Characterization of kallikrein from human saliva isolated by use of affinity chromatography

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Kallikrein was isolated from paraffin stimulated saliva by use of two steps affinity chromatography. A 610-fold purification of the enzyme was achieved by use of Sepharose 4B conjugated with soy bean trypsin inhibitor and pancreatic trypsin inhibitor. The isoelectric point of kallikrein was found to be pH 4.3 and the molecular weight was calculated to be about 38.000 by gel filtration on a AcA 44 Ultrogel column. The enzyme had a pH optimum at pH 8.6 using BAEE and TAME as substrates. The michaelis constant for kallikrein on these two substrates was $4.0 \cdot 10^{-4}$M and $5.5 \cdot 10^{-4}$M. respectively.

Key-words: Biochemistry; physiology

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Kallikreins are proteolytic enzymes included in the group called kininogenases (EC 3.4.4.21). They are endogenous enzymes which liberate vasoactive peptides named kinin from plasma precursors called kininogens (*Webster*, 1970). Kinin has also been reported to increase vascular permeability, produce pain and modify the migration of leucocytes (*Kellermeyer and Graham*, 1968).

Multiple forms of kallikrein have been demonstrated in human plasma *Colman, Mattler & Sherry* (1969), *Eisen and Glanville* (1969) and also in human urine. (*Hial, Diniz & Mares Guia*, 1974, *Silva, Diniz & Mares Guia*, 1974). *Hilton & Lewis* (1957) *& Lewis* (1959) demonstrated that many glandular secretions contained a plasma kininforming enzyme. The presence of kallikrein in human saliva has been demonstrated by *Moriya et al.* (1965), *Liebermann & Littenberg* (1969), *Wotman, Greenbaum & Mandel* (1969).

It has been suggested that the enzyme may be involved in the regulation of blood flow in the salivary glands (*Hilton & Lewis*, 1956, *Gautvik*, 1970).

The method of affinity chromatography has been applied for the purification of hog and human serum kallikrein (*Fritz Wunderer & Dittmann*, 1972, *Fritz & Förgbrey*, 1972) and the isolation of trypsin-like enzymes from human saliva *Söder & Modéer* (1977). Recently *Fujimoto et al.* (1973 a, b,) purified kallikrein from human saliva and determined some properties. However, the purification proce-

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dure involved a great number of laboratory steps.

The present communication deals with the isolation of kallikrein from human saliva by use of affinity chromatography and the determination of some biochemical characteristics. The biological properties of the kallikrein will be reported in another paper (*Modéer*, 1977).

MATERIAL AND METHODS

Saliva

Paraffin stimulated saliva was collected from each of 5 men and 3 women (22–30 years old). 3–4 hours after oral hygiene the subjects had to rinse their mouths before the collection procedure. The saliva samples were pooled after the collection (1300ml) and centrifuged at 10.000 g for 15 min at 4 °C. The sediment was washed twice with 0.05 M tris HCl buffer pH 8.1 and centrifuged. The supernatants were pooled (1500 ml). This pool was concentrated to 134 ml 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.

Enzyme assays

Kallikrein activity was tested towards following substrates: α-N-benzoyl-L-arginine ethyl-HCl (BAEE) (Sigma Chemical Company, St. Louis, Mass, USA). (*Ketzdy, Lorand & Miller*, 1965), p-tosyl-L-arginine methyl ester HCl (TAME) (Nutritional Biochemical Corp., Cleveland, Ohio, USA) (*Hummel*, 1959), L-lysine-p-nitroanilide dihydrobromide (LPA), α-N-benzoyl-L-arginine-p-nitroanilide (BAPA) (Nutritional Biochemical Corporation) (*Erlanger, Kokowsky & Cohen*, 1961) and N-acetyl-L-tyrosine ethyl ester-HCl (ATEE) (Sigma). The enzyme hydrolysis of ATEE was measured at 237 nm. The reaction mixture consisted of 3.0 ml of 1 nM ATEE in 0.2 M tris HCl buffer pH 8.1 (containing 0.02 M $CaCl_2$) and 0.20 ml enzyme solution. The activity was expressed as μmoles of substrate converted per minute (*Patat & Hirsch*, 1966). The enzyme hydrolysis of BABA was measured at 410 nm in an assay system like LPA described previously (*Lindqvist et al.*, 1974). The other assay systems have been described earlier (*Lindqvist et al.*, 1974).

