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PLASMA-KININ-FORMING ENZYME IN HUMAN SKIN: EXTRACTION AND COLUMN CHROMATOGRAPHIC SEPARATION OF PLASMA-KININ-FORMING ENZYME AND ITS INHIBITOR

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Abstract. The optimal salt concentration for extraction of plasma-kinin-forming enzyme from the human skin was investigated. It was confirmed that a salt concentration higher than 1.5 M in the extraction buffer was required for optimal extraction of this enzyme. An inhibitor of this enzyme was detected in the extract with buffer alone, and the coexistence of this enzyme with the inhibitor in buffer extract was also confirmed by using G-200 gel chromatography.

Key words: Kinin; Kallikrein; Skin; α1-antitrypsin

It has been reported that plasma-kinin-forming (PKF) enzyme, or bradykinin, assumes the role of an endogenous mediator in some acute inflammatory reactions of the skin (1, 6, 9, 12, 14). Lewis extracted PKF enzyme from the skin of a rat and suggested that this enzyme may play a part in the inflammatory reaction to injury (8). Rocha e Silva & Rosenthal observed the appearance of histamine and a bradykinin-like agent after inducing burns to rat skin (12). The role of these enzymes in biological or pathological states of human skin, however, is not clear yet. In a preliminary report, the authors indicated that at least two enzymes could be extracted from normal human skin and that one of them released kinin from the heated human plasma, suggesting a kallikrein-like enzyme (15). Seppä et al. demonstrated that several proteases could be extracted from the human skin homogenate with a high salt concentration (13). It is also known that the human skin contains extractable inhibitors for exogenous proteases, i.e. trypsin, elastase (7), collagenase (2) and fibrinolytic enzymes (16). In this study, the extractability and chromatographic behaviour of PKF enzyme were investigated. In addition, an inhibitor of this enzyme was confirmed to be present in human skin.

MATERIALS AND METHODS

Extraction of PKF enzyme from human skin

Samples of human skin were obtained from patients who had undergone mammectomy. The skin was removed and cleaned absolutely free of subcutaneous fat, washed in cold de-ionized water and frozen at −20°C for 1–4 days. The frozen skin (15.4–32.7 g) was minced with scissors and homogenized with Virtis homogenizer (Virtis Co., Ltd.) in 10 volumes (w/v) of 0.1 M phosphate buffer, pH 7.4, containing potassium chloride (from 0 to 2 M). The homogenate was allowed to stand at 0°C overnight and then centrifugated at 4°C for 30 min at 15 000 *g*. The supernatant was filtered with glass fiber and dialysed for 3 days at 4°C with 0.1 M phosphate buffer, pH 7.4, which was changed four times daily. The dialysed filtrate was named HSE in this study.

Evaluation of PKF activity

The ability of samples to generate kinin from substrate was tested by bioassay, using isolated rat uterus suspended in a 2 ml organ bath in oxygenated de Jalon solution (28°C) containing atropine sulfate (10^{-7} M). One-tenth ml of the sample was incubated with 0.1 ml of substrate for 2 min at 37°C and the incubated mixture was tested on rat uterus. Suitable dilutions of the incubated mixture were prepared with de Jalon solution containing atropine sulfate at a concentration of 10^{-7} M. The kinin concentration in incubated mixture was determined by comparing the concentration it produced with that produced by kinin amounts of synthetic bradykinin standard (Sand Pharm Co., Ltd.)

Preparation of kininogen

Fresh heparinized human plasma (heparin 5 units/ml) was heated at 56°C for 3 hours according to the method of Eisen (3) and used as a substrate of PKF enzyme.

Gel-filtration

HSE, prior to gel filtration, was concentrated to one-tenth of its original volume by ultrafiltration. A 2.4×60 cm column of Sephadex G-200 was equilibrated with 0.1 M phosphate buffer at pH 7.4. Six ml of concentrated HSE was applied to the column which was run at a flow rate of 13 ml/hr, and 5 ml fractions were collected.

