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STUDIES OF THE PEPTIC DEGRADATION OF BOVINE BRADYKININOGEN*

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Abstract—Investigations into the manner in which bradykinin is linked in the bradykininogen molecule has led to the degradation of bovine bradykininogen by the enzyme pepsin. The degradative products include dialyzable polypeptides which contain bradykinin of bradykinin-like peptides as part of their structure. Partial purification of these peptides has been accomplished by Dowex 1-X2 chromatography. Purification has been aided by the use of *p*-toluenesulfonic acid, an agent found to protect bradykinin and bradykinin-like peptides during concentration procedures.

IT IS well established through the efforts of Rocha e Silva[1] that trypsin releases the nonapeptide bradykinin from kininogen(s) in the blood. The structure of bradykinin has been elucidated by Elliott *et al.*[2] and by Boissonas *et al.*[3] Webster and Pierce[4] demonstrated that human plasma kallikreins also release bradykinin from acid-treated plasma, whereas urinary and glandular kallikreins release from kininogen(s) the decapeptide lysylbradykinin. The isolation of methionyl-lysylbradykinin by Elliott *et al.* from autolysates of plasma,[5] in addition to the above findings, lends support to the idea that urinary and pancreatic kallikreins cleave a methionyl-lysyl bond in the kininogen molecule to produce lysylbradykinin, while trypsin and plasma kallikrein cleave a lysylarginine in the kininogen to release bradykinin. This is illustrated in Fig. 1. It is not known, however, if any bonds need be cleaved by these

FIG. 1. Probable action of urinary and glandular kallikrein (k) and trypsin and plasma kallikrein (tr) on bradykininogen to release lysylbradykinin and bradykinin respectively; ? refers to the unknown sequence of amino acids, if any, linked to the carboxyl-terminal arginine of bradykinin in the bradykininogen molecule.

enzymes at the carboxyl-terminal moiety of bradykinin to release the active peptide from the protein, since it is not known whether bradykinin is the carboxyl-terminal

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peptide of the kininogen or is covalently linked at its carboxyl-terminal arginine group. Such knowledge is of importance in understanding the mechanism by which kallikrein or other enzymes release the kinins from the kininogen molecule.

Investigations have been designed in this laboratory to find a procedure to degrade bradykininogen into small molecular weight polypeptides which contain the bradykinin moiety intact and in covalent linkage. The purpose of the experiments was to isolate eventually such polypeptides and, by analysis of their structure, elucidate various aspects of bradykinin linkage and enzymatic release from the kininogen molecule. In the current communication it is demonstrated that pepsin degrades bovine bradykininogen into low molecular weight (dialyzable) peptides, one or more of which contain kinins in covalent linkage. Various pharmacological properties of these peptides and experiments concerning their purification are reported; preliminary accounts of some of them have been given.[6] In addition, experiments demonstrating the protective action of *p*-toluenesulfonic acid and oxalic acid on bradykinin are reported.

MATERIAL AND METHODS

Bovine bradykininogen was prepared by the methods previously described.[7] The guinea pig ileum assays were also carried out, as previously described, with Tyrode's solution containing 1 μg diphenhydramine/ml and 1 μg atropine sulfate/ml used as the muscle-bathing medium. Synthetic bradykinin was the generous gift of Parke, Davis Ltd. and The Sandoz Pharmaceutical Co., N.J. Blood pressure assays were carried out on rabbits which had been anesthetized with pentobarbital. The pressure was recorded from the common carotid artery with a Statham strain gauge in conjunction with a Gilson polygraph. Pepsin and trypsin were the twice and thrice-crystallized preparations, respectively, of the Worthington Biochemical Co., Freehold, N.J. Trypsin was dialyzed free from $MgSO_4$ before it was used.[8] *p*-Toluenesulfonic acid was obtained as the reagent grade from the Fisher Scientific Corp., N.Y. Tubing for the dialysis experiments was the 8/32 casing obtained from the Visking Co., Chicago, Ill. It was placed in 50% acetic acid for 12 hr before use and washed thoroughly with tap water and distilled water. This treatment was necessary to remove material which leached from the casing during the experiment and affected the assays.

