Human Saliva Kallikrein

Biological properties of saliva kallikrein isolated by use of affinity chromatography

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Kallikrein purified from whole human saliva was studied for its influence on leucocyte migration and kinin generating capacity. The kallikrein had a leucotactic effect on neutrophil leucocytes in the presence of 2% serum albumin. This leucotactic effect could be inhibited by Trasylol. The study showed that kallikrein liberated kinin from the precursor kininogen in dog plasma. Maximal release of kinin was achieved after 4 min of incubation. The kinin generating capacity of the enzyme was studied at different concentrations of kininogen. A maximum kinin release at 11.2 μg bradykinin per mg enzyme per minute was found.

Key-words: Biochemistry; chemotaxis; kinin

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Kallikreins are proteolytic enzymes included in the group called kininogenases (E.C. 3.4.4.21) (*Webster*, 1970). The enzymes are present in different body fluids such as saliva, urine, plasma and also in organs of different species, (*Pierce*, 1970). Kallikrein from different sources can be separated with regard to the molecular weight, isoelectric point and inhibitors. (*Frey et al.*, 1968; *Eisen & Wogt*, 1970).

The presence and isolation of kallikrein in human saliva have been demonstrated by *Moriya, Yamazaki & Fukushima* (1965), *Moriya et al.* (1966), *Libermann & Littenberg* (1969), *Wotman, Greenbaum & Mandel* (1969) and *Fujimoto, Moriwaki & Moriya* (1973). *Modéer* (1977) isolated kallikrein from human saliva by use of affinity chromatography and determined some biochemical properties of the enzyme.

Kallikreins rapidly liberate vaso-active peptides, kinins from the precursor kininogen present in human plasma (*Webster*. 1970). Plasma kallikrein liberates the nonapeptide bradykinin from the kininogen whereas salivary kallikrein generates the decapeptide kallidin (*Webster & Pierce*, 1963). Animals depleted of the kinin precursor kininogen fail to develope granulomatous inflammatory responses. This has been taken as evidence for the role of kinins in granulomatous inflammation (*Warren*, 1962). Few studies have been carried out concerning the role of kinins in periodontal disease. *Rodin et al* (1972) showed

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a positive correlation between kinin activity of gingiva and the radiographic bone loss scores of patients with periodontal disease. Apart from the kinin generating capacity kallikrein has also been shown to be leucotactic for neutrophil leucocytes but the significance of this observation is not known (*Kaplan, Kay & Austen,* 1972).

The present investigation was undertaken to study the kinin generating capacity and the influence on neutrophil leucocyte migration by purified human saliva kallikrein.

MATERIAL AND METHODS

Saliva. Paraffin-stimulated whole saliva was collected from each of 5 men and 3 women (20–30 years old). Before the collection procedure the subjects had to rinse their mouths, 3–4 hours after oral hygiene. The saliva samples were pooled, centrifuged at 10,000 g for 15 min at 4°C. The sediment was washed with 0.05 M tris HCl buffer pH 8.1 and recentrifuged. The supernatants were pooled. This pool was concentrated by amicon diafilter with a H1DP 10 filter (Amicon, Oosterhaut, Holland) and dialysed against 0.05-M tris-HCl buffer, 0.5 M NaCl pH 8.1 before affinity chromatography.

Animals. For the kinin experiment white spraque Dawley rats (Anticimex, Stockholm, Sweden) weighing 200–250 g were used. All animals were ovariectomized and at least 3 weeks elapsed before they received 10 μg oestradiol 17-β. The injections were given subcutaneously for three days according to a method of *Eliasson, Brzdekiewics & Wiklund* (1969). The animals were killed, the uterine horn was removed and a segment 2 cm long was used for the kinin assay.

For the chemotaxis experiments white spraque Dawley rats 250–300 g were used. Rat neutrophil leucocytes were obtained after intraperitoneal stimulation by sterile isotonic glycogen (*Boyden,* 1962). 400 mg glycogen was injected and after 3 hours the cell suspension was collected with a syringe, centrifuged at 1,000 g for 10 min at 4 °C and suspended in Hanks' balanced salt solution to a concentration of $6.0 \cdot 10^6$ cells/ml.

