Hiroshima Journal of Medical Sciences
Vol. 33, No. 4, 801～808, December, 1984
HIJM 33-116

Purification of Thiol Protease Inhibitor from Human Lung Cancer Tissue*)

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(Received September 25, 1984)

Key words: Thiol protease inhibitor, Human lung cancer

ABSTRACT

It was confirmed that the thiol protease inhibitory activities in human lung cancer extracts were significantly higher than normal lung tissue extracts.

Furthermore the thiol protease inhibitor was extracted from human lung cancer tissue and purified by papain-Sepharose and Sephadex G-100 column chromatographies. The purified inhibitor inhibited papain and ficin, and its molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was about 13,000.

INTRODUCTION

The mechanism of the process by which malignant tumors invade and metastasize is complex, and not yet fully elucidated.

It has been reported that proteolytic enzymes such as plasminogen activator[23] (one of serine proteases), cathepsin B[22] (one of thiol proteases) and collagenase[7] (one of metallo proteases) might be associated with invasiveness and metastasis of malignant tumors. Although an enormous amount of literature has been accumulated on the association of proteolytic enzymes and malignant tumors, there have been few reports about the association of tumors and protease inhibitors[10,12,22,28,32]. In a previous paper[24], the authors reported that human lung cancer tissue contained a urinary trypsin inhibitor-like inhibitor, and proposed that the protease inhibitor might play an important role as an inhibitor of the promoting or metastatic factor in lung cancer.

Only few reports were found about the association of thiol protease inhibitors and tumors[5,17], such as Giraldi et al.[9] reported that the bovine spleen neutral proteinase inhibitor (SNPI-1), which inhibited thiol proteinases cathepsin B and H, reduced the formation of spontaneous pulmonary metastases in mice bearing Lewis lung carcinoma. We thought it worthwhile to study about the presence of thiol protease inhibitor in human lung cancer tissue.

In the present study we extracted and purified a thiol protease inhibitor from human lung cancer tissue.

MATERIALS AND METHODS

Assay of thiol proteases and protein:

The caseinolytic activity of the sample was determined by the method of Lowry et al.[20] as reported previously[21], using 8% casein substrate (caseinolysis method). The amidolytic activity of the sample was determined with chromogenic substrates S-2251 (H-D-Val-Leu-Lys-pNA, Kabi Diagnostica) and S-2302 (H-D-Pro-Phe-Arg-pNA, Kabi Diagnostica) by the method of Bang and Mattler[3] (Testzym method). The inhibitory activities of the sample on thiol proteases were determined from the residual caseinolytic and amidolytic activities of the

*) 奥道恒夫，西亀正之，高杉純好，山根 基，江崎治夫：ヒト肺癌組織からのチオールプロテアーゼインヒビターの精製

proteases after incubation with a mixture of protease and various amounts of inhibitors for predetermined periods at 37°C. The concentration of the thiol proteases were adjusted to 25 μg/ml with 0.1 M phosphate buffer, pH 7.4 containing 75 mM cysteine in caseinolysis method and to 10 μg/ml with 0.05 M Tris-HCl-buffer, pH 7.4 containing 75 mM cysteine in Testzym method and 0.1 ml of the protease was used. One unit (U) of thiol protease inhibitory activity was defined as the amount inhibiting 1 μg of the protease.

Protein concentration was determined by the method of Lowry et al.[20] using bovine albumin (Merck) as the standard.

Extraction of thiol protease inhibitor from lung cancer tissue and normal lung tissue:

Human lung tissue samples were obtained from both sites of cancer tissue and apparently healthy parts of 18 lung cancer patients at the time of surgical operations. Ten patients were diagnosed histologically as adenocarcinoma, 7 were as squamous cell carcinoma and 1 was as large cell carcinoma.

Four patients were diagnosed as Stage I (Stage by p-TNM[31]), 4 were as Stage II, 8 were as Stage III and 2 were as Stage IV.

The extracts of tissues with 2 M KSCN solution were prepared according to the procedure of Astrup et al.[2] with the modification as reported by us previously[24].

Papain-Sepharose affinity chromatography:

Papain-Sepharose gel was prepared by the method of Cuatrecasas[6]; Sepharose 4B (Pharmacia) was activated by treatment with CNBr, and coupled with papain.