Preparation of the gels

The cyanobromide activated Sepharose 4B gel (Pharmacia, Fine Chemicals, Uppsala, Sweden) was prepared according to the instructions from the manufacturer. Pancreatic trypsin inhibitor (Iniprol) (PTI) (Laboratoire Choay, Paris) at a concentration of 200,000 units/ml and soybean trypsin inhibitor (STI) (3x cryst.) (Worthington Biochemical Corporation, Freehold, N.J., USA) at a concentration of 2 mg/ml was dissolved in 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl pH 9.0 and mixed with cyanobromide activated Sepharose 4B (1 gr Sepharose/10 mg STI or 600,000 units Iniprol) for 2 hours at 25 °C. Unbound inhibitor was washed away with the coupling buffer and to block any remaining active groups the gels were mixed with 1.0 M ethanolamine. Non-covalently bound material was removed by washing three times each with 0.1 M acetate buffer pH 4,0 containing 1,0 M NaCl and 0.1 M borate buffer, pH 9.0 containing 1.0 M NaCl.

Purification procedure

Affinity chromatography was used according to the method of *Porath & Sundberg* (1970). The prepared fraction was first introduced into a STI-Sepharose 4B column (3.0 x 4.0) cm at 4 °C to remove the trypsin-like enzymes (*Söder & Modéer*, 1977). The non-adsorbed eluate was then introduced into a PTI-Sepharose 4B column (3.0×2.0 cm), equilibrated with 0.05 M tris-HCl, buffer pH 8.1 containing 0.05 M NaCl. Before desorption the column was washed with the buffer mentioned above and then with 0.05 M tris-HCl buffer pH 8.1 containing 0.1 M NaCl, until the effluent was free from UV adsorbing material. Desorption was effected by 0.05 M glycin-HCl buffer pH 3.6

containing 0.1 M NaCl and 0.02 M $CaCl_2$. The pH of the effluent was adjusted to around pH 8. The absorbance at 260 and 280 nm was measured on all fractions with a Zeiss Spectrophotometer PMQ II and the protein content was determined. Before electrofocusing, the material was dialysed against distilled water.

Isoelectric focusing

Isoelectric focusing according to *Vesterberg & Svensson,* (1966) was performed in an Ampholine column 8100 (LKB, Bromma, Sweden) at 12–14 °C for 48 hours. The pH of the fractions were determined by using a pH-meter type PHM 28 (Radiometer, Copenhagen, Denmark). Other data are given in the figure legends.

pH optimum and stability

The effect of pH on the enzymatic hydrolysis of BAEE and TAME was determined in a range of pH 5–9 using 0.2 M acetate-tris-glycine buffer. The substrate at the appropriate pH was used as a blank and buffer was added instead of enzyme.

To study pH stability the enzyme was incubated for 30 min at 25 °C at different pH values from 2.5 to 9.5. At the end of the incubation period the enzymatic activity remaining was determined using BAEE at pH 8.5.

Heat inactivation was determined by incubating kallikrein at 25 °, 40 °, 50 ° and 60 °C for different periods of time up to 30 min.

Influence of various inhibitors and metal ions

The following substances were tested as inhibitors: Ethylene-diaminetetraacetic acid disodium salt (EDTA), L + cysteinumchlorid, acetic acid anydride (Merck A.G.), iodobenzoic acid (Hopkin and Williams LTD, Chadwell Heath, Essex, England), ovomucoid trypsin inhibitor, lima bean trypsin inhibitor, soybean trypsin inhibitor, p-aminobenzamidine, p-chlormercuribenzoate (PCMB), N-α-tosyl-L-lysine chloromethyl ketone TLCK) and N-tosyl-L-phenylalaline chloromethyl ketone (TPCK)(Sigma).

Following divalent metal ions were tested: Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+} (added as chlorides).

