Isolation of Urinary Trypsin Inhibitor-like Inhibitor from Human Lung Cancer Tissue

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ABSTRACT

In the present study, a trypsin inhibitor was first extracted from lung cancer tissue and purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A final yield of 20 to 60 μg of inhibitor with a specific activity of 2040 units/mg of protein was obtained from 1 g of original lung cancer tissue. This inhibitor inhibited trypsin strongly, plasma kallikrein weakly, and plasmin more weakly, and its molecular weight was approximately 43,000 to 45,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Its antigenicity was confirmed to be quite the same as that of human urinary trypsin inhibitor by double immunodiffusion, immunoelectrophoresis, and neutralization with anti-urinary trypsin inhibitor rabbit immunoglobulin.

INTRODUCTION

Since Bauer and Reich (2) reported the presence of trypsin inhibitor in human urine, an enormous amount of literature has been accumulated on human UTI (1, 8, 10, 18, 19, 21). The origin and many aspects of physiological and pathological roles of UTI have not been clarified yet. In 1973, Proksch et al. (18) and Hochstrasser et al. (10) reported that the antigenicity of UTI might be the same as that of laTI in human serum and, thus, that laTI might be the precursor of UTI. Contrarily, Clavey et al. (6) reported that purified UTI did not show an immunoprecipitin line with anti-laTI, and Chawla et al. (5) reported that EDC1, which is a trypsin inhibitor in urine of a patient with leukemia and different from UTI, was an inhibitor derived from laTI. The authors (14) also reported the presence of a new trypsin inhibitor (PRAUTI) in human plasma, which reacted with anti-UTI rabbit serum but was immunologically distinct from laTI.

On the other hand, Hochstrasser et al. (10) reported that UTI possessed the same antigenicity as trypsin inhibitor in human bronchial mucous membrane and that UTI might be originated from an organ, such as lung, kidney, and liver.

However, there are few reports on isolation of UTI-like inhibitor from organ tissue. This study describes the isolation of UTI-like inhibitor from lung cancer tissue.

MATERIALS AND METHODS

Materials. The following commercial preparations were used: Casein (Sigma), trypsin (type III, Sigma Chemical Co.); chymotrypsin (Sigma); plasmin (Green Cross Co., Ltd., Osaka, Japan); urokinase (Green Cross Co.); bovine thrombin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan); TAMe (Sigma); N-acetyl-L-tyrosine ethyl ester (Sigma); H-o-Val-Leu-Lys-pNA (S-2251; Daiichi Pure Chemical Co., Ltd., Tokyo, Japan); pyro-Glu-Gly-Arg-pNA (S-2444; Daiichi); BANA (Foundation for Promotion of Protein Research, Institute for Protein Research, Osaka, Japan); anti-laTI rabbit serum (Behringwerke); and anti-human serum rabbit serum (Behringwerke). Human plasma kallikrein was obtained in a previous paper (14). One mg of human plasma kallikrein activated with acetone possessed 0.21 TAMe esterolytic units. To determine the stoichiometry of the reaction between inhibitor and trypsin, trypsin solution was titrated with p-nitrophenyl p-guanidinobenzoate as described by Chase and Shaw (4), and its final concentration was adjusted to 25 μg of trypsin/ml of 0.001 n HCl solution containing 0.02 M CaCl₂.

Assay of Enzymes and Protein. The carboxypeptidase activity of the sample was determined by the method of Lowry et al. (13) as reported previously (12), using 8% casein substrate in 0.1 M phosphate buffer at pH 7.4. Esterolytic activity on TAMe and N-acetyl-L-tyrosine ethyl ester was determined by the method of Hestrin (9) as modified by Roberts (20) (substrate concentration, 10 mm in 0.1 M phosphate buffer, pH 7.4). Amidolytic activity of the sample was measured with S-2251 (final concentration, 0.3 mm in 0.05 M Tris-HCl buffer, pH 7.4) and S-2444 (final concentration, 0.3 mm in 0.05 M Tris-HCl buffer, pH 8.8) by the method described in a previous paper (26). The BANA hydrolytic activity was determined as follows: 0.8 ml of sample-phosphate buffer (0.1 M, pH 7.4) mixture was added to 0.1 ml of 30 mm BANA solution. After incubation for 20 min at 37°, the reaction was stopped by addition of 0.1 ml of 50% acetic acid, and the absorbance at 405 nm was read. One caseinolytic unit was defined as the amount of enzyme releasing 60 μg of acid-soluble tyrosine in 15 min on incubation at 37°. One esterolytic unit was defined as the amount of enzyme which can hydrolyze 1 μmol of substrate/min at 37°. One amidolytic unit was defined as the amount of enzyme which hydrolyzes 1 μmol of substrate/min at 37°. One amidolytic unit was defined as the amount of enzyme which hydrolyzes 1 μmol of substrate/min at 37°. The inhibitory activities of the sample on various enzymes were determined from the residual caseinolytic, esterolytic, and amidolytic activities of the enzymes after incubation with a mixture of enzyme and various amounts of inhibitors for predetermined periods at 37°. These results were usually calculated from amounts required for 50% inhibition. Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin (Merck) as the standard.