Protein and peptide concentrations were determined by absorption at 280 mμ or by the method of Lowry *et al.*[9] with the use of the automatic sampler and recorder of a Technicon autoanalyzer. Measurements were done at 660 mμ. Sephadex G-200 was the product of the Pharmacia Corp., Uppsala, Sweden.

Column chromatography with Dowex-1X2 (200–400 mesh) was carried out by the procedures of Schroeder *et al.*[10] with pyridine-acetate at pH 8·3 used as the equilibrating buffer and the initial eluting buffer. The resin was cycled in acid and alkali as described by the authors before pouring. Pouring of the column was carried out by having the resin settle under pressure of the buffer dripping from a height of 24 inches above the column. The flow rate during the actual chromatography was 20 ml/hr; the size of the column was 0·9 cm $\times$ 100 cm. The tubes were analyzed by the Lowry procedure and those tubes contributing to each peak were pooled, concentrated to dryness by rotary evaporation at 37°, and taken up in 2·0 ml of water.

RESULTS

Peptic degradation of bovine bradykininogen

Degradation of bradykininogen was attempted with the aid of the enzyme pepsin. The specificity of pepsin favored its use in these studies, since bradykininogen is acidic in nature[7] (pepsin has a high affinity for peptide bonds of acidic amino acids); in addition pepsin does not hydrolyze synthetic bradykinin. Thus the enzyme should degrade the protein molecule into low molecular weight polypeptides without destroying the bradykinin linked in these peptides. Indeed, the bradykinin moiety contained in the peptides was used to follow them during their purification. This was possible, since the bradykinin could be released from the peptide by treatment with trypsin. It was also found that some of the peptides could be detected by direct assay without prior trypsin treatment. The demonstration that pepsin degraded bradykininogen into smaller molecular weight material was carried out as follows.

Ten mg of bradykininogen was dissolved in 1·0 ml of 0·01 N HCl (final pH 3·0). To 0·5 ml of this solution 0·1 mg of pepsin in 0·1 ml of 0·01 N HCl was added. The remaining 0·5 ml served as the control or "untreated solution." Both solutions were allowed to stand for 12 hr at 22°; 0·5 ml of 0·1 M Tris buffer, pH 8·0, was then added to each sample and the samples chromatographed on 0·9 cm × 30 cm columns of Sephadex G-200, with 0·05 M Tris buffer, pH 8·0, as the eluant. The results are seen in Fig. 2 and demonstrate that the "untreated" sample of bradykininogen was eluted

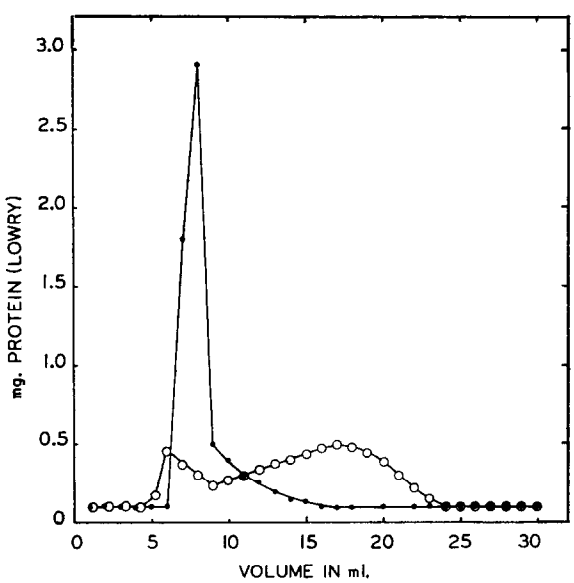

FIG. 2. Chromatography of native bradykininogen (●-●) and pepsin-treated bradykininogen (○-○) on Sephadex G-200 as described in the text. Flow rate was 5 ml/hr.

immediately after the void volume (8 ml) of the column as a sharp peak. On the other hand, the pepsin-treated sample chromatographed as a broad peak, the maximal absorption being eluted at twice the void volume. This indicated that pepsin hydrolyzed bradykininogen into lower molecular weight material. In other experiments it

was demonstrated that the pepsin-treated bradykininogen released as much pharmacologically active material after incubation with trypsin as did native bradykininogen. It was thus demonstrated that although pepsin degrades bradykininogen into lower molecular weight materials it leaves the bradykinin moiety of the peptides intact. Activity was also noted upon the direct assay of the pepsin-treated bradykininogen as seen in the experiments presented below.

