Biochemical Pharmacology, 1963, Vol. 12, pp. 325–330. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE ISOLATION OF BRADYKININOGEN*

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(*Received* 2 *October* 1962; *accepted* 19 *November* 1962)

Abstract—This investigation has demonstrated that a purified protein from bovine blood may be obtained in solid form and in high yield which, when incubated with trypsin, releases pharmacologically active material that appears to be bradykinin or a very similar material. The material liberated contracts guinea pig ileum, causes vasodepression in the cat via vasodilation, and is destroyed by chymotrypsin. This precursor protein is obtained by relatively simple procedures and apparently can be stored without deterioration. The preparation is highly reproducible. Certain physical properties of the protein have been studied as well as the kinetics of its reaction with trypsin. One mg of the average preparation releases 0·8 to 1·0 μg of bradykinin when incubated with trypsin under the conditions described.

BRADYKININ is a nonapeptide that is produced by the action of trypsin or certain snake venom enzymes on the α_2-globulin fraction of blood.[1] A related pharmacologically active peptide, kallidin II, is produced by the action of kallikreins on this fraction.[2] Bradykinin is known to cause vasodepression in various animals and contraction of the guinea pig ileum and rat uterus, as well as other important pharmacological actions.[3] Despite the advances made in the elucidation of the structure and pharmacological properties of this peptide,[3–5] little is known concerning its protein precursor. Indeed it is not known whether the precursors of bradykinin and of kallidin II are the same or different proteins. The current communication† describes a procedure for the preparation of a purified protein from bovine blood which can be obtained in good yield in a solid form and which, when incubated with trypsin, yields bradykinin or a material very similar to bradykinin as determined by several pharmacological assays. The purified protein has been tentatively called bradykininogen in keeping with the nomenclature for the precursor of bradykinin.[6]

MATERIALS AND METHODS

Bradykininogen was assayed during the purification procedures in the following manner. The protein content of each fraction was determined by ultraviolet absorption at 280 mμ in a Beckman spectrophotometer. Crystalline bovine serum albumin was used as a protein standard. Each fraction was then incubated with trypsin (2× crystalline, Worthington Biochemical Corp.) in a ratio of 100 to 1 by weight, at 37° for 30 min in 0·1 M Tris buffer, pH 7·8, containing 0·2 M $CaCl_2$. The latter prevents

* Supported by the United States Public Health Service General Medical Research Fund, I.G.S. 101 and U.S.P.H.S. grant A-1552.

† A preliminary presentation of the results of this investigation was given at the August 1962 Meetings of The American Pharmacology Society, Nashville, Tenn: *Pharmacologist* **4** (1962).

autodigestion of the trypsin[7] and has been found in the current investigation to enhance the rate of bradykinin liberation in various fractions over 100%. The trypsin is then inactivated by the addition of twice its weight of soybean inhibitor (Worthington). A sample of the incubation mixture is then diluted 10-fold with Tyrodes' solution and 0·1 to 0·4 ml of the dilute solution tested for its ability to contract guinea pig ileum. The dilution is such that the final $CaCl_2$ concentration is equal to or less than the concentration of $CaCl_2$ in the Tyrode's solution of the muscle bath. The amount of bradykinin liberated during the reaction is calculated by comparing the height of the contraction produced by the reaction mixture with synthetic bradykinin and trypsin-produced bradykinin. Both bradykinin preparations were generous gifts of Parke, Davis and Co. in England.

The guinea pig ileum assay was carried out in a 20-ml muscle chamber at 35° using Tyrode's solution as a bathing medium. After the muscle was in contact with a sample for 1 min, the kymograph was stopped and the height of the contraction from the base line measured at this point. Samples were assayed with a minimum interval of 5 min. An ink-writing pen was used to follow the contraction.

Blood pressure assays were carried out on cats which had been anesthetized with pentobarbital or chloralose. The pressure was recorded from the carotid artery. Injections were made into the femoral vein. $CaCl_2$ was omitted from reaction mixtures which were to be assayed for their effect on blood pressure since $CaCl_2$ interfered in the interpretation of the results.