The resultant papain-Sepharose gel was inactivated by KSCN and acetic acid and washed with 0.02 M trisodium phosphate containing 3 M KSCN, pH 12.1 and 0.01 M phosphate buffer, 0.1 M NaCl, pH 6.0. A column (2.7 × 10 cm) was packed with the inactivated papain-Sepharose gel and equilibrated with 0.01 M phosphate buffer, 0.1 M NaCl, pH 6.0 (starting buffer). The pooled lung cancer extract was applied to the column at a flow rate of 60 ml/hr and collected fractions of 10 ml. Unadsorbed material was eluted with the starting buffer, non specifically adsorbed proteins were eluted with the starting buffer containing 3 M KCl and washed with the starting buffer. The adsorbed inhibitor was eluted with 0.02 M trisodium phosphate, 0.1 M NaCl, pH 12.1. Finally, the column was washed with 0.02 M acetate buffer, 3 M KSCN, pH 4.0 and the starting buffer. This method of affinity chromatography was accorded to the procedure of Järvinen[13]. The collected fractions containing inhibitor from papain-Sepharose affinity chromatography were concentrated on cellulose tubing (Visking) and applied to a column of Sephadex G-100.

Gel filtration:

A column (2.6 × 42 cm) of Sephadex G-100 (Pharmacia) was equilibrated with 0.1 M phosphate buffer, 0.1 M NaCl, pH 6.0. The separation was performed at 4°C and the effluent was collected in fractions of 3 ml at a flow rate of 15 ml/hr.

The molecular weight of the inhibitor was estimated according to Andrews[1] by using molecular weight standards (Pharmacia).

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was carried out by the method of Weber and Osborn[34] using 7.5% gel with 1% SDS and 8 M urea. After electrophoresis, the gel was stained with Coomassie brilliant blue. Molecular weight was determined from a simultaneous run of molecular weight standards.

RESULTS

Measurement of thiol protease inhibitory activities in tissue extracts:

Thiol protease inhibitory activities in tissue extracts from 18 lung cancer patients were determined using ficin (Sigma) and papain (Sigma) as thiol protease. As shown in Fig. 1, ficin inhibitory activities were measured by both Testzym (S-2251) and caseinolysis (casein) method. The inhibitory activities measured by S-2251 in lung cancer extracts were obviously higher than in normal lung tissue extracts, and the inhibitory activities by casein in lung cancer extracts were also significantly higher than in normal lung tissue extracts (Fig. 1-A). Although, the inhibitory activities of individual patients were widely different, almost all the lung cancer extract had higher inhibitory activity compared with the normal lung tissue extract of the same patient.

On the other hand, there were no significant difference between adenocarcinoma and squamous cell carcinoma in the inhibitory activities

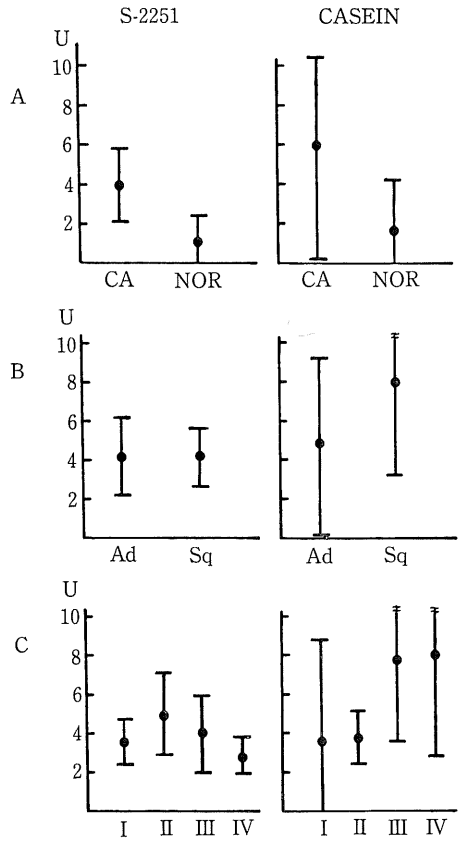

Fig. 1. Ficin inhibitory activities in tissue extracts from lung cancer patients.
Ficin inhibitory activities (FIA) were determined by Testzym (S-2251) and caseinolysis (casein) method, and expressed by U/mg protein (M±SD).
A: FIA in lung cancer extract (CA) and normal lung tissue extract (NOR).
B: The comparison of FIA in lung cancer extract by histologic types (Ad=adenocarcinoma, Sq=squamous cell carcinoma)
C: The comparison of FIA in lung cancer extracts by p-TNM Stage.