Extraction of Trypsin Inhibitor from Lung Cancer Tissue and Normal Lung Tissue. Ten lung tissue samples were obtained from both sites of tumor and apparently healthy parts of 5 lung cancer patients at the time of surgical operation. Two patients were diagnosed histologically as having squamous cell carcinoma, and the others, as adenocarcinoma. The lung tissue sample was washed in cold phosphate buffer (0.1 M, pH 7.4) to remove contaminating blood and frozen at −20°. The frozen tissue was minced with scissors and homogenized with Virtis homogenizer (Virtis Co., Ltd.) in 10 volumes (w/v) of 2 M KSCN solution. The homogenate was maintained at 4° overnight and then centrifuged at 4°

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for 1 hr at 20,000 × g. The supernatant was dialyzed for 12 hr at 4°C against phosphate buffer (0.1 M, pH 7.4) and recentrifuged at 4°C for 1 hr at 20,000 × g to remove the precipitate during dialysis.

Preparation of the IgG Fraction of Anti-UTI Rabbit Serum. UTI (M, 44,000) was prepared according to the method described in a previous paper (22) in which trypsin-Sepharose affinity chromatography and Sephadex G-100 gel filtration were used. Its specific activity was 1,850 units/mg protein, and its homogeneity was confirmed by SDS-PAGE. One unit of UTI activity was defined as the amount of inhibitor which can inhibit completely 1 μg of trypsin after preincubation with trypsin-inhibitor mixture for 5 min at 25°C. A rabbit was immunized by footpad injections with 5 mg of purified UTI in complete Freund’s adjuvant and given a single booster injection i.v. of the same amount 4 weeks later. After 4 weeks, a blood sample was drawn from the rabbit and incubated at 37°C for 3 hr and at 4°C for 18 hr before separating the serum by centrifugation at 2500 × g. The IgG fraction was separated from serum by ammonium sulfate fractionation (35% saturation) and DEAE-cellulose column chromatography resulting in 10-fold concentration, as compared to the original antisera. Specificity of the anti-UTI rabbit IgG had been confirmed earlier by double immunodiffusion as well as its neutralization effect for purified UTI. The anti-UTI rabbit IgG gave only a single immunoprecipitin line on double immunodiffusion against purified UTI. Also, 1 mg of IgG could neutralize almost completely 32 units of purified UTI when BAÑA and casein. As shown in Table 1, the anti-UTI rabbit IgG gave only a single immunoprecipitin line with purified UTI, 7-1, T-2, 7-3, and 7-4. Anti-UTI rabbit IgG, however, did not form any immunoprecipitin line with normal lung tissue extracts (AM, 2, 3, 4, 5; concentration, 0.05 to 10 μg/ml; 2, Tumor Extract 1 (0.51 μg/3 μl); 3, Tumor Extract 2 (0.51 μg/3 μl); 4, Tumor Extract 3 (0.61 μg/3 μl); 5, Tumor Extract 4 (0.64 μg/3 μl); 6, veronal buffer (3 μl)).

Electrophoretic Technique. SDS-PAGE was carried out by the method of Weber and Osborn (27) using 7.5% gel with 1% SDS and 8 M urea. Electrophoresis was performed at 8 ma/gel for 3 hr. Gels were stained for 2 hr with 2% Coomassie brilliant blue and destained electrophoretically.

Immunological Experiments. Double-immunodiffusion analysis (Ouchterlony method) (17) was performed at room temperature for 3 days in 1% agarose (Agar Special Noble; Difco) in veronal buffer (pH 8.6) containing 0.02% NaN3. The distance between antigen and antibody wells was 5 mm, and the diameter of the wells was 2 and 4 mm, respectively. Immunoelectrophoresis was run in 1.2% agarose (agarose L; Behringwerke) mixed gel in the same buffer at pH 8.6, and 3-ma/cm glass plate width was applied. After the reactions were continued at room temperature for 2 days, plates were washed in phosphate-buffered saline (0.15 M, pH 7.4) for 2 days, dried, stained with Amido-black, and destained in 5% acetic acid.