The procedure of Lindgren[8] was used to study blood flow through the hind limb of the cat. Injections were made into the femoral artery, and the rate of blood flow was measured at the femoral vein by means of a photoelectric blood-drop counter.

Chymotrypsin was the 3× crystalline, salt-free preparation of Worthington. Dialysis tubing was the product of the Visking Co.

EXPERIMENTAL AND RESULTS

Purification procedure

The purification procedure can be divided into three steps: (1) preparation of a crude globulin solution, (2) heat treatment in the presence of Ca^{2+} ions, (3) an isoelectric precipitation.

Crude globulin solution. Bovine blood (3·5 L obtained during slaughter) is immediately defibrinated by whipping and then chilled during transport to the laboratory. It is centrifuged at 7,000 × *g* for 15 min at 2°. The 1,250 ml of serum is siphoned off and the serum warmed to 57·5° for 3 hr to inactivate various peptidases as described by Holdstock *et al.*[9] and Werle *et al.*[10] The heated serum is then treated by a modified procedure of Holdstock to obtain the crude globulin solution as follows. The serum is centrifuged as above to remove a small amount of sediment, and the supernatant fluid is dialyzed in several sacks against running tap water overnight. It is then dialyzed against cold distilled water (10 L) for 24 hr, with two changes of water. The dialyzed solution is treated with an equal volume of saturated ammonium sulfate (767 g/L) at room temperature and after 1 hr of stirring is centrifuged as above. The precipitate is dissolved in water (one-half the volume of the dialyzed serum) and is dialyzed against running tap water overnight. This is followed by dialysis against several changes of cold distilled water for 48 hr. The resulting suspension is brought

to pH 7·5 by the dropwise addition of 2 N NaOH to give 2 L of a brown opalescent solution.

Heat treatment. The globulin solution is divided into two equal parts to facilitate handling. To each portion, an equal volume of 0·4 M $CaCl_2$ in 0·2 M Tris, pH 7·7, is added. The solution is then placed in a boiling water bath for 15 min, the temperature of the solution reaching 70° to 80° in about 8 min. At this temperature a huge mass of "inactive protein" precipitates from solution. The suspension is chilled and then centrifuged as above; the residue is discarded. The "boiled supernatant" is dialyzed against 0·001 M Versene (1 L supernatant to 12 L Versene) for 24 hr in the cold room with one change of Versene to remove the Ca^{2+} ions. The solution is then dialyzed against several changes of distilled water for an additional 24 hr.

Isoelectric precipitation. The dialyzed solution (or suspension if the pH of the distilled water is 5·3) is then stirred at room temperature and brought to pH 5·3 by the dropwise addition of 2 N acid or base and stirred for an additional 30 min. The resulting "isoelectric precipitate" contains the "bradykininogen". The suspension is centrifuged at 7,000 × *g* for 15 min at room temperature. The supernatant is discarded and the residue suspended in 400 ml of water and brought to pH 7·5 to 8·0 to redissolve the precipitate. After 30 min of stirring, the insoluble material is spun off. The isoelectric precipitation and re-solution in fresh water are repeated twice. The final solution is brought to pH 5·3, centrifuged, and the final residue is suspended in 80 ml of ice-cold acetone. It is then centrifuged for 5 min and the supernatant discarded. This washing procedure is repeated twice. The final acetone suspension is filtered by gentle suction over Whatman no. 1 filter paper, air-dried, and then placed in a vacuum desiccator to complete the drying.

The yield from various preparations has ranged from 0·6 to 2·5 g. Table 1 illustrates

TABLE 1. PURIFICATION OF BRADYKININOGEN

Fraction	Volume (ml)	Total units*	Total protein (mg)	Specific activity†
Heated dialyzed serum	1,650	4,130	132,000	0·03 (0·026)
Globulin	1,450	17,400‡	97,000	0·18 (0·10)
"Boiled" supernatant	1,820	2,190	5,460	0·42 (0·30)
Acetone powder		540	600	0·90 (0·80)

* One unit of bradykininogen will produce 1 μg of bradykinin under the conditions outlined in Materials and Methods.

† Specific activity is defined as units of bradykininogen/mg of protein. The numbers in parentheses refer to a second independent preparation.

