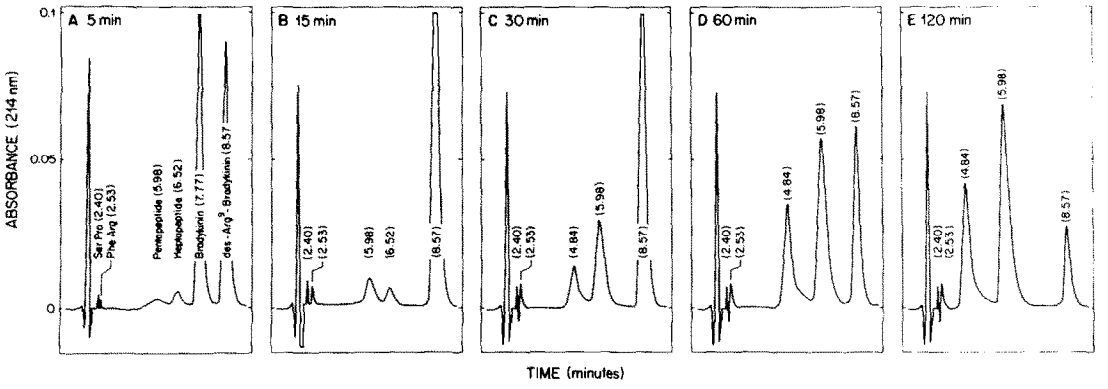

Fig. 3. Elution pattern of the degradation of BK by a mixture of ACE and CPB as assessed by HPLC. Time points at 5, 15, 30, 60 and 120 min are shown in panels A–E.

Panel D of Fig. 1 shows complete conversion of BK into Ser-Pro, Phe-Arg, and Arg-Pro-Pro-Gly-Phe; a new peak eluting at 4.84 min was Ser-Pro-Phe (tripeptide), a product of DBK digestion by ACE [6]. ACE, therefore, appeared to be the dominant kininase when BK was digested by a mixture of purified kininases at physiologic proportions.

Next we compared this pattern of degradation with that seen in whole human serum (Fig. 2, A–F). Panel 2A shows HPLC peaks of serum components plus added bradykinin. Among the serum peaks relevant to our data, there was a small peak identified as free phenylalanine (confirmed by amino acid analysis) at 2.84 min which was present in all the samples assayed. By 5 min approximately 50% of BK was converted to DBK, and by 15 min conversion was over 90% (panels 2B and 2C). There was no evidence of BK digestion by ACE-like activity; thus, the result appeared to be the reverse of that seen with a mixture of these purified enzymes in buffer (Fig. 1). There was no evidence of DBK digestion by ACE within the first 15 min. However, by 30 min (Fig. 2D), BK was digested completely and the known products of DBK digestion by ACE were seen, i.e. Arg-Pro-Pro-Gly-Phe plus Ser-Pro-Phe. This observation confirms earlier findings [8] that DBK cannot be digested by ACE in the presence of BK but can proceed once all the BK is converted. The presence of Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe in serum was transient, and they did not accumulate although tripeptide was digested more rapidly than pentapeptide. We also noted an increase in the level of free Phe between 15 and 30 min. Since no heptapeptide was produced, removal of the C-terminal Phe from DBK seems unlikely; rather Phe can be formed by removal of the C-terminus from either pentapeptide, tripeptide, or both. By 120 min (panel 2E), almost complete depletion of DBK was seen. After 180 min, Phe continued to increase and only traces of pentapeptide and tripeptide remained. This experiment was repeated ten times with sera from different normal volunteers, and in each case the same qualitative result was obtained. A serum control containing no added bradykinin gave no increase in Phe during a 24-hr incubation.