RESULTS

Measurement of LCATI Extract. Trypsin-inhibitory activity was determined using BANA and casein. As shown in Table 1, lung tissue extract from the cancer site possessed obviously higher trypsin-inhibitory activity in spite of the wide difference in activity among 5 patients, whereas only a trace amount or no activity was detected in extraction from the normal site. In Cases 1 to 3, trypsin-inhibitory activity by the caseinolysis assay method was higher than that by the BANA assay method, but the cause for this difference is not clear now.

Immunological Identity of LCATI with UTI and laTI. The identity of LCATI was investigated by a double-immunodiffusion method using anti-UTI rabbit IgG and anti-laTI rabbit serum. As shown in Fig. 1, anti-UTI rabbit IgG formed a single immunoprecipitin line with purified UTI, T-1, T-2, T-3, and T-4. Anti-UTI rabbit IgG, however, did not form any immunoprecipitin line with normal lung tissue extracts (N-1, 2, 3, 4, 5; concentration, 0.05 to 10 μg/3 μl). As shown in Fig. 2, on immunoelectrophoresis, all lung cancer extracts formed a single immunoprecipitin arc with anti-UTI rabbit IgG and were found to have a mobility similar to that of UTI (prealbumin position). On the other hand, anti-laTI rabbit serum did not form any immunoprecipitin line with lung cancer and normal lung tissue extracts.

Inhibitory Effect of Anti-UTI Rabbit IgG on LCATI. To ex-

![Fig. 1. Double immunodiffusion of anti-UTI rabbit IgG against lung cancer extracts.
Center well (AS), anti-UTI IgG (50 μg/10 μl); peripheral wells: 1, purified UTI (1.5 μg/3 μl); 2, Tumor Extract 1 (0.51 μg/3 μl); 3, Tumor Extract 2 (0.51 μg/3 μl); 4, Tumor Extract 3 (0.61 μg/3 μl); 5, Tumor Extract 4 (0.64 μg/3 μl); 6, veronal buffer (3 μl).](image1)

![Fig. 2. Immunoelectrophoresis of lung cancer extracts against anti-UTI rabbit IgG. 1, standard human serum; 2, purified UTI; 3, lung cancer extract (Tumor Extract 1); 4, lung cancer extract (Tumor Extract 2); AS-1, anti-human serum rabbit IgG; AS-2, anti-UTI rabbit IgG.](image2)
amine the true UTI activity in LCATI, the inhibitory effect of anti-UTI rabbit IgG was measured on 1 unit of LCATI activity capable of completely inhibiting BANA hydrolytic activity of 1 μg of trypsin. As shown in Chart 1, 1 unit of LCATI activity was almost completely inhibited by 30 μg of anti-UTI rabbit IgG, suggesting that antigenicity of LCATI was the same as that of UTI.

**Purification of LCATI by SDS-PAGE.** To estimate the molecular weight of LCATI and to clarify its inhibitory spectra, LCATI was purified by SDS-PAGE. LCATI (5 μg of protein) was run on each polyacrylamide gel in the presence of SDS. One gel was stained, and the others were sliced into 2-mm segments, each of which was eluted overnight at 4° with 0.1 ml of Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2% Triton X-100. The profile of protein bands and trypsin-inhibitory activity in each gel band are shown in Fig. 3 and Chart 2. LCATI showed several protein bands, including a weak and broad band corresponding to the protein band of UTI. Trypsin-inhibitory activities of LCATI were recognized in 2 protein bands M, 43,000 to 45,000 and M, 20,000, which were completely neutralized by anti-UTI rabbit IgG. The 5 sliced gel segments (Nos. 17 to 21) of LCATI were collected, eluted, and lyophilized. By these procedures, a final yield of 20 to 60 μg of LCATI with a specific activity of 2040 units/mg of protein was obtained from 1 g of original lung cancer tissue.

**Properties of LCATI.** The inhibitory effects of purified LCATI (M, 43,000 to 45,000) on 6 different enzymes were examined and compared with those of UTI. As shown in Table 2, about
0.61 μg of LCATI could inhibit completely 1.25 μg of trypsin. This indicates that the molar binding capacity of LCATI for trypsin is 4 mol, assuming that the molecular weights of trypsin and LCATI are 24,000 and 44,000, respectively. In order to inhibit equivalent amounts of these enzymes, it is necessary to use about 3 times the amount of LCATI for chymotrypsin than for trypsin, about 2 times the amount for kallikrein, and 10 to 15 times the amount for plasmin. When the inhibitory spectra of LCATI were compared with that of UTI, it was confirmed that almost the same amounts of LCATI as UTI were required for complete inhibition of trypsin. On the other hand, LCATI could more strongly inhibit chymotrypsin and kallikrein than UTI, whereas its inhibitory effects on plasmin were almost the same.