‡ The increase in units in this fraction over the heated dialyzed serum fraction is due to the presence of interfering substances in the heated dialyzed serum which makes quantitation of bradykinin production by our procedures difficult. A similar problem is encountered in defibrinated blood (not listed here). Recovery data should be considered quantitative only from the globulin fraction on.

the purification in terms of specific activity of various fractions of two typical preparations.

Physical properties

The final "acetone powder" has a slightly tan cast. It is easily handled and has an opalescent appearance when in solution. It is soluble to at least 1% from pH 7·0 up in the alkaline range and from pH 4·0 down in the acid range. It absorbs

maximally at 276 mμ at pH 2·0. It appears to be an acidic protein in view of its isoelectric point of 5·3 (this should be considered approximate at this time) and because, at neutral pH, it is absorbed on the anion exchange resin diethylaminoethyl cellulose but not on the cation exchange carboxymethyl cellulose. It appears to be indefinitely stable when kept in a desiccator over $CaCl_2$.

Moving-boundary electrophoresis. The appearance of a 1% solution of the acetone powder in the moving-boundary electrophoresis at pH 8·6 is seen in Fig. 1. A similar pattern is obtained at pH 7·5. It is apparent that under the conditions of electrophoresis so far employed, the material is quite homogeneous.

Pharmacological properties

Guinea pig ileum. When the acetone powder is incubated with trypsin and tested for its ability to contract the guinea pig ileum; the results are seen in Fig. 2. In the absence

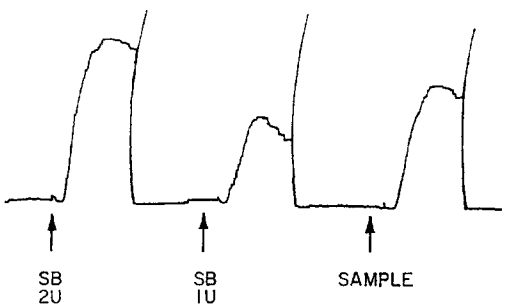

FIG. 2. Effect of the incubation mixture on the guinea pig ileum; 5·0 mg acetone powder and 0·05 mg trypsin in 0·1 M Tris buffer, pH 7·8, containing 0·2 M $CaCl_2$, at 37° for 30 min in a volume of 1·0 ml. The reaction was stopped by the addition of 0·1 mg soybean inhibitor; 0·2 ml of the reaction mixture was diluted to 2·0 ml with Tyrode's solution and 0·4 ml used for the assay. 1U, 0·1 μg synthetic bradykinin; 2U, 0·2 μg synthetic bradykinin. Controls, with acetone powder alone and trypsin alone, showed no effect.

of trypsin no response is obtained. Indeed, in the absence of trypsin, no response is obtained by boiling or by treatment with acid or alkali. Soybean inactivated incubation mixtures of the acetone powder and trypsin have no ability to catalyze the formation of pharmacologically active material.

Cat blood pressure. The effect of an incubation mixture of the acetone powder and trypsin on the blood pressure of the cat is seen in Fig. 3. It will be observed that only the product of the incubation mixture exerts a hypotensive effect.

Effect of chymotrypsin. It is well known that chymotrypsin inactivates bradykinin.[4, 11] Fig. 4 demonstrates that chymotrypsin inactivates the product of the reaction mixture of the acetone powder and trypsin when assayed against guinea pig ileum. Chymotrypsin also destroys the hypotensive effect in the cat (Fig. 5).

Vasodilation effects. Bradykinin is believed to lower the blood pressure in the cat by causing peripheral vasodilation. The effect of the reaction mixture on peripheral blood flow is seen in Fig. 6.

