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A cytocidal tissue kallikrein isolated from mouse submandibular glands

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A cytocidal factor against mouse thymocytes was purified from the submandibular glands of female BALB/c mice using Sephadex G-50 gel filtration chromatography and reverse-phase HPLC. SDS-PAGE and amino acid sequence analysis revealed that the cytocidal factor was mouse glandular kallikrein (mGK)-6. mGK-6 showed an optimal enzyme activity at pH 10 and a cytocidal activity against thymocytes in a dose-dependent manner.

Kallikrein; Serine proteinase; Cytocidal factor; (Mouse submandibular gland)

1. INTRODUCTION

Tissue kallikreins (TK) are known to be serine proteinases which cleave kininogens to release Lys-bradykinin (kallidin) with vasoactive property [1]. Recently, TK have been demonstrated to localize in many other tissues in addition to previous known sites such as submandibular gland and pancreas [1]. Therefore, TK have been suggested to have other functions than kinin production [1]. Mouse glandular kallikreins (mGK) are encoded as a family of closed linked genes [2] and the substrate specificity of members of mGK family is very high [3]. We have recently found that a cytocidal factor(s) against lymphocytes is isolated from the submandibular glands of male mice [4]. The cytocidal factor is the mixture of mGK-6, mouse renal kallikrein and mGK-9, epidermal growth factor-binding protelin *c*. The question whether mGK-6 and/or mGK-9 are cytocidal remains to be answered. Because the expression of mGK-6 is slightly different between the sexes [5,6] and the extracts of submandibular glands of female mice have cytocidal activity [4], we have tried to purify mGK-6 from female mice. We report here that mGK-6 purified from the submandibular glands of female mice is cytocidal to lymphocytes.

2. MATERIALS AND METHODS

2.1. *Mice*

Male and female BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). Mice were 4–6 weeks of age.

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2.2. *Purification of cytocidal factor*

Extracts of submandibular glands of 5-week-old female BALB/c mice were prepared as described previously [4]. After dialysis of the extracts with 10 mM Hepes/150 mM NaCl (pH 7.2), purification of a cytocidal factor was performed as described [4]. Briefly, the extract was loaded on a Sephadex G-50 column (4.0 × 100 cm; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The active fraction was collected and further purified by reverse-phase HPLC. The fraction was directly applied on a ODS-120T column (7.8 mm ID × 30 cm, Tosoh, Tokyo, Japan) equilibrated with 0.1% trifluoroacetic acid (TFA). A linear gradient of 70% acetonitrile (v/v) containing 0.1% TFA was used at flow rate of 1 ml/min.

2.3. *SDS-PAGE*

Polyacrylamide-SDS gel (20%, 2 mm thickness) was run as described elsewhere [7]. The purified samples and carboxymethylated samples were dissolved in 2% SDS sample buffer with or without 2-ME and boiled for 5 min. SDS-6 (Sigma, St. Louis, USA) and low molecular weight marker (Sigma) were used as M_r marker. After electrophoresis gel was stained with silver.

2.4. *Reduction and carboxymethylation*

The sample was dissolved in 1 ml Tris-HCl, pH 8.0, containing 6 M guanidine hydrochloride, 2 mM EDTA and 40 mM dithiothreitol. The solution was flushed with nitrogen gas, and then incubated at 37°C overnight. After incubation 250 μg of 500 mM iodoacetoamide was added. The reaction mixture was further incubated for 45 min on ice, and then dialyzed against water 2 times for 2 h.

2.5. *Amino acid sequence analysis*

After reduction and carboxymethylation, automated sequence analysis of the subunits isolated by reverse-phase HPLC were performed with a gas-phase protein sequencer (Model 470A, Applied Biosystem, Foster, USA). Phenylthiohydantoin derivatives of amino acids were determined using HPLC as described elsewhere [9].