Dialysis experiments

The general size of the peptides produced by pepsin and which retained the bradykinin moiety as part of the structure was investigated by dialysis. A peptic digest of bradykininogen was prepared as follows: 40 mg bradykininogen and 0·4 mg pepsin were incubated in a volume of 2·0 ml at pH 3·0 for 12 hr at 22° in the presence of a few drops of toluene. The solution was then brought to pH 5·3 (the isoelectric point of bovine bradykininogen) and centrifuged at 10,000 *g* for 10 min to remove any undigested bradykininogen. The supernatant solution was dialyzed against 25 ml of distilled water for 6 hr. The dialysate was then concentrated *in vacuo* at 40° to 2·0 ml. A sample was assayed directly on the guinea pig ileum. A second 1·0-ml sample was brought to pH 7·5 and was incubated with 0·25 mg trypsin for 30 min at 37°. The reaction was stopped with 0·5 mg soybean inhibitor, and an aliquot was assayed for activity. An exact duplicate of the above experiment was carried out with bradykininogen that was not treated with pepsin (untreated bradykininogen). The results of these experiments are seen in Table 1. It may be noted that pepsin liberated from

TABLE 1. GUINEA PIG ILEUM ASSAY OF DIALYZABLE FRAGMENTS PRODUCED BY PEPTIC ACTION ON BRADYKININOGEN

Solution	Activity in terms of bradykinin (μg/ml concentrate)	Optical density (280 mμ)
Pepsin-treated bradykininogen		
Dialysate	0·25	3·40
Dialysate + trypsin	4·00*	
Untreated bradykininogen		
Dialysate	0·00	0·50†
Dialysate + trypsin	0·00	

* Represents essentially 100% of the activity released by direct action of trypsin on bradykininogen. In larger-scale experiments 20–50% of the activity produced by trypsin action on bradykininogen is obtained (see text).

† Despite routine 12-hr treatment of dialysis sacks with 50% acetic acid before use, a small amount of material absorbing at 280 mμ was always liberated into solution from the sacks. A much larger quantity is liberated if the acetic acid treatment is not employed.

bradykininogen dialyzable polypeptides which release pharmacologically active material after treatment with trypsin. A small but significant amount of activity was also found by direct assay of the peptic-produced dialyzable peptides It may also be seen that the bradykininogen solution that was not treated with pepsin did not liberate any dialyzable material that could be activated. Pepsin controls (not shown here) also liberated no active material. The results of the above experiments are in keeping with the idea that pepsin liberates from bradykininogen dialyzable polypeptides that contain bradykinin or bradykinin-like peptides as part of their structure.

Purification of the peptides

The dialyzable peptides that were produced by pepsin digestion of bradykininogen were purified by column chromatography on the basic resin Dowex-1X2 as described in Materials and Methods.* In these studies the chromatography of the dialysate was carried out at pH 8·3, followed by acidic eluting agents as shown in Fig. 3. As can be

