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Studies on Thiol Protease Inhibitor Isolated from Human Breast Cancer Tissue

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ABSTRACT

Protease activity is considered to be involved in malignant tumor growth, invasion and metastasis. Although, protease activity is thought to be controlled by protease inhibitors, there are a few reports concerning the relationship between cancer and protease inhibitor derived from cancer tissue.

In the present study, thiol protease inhibitors (TPIs) were isolated from human breast cancer tissue, normal mammary gland tissue and the human breast cancer cell line (YMB-1). Their biochemical properties were investigated, giving the following results.
1) TPIs in human breast cancer extracts were significantly higher than in normal mammary gland extracts.
2) TPI was purified from both human breast cancer and human normal mammary gland extracts by papain-Sepharose affinity chromatography and Sephacryl S-200 gel filtration. Two kinds of TPIs (low-molecular weight TPI and high-molecular weight TPI) were purified from both tissues. Their molecular weights were 14,000 and 90,000, respectively as determined by gel filtration.
3) Low-molecular weight (LMW-) TPI had higher specific activity than high-molecular weight (HMW-) TPI. In breast cancer tissue extracts, LMW-TPI was dominant. Contrarily, HMW-TPI was dominant in normal gland tissue extracts.
4) Only HMW-TPI reacted with anti-urinary thiol protease inhibitor (UTPI) rabbit IgG by double immunodiffusion and immunoelectrophoresis.
5) LMW-TPI inhibited papain competitively using S-2302 as substrate. Its Km and Ki were 1.3×10^{-3}M and 6.1×10^{-8}M, respectively.
6) LMW-TPI was found to be stable to heat and pH variation.
7) TPIs were also purified from the human breast cancer cell line (YMB-1). Both TPIs which were extracted from cultured cells and released into the medium, were confirmed to be LMW-TPI.

Breast cancer cells may have lower ability to produce HMW-TPI than normal mammary gland cells. The difference of antigenicity to anti-UTPI IgG between HMW-TPI and LMW-TPI may be useful for diagnosis in near future.

Many reports[8,9,17,23,25,54,58] have been published on the protease activities involved in malignant tumor growth, invasion and metastasis. Recently, investigations have been focused[18,26,37,41] on thiol protease (mainly cathepsin B). Although protease activity is thought to be controlled by protease inhibitors, there are a few reports[30,33,49] concerning the relationship of cancer and TPI.

Various types of TPI have been isolated from plasma, urine[43,46] and other tissues[12,13,16,20,21,51]. These TPIs have been classified into serum type

TPI and tissue type TPI. Serum type TPIs have high molecular weights (MW above 60,000)[14,39,40,47,53] and tissue type TPIs have low molecular weights (MW 10,000-14,000)[10,15,22,44].

Recently, TPI (MW 66,000 or 65,000) in human plasma was proven to be identical to LMW-kininogen by amino acid analysis[29]. Although various tissue type TPIs have been studied by amino acid analysis[6,17,19,29,44,50], there are no reports on TPI derived from cancer tissue.

Previously, we reported the purification of TPI from the lung cancer tissue[33]. In this study, the author succeeded in extracting and purifying TPI from breast cancer and normal mammary tissues. Also, the author succeeded in purifying the same TPI from a human breast cancer cell line.

MATERIALS AND METHODS

Chemicals:

The following chemicals were used;

Ficin, papain, trypsin(Sigma), plasmin, urokinase(Green Cross Co., Japan), thrombin(Mochida Pharmaceutical Co., Japan), H-D-Val-Leu-Lys-pNA(S-2251, Kabi), H-D-Pro-Phe-Arg-pNA (S-2302, Kabi), pyro-Gru-Gly-Arg-pNA(S-2444, Kabi), α-N-benzoyl-DL-argine-P-nitroanilide (BANA, Nakarai Chemicals), P-Tosyl-L-arginine methyl ester(TAMe, Sigma), casein(Merk), Carbobenzoxy(Z)-Arg-Arg-MCA(Peptide Institute), Sephacryl S-200(Bio-Rad) and Sephadex G-25, Sephadex G-100, Sepharose 4B(Pharmacia). Human liver cathepsin B was purified according to the procedure of Towatari[48] and donated by Dr Sumi from Miyazaki Medical College (Specific activity 386.6 U/mg·protein). Urinary thiol protease inhibitor (UTPI) and anti-UTPI rabbit IgG was kindly donated by Dr Sato from Zeria Pharmaceutial Co. Ltd.

Assay of thiol protease activity:

1) Caseinolysis method: The caseinolytic activity of the enzyme was determined by the method of Lowry. et al[24] as reported previously[27], using 8% casein as substrate.

2) Testzym method: The amidolytic activity of the enzyme was determined with chromogenic substrates S-2251 and S-2302 by the method of Bang and Mattler[3]. S-2251 and S-2302 were used for ficin amidolytic activity and papain amidolytic activity, respectively.