Kinetics of the reaction

The release of active material with time from an incubation mixture of the acetone powder and trypsin is seen in Fig. 7A. In this experiment a substrate to trypsin ratio

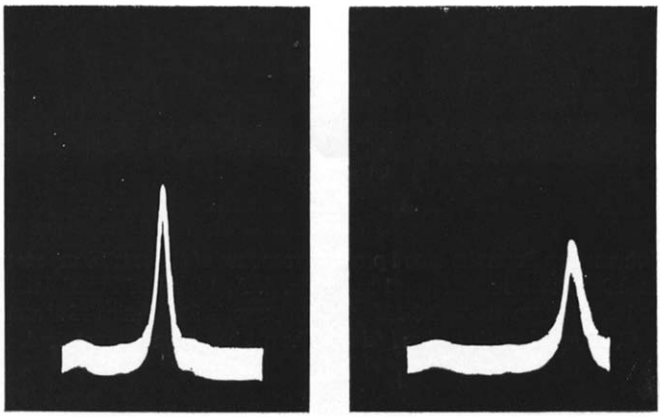

FIG. 1. Moving-boundary electrophoresis of the acetone powder; 1% solution of the acetone powder in veronal buffer, pH 8·5, ionic strength 0·1. The solution was dialyzed against the buffer for 15 hr before subjecting it to electrophoresis which was carried out in a Perkin Elmer machine using 150 v at 15 ma, at 2° in a 2-ml cell. Left, ascending limb after 70 min; right, ascending limb after 115 min.

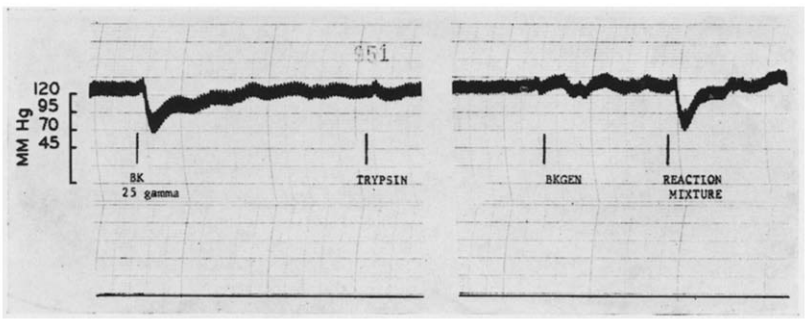

FIG. 3. Effect of the incubation mixture on the blood pressure of the cat; 25 mg acetone powder and 0·25 mg trypsin in 1·0 ml of 0·1 M Tris buffer at pH 7·8 for 240 min at room temperature. Trypsin inactivated with 0·5 mg soybean inhibitor. BK, 25 μg synthetic bradykinin. Trypsin, 1·0 ml of a control solution of trypsin in buffer after 240 min and inactivation by soybean inhibitor. BKGN, 1·0 ml of a control solution of the acetone powder in buffer after 240 min. Reaction mixture, 1·0 m. of the complete reaction mixture.

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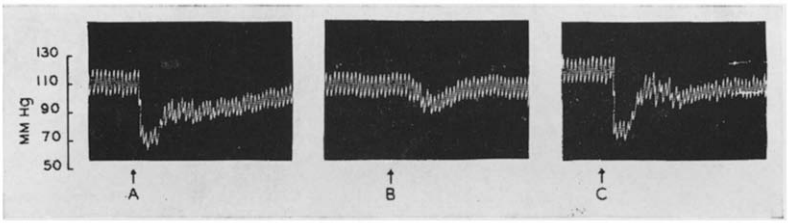

FIG. 5. Effect of chymotrypsin on the blood pressure response of the cat; 50 mg acetone powder and 0·5 mg trypsin in 2·0 ml Tris buffer treated as in Fig. 4. A, 1·0 ml of boiled solution injected; B, 1·0 ml of the boiled solution treated with 0·125 mg chymotrypsin for 30 min at 37° and 1·0 ml injected; C, 20 μg of synthetic bradykinin. The cat had been vagotomized.

