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Action of proteolytic enzymes upon horse urinary kallikrein*

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Horse urinary kallikrein (HUK) is an enzyme that releases kallidin (lysylbradykinin) from horse plasma substrate [1]. This kallikrein [2], as well as purified kallikreins (EC 3.4.4.21) from other sources [3], possesses esterolytic activity toward synthetic *N*-substituted arginine esters. The resistance of kallikrein to proteolytic enzymes was observed for human urinary [4] and hog pancreatic kallikreins [5], but human plasma kallikrein was inactivated by several proteolytic enzymes [4].

HUK (sp. act., 22 TAME units/mg) was obtained by a previously described procedure [2, 6] up to the DEAE-cellulose chromatography step. The kinin-releasing activity was estimated on the isolated guinea pig ileum using heat-treated horse plasma as substrate [2, 6]. The esterolytic activity was measured after the hydrolysis of TAME or BAEE (0·004 M, pH 8·5, 37°) in a pH-stat. When these activities had to be determined in the presence of proteases that possessed esterolytic and kinin-releasing action, the proteases were previously inhibited with specific inhibitors without effect on HUK.

After 6 hr of incubation of HUK with pronase (B grade, Calbiochem.; 1:10 enzyme:substrate weight ratio in 0·05 M Tris–HCl, pH 8·0, 37°), 75 per cent destruction of both activities of kallikrein was observed (Fig. 1). No further inactivation was obtained by prolonging the incubation period, unless a subsequent addition of pronase was made, indicating an auto-inactivation of pronase. Pronase is known to be a mixture of proteases with very low specificity [7], and it was probably able to cleave one or more exposed bonds important for the activity of kallikrein.

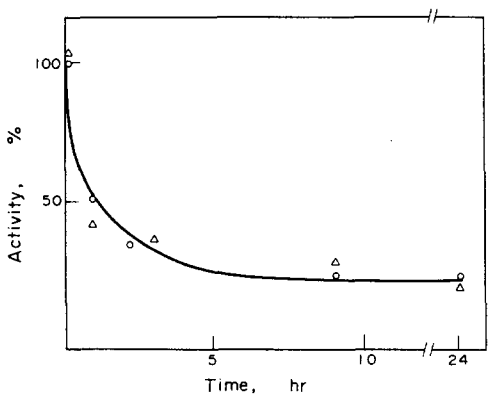

Fig. 1. Inactivation of kallikrein by pronase at pH 8·0, 37°. The concentration of HUK was 0·5 mg/ml. The activity of pronase was interrupted by EDTA, 4×10^{-3} M. —O—O—, Esterolytic activity; —△—△—, kinin-releasing activity.

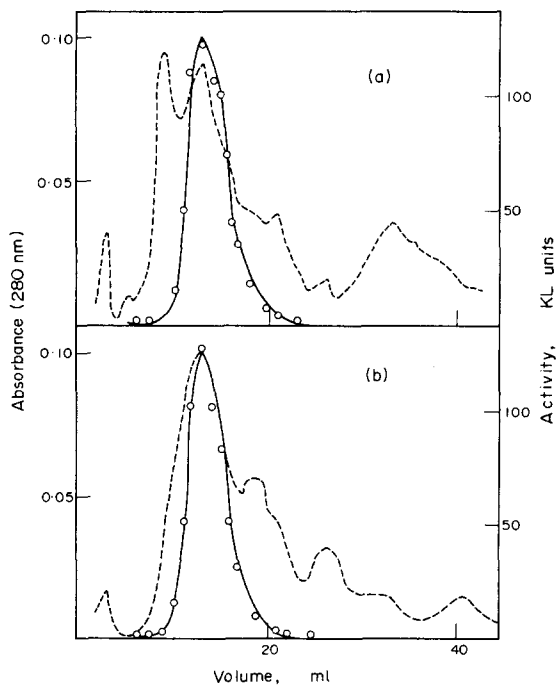

Fig. 2. Gel filtration of kallikrein. A 1·0 × 40 cm Sephadex G-75 column equilibrated with 0·1 M phosphate buffer, pH 6·0, was used. Flow rate: 8 ml/hr. Fractions of 1 ml. (a) HUK not treated by trypsin; (b) HUK treated by trypsin in a weight ratio of 1:20 for 20 hr at 37°. 0·05 M Tris–HCl, pH 8·0. (---) Represents the absorbance at 280 nm and (O—O) the kinin-releasing activity in KL units.