3) Peptidase activity with fluorescence substrate: Human cathepsin B activity was determined with fluorescent peptide substrate (Z-Arg-Arg-MCA) according to the procedure of Barret et al[4] and Vasishta et al[52]. After incubation of 0.85ml of L-cystein(final concentration;1.5mM), cathepsin B(1μg) and 0.1M phosphate buffer (pH 6.0) mixtures for 15min at 40°C, 0.15ml of Z-Arg-Arg-MCA (0.5mM) was added and incubated for 1 hr at 40°C. Then the reaction of test sample was stopped by the addition of 1ml of acetate buffer (pH 4.3) and released 7-amino-4-

Table 1. The assay of thiol protease inhibitory activity.
Inhibitory activity was assayed from the residual caseinolytic and amidolytic activity of the thiol protease.

Caseinolytic activity (Muramatsu method[27])				
Buffer 0.65-X ml Sample X ml Protease* 0.1 ml Casein (8%) 0.25 ml	37°C, 20 min incubation	Reaction was stopped with 10% Perchloric acid	———	Measured by Lowry method
Chromozyme hydrolytic activity (Testzym method[3])				
Buffer 0.8-Y ml Sample Y ml Protease** 0.1 ml Chromozyme*** 0.1 ml	37°C, 20 min incubation	Reaction was stopped with 50% Acetic acid	———	Measured at OD405 nm

Protease* : Ficin and Papain 25 μg/ml containing 75 mM Cysteine
Protease** : Ficin and Papain 5 - 10 μg/ml containing 75 mM Cysteine
Chromozyme*** : S-2251 (H-D-Val-Leu-Lys-pNA; 3 mM) for Ficin
S-2302 (H-D-Pro-Phe-Arg-pNA; 3 mM) for Papain
1 U(unit) of Thiol-protease inhibitory activity
= the amount inhibiting 1 μg of the protease

methyl-coumarin was assayed at excitation of 360 nm and emission of 460 nm.

Assay of thiol protease inhibitory activity:

As summarized in Table 1, thiol protease inhibitory activity was assayed from the residual caseinolytic and amidolytic activities of the thiol protease after incubation with inhibitors at 37°C. The concentration of the thiol protease was adjusted to 25μg/ml with 0.1 M phosphate buffer, pH 7.4 containing 75 mM cystein in the caseinolysis method. Thiol protease concentration was adjusted to 10μg/ml with 0.05 M Tris-HC1 buffer containing 75 mM cystein in Testzym method[3]. In the assay, 0.1 ml of the enzyme solution was used. One unit(U) of TPI was defined as the amount which inhibits 1μg of the protease.

Assay of serine protease inhibitory activity:

The assay was determined from the residual enzymatic activities of the various serine protease after incubation with sample solutions at 37°C. Trypsin, plasmin and urokinase inhibitory activities were determined with BANA, S-2251 and S-2444 as substrate, respectively by Testzym method according to Bang and Mattler[3]. Thrombin inhibitory activity was determined with TAMe as substrate by Hestrin's method[11] as modified by Roberts[38].

Assay of protein concentration:

Protein concentration was determined by the method of Lowry et al[24] using bovine albumin (Merk) as a standard.

Extraction of TPI from breast cancer and normal mammary gland tissue:

The tissue extracts with 2M KSCN solution were prepared according to the procedure of Astrup et al[2] as previously modified by Okumichi[30-33]. The breast tissue sample was washed in cold phosphate buffer (0.1M, pH 7.4) to remove the contaminating blood and frozen at −20°C. The frozen tissue was minced with scissors and homogenized with bio-mixer (Nissei) in 10 volumes (w/v) of 2M KSCN solution. The homogenate was maintained at 4°C overnight and then centrifuged at 4°C for 1 hr at 20,000 *g*. The supernatant was dialyzed for 12 hr at 4°C against phosphate buffer (0.1M, pH 7.4) and recentrifuged at 4°C for 1 hr at 20,000 *g* to remove the precipitate during dialysis.

Papain-Sepharose affinity chromatography:

Papain-Sepharose gel was prepared by the method of Cutrecasas[7]. Sepharose 4B (Pharmacia) was activated by CNBr and coupled with papain. The resultant papain-Sepharose gel was inactivated by KSCN and acetic acid and washed with 0.02M trisodium phosphate containing 3M KSCN, pH 12.1, and 0.01M phosphate buffer, 0.1M NaCl, pH 6.0. A column (2.7×20cm) was packed with the inactivated papain-Sepharose gel and equilibrated with 0.01M phosphate buffer, 0.1M NaCl, pH 6.0 (starting buffer). Extracts of the solution were placed on the column and eluted with eluting buffer at a flow rate of 50 ml/hr. Fractions of 10 ml were collected. Unadsorbed fractions were eluted with the starting buffer and non specifically adsorbed protein was eluted with the starting buffer containing 3M KCl. After washing the column with excess amounts of the starting buffer, the adsorbed inhibitor fraction was eluted with 0.02M trisodium phosphate buffer, 0.1M NaCl, pH 12.1. TPI fractions were collected, dialyzed against phosphate buffered saline (pH 7.4) and concentrated to 3.5ml by ultrafiltration.