Kininogen. Kininogen was prepared by heating dog plasma at 60 °C for 1.5 hour (*Webster,* 1970). After heating, the plasma was centrifuged at 4,000 g for 15 min at 4 °C. The supernatant containing kininogen was diluted 1:2 with 0.1 M tris-HCl buffer pH 7.8 supplemented with 10^{-3} M EDTA and used as substrate.

Isolation of kallikrein. Kallikrein of human saliva was isolated by a method described earlier (*Modéer,* 1977). The concentrated saliva supernatant was first introduced into a soy bean trypsin inhibitor Sepharose 4 B column (STI-Sepharose) to remove trypsin-like enzymes (*Söder & Modéer,* 1977). The nonadsorbed fraction was then introduced into a pancreatic trypsin inhibitor Sepharose 4 B column (PTI-Sepharose) (2.0 x 3.0 cm). Desorption was effected by 0.05 M glycin-HCl buffer, pH 3.6 containing 0.02 M $CaCl_2$ and 0.1 M NaCl. The adsorption at 280 and 260 nm was determined on all fractions. All spectrophotometric measurements were carried out with a Zeiss spectrophotometer PMQ II. Kallikrein used in this study had a specific activity of 4.800 units per A_{280}. One unit is expressed as one nmole of BAEE liberated per ml per minute at 25° pH 8.5.

Enzyme assay. The enzymatic activity was determined on α-benzoyl-L-arginine ethyl ester – HCl (BAEE) (Sigma Chemical Company St. Louis, Mass. USA) (*Ketzdy, Lorand & Miller,* 1965; *Lindqvist et al.,* 1974; *Modéer,* 1977).

Measurements of chemotactic activity. The chemotactic activity elaborated by human saliva kallikrein was studied by use of Boyden-chambers (*Boyden,* 1962). The chamber consists of an upper and lower compartment separated by a Milipore filter (1.2 μm pore aize, 13 mm diameter) (Milipore Filter corp., New Be-

dford, USA). The cell suspension (0.5 ml) was placed in the upper compartment. The test solution or the control (0.5 ml) and 1.4 ml Hanks balanced salt solution in 2% bovine serum albumin (final concentration) were placed in the lower compartment. The chambers were incubated for 3 hours at 37 °C in a humidity atmosphere of 95% air plus 5% CO_2. After the incubation period the filters were removed and stained with Ehrlich hematoxylin, cleared and mounted on glass slides according to *Boyden* (1962). Neutrophil leucocytes present on the lower side of the filter were counted in a light microscope with the aid of a 40 x objective and a 10 x ocular. The chemotactic activity was expressed as the number of cells counted in 10 randomly selected fields. In one series of experiments the chemotactic activity elaborated by kallikrein was inhibited by addition of pancreatic trypsin inhibitor at a concentration from 5 KIU up to 40 KIU (*Frey, Kraut & Werle,* 1950).

Kininogenase activity. The kallikrein was incubated with the substrate kininogen at 37° for different periods of time. Aliquots were removed and assayed at 3 min intervals according to *Eliasson et al* (1969). The kinin releasing activity was assayed on a segment of isolated uterus horn. The uterus was mounted to a registeration device in an organ bath (8.0 ml) containing Tyrodes balanced salt solution pH 7.8 at 30 °C. (*Trautschold,* 1970). The muscle contractions were recorded on a Grass polygraph (Grass instrument Co., Quincy, Mass., USA) with a hearth smooth muscle transducer (Harward Apparatus Co., Inc. Massachusetts, USA) against optimal load (0.5 g).

Different amount of bradykinin (Sigma) was used to calculate a standardization curve (Fig. 1). When the reciprocal of the concentration of the uterus was plotted against the reciprocal of the amount of bradykinin, a straight line was obtained (Fig. 2). By extrapolating this line to the intercepts on the ordinate and abscissa the apparent amount of bradykinin giving 50% of maximal contraction (B_{C50}) and maximal contraction (C_{max}) could be calculated. Before every experiment was started the

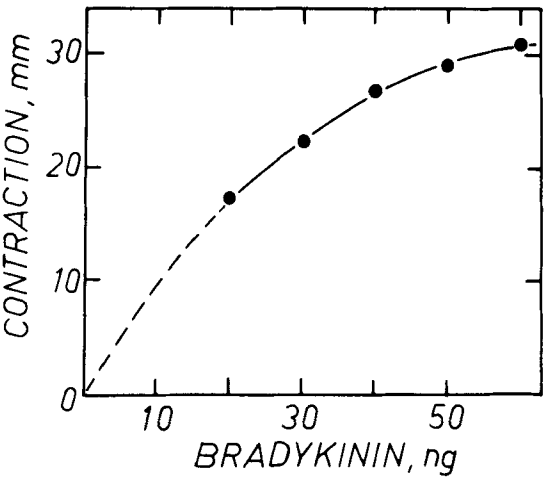

Fig. 1. Standardization curve with synthetic bradykinin. Ordinate = The height of contraction of the uterus (mm). Abscissa = The amount of bradykinin added to the musclebath solution (8.0 ml).