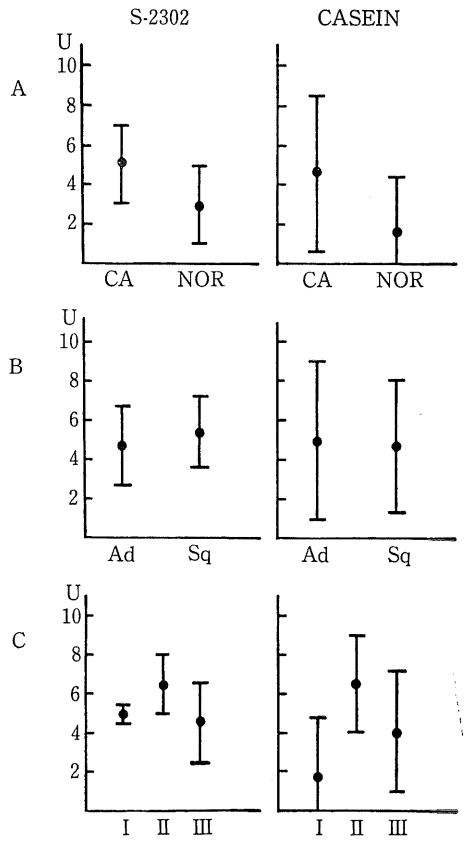

Fig. 2. Papain inhibitory activities of tissue extracts from lung cancer patients.
Papain inhibitory activities (PIA) were determined by Testzym (S-2302) and caseinolysis (casein) method, and expressed by U/mg protein (M±SD).
A, B, C were the same as described in Fig. 1.

using both substrates (Fig. 1-B). Furthermore, the differencies of the inhibitory activities in lung cancer extracts from various stages classified by p-TNM Stage were not found by both methods (Fig. 1-C).

Fig. 2 shows the papain inhibitory activities measured by Testzym (S-2302) and caseinolysis (casein) method. The inhibitory activities in lung cancer extracts measured by both methods were significantly higher than in normal lung tissue extracts (Fig. 2-A).

There were no significant difference of inhibitory activities between adenocarcinoma and squamous cell carcinoma, nor among various lung cancer stages using both methods (Figs. 2-B, C).

Purification of thiol protease inhibitor from human lung cancer extract:

As shown Figs. 1-A and 2-A, the thiol protease inhibitory activities in lung cancer extracts were significantly higher than in normal lung tissue extracts.

Then we tried to purify the thiol protease inhibitor from pooled lung cancer extract. The pooled lung cancer extracts were collected from 8 lung cancer patients who were diagnosed histologicaly as adenocarcinoma, and applied to the papain-Sepharose column. The elution pattern of the papain-Sepharose column is shown

in Figs. 3 and 4. Papain inhibitory activities in each fraction was determined by caseinolysis method. Nonspecifically adsorbed proteins were eluted with eluent 2. and the papain inhibitory activities in these fractions were apparently high, however, almost of the increased activities were due to the eluent 2 itself. So the inhibitory activities of these fractions after dialysis against 0.1 M phosphate buffer, 0.1 M NaCl, pH 6.0 were almost disappeared. The adsorbed inhibitor was eluted with eluent 4 (0.02 M trisodium phosphate, 0.1 M NaCl, pH 12.1) (Fig. 3). Papain inhibitory activity in each fraction determined by Testzym method was almost the same as caseinolysis method.