Gel filtration:

A column (2.5×96cm) of Sephacryl S-200 (Bio-Rad) was equilibrated with phosphate buffered saline (pH 7.4). Fractions of 2.5ml were collected at a flow rate of 20ml/hr. The molecular weight of the inhibitor was estimated by the method of Andrews[1] using a low molecular weight standard (Bio-Rad). Fractions of 2.0ml were collected on a column (2.6×64cm) of Sephadex G-100 (Pharmacia) equilibrated with phosphate buffer (0.1M, pH 7.4) at a flow rate of 15ml/hr.

Immunological experiments of TPI:

1) Double immunodiffusion method

Double immunodiffusion was carried out according to the Ouchterlony method[34]. Agar (Agar Noble, Difco) was suspended in veronal buffer (pH 8.6, μ=0.05) and was poured on glass plates (gel thickness 1.5mm). After irrigation with PBS and distilled water, the dried gel was dyed with Amidoblack.

2) Immunoelectrophoresis

The same volume of agar (Agar Noble, Difco) and agarose (Agarose L, Behringwerke) were suspended in veronal buffer (pH 8.6, μ=0.025) and poured on glass plate (gel thickness 1.3mm). Electrophoresis was carried out with a constant current (3mA/cm width of glass plate).

pH and heat stability test:

1) pH stability test

Aliquots of TPI were dialyzed against acetate buffer (pH 4.0), 0.01M phosphate buffer, 0.1M Nacl (pH 6.0), 0.1M phosphate buffer (pH 7.4), tris HCl buffer (pH 8.0) and 0.02 M trisodium phosphate buffer (pH 12.1) for 2 hr at 4°C. Then the samples were dialyzed against 0.1M phosphate buffer (pH 7.4) for 6 hr at 4°C and inhibitory activities were assayed.

2) Heat stability test

Aliquots of TPI were heated at 20°C, 40°C, 60°C and 80°C for 30 min, the samples were cooled to 4°C and inhibitory activities were assayed.

Measurements of inhibition constant:

Inhibition constants were calculated using the enzyme, papain, the substrate, S-2302, and LMW-TPI as the inhibitor. The concentration of papain and S-2302 were 1μg/ml and 0.3 mM, respectively. The Michaelis constant (Km) and the inhibition constant (Ki) were calculated using a Lineweaver-Burk plot.

Establishment of human breast cancer cell line (YMB-1)[59]:

New cell line (YMB-1) derived from human breast cancer was established in our laboratory. This cell line has both estrogen and progesterone receptors and is considered to be a useful model of breast cancers. This cell line was used for TPI study.

Assay of TPI in the serum free medium released from YMB-1:

Cultured cells (YMB-1) were suspended in RPMI 1640 (2.5% Fetal Calf serum : FCS) and seeded into plastic dishes (Falcon). After 48 hrs, dishes were rinsed with serum free medium (HB102™, Hana). The cell culture was continued in serum free medium for 3, 4 or 6 days. After medium exchange, TPIs in the media were assayed by Testzym method[3]. Before TPI assay, samples were dialyzed against phosphate buffer (0.1M, pH 7.4) and medium free of cultured cells was used as a control.

Purification of TPI from cultured cells (YMB-1):

After washing and homogenizing the cultured cells, TPI was extracted with 2M KSCN according to Astrup et al[2]. Papain-Sepharose affinity chromatography and Sephadex G-100 gel filtration were used as purification methods.

Table 2. Protease inhibitory activity of tissue extracts from breast cancer patients. Ficin, papain and trypsin inhibitory activities of extracts (cancer tissue and normal mammary gland tissue) were measured using S-2251, S-2302 and BANA as substrate, respectively. Ficin and papain inhibitory activities in breast cancer extracts were significantly higher than those in normal mammary gland extracts ($p<0.05$; a-b, c-d by t-test).

Case	Ficin inhibitory act. (S-2251)		Papain inhibitory act. (S-2302)		Trypsin inhibitory act. (BANA)	
	Cancer	Normal	Cancer	Normal	Cancer	Normal
1	4.0	1.3	6.6	1.5	0	0
2	3.3	0	6.3	3.4	0	0
3	4.1	1.7	4.9	1.3	0.1	0
4	2.7	0	5.8	0	0	0
5	6.7	0.7	22.2	0	0	0
6	2.0	0.3	2.5	0	0	0.1
7	2.3	0	2.1	0	0	0
8	1.9	ND*	2.3	ND	0	ND
9	6.4	ND	0	ND	0	ND
10	1.8	0.5	3.8	0	ND	ND
11	2.8	ND	1.9	ND	ND	ND
12	3.9	0.9	4.8	0.3	ND	ND
13	3.1	1.7	3.5	2.3	ND	ND
14	0.9	0.9	1.1	1.0	ND	ND
15	1.6	ND	2.4	ND	ND	ND
16	1.8	ND	2.7	ND	ND	ND
17	2.0	0.8	3.6	2.4	0	0
18	1.5	1.8	2.9	1.1	0	0
19	2.4	1.6	4.7	1.5	0	0
Mean ± SD	2.9 ± 1.6^{a}	0.9 ± 0.7^{b}	4.4 ± 4.6^{c}	1.1 ± 1.1^{d}		

(U/mg·protein)

ND* = not determined

RESULTS

Measurement of TPI from crude extract:

As shown in Table 2, ficin, papain, and trypsin inhibitory activities were measured using S-2251, S-2302 and BANA as substrate, respectively. Ficin and papain inhibitory activities in breast cancer extracts were significantly higher than those in normal mammary gland extracts ($p < 0.05$). On the other hand, trypsin inhibitory activity was not detected using BANA as substrate in either breast cancer or normal gland tissue extracts.