The final concentrations of the above mentioned substances were 10^{-3}M, $2 \cdot 10^{-4}$M and $4 \cdot 10^{-5}$M. Lima bean trypsin inhibitor and ovomuciod trypsin inhibitor were used at final concentrations of $5 \cdot 10^{-3}$%, $1 \cdot 10^{-3}$% and $2 \cdot 10^{-4}$% (w/v).

Molecular weight

Determination of molecular weight of the kallikrein was estimated by gel filtration experiments at 4 °C. A column of Ultrogel AcA 44 (LKB, Stockholm, Sweden) (1,93 x 76 cm) was equilibrated and eluted with 0.05 M tris HCl buffer pH 8.1 containing 0.1 M NaCl. The void volume (V_o) of the column was taken as the elution volume of Blue Dextran. The total volume (V_t) of the column was taken as the elution volume of 2,4-DNP-alanine. The column was calibrated with myoglobulin (mol wt 17,800) chymotrypsinogen A (6 x cryst.) (mol. wt 25.000), ovalbumin (mol wt 45.000) and bovine serum albumin (mol wt 67.000) (Sigma) as reference proteins. The partition coefficient (K_{av}) was calculated for the proteins *Laurent & Killander,* C1964).

Kinetic determinations

Michaelis-Menten constants (K_m values) were derived from Lineweaver-Burk diagrams. For these estimations BAEE and TAME were used as substrates at concentrations between 0.1 nM to 1.6 mM at 25 °C.

RESULTS

Isolation

The trypsin-like enzymes adsorbed to STI-Sepharose could be eluted by an abrupt change of the eluant buffer. (Fig. 1A). The

kallikrein from the nonadsorbed fraction was adsorbed to PTI-Sepharose and released also by an abrupt change of the eluant buffer to 0.05 M glycin-HCl, 0.1 M NaCl, 0.02 M $CaCl_2$. (Fig. 1B). The kallikrein activity measured as hydrolysis of BAEE appeared in tubes numbered 6–17. From table 1 it can be seen that the trypsin-like enzymes eluted from STI-Sepharose contributed to 11% of the TAME hydrolytic activity found in the applied saliva supernatant, but only 0.03% of the protein content. After chromatography of the nonadsorbed fraction on PTI-Sepharose around 35% of the BAEE activity in the saliva supernatant was recovered, which represented kallikrein activity. The specific activity of kallikrein was increased 610 times by the technique used. (Table I).

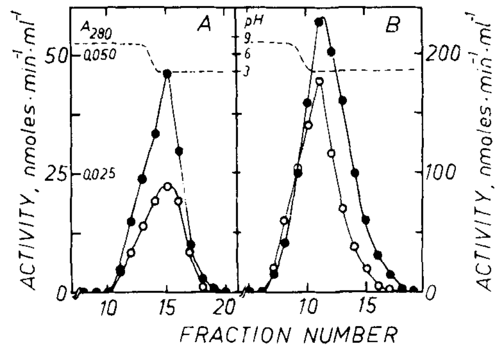

Fig. 1A. Affinity chromatography of human saliva (1300 ml). The saliva was centrifuged and the supernatant was concentrated to 134 ml. This fraction was applied on a STI-sepharose 4B column (4.0 x 3.0 cm).

Fig. 1B. 130 ml of the nonadsorbed fraction from Fig 1A was introduced on a PTI-sepharose 4B column (3.0 x 3.0 cm). The columns were washed with 0.05-M tris-HCl, 0.1M NaCl buffer, pH 8.1 before elution. Eluants were 0.05 M glycine-HCl, 0.1 M NaCl, 0.02 M $CaCl_2$ buffer pH 3.6 marked with arrows in the figure. Flow rate 12 ml/h. Fraction volume 3.0 ml.
The enzyme activity of the eluted fractions was assayed on BAEE at pH 8.2 for STI and at pH 8.5 for PTI (–•–). The absorbance at 280 nm (–o–). The pH of the effluent = - - - - - -

Isoelectric focusing

The isoelectric pattern of pooled fractions from Fig. 1B is shown in Fig. 2. Only one enzymatic peak was found with the pI 4.3.

pH optimum

The optimum for the hydrolysis of both BAEE and TAME by kallikrein was found to be pH 8.6 (Fig. 3). At pH values lower than 6.0 there was no hydrolysis of these substrates.