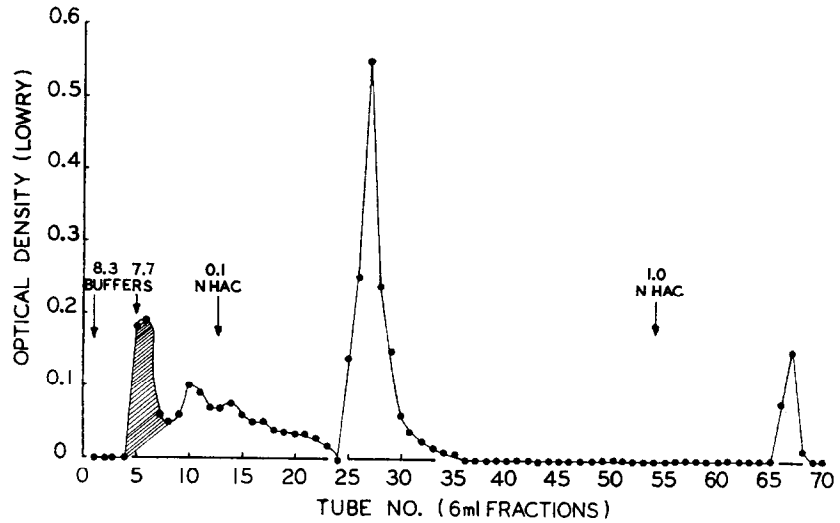

FIG. 3. Partial purification of dialyzable peptides produced by peptic digestion of bradykininogen on Dowex 1-X2. 400 mg bovine bradykininogen was digested with 4 mg pepsin for 18 hr. After an isoelectric precipitation at pH 5·3, dialysis was carried out against distilled water (2 × 200 ml) for 3 hr in 8/32 Visking casing. The dialysate was concentrated by rotary evaporation in the presence of a few drops of caprylic alcohol to approximately 2·0 ml. The concentration was placed on the column and chromatographed as described in Material and Methods. The shaded portion represents the area in which pharmacological activity was found. The pH 8·3 and 7·7 buffers are described by Schroeder *et al.*[10] Tubes 5–8 were pooled as peak 1; 9–20 as peak 2; 25–35 as peak 3; 65–70 as peak 4.

seen, four peaks were obtained. The tubes contributing to each peak were pooled, concentrated to dryness by rotary evaporation, and taken up in 2·0 ml water. The solution was brought to pH 7·5 with 1 N NaOH. A 0·1-ml sample was assayed directly on the isolated ileum. An additional 0·1-ml sample was incubated with 0·02 mg trypsin for 30 min at 37° and assayed against the ileum after stopping the reaction with 0·1 mg soybean inhibitor. The results as recorded in Fig. 4 demonstrate that only peak 1 (the most basic fraction) had activity. When the peaks were treated with trypsin, only peak 1 showed increased activity, which was about 9-fold over the pretrypsin activity. Chymotrypsin was found to abolish the activity of peak 1. The blood pressure response of the rabbit to peak 1 before and after treatment with trypsin is shown in Fig. 5. As may be seen, vasodepression results from an i.v. injection of 0·02 ml of peak 1 directly. As in the case of the smooth-muscle stimulating activity on the ileum, trypsin liberated additional pharmacological activity from peak 1 as compared to the activity of peak 1 itself.

* Attempts to chromatograph the peptides on negatively charged materials such as CM-cellulose or Dowex 50-X2 proved impractical, since the recovery of the peptides was almost nil. The probable reason for this is the very basic nature of the peptides.

The protective action of p-*toluenesulfonic acid*

It was noted that during the purification of the active peptides a low order of recovery of activity was obtained in the partially purified fraction as compared to the original dialysate of the peptic digest. Further investigation demonstrated that most of the activity could be recovered from the Dowex-1 column but, that in concentrating