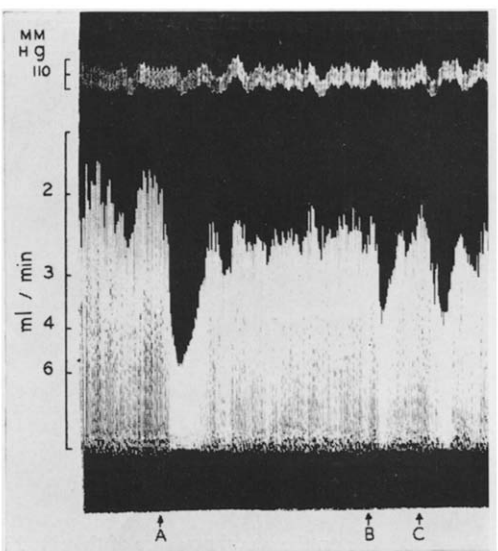

FIG. 6. Effect of the reaction mixture on blood flow in the hind limb of the cat. The procedure of Lindgren[8] was used at the femoral vein to measure blood flow; 5·0 mg acetone powder and 0·05 mg trypsin in 1·0 ml Tris buffer for 30 min at 37°; 0·1 mg soybean inhibitor was then added. A, 0·1 ml injected into the femoral artery; B, C, controls of the acetone powder alone and trypsin alone. Controls showed typical response of inactive material in this preparation. Decreasing magnitude of the lines represents increasing blood flow by this procedure.

of 500 : 1 (as opposed to the usual 100 : 1) was used so that the course of the reaction might be followed. The rate of the reaction, when the substrate is kept constant but increasing trypsin concentrations are used, is seen in Fig. 7B. The preparation used for these experiments had a specific activity of 0·5.

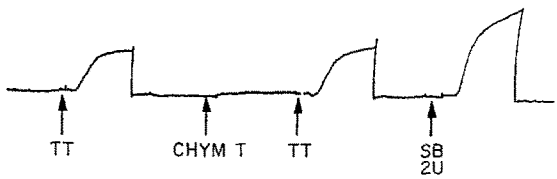

FIG. 4. Effect of chymotrypsin on the guinea pig ileum response; 10 mg acetone powder and 0·1 mg trypsin in 2·0 ml Tris–Ca^{2+} buffer under the conditions of Fig. 2. After 30 min, the tube was placed in a boiling water bath to inactivate the trypsin (about 90%). TT, a sample of the boiled solution was diluted 20-fold and 0·4 ml assayed. Chym T, 1·0 ml of the boiled solution was incubated with 0·025 mg chymotrypsin for 30 min at 37°. A sample was diluted 20-fold and 0·4 ml assayed; SB, 0·2 μg of synthetic bradykinin.

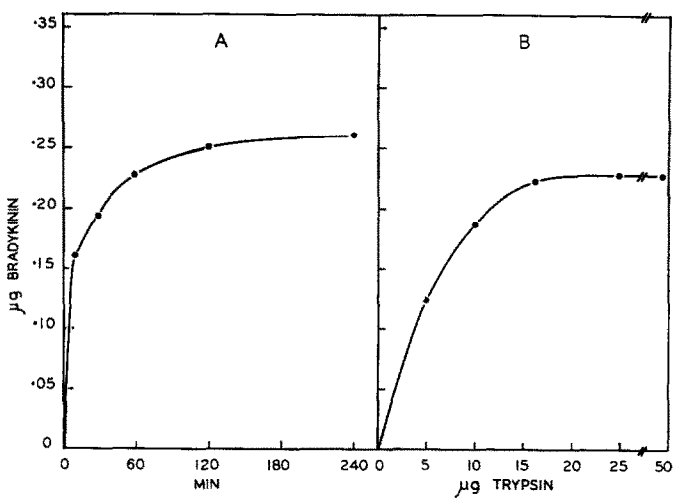

FIG. 7. A, The liberation of bradykinin with time; 10 mg acetone powder and 0·02 mg trypsin under the conditions of Fig. 2. After the various time periods, the trypsin was inactivated and diluted solutions assayed on the guinea pig ileum. In this experiment a ratio of acetone powder to trypsin of 500 : 1 is used as opposed to the usual ratio of 100 : 1. B, The effect of increasing trypsin concentrations on the liberation of bradykinin; 5·0 mg acetone powder and varying concentrations of trypsin incubated under the conditions of Fig. 2 for 30 min In these experiments a preparation having a specific activity of 0·5 was used. μg of bradykinin represent 0·1 ml of the reaction mixture.

