Kallikrein

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I. Determination of Kallikrein

Kallikrein is a compound of high molecular weight which lowers blood pressure and stimulates the movement of the uterus and the intestine, and occurs in the pancreas, the submaxillary glands and the urine of practically all mammals including man[1]. It also occurs as the inactive precursor, kallikreinogen, in serum and pancreas[1]. The excretion in the urine is greatly reduced in essential hypertension, in Addison's disease and in acute nephritis[1]. Previously kallikrein could only be determined by comparison of its hypotensive activity in lowering blood pressure of experimental animals with that of a standard preparation[1]. The discovery of its hydrolytic action on benzoyl-L-arginine ethyl ester (BAEE)[2,3] enables kallikrein to be determined by a non-biological method.

Principle

Kallikrein catalyses the reaction:

(1) Benzoyl-L-arginine ethyl ester $\rightleftharpoons$ benzoyl-L-arginine + ethanol

The equilibrium of the reaction is far on the side of the hydrolysis products. There are 5 possible methods of estimating the hydrolysis of BAEE[4] (see "Other Methods for the Determination of Kallikrein", p. 883). The most suitable is the measurement of the increase of optical density at 253 mμ (due to benzoyl-L-arginine).

The hydrolysis of BAEE by kallikrein has a broad pH maximum at 8.5. The molar extinction coefficient for BAEE at 253 mμ and pH 8.0 is 2.3×10^6 cm.²/mole and for benzoylarginine is 3.45×10^6 cm².mole[4].

Optimum Conditions for Measurements

At 25° C a final concentration of BAEE of 0.5×10^{-3} M is sufficient. The measurements are made at pH 8.0. At higher pH values the ester is hydrolysed non-enzymatically. The Michaelis constant at 25° C and pH 8.0 is between 2×10^{-4} and 8×10^{-4} M according to the source of the kallikrein[4].

Reagents

1. Triethanolamine hydrochloride
2. Sodium hydroxide, A. R., 0.2 N
3. Benzoyl-L-arginine ethyl ester*), BAEE
4. Kallikrein
   standard preparation from pancreas**).

Preparation of Solutions (for *ca.* 20 determinations)

I. Triethanolamine buffer (0.067 M; pH 8.0):
Dissolve 1.24 g. triethanolamine hydrochloride in about 70 ml. doubly distilled water, adjust to pH 8.0 with 19 ml. 0.2 N NaOH and dilute to 100 ml. with doubly distilled water.

*) *e.g.* from Mann Research Laboratories, New York, N. Y., USA.
**) Standard Padutin (Bayer, Leverkusen, Germany) = kallikrein from pig pancreas.

1) *E. K. Frey, H. Kraut* and *E. Werle:* Kallikrein-Padutin. Enke-Verlag, Stuttgart 1950.
2) *E. Werle* and *B. Kaufmann-Boetsch*, Naturwissenschaften *46*, 559 [1959]; *E. Habermann*, Naunyn-Schmiedebergs Arch. exp. Pathol. Pharmakol. *236*, 492 [1959]; *Ch. Contzen, P. Holtz* and *H. W. Raudonat*, Naturwissenschaften *46*, 402 [1959]; *E. Werle* and *B. Kaufmann-Boetsch*, Hoppe-Seylers Z. physiol. Chem. *319*, 52 [1960].
3) *E. Werle*, Angew. Chem. *60*, 53 [1948].
4) *I. Trautschold* and *E. Werle*, Hoppe-Seylers Z. physiol. Chem. *325*, 48 [1961].

II. Benzoyl-L-arginine ethyl ester, BAEE (3×10^{-3} M):
Dissolve 12.9 mg. BAEE in 12.5 ml. buffer (solution I).

III. Kallikrein standard solution:
Dissolve the contents of an ampoule of Padutin*) (*e.g.* 10 kallikrein units**)) in 10 ml. doubly distilled water.

Stability of the solutions

The BAEE solution is usable for 1 to 2 days if stored at 0 to 4° C; it is better to freeze it overnight. A solution of BAEE whose absorption at 253 mμ is so large that it cannot be compensated by using the maximum slit-width is useless. The buffer solution is stable almost indefinitely in the cold. The kallikrein standard solution keeps for *ca.* 1 week at 0 to 4° C.

Procedure

Experimental material

According to the source of the kallikrein the solutions must contain at least: 0.1 kallikrein units (KU)/ml. for pancreas, 0.2 KU/ml. for submaxillary gland, 0.4 KU/ml. for urine and 0.05 KU/ml. for serum kallikrein. The solutions must be optically clear and the amount used must not have a large absorption at 253 mμ. The degree of purity of the kallikrein preparation up to 150 μg. protein/KU has no effect on the assay of activity. The purest preparations so far obtained contain 1—2 μg. protein/KU.

Assay

Preliminary remarks: The amount of kallikrein taken is so arranged that the change in the optical density does not exceed 0.150 per 15 min. at 25° C. The measurements are made against a control cuvette containing 0.5 ml. BAEE solution (II) and 2.5 ml. buffer (solution I). This cuvette can be used for 3 or 4 successive measurements (about 30 min.).