Trypsin (twice crystallized, Worthington Biochemical Corp.), under the same conditions described for pronase in Fig. 1, did not cause inactivation of HUK even at an enzyme:substrate weight ratio as high as 1:1. A trypsin–kallikrein incubate was gel-filtered through a Sephadex G-75 column, the elution pattern was compared to that of untreated HUK, and no difference was detected in the size of the HUK molecule (Fig. 2). Also, the proteases, chymotrypsin, pepsin, papain, carboxypeptidase B (twice crystallized enzymes purchased from Worthington Biochemical Corp.) and subtilisin (Nagarse, Nagase Co. Ltd.), under

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Table 1. Conditions of incubation of horse urinary kallikrein with different proteases at 37°

	Enzyme:substrate wt ratio*	Time (hr)	pH
Chymotrypsin†	1:1	24	8·0
Papain‡	1:10	2	6·0
Subtilisin BPN'†	1:1	18	8·0
Pepsin§	1:1	4	3·0
Carboxypeptidase B†	1:20	4	8·0

* In the concentrations used, none of these proteases affected the kinin-liberating and esterolytic assays.

† In 0·05 M Tris–HCl.

‡ In 0·1 M phosphate buffer, 6×10^{-4} M cysteine.

§ 0·05 M glycine–HCl buffer; at pH values lower than 3·0, the acidity itself inactivated kallikrein.

conditions described in Table 1, did not inactivate kallikrein.

The treatment of HUK by chymotrypsin, followed by inactivation of this enzyme by TPCK [8] and further treatment by trypsin, did not inactivate kallikrein. Previous incubation with pronase under conditions leading to only partial inactivation, or with subtilisin, also did not induce susceptibility to subsequent tryptic attack.

Heating of HUK for 15 min at 70 and 97° led to losses of 30 and 49 per cent, respectively, of the kallikrein enzymatic activities. Incubation at 37° of the heat-treated kallikrein solutions with the above proteases did not induce further inactivation. Treatment of HUK with 8 M urea for 30 min, followed by dialysis, reduced the original HUK activity by 50 per cent; this material was also resistant to proteolysis by trypsin. Thus, only pronase was able to inactivate HUK under the usual conditions for proteolytic digestion, indicating a poor susceptibility of HUK to proteolytic attack. In this respect, kallikrein, which has several similarities to trypsin [9], shows a completely different behavior, since trypsin is highly susceptible to auto-digestion and to hydrolysis by other proteases, even in its native state.

When HUK was heated at 55 or 60° for 30 min and then incubated with trypsin at these high temperatures, both esterolytic and kinin-releasing activities were destroyed (Fig. 3). If kallikrein and trypsin were heated simultaneously, no inactivation of HUK was observed, indicating the need for pre-heating kallikrein. Incubation of kallikrein with subtilisin and chymotrypsin at these high temperatures and with same conditions as in Fig. 3 gave results identical to those with trypsin incubation.

The inactivation of HUK by trypsin, chymotrypsin and subtilisin at high temperatures may be explained by proposing that unfolding of the HUK molecule would expose to proteolytic attack peptide bonds that are important for kallikrein activity. Inactivation due only to the increase of proteolytic activity produced by the temperature rise was excluded because, when HUK was not preheated, the proteases were ineffective. The temperature-dependent modification in the kallikrein molecule is partially reversible, for the enzyme activity recovered after heat treatment alone was not inactivated when it was incubated with proteases at 37°.

The resistance of kallikrein to proteases can help in its purification, some of its contaminants being removed by proteolysis. On the other hand, the conditions for hydrolysis of this enzyme by trypsin and other proteases at high temperatures may be a tool in the study of the structure of kallikreins.

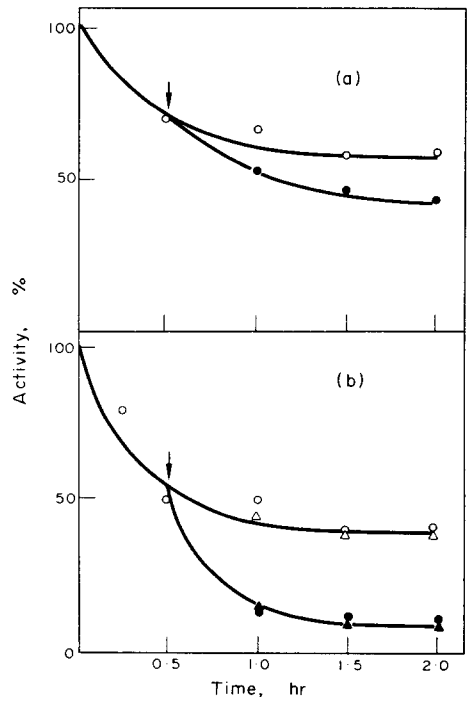

Fig. 3. Inactivation of kallikrein by trypsin at 55° (a) and 60° (b). A solution of HUK (0·15 mg/ml) in 0·025 M Tris–maleate buffer, pH 6·5, 0·01 M $CaCl_2$. The arrow indicates the addition of trypsin to a final concentration of 0·03 mg/ml. The values shown represent the esterolytic activity (○) and kinin-releasing-activity (△) of the controls, and the esterolytic (●) and kinin-releasing activities (▲) of the trypsin-treated kallikrein.

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