DISCUSSION

In order to obtain information as to the chemical, structural, and physical properties of bradykininogen, it is necessary to obtain the material in homogeneous form and in good yield. The current experiments are initial steps toward this end. Although further evidence is necessary to determine the purity of the preparation, the electrophoretic pattern of the 30- to 40-fold purified acetone powder at pH 8·6 and 7·5 demonstrates a rather homogeneous protein under these conditions. The amounts of bradykininogen obtained with the current procedure provide sufficient stable material for further

experimentation and analysis. The preparation is very reproducible, with specific activities from 0·8 to 1·0 usually being obtained. One preparation gave a specific activity of 0·5. The preparation takes about 2 weeks to prepare.

The experiments concerning the pharmacological properties of the product of the acetone powder and trypsin have been done in detail since the various procedures employed for purification necessitate the study of more than one pharmacological parameter to be certain of the nature of the active material. In this regard, the experiments have indicated that the product of the acetone powder and trypsin is bradykinin or very similar to bradykinin so far as its pharmacological properties toward guinea pig ileum and the blood pressure response in the cat are concerned. The inactivation of the active product by chymotrypsin indicates its peptide nature. It is possible, however, that more than one active peptide is released during the reaction. Chemical studies are now being undertaken to determine this point.

An important property of bradykininogen which aids in its purification is its solubility and stability when the crude globulin solution is brought to high temperatures in the presence of Ca^{2+} ions. The presence of Ca^{2+} ions and heating causes the precipitation of over 90% of the protein from the globulin fraction. The supernatant containing the bradykininogen is thus sufficiently purified so that an isoelectric precipitation is possible. Indeed, in the absence of Ca^{2+}, no precipitation of inert protein occurs during heating, nor is an isoelectric precipitation possible.

To our knowledge, this is the first preparation of bovine bradykininogen that has been obtained in solid form, in high purity, and in such high yield. Recently, Henriques *et al.*[12] isolated a purified protein fraction from horse blood which, when incubated with trypsin or *Bothrops jararaca* venom liberates an active material which contracts guinea pig ileum. They have termed the precursor fraction "bradykininogen".

Studies are currently being conducted on further possible purification of the bovine precursor of bradykinin and the chemical structure of this material. Investigations are also being pursued to determine whether the current preparation can serve as a substrate for the production of pharmacologically active material(s) by enzymes other than trypsin.

Acknowledgement—We would like to thank Mr. Michael Peters and Mr. Albert Ellman for their excellent assistance in various phases of this work. We are also grateful to Drs. J. Belford, P. Cervoni, P. Lindgren, and L. Procita of the Department of Pharmacology for their cooperation in aiding us in several phases of the work.

REFERENCES

1. M. ROCHA E SILVA, W. T. BERALDO and G. ROSENFELD, *Amer. J. Physiol.* **156**, 261 (1949).
2. J. V. PIERCE and M. E. WEBSTER, *Biochem. biophys. Res. Commun.* **5**, 353 (1961).
3. H. KONZETT and E. STÜRMER, *Brit. J. Pharmacol.* **15**, 544 (1960).
4. D. F. ELLIOTT, G. P. LEWIS and E. W. HORTON, *Biochem. biophys. Res. Commun.* **3**, 87 (1960).
5. R. A. BOISSONNAS, S. GUTTMAN, P. A. JAQUENOUD, H. KONZETT and E. STÜRMER, *Experientia, Basel* **16**, 326 (1960).
6. M. ROCHA E SILVA, *Polypeptides*, M. SCHACHTER, Ed., p. 210. Pergamon Press, London (1960).
7. J. H. NORTHROP, M. KUNITZ and R. M. HERRIOTT, *Crystalline Enzymes*, 2nd ed. Columbia Univ. Press, New York (1948).
8. P. LINDGREN, *Acta physiol. scand.* **42**, 5 (1958).
9. D. J. HOLDSTOCK, A. P. MATHIAS and M. SCHACHTER, *Brit. J. Pharmacol.* **12**, 149 (1957).
10. E. WERLE, W. GÖTZE and A. KEPPLER, *Biochem. Z.* **289**, 217 (1937).
11. R. A. BOISSONNAS, S. GUTTMAN and P. A. JAQUENOUD, *Helv. chim. Acta* **43**, 1349 (1960).
12. O. B. HENRIQUES, Z. P. PICARELLI and M. C. FARRAZ DE OLIVEIRA, *Biochem. Pharmacol.* **11**, 707 (1962).