2.6. *Assay of enzyme activity*

Enzyme activity of the purified sample was determined using tripeptide derivative, 2 AcOH·H-D-Val-CHA (L-β-cyclohexylalanin)-Arg-pNA(paranitroaniline) as a specific substrate of TK. 900 μl of the sample (4.2 μg in 100 mM phosphate buffer, pH 8.0) was mixed with 100 μl of substrate (10 μmol/ml in super pure-water), and incubated for 30 min at 37°C. To stop enzyme activity, 100 μl of 50% acetate was added to the mixture. Absorbance of the mixture was measured at 405 nm, using pNA as the standard.

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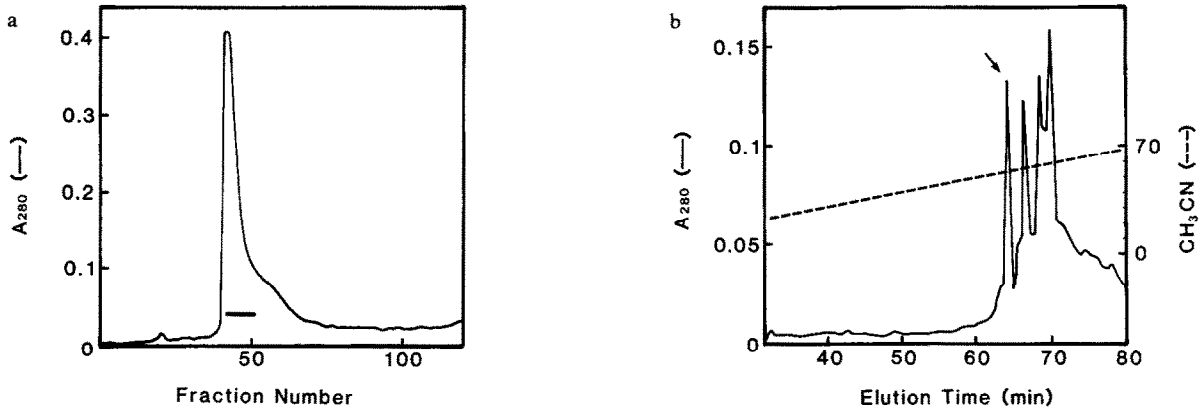

Fig.1. Purification of the cytocidal factor. (a) Gel filtration chromatogram of an extract of submandibular glands. The active fraction is designated by a solid bar. (b) Reverse-phase HPLC. Cytocidal activity is coeluted with the absorbance peak designated with an arrow.

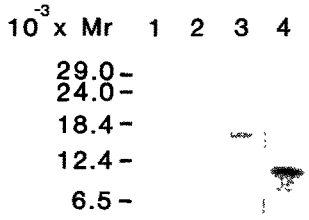

Fig.2. Silver-stained gel pattern after SDS-PAGE. Lanes: (1) the purified sample in the absence of 2-ME; (2) the sample in the presence of 2-ME; (3,4) the purified subunit after reduction and carboxymethylation of the sample.

2.7. *Assay of cytocidal activity*

The purified sample was dialyzed against RPMI-1640 medium (pH 7.0) containing 25 mM Hepes and L-glutamine at 4°C for overnight. 20 μl of various concentrations of the sample was added to syngeneic thymocytes in the same medium containing 10% heat-inactivated FCS (Hyclone, UT, USA), to a final volume of 100 μl (1×10^5 cells) per well of a 96-well microplate (Corning, New York, USA). 70% acetonitrile containing 0.1% TFA dialyzed against the same medium was added to control culture. The cultures in triplicate were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 days. Viable cells in each culture were counted by Trypan blue exclusion. Cytocidal activity of samples was expressed as % specific cytocidal activity which was calculated with the following formula:

% specific cytocidal activity =
(1 − number of viable cells of experimental culture/ number of viable cells of control culture) × 100