Among the differences between serum and PBS which might have contributed to the differing results obtained when Fig. 1 and Fig. 2 are compared is the presence of many additional ions in serum, including a variety of trace metals. We therefore obtained a "physiologic buffer" prepared by filtration of serum through CF-25A Amicon membranes as described in Materials and Methods. We then digested bradykinin in this buffer using a mixture of purified CPN

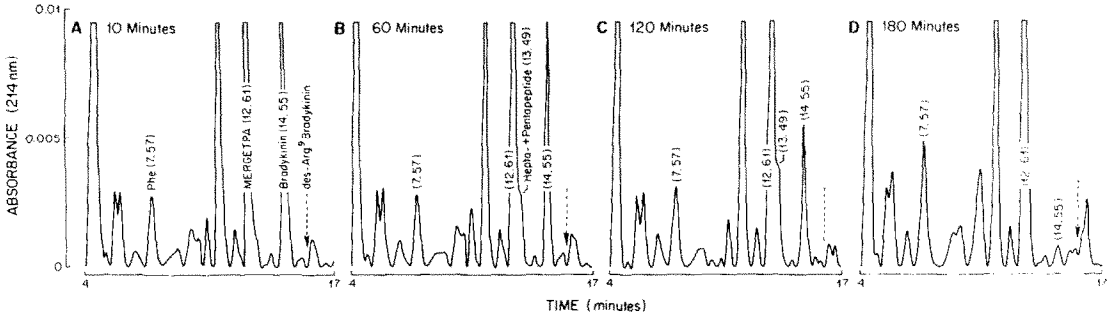

Fig. 4. Elution patterns of the degradation of bradykinin in human serum in the presence of the carboxypeptidase inhibitor MERGETPA. Time points at 10, 60, 120, and 180 min are shown in panels A–D. The MERGETPA peak is identified as are the positions of Phe and bradykinin. Any heptapeptide and/or pentapeptide formed overlapped the MERGETPA peak. An arrow shows the expected time of des-Arg^9-bradykinin elution.

and ACE in the proportions present in plasma. The results were indistinguishable from the kinetics and products of BK digestion seen in Fig. 1 in which ACE was the dominant kininase. We then altered the ratio of CPN/ACE to attempt to reproduce the results seen in serum. At 200 ng/μl CPN (i.e. a 5-fold increase above its concentration in serum), the conversion of BK to DBK was complete by 15 min. Since carboxypeptidase B removes the C-terminal Arg of bradykinin far more readily than CPN [10, 11], we substituted purified CPB for CPN in the aforementioned experiment. As little as 0.03 ng/μl CPB converted bradykinin into DBK in 15 min (Fig. 3). Heptapeptide, indicative of digestion of bradykinin by ACE, was seen at 5 and 15 min and was then converted to pentapeptide. Trace quantities of the dipeptides Ser-Pro and Phe-Arg were seen throughout but did not accumulate. Rather, the major final products were pentapeptide (5.98 min) and tripeptide (4.84 min), indicative of DBK digestion by ACE.

To investigate this observation further, we next digested bradykinin in serum in the presence of a 1 mM concentration of the carboxypeptidase inhibitor MERGETPA (D-L-mercaptomethyl β-guanidine-ethylthioproprionic acid); the results are shown in Fig. 4. At the start, bradykinin was seen at 14.5 min, and the added MERGETPA (determined separately) eluted at 12.6 min. The large peak preceding the MERGETPA peak was a serum component which remained constant throughout. With time, the bradykinin peak gradually diminished, and no significant accumulation of des-Arg^9-bradykinin (expected at 15.7 min, dotted arrow) was seen. A peak that overlapped the MERGETPA peak (shoulder along the downstroke) increased within the first 2 hr of incubation (Fig. 4, A–C) and then disappeared by 180 min (Fig. 4D); this represents heptapeptide and/or pentapeptide which were not resolved from one another or the MERGETPA. The peak of free phenylalanine progressively increased between 1 and 3 hr. Thus, des-Arg^9-bradykinin was not formed in the absence of carboxypeptidase activity but slow degradation of bradykinin proceeded nevertheless.

