Acta Dermatovener (Stockholm) 50: 321–326, 1970

HUMAN SWEAT KALLIKREIN

Biochemical Demonstration and Chromatographic Separation from Several Other Esteropeptidases in the Sweat

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Abstract. Human thermal sweat from healthy young males in a sauna bath was collected, concentrated by ultrafiltration and fractionated by using gel filtration. Five esteropeptidases were identified in the chromatographic fractions by using BAEE, BAPA and BANA as substrates. One of the enzymes was characterized by pH-optimum at 8.0, preferential hydrolysis of BAEE, independence of sulfhydryl groups and by strong inhibition in the presence of Trasylol®. This enzyme represents human sweat kallikrein. One of the other enzymes was an alkaline esteropeptidase hydrolysing BAPA, while the three others were acidic esteropeptidases of the cathepsin B type. None of these acidic enzymes was significantly inhibited by Trasylol®.

On the basis of biological assays, Fox & Hilton (1) demonstrated the release of vasoactive substances from dog pseudoglobulin fraction by a human sweat preparation. This finding suggests the presence of kinin forming enzyme(s) i.e. kallikrein(s) in the human sweat. However, no direct enzymatic proof for the existence of kallikrein in human sweat has ever been presented, nor have any efforts been made to purify the enzyme or to characterize it chemically. The use of synthetic kallikrein substrates, e.g. benzoylarginine ethyl ester (BAEE) and tosylarginine methyl ester (TAME), would serve as a useful method for the identification and tracing of kallikrein-like enzymes to allow a more thorough study of these enzymes in the sweat.

We wanted to find out whether these substrates are split by human sweat, and further, to separate the enzymes active toward these substrates in order to characterize them chemically. This kind of study could be hoped to answer the question of the presence of kallikrein(s) in human sweat as well as to allow a differentiation of the enzymes from other possible trypsin-like enzymes present in sweat.

MATERIAL AND METHODS

Collection of sweat. Thermal sweat was collected from young males (aged 22–35) in Finnish sauna bath at 80°C, essentially as made by Page & Remington (11). The test persons were sitting in plastic bags for 20–30 min and the sweat from the whole body was collected on the bottom of the bag, which was kept in crushed ice. For protection of the possible SH-dependent enzymes, 1 ml of 20 mmol/l cystein solution was present in the plastic bags. All of the test persons were thoroughly washed using soap and water, immediately prior to sweat collection. The usual amount of sweat collected was 200–300 ml per person.

Pretreatment and concentration of the sweat. Sweat sample was passed through a Millipore® filter (AAWP 07040 25 ea 0.8 μm) in order to remove particle material, dead cells etc. Because of the low protein concentration of sweat (0.25–0.5 mg/ml) the sweat was concentrated 20–30 fold using ultrafiltration through Diaflo® membrane UM-10 which retains molecules with molecular weight higher than 10 000. The opalescent concentrated sweat was again filtered through the Millipore® filter, and the clear filtrate used for analysis. The essential steps of the procedure are summarized in Fig. 1.

Substrates, modifiers and buffers. Nα-benzoyl-L-arginine ethyl ester·HCl (BAEE), Nα-benzoyl-DL-arginine *p*-nitroanilide·HCl (BAPA) and Nα-benzoyl-DL-arginine β-naphtylamide·HCl (BANA) were obtained from Sigma Chemical Company (Missouri USA). All of the substrates were dissolved in deionized distilled water (or in buffer), BAEE at 10 mmol/l, BAPA at 1 mmol/l and BANA at 5 mmol/l.

Kallikrein inhibitor Trasylol® from beef lung (Bayer, Leverkusen, BRD) was added in an amount of 100 IU/ml into the assay system in studies on the inhibition of the enzyme preparations by natural kallikrein inhibitor. *p*-Chloromercuric benzoate (Fluka AG, Switzerland), L-cystein·HCl (Nutritional Biochemical Company, Cleve-

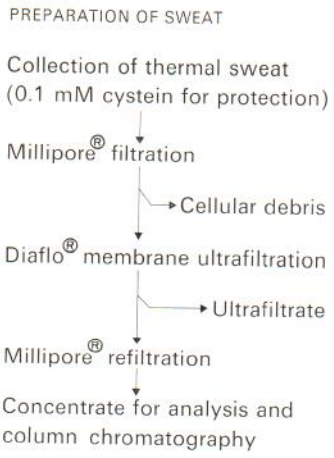

Fig. 1. Summary scheme of the methods for collection and pretreatment of sweat prior to analysis.

land, Ohio) and ethylenediamine tetraacetic acid (EDTA, E. Merck AG, Darmstadt, BRD) were added to the assay system to obtain a final concentration of 0.1, 1.0, and 10 mmol/l, respectively. In all experiments on affector substances, the affector was preincubated with the enzyme preparation for 10 min, before adding the substrate.