Stability

The kallikrein was found to be stable over a pH range between pH 7 to 9 (Fig. 4). At lower pH values there was a slow decrease of enzyme activity; as much as 80% of the initial enzyme activity remained after exposure to pH 4.0 for 30 min.

There was no significant loss of activity when the enzyme was incubated for 30 min at 25 °C, 40 °C, 50 °C and 60 °C (data not shown).

The kallikrein could be stored at 4 °C, pH 8 for several weeks without significant loss of activity.

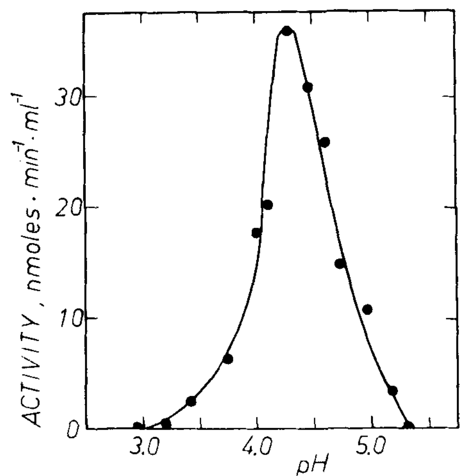

Fig. 2. Isoelectric focusing of kallikrein on a 110 ml column. Range of carrier ampholine (50% pH 3–10 and 50% pH 5–7). Time for isoelectric focusing: 60 hr, voltage 500 V, current ~1.0 mA. The kallikrein activity was assayed on BAEE at pH 8.5 (–•–).

Table I. *Purification of kallikrein from human saliva (1300 ml). One unit (U) = 1 nmole of BAEE or TAME converted per ml per minute. The enzyme activity was determined at pH 8.2 for STI eluted fractions and at pH 8.5 for PTI fractions.*

Fraction	ml	A_{280}	BAEE	TAME	BAEE	Yield %	
			U	U	U/A_{280}	BAEE	Protein
Concentrated saliva supernatant	134	7.9	9500	6550	9.0	100	100
Non-adsorbed fractions STI	134	6.0	7250	4750	9.0	87	85
Eluted fractions STI	24	0.014	560	730	1660	6	0.03
Non-adsorbed fractions PTI	130	5.0	3050	2300	4.7	35	69
Eluted fractions PTI	27	0.022	3050	1520	5500	32	0.06

Molecular weight

From the linear relationship between the elution volume of the reference proteins and the logarithm of their molecular weights, the approximate molecular weight of kallikrein was estimated to be 38.000 (Fig. 5).

Influence of various metal ions

The effect of various metal ions on the kallikrein is shown in Table II. From the table it can be seen that Co^2 had the strongest inhibitory effect at the concentration of $2 \cdot 10^{-4}$M. After treatment with 10^{-3}M Fe^{2+} and Zn^{2+}, 43% and 71%, respectively, of the activity remained. The same concentration of Hg^{2+} resulted in an inhibition of only 19%.

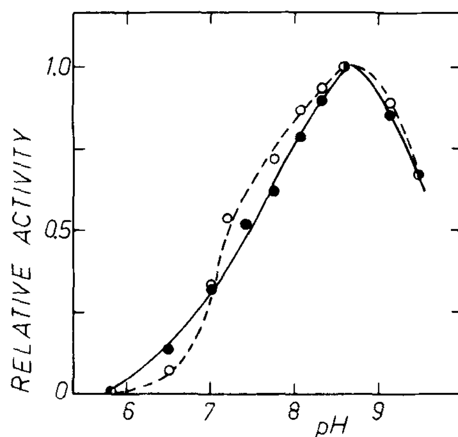

Fig. 3. The effect of pH on the hydrolysis of BAEE (–•–) and TAME (–o–) by saliva kallikrein.