When ficin inhibitory activities were measured using casein as substrate, they were significantly higher in breast cancer extracts compared to normal mammary gland extracts (Table 3). However, no significant differences of TPI activities were detected either among different clinical stages (Table 4) or between estrogen receptor positive and negative groups (Table 5).

Purification of TPI:

The pooled breast cancer extract solutions were collected and applied on papain-Sepharose column. The elution pattern of the papain-Sepharose column is shown in Fig.1. The elution patterns of Sephacryl S-200 is shown in Fig.2. Almost all of proteins were eluted as two main peaks, and TPI activities were eluted as two different peaks with molecular weights that were confirmed to be about 14,000 and 90,000. The specific activities of TPIs with 14,000 and

Table 3. Ficin inhibitory activity of tissue extracts from breast cancer patients by caseinolysis. Assay was made using casein as substrate.

Case	Ficin inhibitory activity (U/mg·protein) Cancer	Normal
1	13.0	3.7
2	13.2	0
3	7.5	4.3
4	27.9	2.0
5	6.1	0
6	11.2	14.9
7	9.9	0
8	11.3	9.5
9	7.2	ND
10	4.6	1.6
11	3.7	5.1
12	5.3	4.1
Mean ± SD	10.1 ± 6.5*	4.1 ± 4.6*

* $p < 0.05$
ND = not determined

Table 4. Relationship between thiol protease inhibitory activities and clinical stage.

Clinical stage*	No. of cases	ficin IA**		papain IA	
		Cancer[a]	Normal[a]	Cancer[a]	Normal[a]
I	4	3.3 ± 1.1	1.3 ± 0.6	4.7 ± 1.4	1.3 ± 1.0
II	6	3.1 ± 1.9	0.8 ± 0.9	7.6 ± 7.3	1.4 ± 1.3
III	5	2.4 ± 1.1	0.5 ± 0.5	2.5 ± 1.4	0.3 ± 0.5
IV	4	2.9 ± 2.3	——	1.9 ± 1.2	——

The values are expressed as mean ± SD
[a] No significant differences among clinical stages by pairwise t-test
* According to General Rule for Clinical and Pathological Record of Mammary Cancer (The 8th Edition) edited by Japan Mammary Cancer Society
** Inhibitory activity by Testzym mothod[3] (U/mg·protein)

Table 5. Relationship between thiol protease inhibitory activities and estrogen receptor.

Estrogen receptor	ficin IA*		papain IA	
	Cancer[a]	Normal[a]	Cancer[a]	Normal[a]
Positive	3.0 ± 1.5 (n = 13)	1.0 ± 0.7 (n = 11)	5.7 ± 5.1 (n = 13)	1.3 ± 1.1 (n = 11)
Negative	2.7 ± 2.1 (n = 5)	0.4 ± 0.5 (n = 3)	1.6 ± 1.0 (n = 5)	0.3 ± 0.6 (n = 3)

The values are expressed as mean ± SD
[a] No significant differences between estrogen receptor positive and negative groups
* Inhibitory activity by Testzym mothod[3] (U/mg·protein)

90,000 molecular weight were 113 and 17 U/mg·protein, respectively. To compare TPI from breast cancer tissue with TPI from normal mammary gland tissue, the pooled normal mammary gland tissue solutions were applied on papain-Sepharose and Sephacryl S-200 gel column by the same methods as cancer tissue extract solutions. The column pattern of papain-Sepharose was almost the same as that of cancer extract. The column pattern of Sephacryl S-200 is shown in Fig.3. The molecular weights of purified TPI were about 14,000 and 90,000 showing the same tendency as purified TPI from breast cancer extracts. As clarified by the comparison of Fig.2 with 3, LMW-TPI (MW14,000) was dominant in breast cancer extracts (60.0% in total activity), and HMW-TPI (MW90,000) was dominant in normal mammary gland extracts (57.4% in total activity). The results of overall purification and yield of TPI from cancer extracts are summarized in Table 6. A final yield of 1.6 mg of TPI with specific activity of 113 U/mg·protein was purified from about 26g of original cancer tissues.

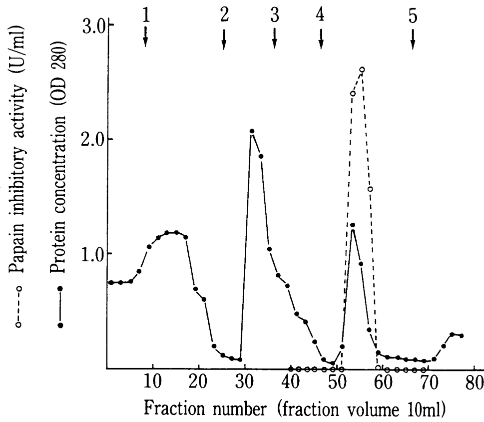

Fig. 1. Papain inhibitory activity on papain-Sepharose affinity chromatography of pooled breast cancer extracts. Pooled breast cancer extracts (adenocarcinoma) were applied to papain-Sepharose affinity chromatography. Papain inhibitory activity in each fraction (10ml) was determined by Testzym method. 1-5 were eluents (1=0.01M phosphate buffer, 0.1M NaCl, pH6.0, 2=0.01M phosphate buffer, 3M KCl, pH 6.0, 3=1, 4=0.02M trisodium phosphate buffer, 0.1M NaCl, pH12.1, 5=0.02M acetate buffer, 3M KSCN, pH 4.0,)