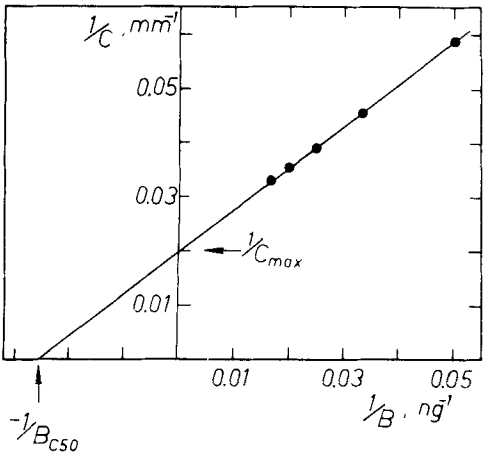

Fig. 2. Standardization curve with synthetic bradykinin. Absicissa = reciprocal of the amount of bradykinin in nanogram, added to the uterus-bath solution (8.0 ml). Ordinate = reciprocal of the uterus contraction (C) in millimeter. C_{max} = Extrapolation of maximal contraction. B_{C50} = Amount of bradykinin giving 50% of maximal contraction.

two parameters B_{C50} and C_{max} was determined. Using these parameters the kininogenase activity was calculated from the formula:

$$B = \frac{C \cdot Bc_{50}}{C_{max} - C}$$

C = The height of the contraction in mm.
B = The kininogenase activity expressed as ng bradykinin.

In another series of experiments the kinin generating capacity of kallikrein was tested when different concentration of kininogen was used.

RESULTS

Chemotactic activity associated with saliva kallikrein. A typical elution pattern of saliva kallikrein from a PTI Sepharose 4 B column is shown in Fig. 3. The kallikrein activity was measured by the hydrolysis of BAEE and appeared in the tubes numbered 4-11. The chemotactic activity mediated by the enzyme appeared in the same fraction (Fig. 3). The highest chemotactic activity generated by kallikrein was found in the same fraction as that with the highest enzymatic hydrolysis of BAEE.

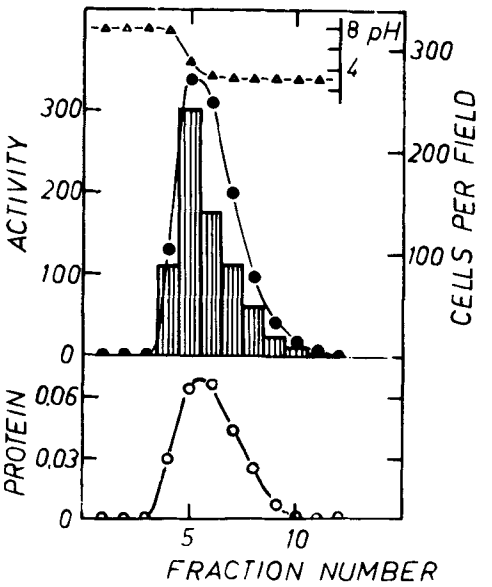

Fig. 3. Affinity chromatography of human saliva (1200 ml). The saliva was centrifuged and the supernatant was concentrated to 245 ml and applied to a STI-Sepharose 4 B column. The non-adsorbed fraction was applied to a PTI-Sepharose 4 B column (2.0 x 3.0 cm). Eluant was 0.05 M glycin HCl, 0.1 M NaCl, 0.02 M $CaCl_2$ buffer pH 3.6. Flow rate 13.0 ml/h. Fraction volume 3.0 ml. The kallikrein activity U/ml was assayed on BAEE at pH 8.5 and expressed as nmoles/min/ml (–●–). The adsorbance at 280 nm (–O–). The pH of the effluent (–▲–). The chemotactic activity cells per field generated by kallikrein is marked with shaded bars.