Influence of various inhibitors

The effect of various inhibitors on the kallikrein is seen in Table III.

From the Table it can be seen that p-aminobenzamidine had the strongest inhibitory effect at the concentration of $2 \cdot 10^{-4}$M. After treatment with 10^{-3}M iodobenzoic acid, about 51% of the enzyme activity remained. Cysteine at a concentration of 10^{-4} M inhibited the enzyme so that 72% of the activity remained. Soy bean trypsin inhibitor had some inhibitory effect on the enzyme activity but lima bean trypsin and ovomucoid trypsin inhibitor had no effect. Incubation of the enzyme with 10^{-3}M TLCK resulted in a slight inhibition of the activity.

Kinetic determinations

Lineweaver-Burk plots of the initial rates of hydrolysis of BAEE and TAME by kallikrein is shown in Fig. 6. The linearity of these plots indicates that the reaction follows Michaelis-Menten kinetics. By extrapolation the K_m for kallikrein against TAME and BAEE at pH 8.5 is estimated to be $5.5 \cdot 10^{-4}$ M and

Table II. *Influence of metal ions on saliva kallikrein. The enzyme activity was assayed on BAEE at pH 8.5.*

Metal ions	Final conc.	Enzyme activity as % of control
Ca^{2+}	$4 \cdot 10^{-5}$ M	99
	$2 \cdot 10^{-4}$ M	100
	$1 \cdot 10^{-3}$ M	100
Mn^{2+}	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	99
	$1 \cdot 10^{-3}$ M	96
Mg^{2+}	$4 \cdot 10^{-5}$ M	99
	$2 \cdot 10^{-4}$ M	103
	$1 \cdot 10^{-3}$ M	104
Ni^{2+}	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	99
	$1 \cdot 10^{-3}$ M	99
Co^{2+}	$4 \cdot 10^{-5}$ M	92
	$2 \cdot 10^{-4}$ M	45
Cu^{2+}	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	90
Zn^{2+}	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	87
	$1 \cdot 10^{-3}$ M	71
Fe^{2+}	$4 \cdot 10^{-5}$ M	93
	$2 \cdot 10^{-4}$ M	74
	$1 \cdot 10^{-3}$ M	43
Hg^{2+}	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	93
	$1 \cdot 10^{-3}$ M	81
Cd^{2+}	$4 \cdot 10^{-5}$ M	98
	$2 \cdot 10^{-4}$ M	96
	$1 \cdot 10^{-3}$ M	92

$4.0 \cdot 10^{-4}$M, respectively. At the concentrations tested, there was no substrate inhibition on the reaction velocity.

Substrate specificity

The relative hydrolysis rates of BAEE, TAME, ATEE, BAPA and LPA by kallikrein at pH 8.5 are summarized in Table IV. The kallikrein readily hydrolysed BAEE. TAME was hydrolyzed at a slower rate and the hydrolysis of BAPA was very low. The enzyme did not hydrolyse LPA or ATEE.

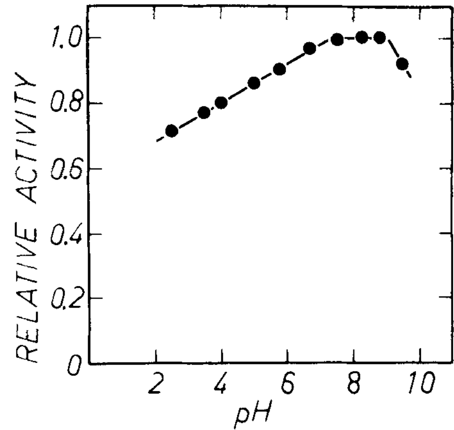

Fig. 4. The effect of pH on the stability of kallikrein. The enzyme was incubated for 30 min at 25 °C with buffer at different pH values and the enzyme activity remaining was determined on BAEE at pH 8.5 (-•-).

DISCUSSION

Trypsin-like enzymes have been separated from the total pool of proteolytic enzymes present in human saliva (*Söder & Modéer* (1977) in one step by use of affinity chromatography. In the present study kallikrein was extracted from the remaining proteolytic enzymes in human saliva supernatant by a two step affinity chromatography technique. In the first step the trypsin-like enzymes were removed by use of a STI-Sepharose 4B column (Fig. 1A and Table I).