Antigenicity of TPI:

1) Double immunodiffusion method

The antigenicity of breast cancer and normal mammary gland extracts was evaluated against UTPI by the Ouchterlony method[34]. As shown in Fig.4, both cancer tissue and normal mammary gland tissue extracts formed a single im-

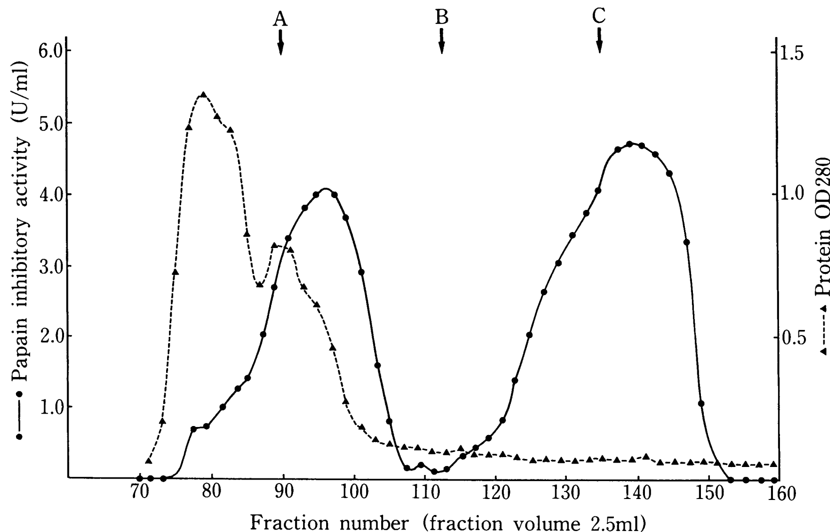

Fig. 2. Sephacryl S-200 chromatograohy of the inhibitor in pooled breast cancer extracts from papain-Sepharose affinity chromatography. Papain inhibitory activities were determined by Testzym method. A-C were molecular weight standards (A=r-globulin 158,000, B=Ovalbumin 44,000, C=Myoglobulin 17,000).

TPI activities were eluted as two different peaks with MW confirmed to be about 14,000 and 90,000. LMW-TPI(14,000) was dominant in activity (60% in total activity).

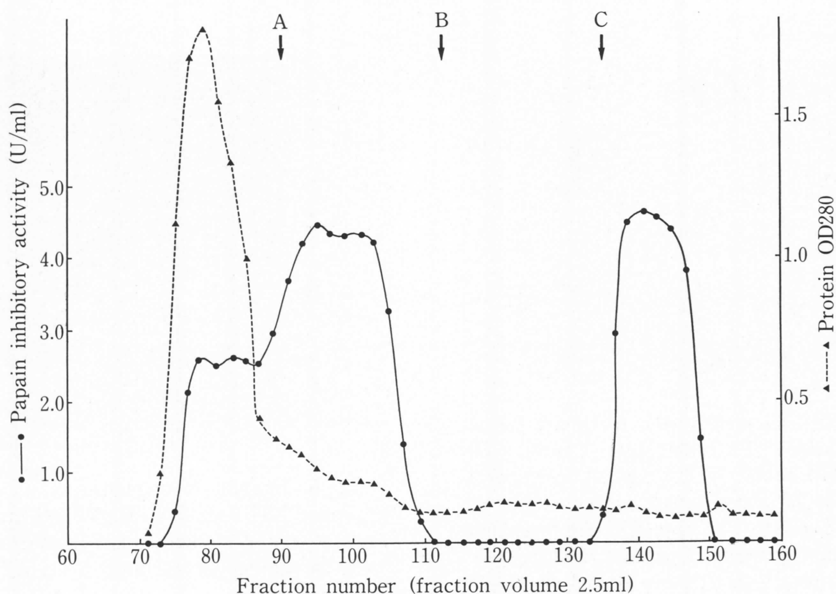

Fig. 3. Sephacryl S-200 chromatography of the inhibitor in pooled normal mammary gland extracts from papain-Sepharose affinity chromatography. Papain inhibitory activities were determined by Testzym method. A-C were molecular weight standards. (A = r-globulin 158,000, B = Ovalbumin 44,000, C = Myoglobulin 17,000).

The MW of TPI were about 14,000 and 90,000 showing the same tendency as TPI from breast cancer extracts. Although, HMW-TPI(90,000) was dominant in activity (57.4% in total activity).

Table 6. Purification procedure of LMW-TPI from breast cancer extracts.