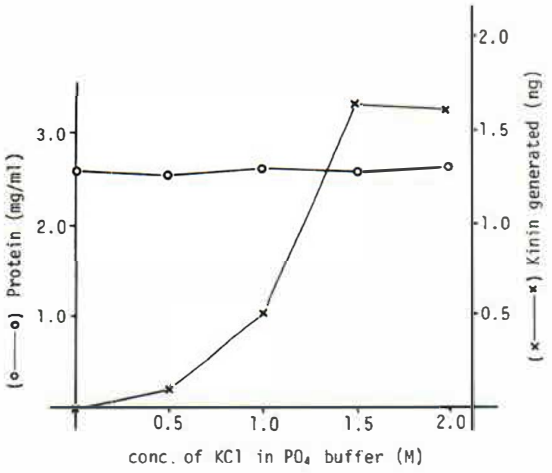

Fig. 1. Optimal salt concentrations for extraction of plasma-kinin-forming enzyme. The amount of proteins (O—O) and plasma-kinin-forming activity (×—×) in HSE extracted by buffer alone or buffer containing various concentrations of KCl. One-tenth ml of HSE was incubated with 0.1 ml of substrate for 2 min at 37°C and kinin generated was determined by bioassay. Each value represents the mean of five experiments.

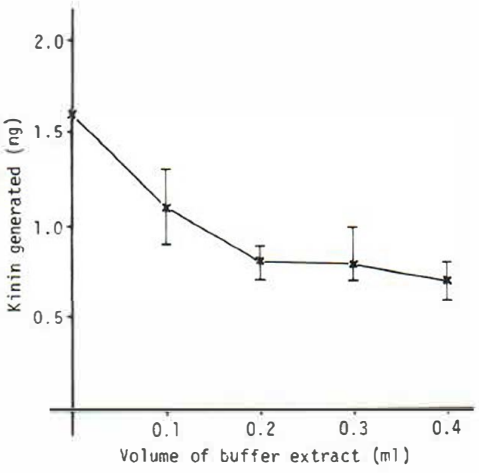

Fig. 2. The inhibitory effect of buffer extract towards plasma-kinin-forming enzyme. One-tenth ml of active HSE extracted with PO_4 buffer containing 2 M KCl was incubated for 5 min at 37°C with various amounts of inactive HSE extracted with buffer alone. The mixture was incubated with 0.1 ml of substrate for 2 min and tested on rat uterus. Each value represents the mean of three experiments.

Protein determination

The protein concentration was estimated according to the method of Lowry et al. (10) using bovine serum albumin (Merck Co., Ltd.) as reference protein.

RESULTS

Optimal salt concentrations for extraction of PKF enzyme

Seppä et al. reported that proteases of human skin could be extracted from homogenate with a high salt concentration (13). In order to determine the concentration of salt required for most effective extraction of PKF enzyme, the frozen and minced skin sample was separated into equal parts and each was homogenized with 0.1 M phosphate buffer at pH. 7.4 containing different concentrations of potassium chloride (from 0 to 2 M). Protein and PKF activity of HSE prepared as described under Materials and Methods were measured. As shown in Fig. 1, the protein concentration of the extract remained constant from 0 to 2 M concentrations of potassium chloride. A salt concentration higher than 1.5 M in the extraction buffer was required for optimal extraction of PKF enzyme. This enzyme, which can generate approximately 160 ng of kinin from substrate, was extracted with 0.1 M phosphate buffer containing a salt concentration higher than 1.5 M from 1 g of original skin. A salt concentration lower than 1.5 M gave a lower yield of enzyme, and HSE extracted with buffer alone did not possess PKF activity. It was also confirmed that none of the HSE caused contraction of the rat uterus, nor did they effect the contraction caused by a standard preparation of synthetic bradykinin.