Universal buffer of Britton & Robinson (12) pH 3.0–12.0 was used throughout the study, except that Tris-HCl buffer (0.01 mol/l pH 7.0) was used in the column chromatographies.

Enzyme and protein assays. All incubations were carried out in a water bath at 37°C. The incubation mixture consisted of buffer 100 μl, substrate solution 100 μl and enzyme sample 100 μl. In studies on the effects of various modifying ions, the modifier was included in the buffer or in the enzyme sample. Hydrolysis of BAEE was assayed according to Hestrin (4) measuring the colour intensity at 540 nm using a Beckman DB spectrophotometer. For assay of the hydrolysis of BAPA the amount of liberated *p*-nitroaniline was measured at 383 nm after the addition of 200 μl of 1 mol/l acetate-HCl buffer, pH 4.2. Hydrolysis of BANA was measured using diazonium salt Garnet GBC in 1 mol/l acetate-HCl buffer containing 10% of Tween 20 (6). However, in assays with reducing substances in the incubation mixture, Erlich reagent was used (7).

Incubation times varied for BAEE 0.5–5 hours, for BAPA 1.5–10 hours and for BANA 2–10 hours. When required, protein was removed using precipitation with 10% zinc sulphate followed by centrifugation, prior to photometry.

Protein concentrations were usually determined according to Lowry et al. (8), using bovine serum albumin (Sigma) as standard. Protein distribution in the column chromatography fractions was estimated using absorbancy at 280 nm.

Column chromatography. Samples of 5 ml of concentrated sweat (total protein content 30–40 mg) were added to a column (2 × 52 cm) filled with Sephadex® G-100 gel, pretreated according to the manufacturers' orders (Pharmacia, Uppsala, Sweden). Tris-HCl buffer (0.01 mol/l, pH 7.0) containing ethylenediamine tetraacetic acid 0.1 mmol/l, was used for elution. Elution rate was 20 ml/hour and fractions of 2.5 ml were collected at +4°C.

RESULTS

Hydrolysis of the substrates by the concentrated sweat. All of the tested substrates were hydrolysed readily by the human sweat concentrate. As shown in Fig. 2 the optimal hydrolysis of BAEE took place on the acidic side of neutrality (optimally at pH 6.0), with a lower optimum at alkaline pH, somewhere around pH 8.0. The hydrolysis of BAPA, on the other hand, took place exclusively at alkaline pH, with an optimum at pH 8.0. The rate of hydrolysis of BANA was considerably lower, and showed two equally high optima, one acidic (pH 5.5) and one alkaline (pH 8.0) as seen in Fig. 2. It is to be noted, however, that the pH-curves obtained from samples collected on differ-

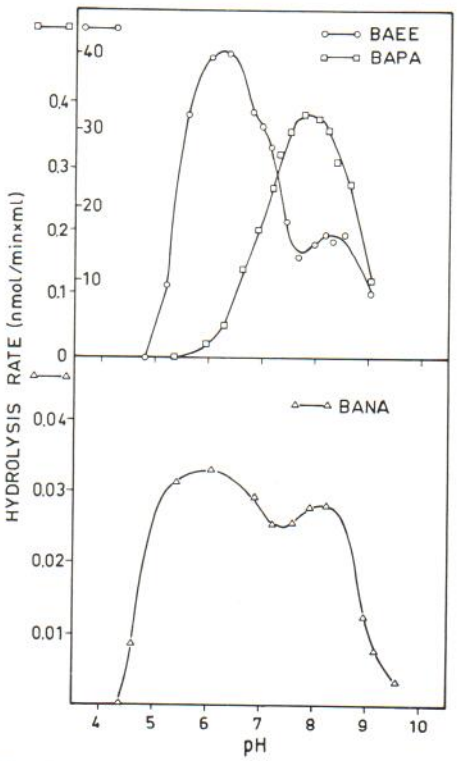

Fig. 2. Dependence on pH of the hydrolysis of BAEE, BAPA and BANA by the concentrated preparation of human sweat. Experimental conditions as given in Material and Methods.