Purification step	Volume ml	Protein mg/ml	Papain inhibitory activity U/ml	Papain inhibitory activity Total(U)	Specific activity U/mg	Recovery %
Extract	276.0	2.60	6.01	1658.8	2.3	100
Papain-Sepharose	190.0	0.22	4.42	839.8	20.1	50.6
Sephacryl S-200	40.0	0.04	4.52	180.8	113.0	10.9

munoprecipitin line with anti-UTPI rabbit IgG. Also, the antigenicity of purified LMW-TPI and HMW-TPI was examined against UTPI. As shown in Fig. 5, HMW-TPI reacted with anti-UTPI IgG, but LMW-TPI didn't react with anti-UTPI IgG.

2) Immunoelectrophoresis

As shown in Fig. 6, on immunoelectrophoresis, purified UTPI formed an immunoprecipitin arc in the α_2-β position with anti-UTPI IgG. But crude extracts from breast cancer and normal mammary gland tissues, purified HMW-TPI and partially purified TPI after papain-Sepharose formed an immunoprecipitin arc in the γ position.

These results indicate that HMW-TPI possesses the same antigenicity with UTPI, despite the difference in electrophoric motility.

Heat and pH stability test:

LMW-TPI (113 U/mg·protein) from breast cancer tissue extract was used for stable test. After keeping for 1 month at 4°C, about 100% of the activity of TPI remained. The TPI was relatively stable below 60°C but gradually inactivated above 70°C (Fig. 7). As shown in Fig. 8, the TPI was relatively stable in wide pH range (pH 4.0-pH 12.0).

Measurement of inhibition constant:

As shown in the Lineweaver-Burk plot (Fig. 9), LMW-TPI competitively inhibited the thiol proteases. The Km and Ki were 1.3×10^{-3}M and 6.1×10^{-8}M, respectively.

TPI from human breast cancer cell line (YMB-1):

The following experiments were conducted to clarify whether or not breast cancer cell produce TPI.

1) The release of TPI into medium from YMB-1

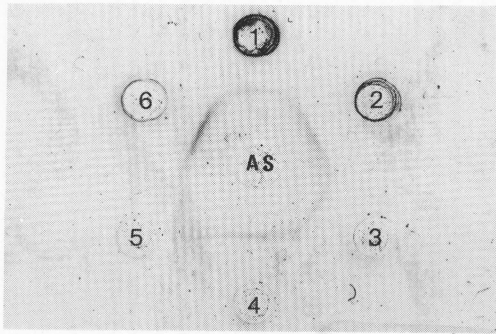

Fig. 4. Double immunodiffusion of breast tissue TPI against anti-UTPI rabbit IgG. Center well (AS): anti-UTPI rabbit IgG (80μg/8μl), peripheral wells: 1=Crude TPI from breast cancer extract of patient A (8.5μg/4μl), 2=Crude TPI from normal mammary gland extract of patient A (12μg/4μl), 3=Crude TPI from breast cancer extract of patient B (4.0μg/4μl), 4=Crude TPI from normal mammary gland extract of patient B (8.5μg/4μl), 5=Crude TPI from breast cancer extract of patient C (5.0μg/4μl), 6=Crude TPI from normal mammary gland extract of patient C (7.0μg/4μl).

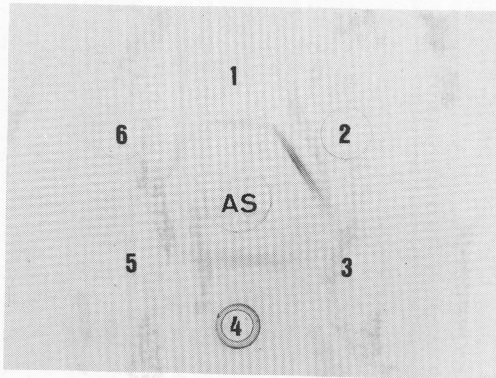

Fig. 5. Double immunodiffusion of breast tissue TPI against anti-UTPI rabbit IgG. Center well (AS): anti-UTPI rabbit IgG (80μg/8μl), peripheral wells:1=UTPI (4.8μg/4μl), 2=HMW-TPI (5.6μg/4μl), 3=LMW-TPI (4.0μg/4μl), 4=semi-purified TPI after papain-Sepharose (1.2μg/4μl), 5=veronal buffer (4μl), 6=Crude breast cancer extract (8.5μg/4μl).

The antigenicity of purified LMW-TPI and HMW-TPI was examined against UTPI. Although HMW-TPI reacted with anti-UTPI IgG, LMW-TPI didn't react with anti-UTPI IgG.

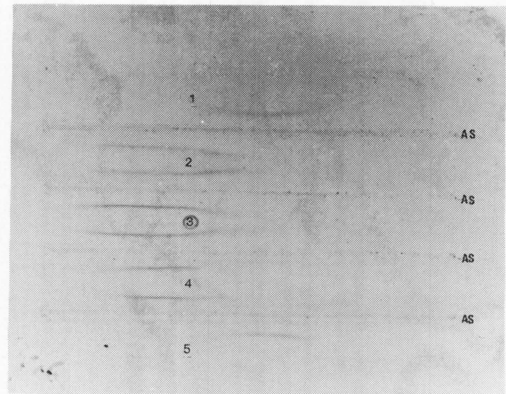

Fig. 6. Immunoelectrophoresis
1=pure UTPI (4μl), 2=HMW-TPI (4μl), 3=Crude breast cancer extract (4μl), 4=semi-purified TPI after papain-Sepharose (4μl), 5=1, AS=Anti-UTPI IgG (80μl).