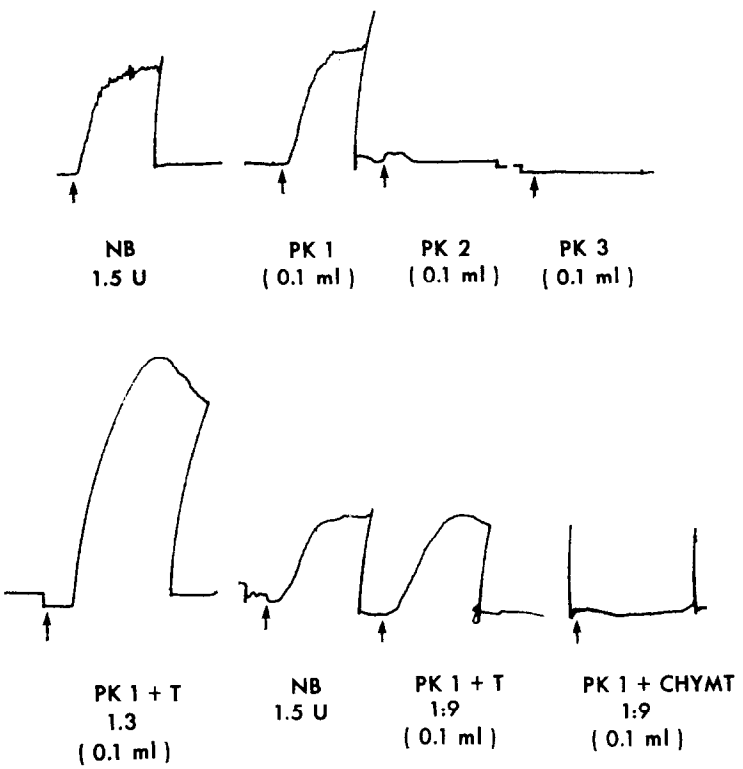

FIG. 4. Guinea pig ileum activity of dialyzable peptic products (Dowex-1). Upper: NB, 0·15 μg bradykinin added to a 20-ml bath; PK 1, 2, 3 refer to the pooled tubes described in the legend to Fig. 3. Lower: PK 1 + T, peak 1 treated with trypsin as described in the text; a sample was diluted 1 : 3 as compared to untreated peak 1 in the upper tracing. PK 1 + T, 1 : 9, as above but diluted 1 : 9; PK 1 + CHYMT, 1 : 9, the above after treatment with 0·025 μg chymotrypsin.

the eluate of the column to dryness by rotary evaporation or by lyophilization, a large percentage of the activity was irreversibly lost. When synthetic bradykinin solutions were also investigated as to the possible loss in activity when concentrated to dryness by the above methods it was also found that 50–95 per cent loss in bradykinin activity occurred, as seen in six experiments reported in Table 2. A study was consequently made of various agents that might be added to solutions of peak 1 or to solutions of synthetic bradykinin that might protect against losses during concentration procedures.

As may be seen in Table 3, two agents, *p*-toluenesulfonic acid (*p*-TSA)[11] or oxalic acid, completely prevented loss in synthetic bradykinin activity during concentration

by rotary evaporation. Other experiments demonstrated that similar protection was obtained during lyophilization. It should be noted that neither *p*-TSA nor oxalic acid, in the concentrations used, had any detectable effects on the guinea pig ileum when tested in the absence of bradykinin. Neither protecting compound enhanced ileum responses when added to the muscle bath in the presence of known concentrations of bradykinin. The ability of these compounds to protect (at a wide pH range)