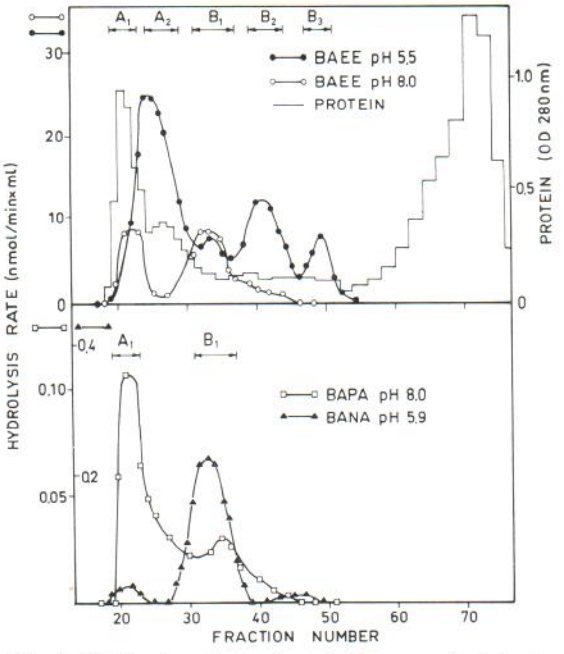

Fig. 3. Distribution of protein and of enzymes hydrolysing BAEE, BAPA and BANA by the chromatographic fractions on the Sephadex® G-100 column run. Experimental conditions as given in Material and Methods.

ent occasions showed some variation in the form of the pH-curves, especially the hydrolysis of BANA at acidic pH often showing low values.

Fractionation of the enzymic activities

The result of the fractionation of the human sweat concentrate on the Sephadex® G-100 gel filtration column is shown in Fig. 3. Two protein peaks containing large molecules (peaks in fractions 31 and 27) and one low molecular weight peak (fraction 70) were obtained. Enzymes hydrolysing the tested substrates were eluted in several fractions and several separate activity peaks could be repeatedly identified. Enzyme assay at acidic pH with BAEE as substrate revealed four separate enzymic peaks designated as A_2, B_1, B_2 and B_3 (see Fig. 3). When assaying the hydrolysis of BAEE at alkaline pH, a peak of hydrolysis in the fractions of peak B_1 was obtained, and in addition, an activity was recorded coincidental with the first main protein peak. This activity peak was named A_1. Hydrolysis of BAPA at pH 8.0 took place preferentially in the fractions of peak A_1, and to a considerably lower degree in the fractions of peak B_1, while the reverse was true for the hydrolysis of BANA when assayed at pH 5.9 (Fig. 3). When assayed at alkaline pH, the curve obtained with BANA was identical to that obtained for alkaline hydrolysis of BAPA, while the hydrolysis of BAPA in acidic conditions was minimal in all fractions. The active fractions were pooled as shown in Fig. 3 to obtain enzyme preparations A_1, A_2, B_1 and B_2, which were characterized further.

pH-Optima of enzymic activities in the pooled preparations

pH-Curves for the hydrolysis of BAEE, BAPA and BANA by the pooled enzyme preparations are presented in Figs. 4 and 5. As can be seen in Fig. 4, hydrolysis of both BAPA and BAEE by pool A_1 showed a pH-optimum of about 8.0. Hydrolysis of BAEE by A_1 was additionally recorded at pH 5.5, i.e., the same optimum as recorded for BAEE by pool A_2. The enzyme active at pH 5.5 is to be considered characteristic for

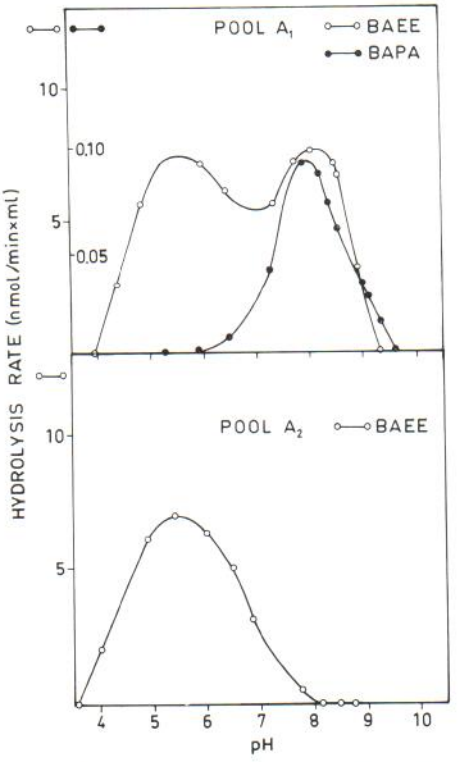

Fig. 4. Dependence on pH of the hydrolysis of BAEE and BAPA by the pooled enzyme preparations A_1 and A_2 of the Sephadex® G-100 chromatography run. Experimental conditions as given in Material and Methods.

pool A_2, and its presence in pool A_1 is probably due to incomplete separation of the two enzyme peaks (cf. Fig. 3).