Method: Wavelength: 253 mμ; light path: 1 cm.; final volume: 3.0 ml.; temperature: 25° C.; duration of measurements: 15 min.

Pipette successively into the cuvettes:

0.50 ml. BAEE solution (II)
2.45—1.50 ml. buffer (solution I).

Warm to 25° C within *ca.* 5 min. in a constant temperature cuvette holder or water bath.

Mix in

0.05—1.00 ml. sample or kallikrein standard solution (III),

after exactly 1 min. start a stopwatch and simultaneously read the optical density E_1 against the control cuvette. After exactly 15 min. read the optical density E_2. The optical density difference $\Delta E = E_2 - E_1$ gives the optical density change due to the hydrolysis of BAEE by kallikrein. This method eliminates the immediate increase in optical density on addition of kallikrein, which can be quite considerable because of the high protein content, and also corrects for the continual spontaneous decomposition of BAEE.

Calculations

A kallikrein unit (KU) is the amount of kallikrein which on intravenous injection into a dog has the same effect on the carotid blood pressure curve as 5 ml. of human urine, which has been taken from a 50 l. sample and has been dialysed for 24 hours against running water.

*) See footnote **) on p. 880.
**) For the definition of a kallikrein unit (KU), see under "Calculations".

According to the source of the kallikrein, BAEE is hydrolysed at different rates per kallikrein unit. Therefore a factor must be obtained for each type of kallikrein, so as to determine how much faster or slower the particular kallikrein hydrolyses BAEE in comparison to the standard kallikrein preparation. The factor can only be determined by simultaneously measuring the hydrolytic activity on BAEE and the hypotensive activity on an experimental animal of both the test material and a standard preparation. The factor is virtually the same for different preparations from the same source. Factors which have been measured so far are given below[4]:

Kallikrein from	Factor F
Pig pancreas	1.0
Pig submaxillary glands	1.25
Pig urine	2.3
Pig serum	0.8
Human serum	8.8

$$\frac{\Delta E_S \times V_1 \times F}{\Delta E_{St} \times V_2} = \text{KU/ml. sample}$$

where

ΔE_S = optical density change with the sample
ΔE_{St} = optical density change with the standard kallikrein
V_1 = amount of standard kallikrein taken for the assay [KU]
V_2 = volume of sample taken for the assay [ml.]
F = factor for the kallikrein contained in the sample.

For calculations with urine samples:

$$\frac{\Delta E_S \times V_1 \times V_3 \times F}{\Delta E_{St} \times V_2 \times V_4} = \text{KU/ml. crude urine}$$

where

V_3 = volume of the urine sample after dialysis [ml.]
V_4 = volume of the crude urine taken for the acetone precipitation [ml.]

Example for human urine

1. Add 100 ml. acetone to 50 ml. of a 24-hour specimen of urine (pH 6.0) and after 5 min. centrifuge off the precipitate. After evaporation of the acetone, take up the residue in 10 ml. water. Centrifuge off any insoluble material. Dialyse the clear supernatant for *ca.* 20 hours against running water (centrifuge off the precipitate) and use a portion for the assay. Volume after dialysis: 15 ml. Amount taken for assay: 1.0 ml. $E_1 = 0.080$; $E_2 = 0.124$; $\Delta E_S = 0.044$.
2. Assay with a standard kallikrein solution (0.45 ml. = 1 KU): volume taken: 0.06 ml. (= 0.134 KU). $E_1 = 0.012$; $E_2 = 0.112$; $\Delta E_{St} = 0.100$.

Substitution in the above formula for urine samples gives 0.156 KU/ml. crude urine.

Stability of Kallikreins

The kallikreins are stable in the lyophilized state in the cold. Solutions of kallikrein, especially urine kallikrein, are also stable at neutral pH in the cold, but on prolonged storage they must be protected from bacterial contamination by a layer of toluene or by the addition of a bacteriostatic agent (*e.g.* ®Cialit*) 1 : 20000). The stability of serum kallikrein decreases with increasing purity.

Sources of Error

The measurement of kallikrein in crude extracts is often impossible because the solutions have too high an absorption at 253 mμ. In this case, assay by the alcohol dehydrogenase method[4] is possible or a crude fractionation of the extract is necessary. Fractionation is also necessary when other

*) Na salt of 2-ethylmercurithio-benzoxazole-5-carboxylic acid.

enzymes which hydrolyse esters (*e.g.* trypsin or esterases) are present. The contaminating enzymes can be excluded by specific inhibitors which do not affect the esterase activity of kallikrein (*e.g.* trypsin by an inhibitor from soya beans). A non-tryptic or chymotryptic proteolytic activity, which accompanies all kallikreins and which is removed to a large extent on purification, does not affect the hydrolysis of BAEE.

Other Methods for the Determination of Kallikrein

1. Comparative assay of activity by measurement of the hypotensive activity on the carotid blood pressure of the dog[1].
2. On incubation with serum, kallikrein liberates kallidin which can be determined with isolated guinea pig intestine[5,6]. This very sensitive method has only been described for urine kallikrein and it has all the disadvantages associated with methods employing isolated organs.
3. Determination of ethanol liberated on hydrolysis of BAEE with alcohol dehydrogenase[4].
4. Determination of the carboxyl groups liberated on hydrolysis of BAEE by microtitration or by manometric measurements in the Warburg apparatus with bicarbonate-CO_2 buffer[4].
5. Determination of the BAEE before and after the action of kallikrein by measurement of the ester concentration according to *Hestrin*[4–7].