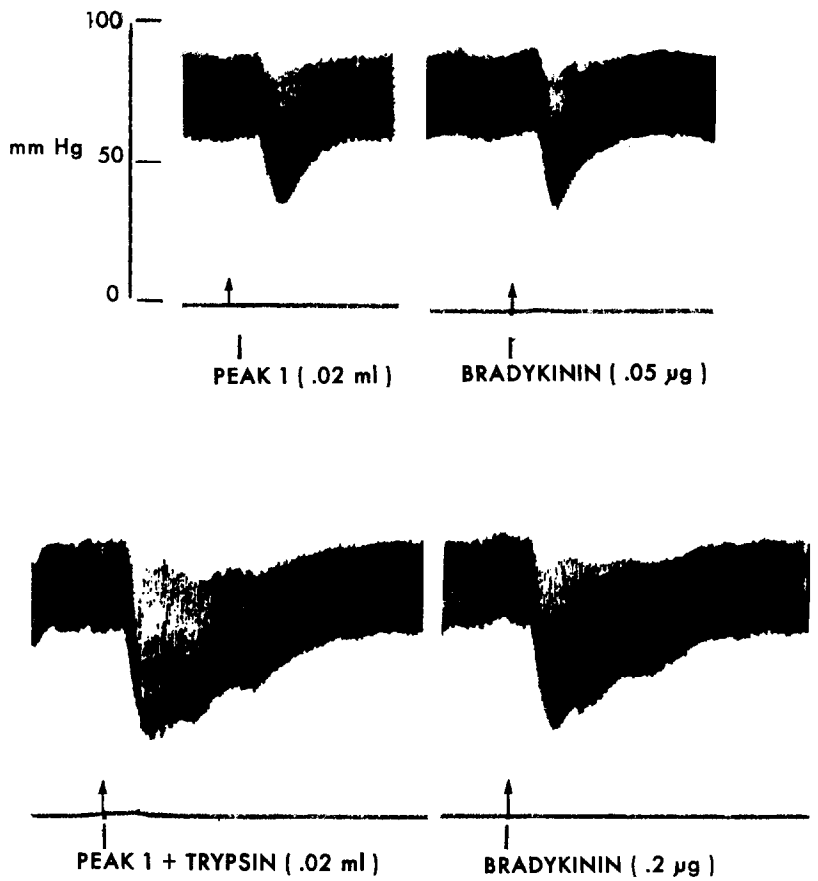

FIG. 5. Blood pressure response of the rabbit to peak 1. Upper, intravenous injection of peak 1 into an anesthesized rabbit that had been pretreated with 0·1 mg atropine. Duration of the response was 20 sec. Lower, 4-fold increase in activity after trypsin treatment of the peptides. The duration of the response was 70 sec. Trypsin controls, 0·5 μg, showed no effect on the blood pressure response.

is probably related to the fact that they are highly negatively charged in solution and probably form a salt with the basic peptides, which prevents either adsorption to glass or molecular rearrangement during the concentration procedures. Weaker acids such as citric and acetic evidently do not bind to the positively charged peptide as do the protecting compounds which are strong acids. Experiments also demonstrated that the minimal concentration of *p*-TSA required for protection was ten times the molar concentration of bradykinin present or two times on a weight basis

(Fig. 6). *p*-TSA has an advantage over oxalic acid for protection in that *p*-TSA absorbs light maximally at 261 mμ (in water); consequently its concentration may be determined rather easily. This property has been used to follow the removal of *p*-TSA from solutions of bradykinin by allowing the solution of the peptide and protecting agent to pass through a 0·9 cm × 1·0 cm column of DEAE-Sephadex. The *p*-TSA is adsorbed on the Sephadex while the bradykinin passes through.

TABLE 2. EFFECT OF ROTARY EVAPORATION ON THE ACTIVITY OF BRADYKININ

Twenty μg bradykinin was dissolved in 10 ml water, and brought to dryness in a rotary evaporator *in vacuo* at 35–37°. The dried material was redissolved in 4 ml water and 0·02 to 0·05 ml tested directly on the guinea pig ileum. Per cent reocvery refers to the activity after evaooration as compared to an untreated sample of synthetic bradykinin in a concentration of 5 μg/ml.

Experiment	Recovery %
1	45
2	40
3	50
4	50*
5	30
6	35
7†	0–5

* Silicone-treated flask.
† Twenty μg bradykinin dissolved in 100 ml water before bringing to dryness.

TABLE 3. EFFECT OF VARIOUS AGENTS ON THE RECOVERY OF BRADYKININ

Twenty μg bradykinin dissolved in 10 ml water (2×10^{-6} M) containing various additions in an initial concentration of 0·001 M. Rotary evaporation and assay as in Table 2.

Additions (0·001 M)	Recovery %
None	45
Sodium chloride	50
Acetic acid	45
Citric acid	60
Versene	70
p-Toluenesulfonic acid	100
Oxalic acid	100

The above findings were duplicated when *p*-TSA was added to solutions of peak 1 which were concentrated by rotary evaporation. A 5- to 10-fold increase in the recovery of peptide (in terms of activity released by trypsin) was obtained in "protected" solutions as compared to solutions to which the protecting compound was not added. Consequently, 0·0002 M *p*-TSA was added to all tubes of the eluate from the Dowex-1 column before concentrating to dryness. Recovery experiments under these optimal conditions have demonstrated that 100 to 250 μg of activity (peak 1 + trypsin) con-

tained in the dialyzable fraction results from digestion of 500 mg of bradykininogen by pepsin. An equal quantity was undialyzable. It should be noted that the total activity that is usually obtained from 500 mg of bradykininogen by treatment with *trypsin* is 250 to 500 μg of bradykinin; thus pepsin releases 20–50% of the activity of bradykininogen as dialyzable polypeptides.