Cell migration in response to kallikrein. A dose-respone relationship between the amount of kallikrein and the chemotactic activity is shown in Fig. 4. There was a linear relationship between the concentration of kallikrein and the increased migration of PMN' cells.

Inhibition of chemotactic activity. A dose response inhibition of chemotactic activity of kallikrein was obtained with pancreatic trypsin inhibitor Trasylol over a concentration range of 5 KIU to 40 KIU (Fig. 5). At 40 KIU the chemotactic activity was at the same level as the control (random migration).

The influence of incubation time on the release of kinin. The effect of incubation time on the generation of kinin from kininogen by kallikrein is shown in Fig. 6. There was a rapid

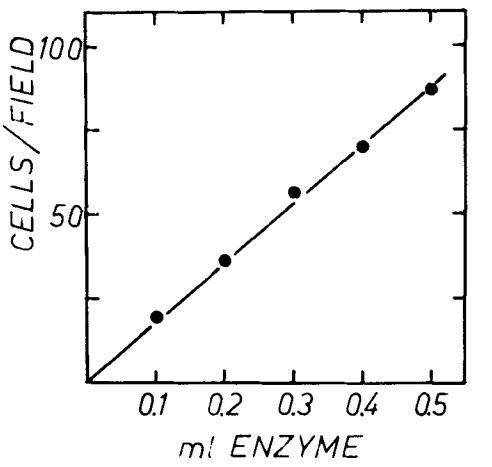

Fig. 4. A dose response relationship between the concentration of kallikrein (230 U/ml) and the generation of chemotactic activity. The activity was expressed as the number of PMN cells per field of view.

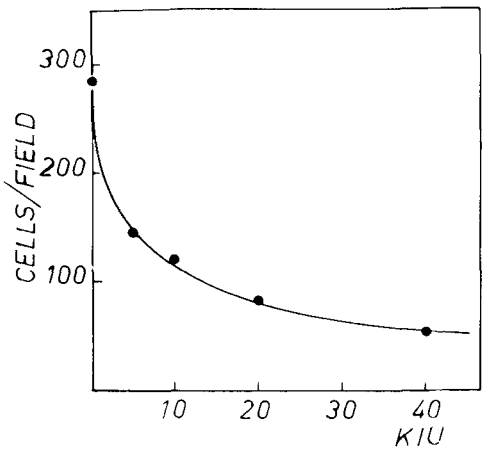

Fig. 5. Dose response inhibition of the chemotactic activity generated by kallikrein with the pancreatic trypsin inhibitor Trasylol. The enzyme (240 U) and different concentrations of Trasylol were preincubated for 45 min at 25 °C.

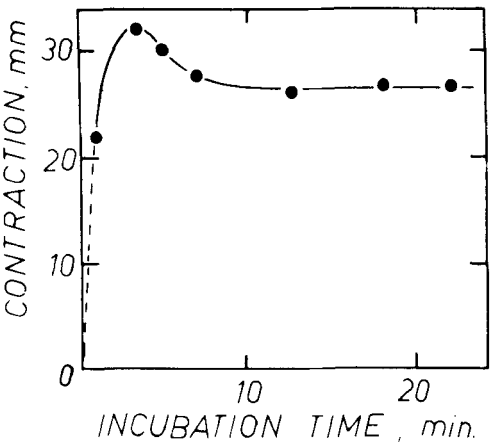

Fig. 6. The effect of incubation time between kallikrein and kininogen on the generation of kinin at 37 °C. Aliquots were removed at various time and assayed in the uterus-bath solution (8.0 ml).

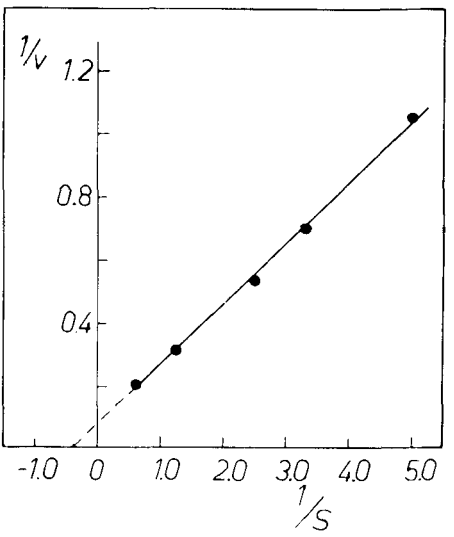

Fig. 7. Lineweaver-Burk plot for the kinin releasing activity of saliva kallikrein. The enzyme (120 U) was preincubated with different concentrations of dog plasma kininogen at pH 7.8 for 4 min at 37 °C. Aliquots were removed and assayed in the uterus-bath solution (8.0 ml). The heights of contraction was registered and kininogenase activity expressed as ng bradykinin was calculated from the formula in Material and Methods. Abscissa = reciprocal of the kininogen concentration. Ordinate = reciprocal of the rate of kinin released.