II. Determination of Kallikrein Inhibitors

The method described above also allows the quantitative estimation of kallikrein inhibitors. These are naturally occurring inhibitors, which have, for example, been isolated from parotid gland[1], lung[8] and other ruminant tissues[9], and also from plants[10]. They are used, for example, in the therapy of pancreatitis.

Principle

A kallikrein solution of known concentration is pre-incubated with the inhibitor for 1 hour at pH 8.0 and 37°C, and the residual activity is determined by means of the hydrolysis of BAEE. A kallikrein control is prepared containing buffer instead of the inhibitor and is incubated in the same way.

Reagents and Preparation of Solutions

See p. 880.

Procedure

Preliminary remarks: A kallikrein inhibitor unit (KIU) is the amount of inhibitor which causes a 50% inhibition of two kallikrein units (KU) in 1 hour at pH 8 and 37°C. The amount of inhibitor solution taken for assay must be so arranged that the inhibition of kallikrein activity is below 50%, since the relationship between the inhibition and the concentration of inhibitor is practically linear only up to 50% inhibition or to a ratio of KU : KIU of 1 : 0.5. With higher concentrations of inhibitor the inhibition increases rapidly and is, for example, 80% when KU : KIU is 1 : 0.625.

Experimental material

To determine the inhibitor content of tissue homogenates and blood samples proceed as follows: deproteinize 5 ml. blood (or tissue homogenate) by mixing with the same volume of

5) *E. Werle*, Biochem. Z. *289*, 217 [1937].
6) *E. W. Horton*, J. Physiology *148*, 267 [1959].
7) *S. Hestrin*, J. biol. Chemistry *180*, 249 [1949].
8) *E. Werle*, unpublished.
9) *E. Werle* and *W. Appel*, Z. f. Naturforsch. *14b*, 648 [1959].
10) *E. Werle*, *W. Appel* and *E. Happ*, Z. Vitamin-, Hormon- u. Fermentforsch. *10*, 127 [1959].

cold 6% (w/v) perchloric acid and centrifuge for a short period. Neutralize a portion of the clear supernatant (V_1 = 4.5 ml.) with 2 N KOH (V_2 = 0.5 ml.) and allow to stand for at least 10 min. at 0°C. Decant off the solution from the precipitated potassium perchlorate and incubate a portion of the supernatant (with blood V_3 = 0.8 ml.) with kallikrein (V_4).

Assay

Pipette into test tubes:

Experimental: 0.1—1.0 ml. kallikrein standard solution (III; 0.5 to 5.0 KU)
0.1—1.0 ml. inhibitor solution (sample)
to 4.0 ml. with buffer (solution I)

Control: 0.1—1.0 ml. kallikrein standard solution (III; as experimental tube)
to 4.0 ml. with buffer (solution I).

Incubate for 1 hour at 37°C. Remove the same portion from each tube for the kallikrein determination (see p. 881).

Calculations

The following formula gives the concentration of kallikrein inhibitor in blood samples:

$$\frac{V_2 \times I \times V_4}{V_1 \times V_3 \times 50} = \text{KIU/ml. blood}$$

where

V_1 = portion taken after deproteinization [ml.]
V_2 = volume of the portion after neutralization with 2 N KOH [ml.]
V_3 = volume incubated with kallikrein [ml.]
V_4 = amount of kallikrein taken [KU]
I = inhibition of BAEE hydrolysis [%]

To obtain the inhibitor content of tissue homogenates per gram of tissue, the measured values must be multiplied by the dilution factor arising from the homogenization of the tissue. The following formula applies to inhibitor solutions which have been incubated with kallikrein without deproteinization:

$$\frac{I \times V_4}{V_3 \times 100} = \text{KIU/ml. sample}$$

Example

Blood (5 ml.) was treated with 5 ml. 6% cold perchloric acid solution and centrifuged; 4.0 ml. of the supernatant was neutralized with 0.55 ml. KOH. After decanting from the potassium perchlorate, 0.8 ml. of the clear supernatant was incubated with 2 units of kallikrein.
The following optical densities were measured:
Experimental: $E_1 = 0.010$; $E_2 = 0.071$; $\Delta E_E = 0.061$.
Control: $E_1 = 0.012$; $E_2 = 0.122$; $\Delta E_C = 0.110$.

The inhibition of the kallikrein standard is $\frac{(\Delta E_C - \Delta E_E)}{\Delta E_C} \times 100 = 44.5\%$ (I = 44.5). From this it follows that the sample contained:

$$\frac{4.55 \times 44.5 \times 2}{4.0 \times 0.8 \times 50} = 2.53 \text{ KIU/ml. blood}$$

Other Methods for the Determination of Kallikrein Inhibitors

See p. 883 under "Other Methods for the Determination of Kallikrein".