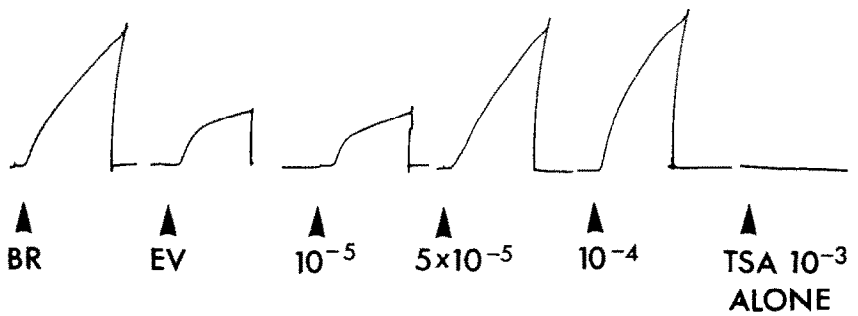

FIG. 6. Effect of varying concentrations of *p*-TSA on bradykinin activity after rotary evaporation; 20 μg bradykinin was dissolved in 10 ml water (2×10^{-6} M); rotary-evaporated to dryness and dissolved in 4 ml water: BR, 0·02-ml sample of 5 μg/ synthetic bradykinin ml added to 10-ml bath; EV, 0·02-ml sample of evaporated sample; 10^{-5}, 5×10^{-5}, 10^{-4}, as in EV except in the presence of these concentrations of *p*-TSA in molarity; TSA 10^{-3} alone, 0·02-ml sample of 10^{-3} M *p*-TSA added to the bath.

DISCUSSION

The demonstration that pepsin degrades bradykininogen into low molecluar weight (dialyzable, ca. 1,000 to 6,000 molecular weight) polypeptides, which contain bradykinin or bradykinin-like peptides in covalent linkage, provides a useful procedure in attempts to elucidate the structure of bradykininogen and the mechanism of release of kinins from the bradykininogen molecule. Investigations are now being carried out in our laboratory to purify the dialyzable peptides further so that structure and sequence studies may be carried on.

It is of interest that the peptides released from partially purified bovine bradykininogen by pepsin do not have the pharmacological properties of "pepsitensin," the latter being released from proteins contained in blood by pepsin as first demonstrated by Croxatto and Croxatto.[12] Whereas pepsitensin activity is *hypertensive* in nature and is not enhanced (actually reported to be destroyed) by trypsin,[13] the peptides obtained in this investigation are *hypotensive* in nature and release activity upon incubation with trypsin.

The action of pepsin on bradykininogen may be explained in several possible ways. One is that pepsin liberates from bradykininogen a dialyzable peptide that contains bradykinin as part of its structure. This would account for the bradykinin-like activity seen by direct assay. The increase in activity after trypsin treatment of the peptide would occur if bradykinin is released from the polypeptide, the bradykinin being pharmacologically more potent than initial polypeptide. Another possibility is that pepsin liberates from bradykininogen dialyzable polypeptides, some having bradykinin-like activity, others having negligible activity per se but releasing bradykinin when acted upon by trypsin.

The loss in activity contained in peak 1 and of bradykinin by current laboratory procedures of concentration posed a serious problem in elucidating various chemical and pharmacological aspects of these peptides. Although *p*-toluenesulfonic acid has been previously used by investigators to aid in the extraction of bradykinin and vasopressin into organic solvents,[14,15] the current finding that *p*-TSA and oxalic acid may be used as a protecting agent for bradykinin-like peptides during concentration procedures is to our knowledge the first report of such a property. In our hands both *p*-TSA and oxalic acids have been found to be indispensable in preventing "activity losses" not only with the peptides of peak 1 but in solutions of bradykinin that are used for assay standards. Twice the weight of the active peptide is sufficient to prevent losses.

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