The hydrolysis of BAEE by enzyme preparation B_1 was maximal at about pH 8.0 (Fig. 5). In addition, another pH-optimum for BAEE, as well as for BANA was demonstrable at about pH 5.5. Hydrolysis of BAEE by pool B_2 was recorded mainly in the acidic pH-range, optimally at about pH 5.5. The low activity recorded at alkaline pH may be contamination from pool B_1. Pool B_3 was not characterized because of its low and variable activity in different chromatographies. Thus the pH-optima characteristic for the above enzyme preparations were as follows: 8.0 for A_1, 5.5 for A_2, 8.0 and 5.5 for B_1 and 5.5 for B_2.

Substrate specificity and modifier characteristics of the enzyme preparations

The rates of hydrolysis of the different substrates at their optimum pH are given in Table I. It appeared that BAEE was the substrate hydrolysed most rapidly by all the enzyme preparations. A fairly good hydrolysis rate of BAPA was characteristic of enzyme preparation A_1 and hydrolysis of BANA at acidic pH was characteristic of enzyme preparation B_1. Enzyme preparations A_2 and B_2, on the other hand, showed only minimal hydrolysis of these peptide-type substrates.

Table I. *Hydrolysis rates of various substrates (nmol/min · ml)*

m = minimal, i.e. <0.05 nmol/min · ml

Substrate	Enzyme preparation (pH of incubation)				
	A_1 (pH 8.0)	A_2 (pH 5.5)	B_1 (pH 5.5)	B_1 (pH 8.0)	B_2 (pH 5.5)
BAEE	8.4	25.0	8.1	7.6	12.0
BAPA	0.1	m	m	m	m
BANA	m	m	0.2	m	m

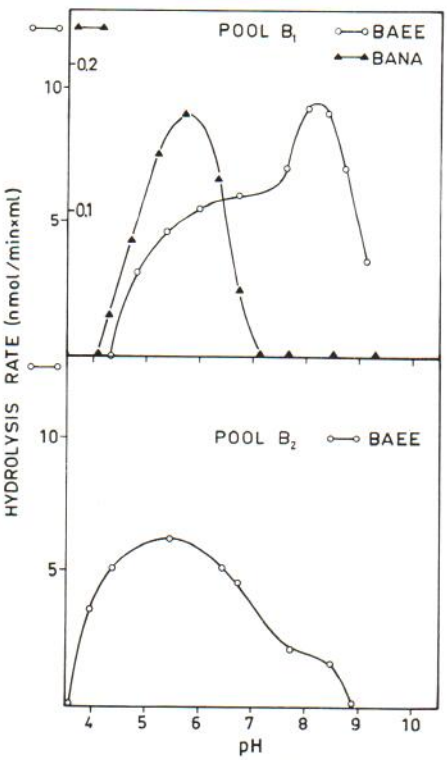

Fig. 5. Dependence on pH of the hydrolysis of BAEE and BANA by the pooled enzyme preparations B_1 and B_2 of the Sephadex® G-100 chromatographic run. Experimental conditions as given in Material and Methods.

The dependence of the enzymatic activities on sulfhydryl groups was assayed by measuring the inhibition caused by the presence of *p*-chloromercuric benzoate (0.1 mmol/l), as well as measuring the activation caused by the presence of cysteine and EDTA (1.0 mmol/l). It appeared that the enzymes optimally active at alkaline pH were not affected by these measures while enzymes active at acidic pH were clearly dependent on intact SH-groups.

The effect of the well known kallikrein inhibitor Trasylol® was tested at a concentration of 100 IU/ml. A total inhibition of the enzyme active at alkaline pH present in preparation B_1 was re-

Table II. *Summary of the characteristics of the pooled enzyme preparations*

Characteristic	Enzyme preparation				
	A_1	A_2	B_1	B_1	B_2
pH-Optimum (approx.)	8.0	5.5	5.5	8.0	5.5
Type substrate	BAPA	BAEE	BANA	BAEE	BAEE
SH-dependence	–	+	+	–	+
Inhibition with Trasylol®	Minimal	Minimal	Minimal	Strong	Minimal

corded, while only a minimal or no inhibition of the other enzymes was observed.