DISCUSSION

In the present study, the authors first succeeded in extracting a trypsin inhibitor from lung cancer tissue and in comparing the immunological and biochemical properties of LCATI with those of UTI. As shown in Figs. 1 and 2 and Chart 1, it was confirmed that the antigenicity of LCATI was quite the same as that of UTI. The molecular weight of LCATI was found to be approximately 43,000 to 45,000 by SDS-PAGE and was also the same as that of UTI. SDS-PAGE of lung cancer tissue extracts showed the presence of another low-molecular-weight LCATI (M, 20,000). The authors assume that it may be an enzymatically degraded product of high-molecular-weight LCATI, because it possesses the same antigenicity as UTI as shown by the neutralization test with anti-UTI rabbit IgG. Such modifications of LCATI are also very similar to those of UTI as reported previously (23, 24). Further experiments have to be carried out for clarification of its biochemical and biophysical properties. In Table 2, the inhibitory spectra of LCATI on several enzymes are compared with those of UTI. One molecule of LCATI as well as UTI could combine with about 4 molecules of trypsin, and this result coincided well with the result of Muramatu et al. (15). Although the inhibitory spectra of LCATI were almost the same as those of UTI, the specific inhibitory activity on chymotrypsin of LCATI was almost 4 times higher than that of UTI. It was of particular interest that its specific inhibitory activity in kallikrein was remarkably higher than that of UTI (about 15 times), whereas its specific inhibitory activity on plasmin was almost the same as that of UTI. The reason for these differences in specific inhibitory activity between LCATI and UTI is still unclear. As the homogeneity of both samples has been confirmed by SDS-PAGE, there is a possibility that the molecular structure of LCATI may be different from that of UTI, despite the fact that they possess the same antigenicity, molecular weight, and electrophoretic mobility. As described in “Results,” LCATI could not be extracted from normal lung tissue (Table 1) and did not form any immunoprecipitin line with anti-UTI rabbit serum on a double-immunodiffusion.

In a previous paper (14), the authors reported that human plasma contained a new trypsin inhibitor which reacted immunologically with anti-UTI rabbit serum but not with anti-UTI rabbit serum. However, the apparent molecular weight of this inhibitor is 90,000 and is obviously different from that of LCATI. In the present study, the authors also isolated the plasma proteins (M, ~44,000) from normal plasma and patient plasma with lung cancer by quite the same isolation method as that of LCATI from lung cancer tissue. When their immunological identities were examined by a double-immunodiffusion method, anti-UTI rabbit serum did not form any immunoprecipitin line with plasma proteins adjusted to various protein concentrations. These results and facts make us speculate strongly that LCATI is being produced in the lung cancer tissue and is not just a plasma inhibitor present in cancer tissue extract. This problem will be solved finally by the lung cancer cell culture in the near future. At any rate, it is very important that a new UTI-like trypsin inhibitor can be extracted from lung cancer tissue, because the origin of UTI is still unclarified as described in the “Introduction.”

Although there are many contradictory discussions as to whether or not the origin of UTI is ιαTI, this study makes us speculate that not only UTI but also ιαTI or an ιαTI-like inhibitor (14) may be originated from organ tissue.

Regarding the isolation of trypsin inhibitor from lung tissue, there is a great deal of literature on Trasylol (bovine pancreatic trypsin inhibitor) which was isolated from bovine lung tissue, but there are few reports on isolation of it from human lung tissue. In the present study, although LCATI could be extracted from both squamous cell carcinoma and adenocarcinoma, it is very important whether or not LCATI could be extracted from only lung cancer tissue. The authors assume that the LCATI-like inhibitor might be extracted from cancer tissue of other organs. This problem is now under investigation in our laboratory. There are many reports on the role of serine protease as a promoting or metastatic factor of tumor (3, 7, 16). However, there are only a few reports on the role of a serine protease inhibitor, such as UTI (11, 25) and LCATI. The authors wish to proceed with their investigations on the role of LCATI as an inhibitor of the promoting or metastatic factor in lung cancer. The authors also consider that the measurement of UTI or LCATI-like inhibitor in serum, if possible, may be utilized for diagnosis or assessment of the severity of lung cancer.

REFERENCES


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