Purified UTPI formed an immunoprecipitin arc in the α_2-β position with anti-UTPI IgG. But purified HMW-TPI, partially purified TPI after papain-Sepharose and crude extracts formed an immunoprecipitin arc in the γ position.

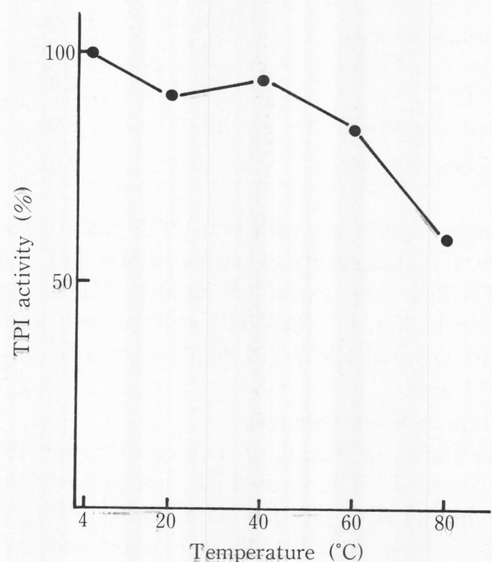

Fig. 7. Heat stability test of LMW-TPI from breast cancer extracts. The TPI was relatively stable below 60°C but gradually inactivated above 70°C.

A 2ml cell suspension (5 × 10^4 cells/ml) was seeded in plastic dishes. On days 3, 4 and 6, the medium was collected and its ficin inhibitory activity was measured. The medium free of cultured cells was used as a control. As shown in Fig. 10, ficin inhibitory activity accumulated in the medium with culture time.

2) Purification of TPI released into medium from

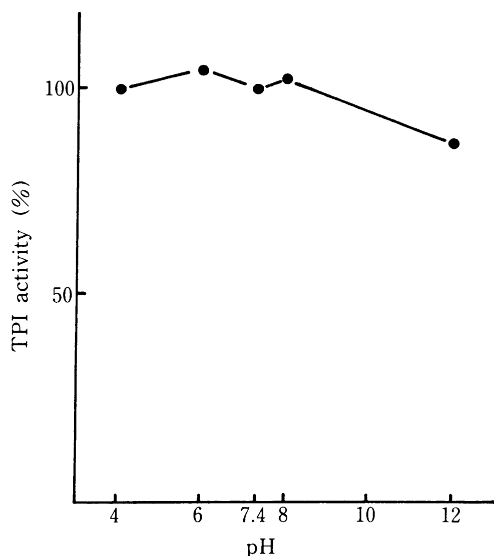

Fig. 8. PH stability test of LMW-TPI from breast cancer extracts. The TPI was stable in wide pH range (pH 4.0-pH 12.0).

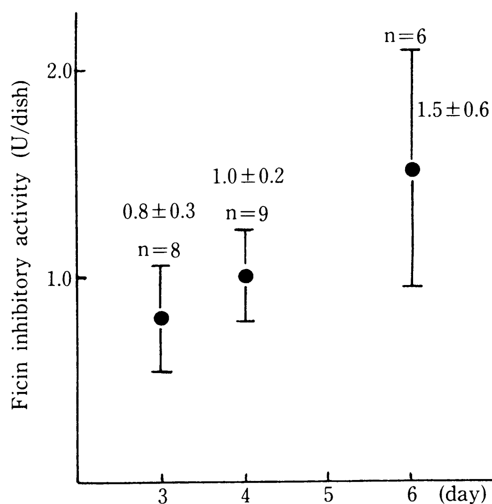

Fig. 10. The release of TPI into medium from cultured cell (YMB-1). A 2 ml cell suspension (5 × 10^4 cells/ml) was seeded in dishes. On days 3, 4 and 6, after medium exchange, ficin inhibitory activity was measured using S-2251 as substrate. The medium free of cells was used as a control. Ficin inhibitory activity accumulated with culture time.

YMB-1

Eight hundred and eighty ml of serum free medium was fractionated by ammonium sulfate (0-70% saturation). After gel filtration with

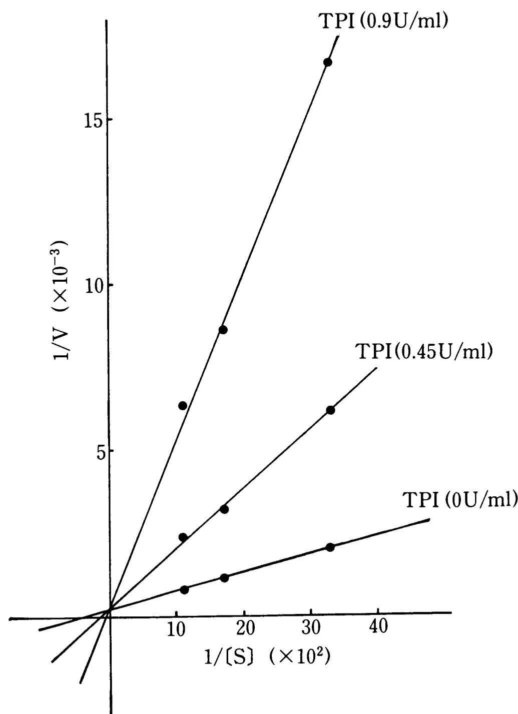

Fig. 9. Lineweaver-Burk plot. Inhibition constant was calculated using the enzyme, papain, the substrate, S-2302 and LMW-TPI as the inhibitor. The Michaelis constant (Km) and the inhibition constant (Ki) were 1.3 × 10^{-3} M and 6.1 × 10^{-8} M, respectively.