Kallikrein was present in the nonadsorbed fraction (Table I) since glandular kallikreins normally not are inhibited by soy bean trypsin inhibitor (*Schachter*, X1969). In the second step the kallikrein could be isolated on a PTI-Sepharose 4B column using pancreas trypsin inhibitor as a substrate analogue inhibitor (Fig. 1B). Earlier investigators (*Moriya et al.*, 1965, *Fujimoto et al.*, 1973) measured the de-

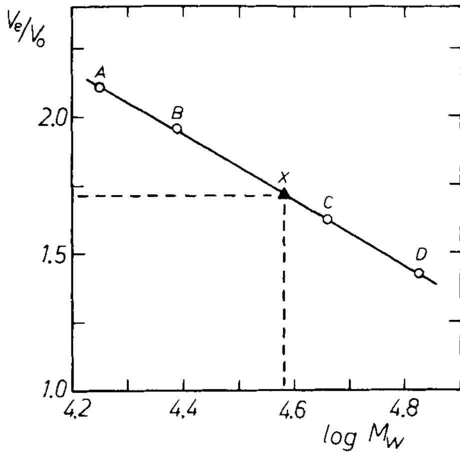

Fig. 5. Estimation of the molecular weight of saliva kallikrein by gel-filtration on AcA 44 Ultrogel column (1.93 · 76 cm).
Blue dextran was used as a marker for the void volume (V_0) and 2.4-DNP-alaine for the total volume (V_t).
Eluant was 0.05 M tris HCl, 0.1 M NaCl pH 8.0. Flow rate 10.0 ml/h. The comparison of the elution volume of these proteins and the void volume was plotted against the logarithm for their molecular weights.
A = myoglobulin, B = α-chymotrypsinogen, C = ovalbumin, D = bovine serum albumin and X = saliva kallikrein.

Table III. *Influence of various inhibitors on saliva kallikrein. The inhibitors TLCK, TPCK and PCMB were preincubated with the enzyme for 30 min at 25 °C. The rest of inhibitors were preincubated for 5 min. The enzyme activity was determined at pH 8.5.*

Substances	Final conc.	Enzyme activity as % of control
Ovomucid	$5 \cdot 10^{-3}$ %	100
trypsin	$5 \cdot 10^{-2}$ %	100
inhibitor	$1 \cdot 10^{-2}$ %	100
Lima bean	$5 \cdot 10^{-3}$ %	100
trypsin	$5 \cdot 10^{-2}$ %	100
inhibitor	$1 \cdot 10^{-2}$ %	96
Soy bean	$5 \cdot 10^{-3}$ %	93
trypsin	$5 \cdot 10^{-2}$ %	91
inhibitor	$1 \cdot 10^{-2}$ %	91
EDTA	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	97
	$1 \cdot 10^{-3}$ M	95
Cysteine	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	90
	$1 \cdot 10^{-3}$ M	72
p-amino	$4 \cdot 10^{-5}$ M	73
benzamidine	$2 \cdot 10^{-4}$ M	56
	–	–
Acetic	$4 \cdot 10^{-5}$ M	88
acide	$2 \cdot 10^{-4}$ M	88
anhydride	$1 \cdot 10^{-3}$ M	88
Iodobenzoic	$4 \cdot 10^{-5}$ M	89
acid	$2 \cdot 10^{-4}$ M	82
	$1 \cdot 10^{-3}$ M	51
TLCK	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	100
	$1 \cdot 10^{-3}$ M	93
TPCK	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	87
PCMB	$4 \cdot 10^{-5}$ M	100
	$2 \cdot 10^{-4}$ M	72

gree of purification of kallikrein in terms of its biological activity which was expressed as ng bradykinin. In the present study the purification of kallikrein was monitored by its esterase activity towards the syntetic substrates BAEE and TAME. The specific activity towards BAEE increased 610 times (Table I).