The most essential characteristics of the enzymes identified in this study are summarized in Table II.

DISCUSSION

Sweat analysed by us should contain the main part of the kallikrein activity secreted in the sweat during thermal stimulation in a sauna bath since it was demonstrated by Fox & Hilton (1) that kinin-forming activity reached a maximum 20–30 min after sweating began and then declined fairly rapidly. The collection of the sweat in the cooled corner of the collecting bag, kept in the crushed-ice bath, should ensure good preservation of the enzymes during the collection of the sweat. Some denaturation of the heat-labile enzymes may, however, have occurred. The presence of cysteine in the collection bags was found to be essential for preservation, especially of the enzymes active at acidic pH-values. The relatively low contribution of the acidic enzymes in the total hydrolytic enzyme pattern in the sweat found by us in preliminary experiments (2), made on sweat collected without cystein-protection, illustrates this fact. The enzymic activities found in human sweat, collected and pretreated as done in this work, are certainly to be considered as originating from the fluid secretions of the skin, and not derived from cellular or bacterial contamination. The ultimate site of origin of the enzymes found in the sweat, e.g. sweat gland or duct, sebaceous glands etc., however, remains to be elucidated.

Even while the enzyme activity of the trypsin-like esteropeptidases studied in this work are too small to be readily measured directly in the sweat, the activities in the concentrated sweat are high enough for ready estimation of the hydrolysis of chromogenic substrates and allow chromatographic separation of these enzymes. Simple pH-curves revealed that more than one trypsin-like esteropeptidase is present in the human sweat, a fact clearly proved by the partial separation of five distinct esteropeptidases of human sweat by column chromatography based on molecular sieving effect.

The three acidic esteropeptidases, present in enzyme preparations A_2, B_1 and B_2 were characterized by their pH optima of about 5.5 and by their dependence of sulfhydryl groups. The enzymological profile of these enzymes thus comes close to the cathepsin B category of enzymes. The enzyme present in peak B_1 which was characterized by hydrolysis of BANA possibly is similar to the so-called cathepsin B′ earlier described in bovine spleen (9, 10). None of these enzymes was inhibited by the kallikrein inhibitor Trasylol®. It may be, as suggested by Greenbaum & Yamafuji (3), that these enzymes are active in the conversion of zymogen (prekallikrein) to active kallikrein. Observations made by Fox & Hilton (1) on inactivation of the kinins formed by sweat during prologed incubation might be explained by the effect of some of these enzymes as well as by the effect of other proteolytic enzymes present in human sweat (to be published).

The enzyme in the pooled preparation A_1 appeared to be characterized by a slightly alkaline pH, capability of hydrolysis BAPA, and nondependence on sulfhydryl groups, as well as noninhibitability by kallikrein inhibitor Trasylol®. An enzyme possessing these characteristics is known to be present in human skin homogenate (13), and appears to resemble closely an enzyme demonstrated in human skin mast cells (5).

The second enzyme present in the B_1 preparation, characterized by an alkaline pH optimum, sulfhydryl independence, and lack of hydrolysis of the peptide type substrates, showed strong inhibition with the kallikrein inhibitor used. On the basis of these characteristics, which are those of glandular kallikrein, this enzyme can be considered to represent sweat kallikrein. The presence of this enzyme in the sweat could explain the findings on kinin-liberating factors of Fox & Hilton (1) carried out on unfractionated human sweat. A direct proof will be the demonstration of the liberation of kinins from physiological kallikrein substrates by the enzyme preparation. Preliminary experiments by us (to be published) on the biological activity of this enzyme preparation, in fact, support this conclusion.

The data presented in this paper definitely demonstrate the presence of several potent esteropeptidases in the human thermal sweat. They can be characterized by differences in their molecular size, pH-optima, substrate specificity and modifier characteristics. The site of origin and the significance in normal skin physiology as well as in pathophysiology of any one of the enzyme species remains to be explored.

ACKNOWLEDGEMENTS

Financial support was obtained as grants from the Sigrid Juselius Foundation and from the Finnish Medical Council to the senior author (V. K. H.-H.).

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Received April 15, 1970

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