release of kinin during the first minute of incubation period followed by a plateau. The maximal generation of kinin occurred after 4 min of incubation.

Kininogenase activity of kallikrein. The generation of kinin from different concentrations of the substrate kininogen by kallikrein is shown in Fig. 7. The enzyme was preincubated with the substrate for 4 min at 37°.

A Lineweaver-Burk plot for the enzyme shows that the reaction followed Michaelis-Menten kinetics. The parameter V_{max} for the kinin release by kallikrein was calculated to correspond to 11.2 μg bradykinin per mg of protein per minute.

DISCUSSION

In a previous study *Modéer* (1977) isolated one active kallikrein from human saliva with a two step affinity chromatography technique and determined some biochemical properties of the enzyme. The results of the present investigation indicate that purified saliva kallikrein

was chemotactic for rat PMN cells in the presence of 2% serum albumin (Fig. 3 and 4). *Kaplan et al.* (1972) showed that conversion of prekallikrein by the Hageman-Factor-fragments was associated with the appearance of chemotactic activity of the enzyme. Neither prekallikrein nor the Hageman-factor alone exhibited such property. The activated Hageman-factor could directly or through its prealbumin fragments convert prekallikrein (*Kaplan & Austen*, 1970). This mechanism is independent of the complement system. A possible explanation of why saliva kallikrein needs the presence of serum albumin to exhibit chemotactic activity towards PMN cells may be that the enzyme preparation used contained prekallikrein which had to be converted to kallikrein by albumin fragements or perhaps the enzyme itself is influenced by albumin fragments. This requirement deserve further studies. The studies of human saliva kallikrein so far show that saliva contains one active kallikrein (*Moriya et al.*, 1966; *Fujimoto et al.*, 1973; *Modéer*, 1977). The capacity of trasylol to inhibit the chemotaxis shows that the chemotactic activity is associated with the active site of kallikrein (Fig. 5).

When the reciprocal of the contraction of the uterus was plotted against the reciprocal of the amount of bradykinin added to the organ bath a straight line was obtained (Fig. 2). Such a «double reciprocal» plot has the advantage that the kinin releasing activity by kallikrein could be more accurately determined than from the interpolation of the standardization curve (Fig. 1). Using ovarieectomized rats and the formula to calculate the activity of kallikrein, the activities could be compared between the different experiments. There was good reproduction using different uteruses.

The kinin generating capacity of kallikrein was studied at different substrate concentration of kininogen (Fig. 7). Maximal release of kinin was calculated to correspond to 11.2 μg bradykinin per mg enzyme per minute. *Hial, Diniz & Mares-Guia* (1974) found the corresponding value for humans urine kallikrein to be 12.5 μg. Since a commercial kininogen was not available the comparison of kinin releasing activities by different kallikreins was difficult. A high and a low molecular weight kininogen have been isolated in highly purified form from human plasma by *Habal, Movat & Burrowes* (1974). There is a well defined functional difference between the two kininogens with respect to the rate by which kinin is generated by plasma kallikrein. The rate of kinin release from the high molecular weight kininogen was faster than from the low molecular weight kininogen. In this study saliva kallikrein generated kinin from kininogen rapidly (Fig. 6) which indicates that kinin to the main part is released probably from the high molecular weight kininogen.

It is still unclear in what way the kinin system is involved in gingival or periodontal disease but *Kroeger & Weatherred* (1966) postulated that kallikrein – plasma kinin system was involved with the inflammatory process of acute periodontitis. *Ito et al.*, (1973) suggested that bradykinin, acetylcholine and catecholamines all cause a release of certain vaso active factors which may regulate the gingival flow. Various types of evidence suggest that glandular kallikrein also play a role in the regulation of blood flow in the salivary glands (*Gautvik*, 1970).

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