Digestion of bradykinin in serum differed from any of the aforementioned enzyme/substrate mixtures because Phe accumulated and both pentapeptide and tripeptide were digested further. To characterize these latter steps, a large excess of pentapeptide was added to serum, the vertical scale was increased to more easily discern small peaks, and its digestion was monitored by HPLC to better resolve its degradation products from the peaks seen in serum. As can be seen in Fig. 5A (zero time), the pentapeptide peak was identified and the position of Phe is indicated. By 60 min and 90 min, Phe had increased and a new peak eluting at 7.31 min was seen. Both peaks accumulated further over the 3-hr time course shown. The amino acid composition of the 7.31-min peak was Arg-Pro-Pro, and we have corroborated this elution position by addition of an excess of Arg-Pro-Pro to normal serum. No digestion of Arg-Pro-Pro was seen after incubation for 24 hr. The accumulation of Phe may have occurred by removal of the C-terminal Phe from pentapeptide to leave the

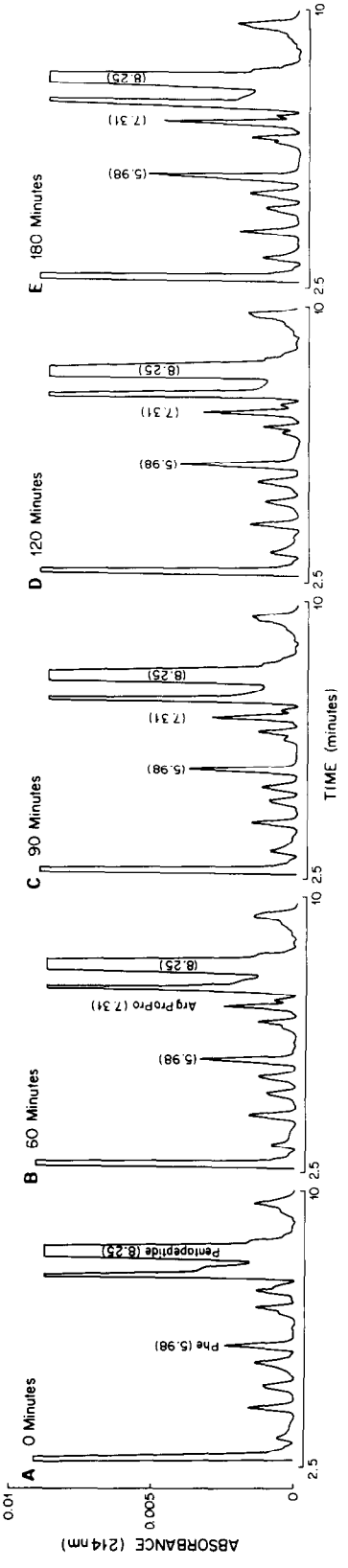

Fig. 5. Elution pattern of the degradation of pentapeptide in serum as assessed at 0, 60, 90, 120, and 180 min. A large excess was added so that depletion was not evident at the sensitivity utilized. However, a gradual increase in Phe plus formation of the tripeptide Arg-Pro-Pro was seen.