In human saliva only one kallikrein peak appeared after isoelectrofocusing (Fig. 2), which is in agreement with *Fujimoto et al.* (1973). However, they found a lower pI value around pH 4.0. *Ekfors et al.* (1967) isolated four forms of peptidases resembling kallikrein from rat submandibular gland. Also human urine kallikrein was shown to be microheterogeneous with three different pI values around pH 4.

Saliva kallikrein was found to be stable when exposed to a wide pH range; as much as 80% of the enzyme activity remained after 30 min at pH 4.0 (Fig. 3). *Zuber & Sache* (1974) reported that porcine pancreas kallikrein was stable down to pH 4.5. The molecular wight of the saliva kallikrein was calculated to be 38,000 (Fig. 4). This value is in agreement with

Table IV. *Substrate specifity of saliva kallikrein.*

Substrates	Conc. mM	Rel. enzyme conc.	pH	Temp.	Relative activity %
BAEE	1	1	8.5	25	100
TAME	1	1	8.5	25	46
ATEE	1	1	8.5	25	0
BAPA	1	2.5	8.5	37	0.02
LPA	1	2.5	8.5	37	0

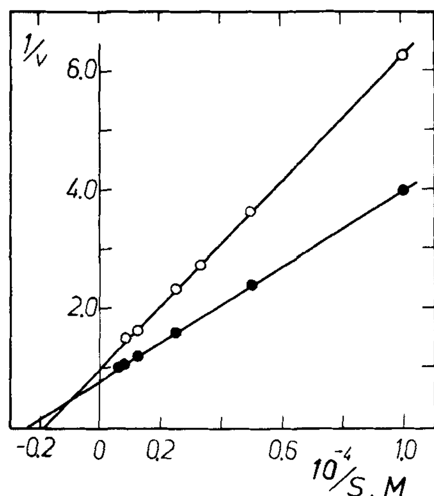

Fig. 6. Lineweaver-Burk plot for the hydrolysis of TAME (-o-) and BAEE (-•-) by saliva kallikrein at pH 8.5 and 25 °C.
Ordinate: reciprocal of rate.
Abscissa: reciprocal of molar concentration of substrate.

those of glandular kallikreins. The molecular weights of these kallikreins varied between 24,000 to 40,000 (*Ekfors et al.*, 1967, *Schachter*, 1969, *Fujimoto et al.*, 1973). The plasma kallikrein have a higher molecular weight around 100,000 (*Colman et al.*, 1969).

The pH-optimum of the saliva kallikrein on BAEE as well as TAME was found to be pH 8.6 (Fig. 5). With the same substrates *Fujimoto et al.* (1973) found the saliva kallikrein had a lower pH-optimum around pH 8.0. The K_m value of the enzyme for BAEE and TAME (Fig. 6) are similar to that found by *Fujimoto et al.* (1973). The ratio of the hydrolysis rate between BAEE and TAME was found to be 2.2 (Table IV). *Fujimoto et al.* (1973) calculated this ratio to be 0.75. The same ratio for serum kallikrein was 1.3 (*Habermann & Klett*, 1966).

The influence of metal ions on the kallikrein showed that Fe, Co, Zn, Hg and Cu acted as inhibitors (Table II). These metal ions have been reported also to inhibit kallikrein from porcine pancreas (*Zuber & Sache,*, 1974).

Neither ovomucoid nor lima bean trypsin inhibitor influenced saliva kallikrein (Table III). Soybean trypsin inhibitor had a very low inhibiting effect on the enzyme; the affinity was too low to permit an adsorption of the enzyme to a STI-Sepharose gel. (Fig. 2). In similarity to the trypsin-like enzymes in human saliva the kallikrein was inhibited by p-aminobenzamidine (*Söder & Modéer*, 1977). The trypsin inhibitor TLCK reduced the enzyme activity slightly (Table III). This effect may depend on the presence of histidine in the active site which is more distant from the center than in trypsin. The saliva kallikrein was also affected by Cysteine (Table III), which indicates the presence of cysteine in the active site.

The biological effect of kallikrein by the generation of kinin from plasma precursors kininogen is reported in a separate paper.

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