3. RESULTS AND DISCUSSION

We purified a cytocidal factor from the extracts of submandibular glands of female mice by collecting active fractions after Sephadex G-50 gel filtration chromatography (fig.1a) and reverse-phase HPLC (fig.1b). 0.7 mg of purified protein was recovered from 530 mg total protein of the extracts of submandibular glands (10 mice), which represents a recovery of approximately 0.13%. SDS-PAGE of the final active fraction, corresponding to the absorbance peak at the arrow in fig.1b showed two protein bands with a M_r of approximately 27000 and 17000 in the absence of 2-ME and 3 protein bands with a M_r of approximately 27000, 17000 and 10000 in the presence of 2-ME (fig.2). Partial N-terminal sequences of 17 kDa protein (fig.2, line 3) and 10 kDa protein (fig.2, line 4), which were isolated by reverse-phase HPLC after reduction and carboxymethylation of purified protein, are shown in fig.3. The amino acid sequences of the 17 kDa subunit and the 10 kDa subunit were identical with that of the N-terminal portion predicted from mGK-6 gene [2,5,10] and that of the portion from 141 amino acid residue [5,10], respectively. SDS-PAGE and amino acid sequence analysis indicated that the purified protein was mGK-6. The enzyme activity of purified pro-

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17K
     1                                        10
Ile-Val-Gly-Gly-Phe-Asn-Cys(CM)-Glu-Lys-Asn-Ser-Gln-Pro-

                        20
xxx-Gln-Val-xxx-Val-Tyr-Arg-Phe-Thr-Lys-

10K
     1                                        10
Tyr-Glu-Tyr-Pro-Asp-Glu-Leu-Gln-Cys(CM)-Val-
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Fig.3. Amino acid sequence of subunits of purified protein.

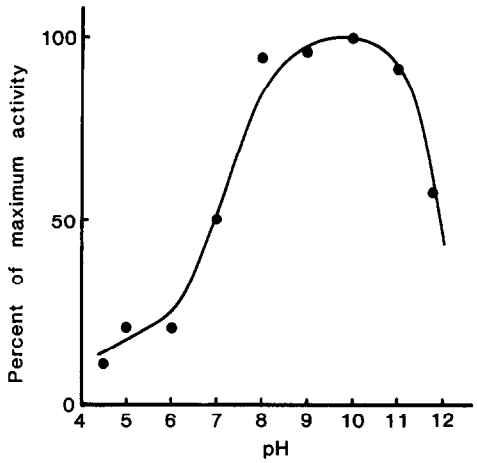

Fig.4. pH dependency of enzyme activity of mGK-6. The enzyme activity was determined in solutions containing 100 mM acetate (pH 4.5 and 5), 100 mM phosphate (pH 6, 7, 8, 11 and 11.8) or 100 mM glycine/NaOH (pH 9 and 10). The enzyme activity was expressed as % of the maximum activity (1.09 μmol/min per mg of protein) at pH 10.

tein using a specific substrate of TK was pH dependent (fig.4). The optimal activity was 1.09 μmol/min per mg of protein at pH 10. The activity dropped fairly steeply below neutrality. The purified protein showed a cytocidal activity to mouse thymocytes in a dose-dependent manner (table 1). Our results indicate that mGK-6 is purified from the submandibular glands of female mice and is cytocidal against lymphocytes, providing a new function of TK. Although a cytocidal activity of mGK-9 is unclear at present, mGK-9 also is assumed to be cytocidal because the enzyme and cytocidal activities of mGK-6 are less than those of the mixture of mGK-6 and mGK-9 reported previously [4]. mGK-6 is considered to be identical with mouse renal kallikrein, which is equivalent to renal kallikrein found in other mammalian species [1]. The term TK has been given to such a kallikrein. TK has been localized in many tissues and various types of cells and is assumed to have other functions than kinin production. The physiological role of cytocidal activity of mGK-6 is

Table 1

Cytocidal activity of mGK-6 against mouse thymocytes

Expt	mGK-6 (μg/ml)	% specific cytocidal activity (mean ± SD)
A	25	12.8 ± 5.1
	50	27.6 ± 2.1
	100	42.4 ± 12.2
B	50	28.6 ± 2.2
	100	50.1 ± 2.0

No. of viable cells of control culture after 3 days were 0.52×10^5 in Expt A and 0.50×10^5 in Expt B

quite unknown at present. This kallikrein, however, may participate in tissue disorders such as inflammation and diabetes mellitus. The cytocidal mechanism of mGK-6 is now under study.

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