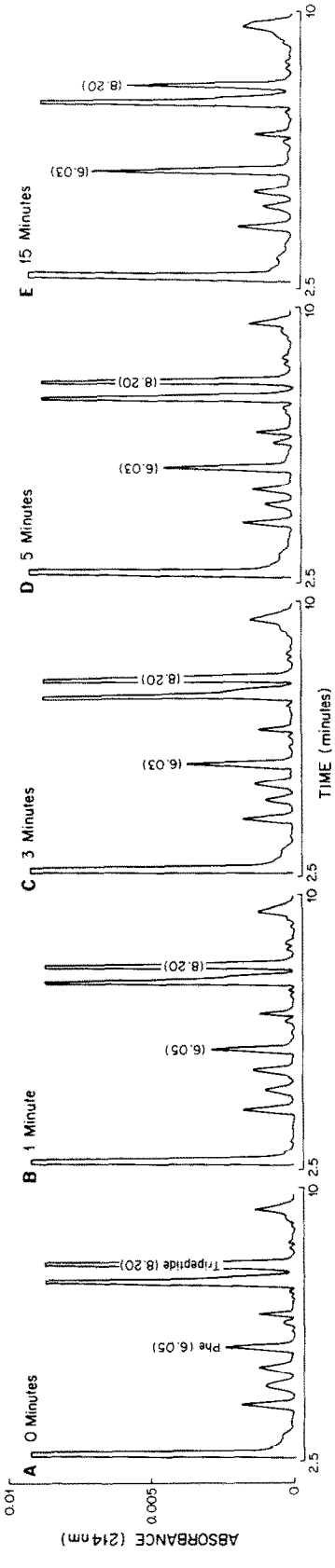

Fig. 6. Elution pattern of the degradation of tripeptide in serum as assessed at 0, 1, 3, 5, and 15 min. Only significant depletion of tripeptide by the 15-min point and accumulation of free phenylalanine was noted.

tetrapeptide Arg-Pro-Pro-Gly followed by cleavage of the C-terminal Gly. Alternatively, pentapeptide may have been cleaved to Arg-Pro-Pro plus the dipeptide Gly-Phe. The latter dipeptide would then be cleaved to Gly plus Phe. We have not distinguished these possibilities; however, in separate experiments in which an excess of Gly-Phe was added to serum, there was rapid digestion with release of Phe. Thus the final degradation of pentapeptide yielded Arg-Pro-Pro, Gly, and Phe.

Tripeptide digestion in serum was far more rapid than pentapeptide digestion, as suggested by the result shown in Fig. 2. We next studied tripeptide digestion separately (Fig. 6). One hundred nanograms of tripeptide was added to serum, and its rate of digestion was monitored over a 15-min period. As tripeptide was depleted, Phe accumulated. Ser-Pro, if stable, eluted with the first plasma protein peak and therefore was obscured. However, by addition of a large excess of Ser-Pro (1–10 μg/ml) so that the plasma contribution to the peak seen was small, we were able to demonstrate a rapid decline of the peak with time (data not shown). Thus, Ser-Pro-Phe appeared to be digested to free amino acids.

Since tissue kallikrein digests kininogen to release lysylbradykinin, we also studied the course of its digestion when added to serum. As shown in Fig. 7A (zero time point), LBK eluted at 7.34 min. By 1 min (Fig. 7B), the LBK was partially converted to bradykinin, des-Arg^{10}-LBK and des-Arg^{9}-BK although conversion to bradykinin predominated. Thus, an aminopeptidase rapidly removed the amino terminal lysine from LBK to convert it to BK, and both LBK and BK were degraded by carboxypeptidase to form the respective des-Arg derivatives. By 2.5 min (Fig. 7C) there was continued depletion of LBK and des-Arg^{10}-LBK with further conversion to BK and DBK. By 5 min (Fig. 7D), only BK and DBK were seen and the digestion then continued to convert BK to DBK followed by the products of ACE digestion and the late acting enzyme(s).

DISCUSSION

Most prior studies have examined bradykinin degradation either by carboxypeptidase N or ACE; however, there were no reports of kinin degradation by a mixture of these enzymes and there are only two prior reports regarding kinin degradation in plasma or serum [12, 13]. The latter workers used 16–20% plasma to monitor bradykinin degradation and, although depletion of kinin was followed, the nature of the degradation products was not determined.

When we studied a mixture of ACE and CPN at their respective serum concentrations, ACE appeared to be dominant. Ryan *et al.* [14] have emphasized the predominance of ACE in studies of cellular or tissue kininases and have questioned whether CPN is a physiologically effective kininase. Using a purified mixture of these enzymes, the same conclusion might be warranted. However, Hiraga *et al.* [13] and Marceau *et al.* [12] have reported that Arg removal occurs first when bradykinin is incubated in human serum. We found that the C-terminal arginine was indeed removed first to form des-Arg^{9}-