Ficin inhibitory activity in each fraction was determined by Testzym method (Fig. 4). The elution pattern of the inhibitory activities were almost the same as the papain inhibitory activities. Though the inhibitory activities were strongly influenced by the eluent 2, the true inhibitory activities were existed in the fraction which were eluted with eluent 4 (Fig. 4). Ficin inhibitory activity in each fraction determined by caseinolysis method was almost the same as Testzym method. The inhibitor eluted with the eluent 4 was collected (fractions Nos. 91–93), dialyzed against 0.1 M phosphate buffer, 0.1 M NaCl, pH 6.0 and concentrated. The concentrated inhibitor was applied to Sephadex G-100 column. The elution pattern of the protein and thiol protease inhibitory activity are shown in Fig. 5. The peaks of papain and ficin inhibitory activities were coincided (molecular weight about 13,000) and were apparently different from the peaks of the proteins.

In this way, the thiol protease inhibitor from lung cancer tyssue was purified and its molecular weight was about 13,000.

A typical purification procedure is summarized in Table 1. The recovery rates of papain-Sepharose affinity chromatography and Sephadex G-100 gel filtration were 5.2% and 2.2%, respectively.

The purified inhibitor from Sephdex G-100 gel filtration gave a single broad and faint

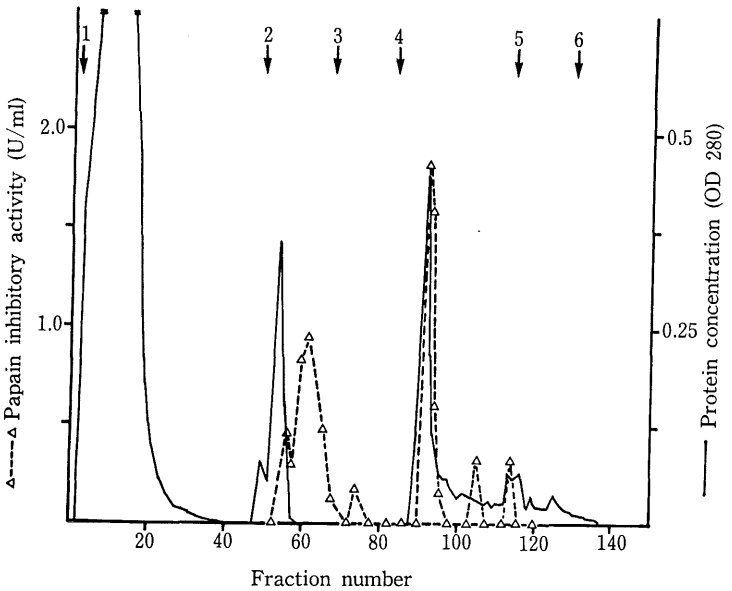

Fig. 3. Papain inhibitory activity on papain-Sepharose affinity chromatography of pooled lung cancer extract.
Pooled lung cancer extract (adenocarcinoma) was applied to papain-Sepharose affinity chromatography. Papain inhibitory activity in each fraction (10 ml) was determined by caseinolysis method. 1–6 were eluents (1=0.01 M phosphate buffer, 0.1 M NaCl, pH 6.0, 2=0.01 M phosphate buffer, 3 M KCl, pH 6.0, 3=1, 4=0.02 M trisodium phosphate, 0.1 M NaCl, pH 12.1, 5=0.02 M acetate buffer, 3 M KSCN, pH 4.0, 6=1).