An inhibitor of PKF enzyme in buffer extract

Lewis reported that PKF enzyme could be extracted from acetone-dried rat skin with a 2 M potassium thiocyanate solution and that some of the potassium thiocyanate extracts contained an inhibitor of PKF enzyme (8). It has been reported that the buffer extract of human skin homogenate contained inhibitors of proteases extracted from homogenates with high salt concentrations (4). In the present study, efforts were made to ascertain whether or not some inhibitors of PKF enzyme are present in HSE extracted with buffer alone. An active HSE extracted with 0.1 M phosphate buffer at pH 7.4 containing 2 M potassium chloride was incubated for 5 min at 37°C with various amounts of inactive HSE extracted with buffer alone. The

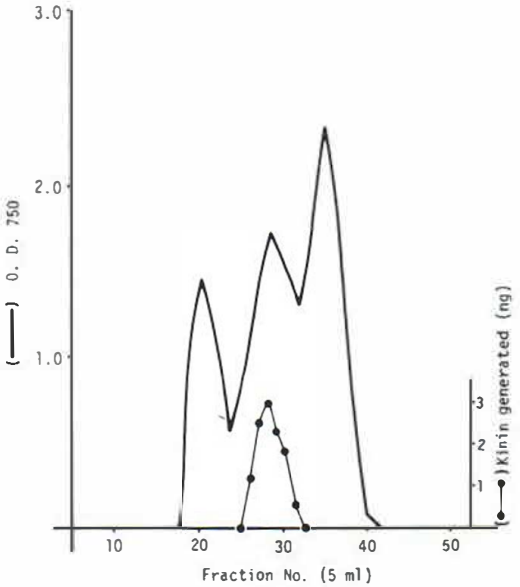

Fig. 3. Gel filtration of HSE extracted with PO_4 buffer containing 2 M KCl. One-tenth ml of each of the fractions obtained was incubated with 0.1 ml of substrate for 2 min at 37°C and kinin generated was determined by bioassay.

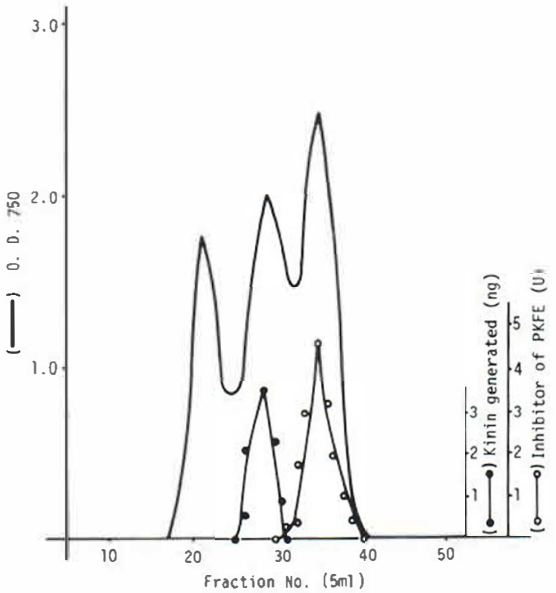

Fig. 4. Gel filtration of HSE extracted with PO_4 buffer alone (○—○). In order to determine the inhibitory activity of the fractions, 0.1 ml of the active HSE which can generate 2 ng of kinin from substrate was incubated with 0.1 ml of the fractions obtained for 5 min at 37°C. Then the mixture was incubated with 0.1 ml of substrate for 2 min at 37°C and kinin generated was determined by bioassay. One unit of the inhibitor was expressed as the amount of inhibitor necessary to completely inhibit 1 unit of plasma-kinin-forming enzyme (PKFE) and one unit of PKFE was defined as the amount of enzyme which can generate 1 ng of kinin from substrate. (●—●) The plasma-kinin-forming activity of the fractions was determined by the same method as that of HSE extracted with PO_4 buffer containing 2 M KCl.

mixture was incubated with 0.1 ml of substrate at 37°C for 2 min and tested on rat uterus. Fig. 2 shows that HSE extracted with buffer alone contains an inhibitor of PKF enzyme.