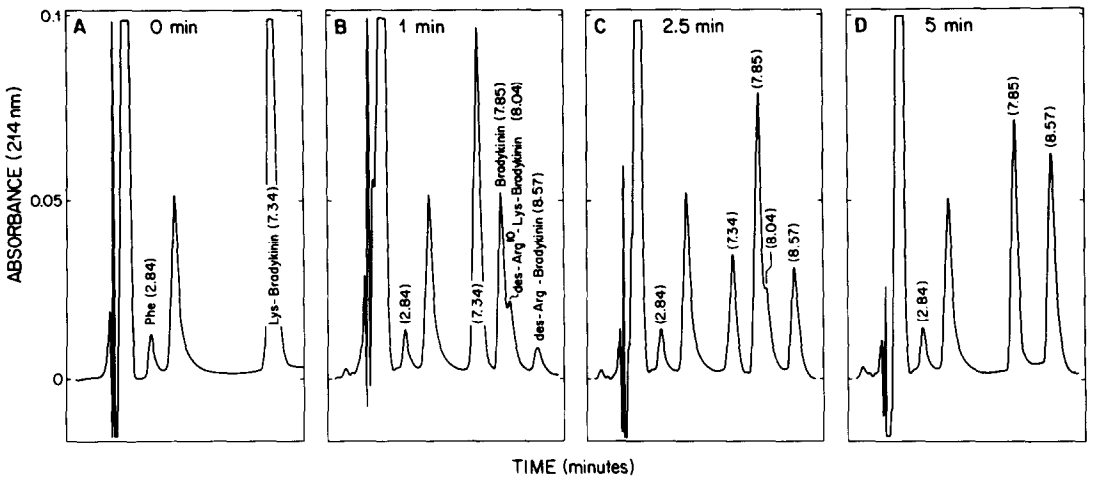

Fig. 7. Elution pattern of lysylbradykinin degradation in human serum as monitored by HPLC at time points of 0, 1, 2.5 and 5 min (A–D respectively). Each peak is identified, and its retention time is given in parentheses the first time it appears; only the retention time is given in the subsequent panels.

BK and that des-Arg^9-BK was the substrate for ACE. We also confirmed prior findings regarding the tripeptidase activity of ACE (Inokuchi and Nagamatsu [15]; Sheikh and Kaplan [11]) whose work has been extended recently to other peptides [16]. Thus, one striking difference seen when the purified enzyme system was compared to serum was the augmented carboxypeptidase activity in serum. Close to five times the normal concentration of CPN was needed to approximate this activity; alternatively, about 1% carboxypeptidase B accomplished the same thing. We therefore propose that serum may contain small quantities of a carboxypeptidase B-like enzyme or it may contain a co-factor which augments the activity of CPN.

Hiraga *et al.* [13] did not detect Ser-Pro-Phe when studying BK digestion in serum but they did observe a peak which they "presumed" to be Arg-Pro-Pro-Gly-Phe (pentapeptide). An identification based on an amino acid composition was not made. Marceau *et al.* [12] reported the serum degradation of DBK into the heptapeptide Arg-Pro-Pro-Gly-Phe-Ser-Pro plus Phe. We cannot confirm this latter observation and instead found that DBK was acted upon by ACE to give pentapeptide and tripeptide, each of which was confirmed by amino acid analysis and coelution with standard peptides. Both pentapeptide and tripeptide were further digested by one or more enzymes that are not yet characterized. These clearly are different from CPN or ACE and possess activities resembling carboxypeptidase A and/or prolylcarboxypeptidase or perhaps plasma neutral metalloendopeptidase [17]. The final degradation products of bradykinin digestion were one mole each of Arg-Pro-Pro, Gly, Ser, Pro, Arg and two moles of Phe.

The N-terminal tripeptide, Arg-Pro-Pro, may be suitable for development of an assay for late kinin-degradation products. Such an assay could be used in conjunction with a radioimmunoassay for bradykinin [18] and a recently developed radioimmunoassay for des-Arg^9-BK [19] for study of kinin metabolism in human disease. However, further work would be required, perhaps in animal models, to assess the *in vivo* stability of this degradation product and to reevaluate kinin degradation through parenchyma such as the lung. Further studies are in progress to identify more specifically the carboxypeptidase activity in serum and to isolate the enzyme(s) responsible for the final cleavage steps.

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