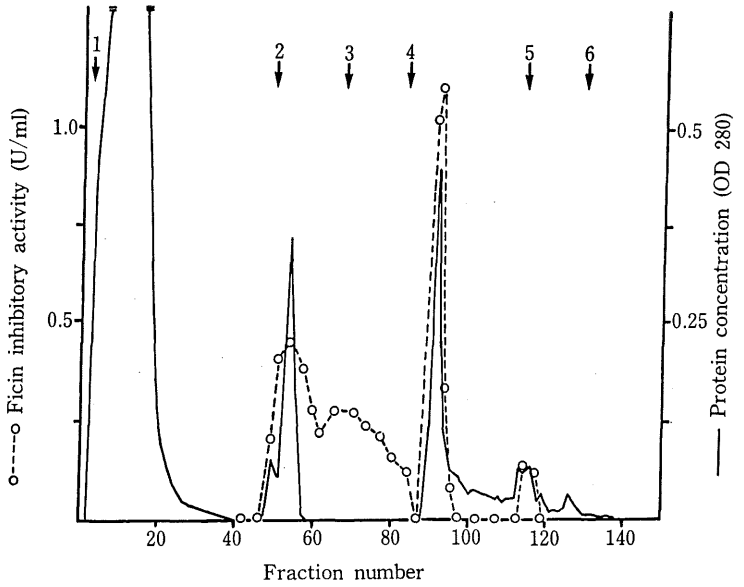

Fig. 4. Ficin inhibitory activity on papain-Sepharose affnity chromatography of pooled lung cancer extract.
Sample was pooled lung cancer extract (adenocarcinoma). Ficin inhibitory activity was determined by Testzym method. 1-6 were eluents which were the same as described in Fig. 4.

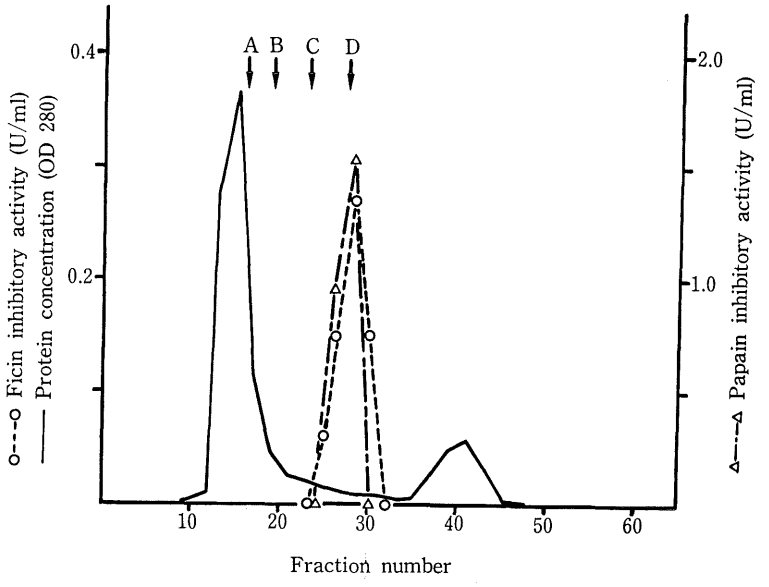

Fig. 5. Sephadex G-100 chromatography of the inhibitor in pooled lung cancer extract from papain-Sepharose affinity chromatography.
Sample was pooled lung cancer extract (adenocarcinoma). Papain and ficin inhibitory activities were determined by Testzym method.
A-D were molecular weight standards (A=Albumin 67,000, B=Ovalbumin 43,000, C=Chymotrypsinogen A 25,000, D=Ribonuclease A 13,700).

Table 1. Purification procedure of TPI from pooled lung cancer extract

Purification step	Volume ml	Protein mg/ml	Ficin inhibitory activity U/ml	Total(U)	Specific activity	Recovery %
Extract	68.0	1.35	3.96	269.0	2.93	100
Papain-Sepharose	6.0	0.51	2.32	13.9	4.55	5.2
Sephadex G-100	7.5	0.01	0.80	6.0	80.00	2.2