Sephadex G-200 gel filtration of HSE extracted with 0.1 M phosphate buffer at pH 7.4 containing 2 M potassium chloride

HSE concentrated by ultrafiltration was fractionated with Sephadex G-200 gel filtration. The distribution of proteins and PKF enzyme is shown in Fig. 3. Proteins were eluted as three main peaks and PKF activity was eluted in the fractions of the second protein peak.

Sephadex G-200 gel filtration of HSE extracted with buffer alone

The concentrated buffer extract—which is known to possess inhibitory activity against PKF enzyme—was fractionated with G-200 gel filtration. Fig. 4 shows that it is eluted in the fractions of the third protein peak, different from the protein peak of PKF enzyme. If the absence of PKF activity in buffer extract is due to coexistence with the inhibitor, the PKF activity in buffer extract should be detected in the second protein peak. Therefore, the ability of each fraction to generate kinin from substrates was also tested by bioassay. As shown in Fig. 4, PKF activity was detected in the second protein peak and the total activity detected was equal to that of HSE extracted with optimal salt concentration.

DISCUSSION

Lewis reported that PKF enzyme could be extracted from acetone-dried rat skin with a 2 M potassium thiocyanate solution, but not with a 2 M potassium chloride solution (8). These results suggest that PKF enzyme of the skin remains firmly bound, making the enzyme insoluble, and therefore a 2 M potassium thiocyanate solution (but not

potassium chloride) had to be employed to free the enzyme from the substructural elements. Contrary to Lewis's results, in the present study PKF enzyme could be extracted from the human skin with 0.1 M phosphate buffer at pH 7.4 when containing a potassium chloride concentration higher than 1.5 M. This difference may be due to the difference in species, extracting method, or sensitivity of bioassay. It has also been reported that some of the potassium thiocyanate extracts contained an inhibitor of PKF enzyme (8). In the present study HSE extracted with optimal salt concentration contained only PKF enzyme, and not its inhibitor. On the other hand, HSE extracted with buffer alone possessed an inhibitor, but no PKF activity could be demonstrated. However, it was confirmed that PKF enzyme coexisted with its inhibitor in HSE extracted with buffer alone. This was confirmed by the finding that the total PKF activity which was equal to that of HSE extracted with optimal salt concentration, was detected in the second protein peak and was different from the third protein peak obtained by gel filtration of HSE extracted with buffer alone. It was also confirmed that the inhibitor content in HSE extracted with buffer alone was at least 1.5-fold greater than that of PKF enzyme. These results suggest that PKF enzyme may be present as an inactive complex together with its inhibitor in normal skin, ans that potassium chloride probably serves to dissociate the enzyme–inhibitor complex, allowing the enzyme to be most effectively extracted. These results, moreover, raise the possibility that the interaction between PKF enzyme and its inhibitor may be reversible. It has been reported that several proteases could be most effectively extracted from the human skin homogenate with a high potassium chloride concentration, and that inhibitors of the proteases were present together with the enzymes in the extract with buffer alone (4). The authors also reported that the protease inhibitor in the extract with buffer alone was identified as α1-antitrypsin by the immunodiffusion method (5). It is considered that the inhibitor of PKF enzyme detected in HSE extracted with buffer alone by the authors is different from the protease inhibitor detected in buffer extract by Fräki & Hopsu-Havu, as α1-antitrypsin inhibits proteases irreversibly (11). In the preliminary report, it was suggested that PKF enzyme in the human skin might be kallikrein-like enzyme which is localized in the sweat glands and plays a role in microcirculation (15). The roles of this PKF enzyme and its inhibitor in the biological and pathological states will be investigated and discussed in a subsequent paper.

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