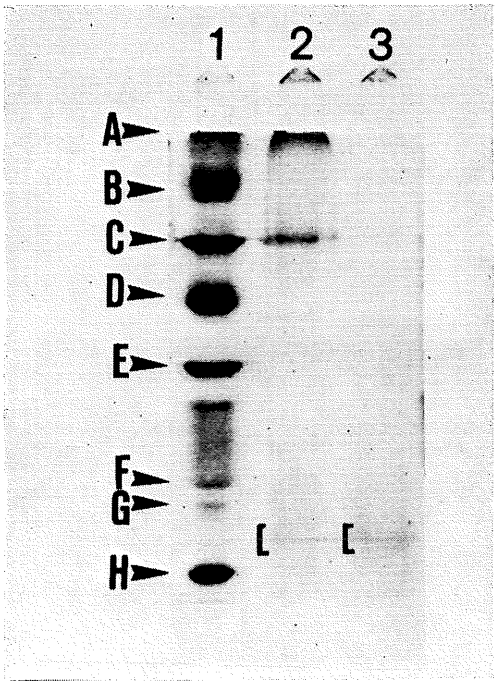

Fig. 6. SDS-polyacrylamide gel electrophoresis of the inhibitor in pooled lung cancer extract (adenocarcinoma).
1=Molecular weight standard (A=Ferritin 440, 000, B=Catalase 232, 000, C=Phosphorylase b 94, 000, D=Albumin 67, 000, E=Ovalbumin 43, 000, F=Chymotrypsinogen A 25, 000, G=Soy bean trypsin inhibitor 20, 100, H=Cytochrome C 12, 500).
2=TPI from the papain-Sepharose affinity chromatography of pooled lung cancer extract (adenocarcinoma).
3=Purified TPI in pooled lung cancer extract (adenocarcinoma) by papain Sepharose affinity chromatography and Sephadex G-100 gel filtration.
([ )=broad and faint band by TPI.

band, and was estimated as molecular weight of about 13, 000 by SDS-PAGE (Fig. 6).

The thiol protease inhibitor from the pooled lung cancer extract which was collected from 6 lung squamous cell carcinoma patients, was also purified through papain-Sepharose affinity chromatography and Sephadex G-100 gel filtration.

The purified thiol protease inhibitor from squamous cell carcinoma had a molecular weight of about 13, 000 same as the inhibitor from adenocarcinoma (data is not shown).

DISCUSSION

In the present study, we first succeeded in purification of a thiol protease inhibitor from human lung cancer tissue.

There has been no reports about thiol protease inhibitor in human lung cancer tissue. As shown in Figs. 1 and 2, it was confirmed that the thiol protease inhibitory activities in lung cancer extracts were significantly higher than in normal lung tissue extracts.

Moreover, the authors could purify a thiol protease inhibitor (TPI) from human lung cancer tissue through papain-Sepharose affinity chromatography and Sephadex G-100 gel filtration (Figs. 3, 4, 5). The purified TPI from human lung cancer tissue gave a single band on SDS-polyacrylamide gel electrophoresis and was estimated as molecular weight of about 13, 000 (Fig. 6). This purified inhibitor inhibited some thiol proteases such as ficin and papain. The further inhibitory spectra of the TPI are now under investigation in our laboratory.

Recently, many investgators have reported the interaction among protease inhibitors and various diseases, and purified some inhibitors from tissue extracts. Blackwood et al.[4] reported that in human gynecological tumors the trypsin inhibitor concentration was lower in advanced tumors and metastases than in the

less malignant tumors or control tissues, and they suggested that the inhibitor might be consumed by proteases. However, in a previous study[24] we confirmed that trypsin inhibitory activity in lung cancer tissue was obviously higher than in normal lung tissue.

On the other hand, since Finkenstaedt[8] found a inhibitor which could inhibit cathepsin B in supernatant of rat liver homogenate, many studies have been reported concerning TPIs in various tissues, plasma and urine.

Järvinen et al.[15] reported that the human epidermal SH-protease inhibior was demonstratable in squamous cell carcinoma of the bronchus, but not in pulmonary adenocarcinoma or small-cell anaplastic carcinoma of the bronchus.

However, we confirmed the TPI activities in both adenocarcinoma and squamous cell carcinoma tissue extracts and could purify the inhibitor from both histologic types.

The molecular weight of the TPI from human lung cancer tissue is similar to the TPI from Arthus lesions of rabbit skin[33], rat liver[11,18], bovine nasal cartilage[26], rat lung and hog kidney[19] and human spleen[14].

It was of particular interest that the molecular weight of TPI from human lung cancer tissue was obviously smaller than human plasma TPIs (90, 000–170, 000)[25,27], and urinary TPIs (22, 500 –76. 000)[29,30]. The reason for these differences in molecular weights among TPIs of tissues, plasma and urine is still unclear.

Although, the regulatory mechanism of thiol protease activities by the inhibitor has been reported[16], various problems concerning the pathophysiological significance of TPI represent subject for future study.

The authors speculate that TPI from human lung cancer tissue may play a protective role against tumor cell, inhibiting the degradation of the extracellular matrix by thiol proteases.

ACKNOWLEDGEMENT

This research was funded in part by Grant-in-Aid for Scientific Research, Project 56480213, of the Japanese Ministry of Education.

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