Sephadex G-25, the sample was purified by papain-Sepharose and Sephacryl S-200 column chromatographies. The recovery rates of TPI were 34.6% and 4.8%, respectively (Table 7). The molecular weight of purified TPI was only 14,000 by Sephacryl S-200 gel filtration, and its specific activity was 165.0 U/mg·protein (Fig. 11). This TPI didn't possess the same antigenicity as UTPI.

3) Purification of TPI from cultured cells (YMB-1)

An attempt was made to isolate TPI from cultured cells. As shown in Fig.12, the molecular weight of TPI was approximately 28,000 and its specific activity was 105 U/mg·protein. This TPI also didn't react with anti-UTPI rabbit IgG.

4) Papain-Sepharose and Sephacryl S-200 rechromatographies of TPI (MW 28,000) from cultured cells (YMB-1)

Table 7. Purification procedure of TPI released into medium from cultured cell (YMB-1). The sample was purified papain-Sepharose and Sephacryl S-200 chromatographies.

Purification step	Volume ml	Protein mg/ml	Papain inhibitory activity U/ml	Papain inhibitory activity Total(U)	Specific activity U/mg	Recovery %
Medium	880.0	—	—	—	—	—
Ammonium sulfate fractionation (0-70%sat.)	100.0	1.44	3.47	347.0	2.4	100
Papain-Sepharose	200.0	0.04	0.60	120.0	15.0	34.6
Sephacryl S-200	12.5	0.01	1.32	16.5	165.0	4.8

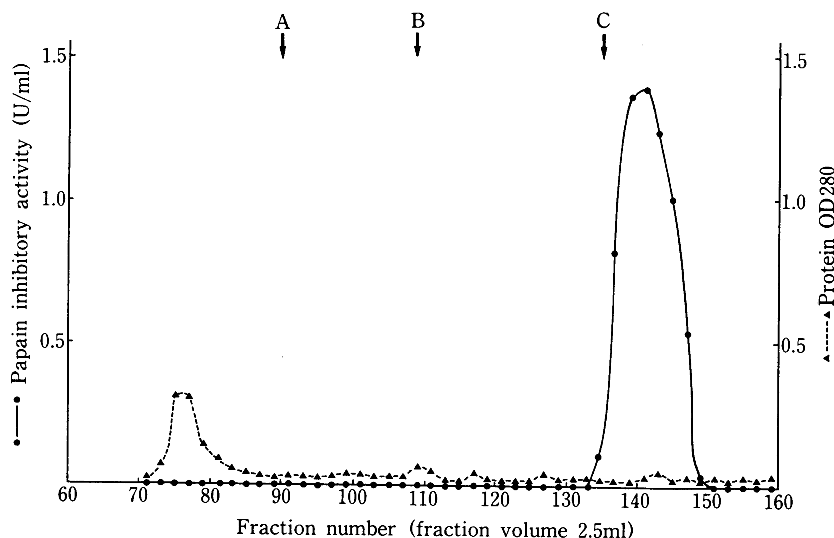

Fig. 11. Sephacryl S-200 chromatography of the inhibitor released from breast cancer cell line after papain-Sepharose affinity chromatography. A-C were molecular weight standards (A=158,000, B=44,000, C=17,000) The MW of purified TPI was only 14,000 by Sephacryl S-200 gel filtration.

TPI with the molecular weight of 28,000 was rechromatographied on papain-Sepharose column. The column patterns were almost the same as those shown in Fig.1. To estimate the molecular weight, TPI fractions were applied on Sephacryl S-200 column. The column patterns are shown in Fig. 13.

DISCUSSION

The author succeeded in isolating and purifying TPI from human breast cancer, normal mammary gland tissues and a breast cancer cell line. Although both HMW- and LMW- TPIs were purified from breast cancer and normal mammary gland tissues, only LMW-TPI was purified from the breast cancer cell line. As shown in Fig. 2 and 3, LMW-TPI was dominant in breast cancer tissue, indicating that breast cancer tissue contains more abundant tissue type[12,13,16,19-21,51] or LMW-TPI than normal mammary gland tissue.

Regarding antigenicity, HMW-TPI possessed the same antigenicity as UTPI which is thought to originate in LMW-kininogen in serum[29]. On the other hand, the antigenicity of LMW-TPI was different from UTPI. Elimination of serum is very important in the purification of TPI from tissue extracts, because serum contains numerous protease inhibitors. However, it is so difficult to eliminate serum completely that research in a cell culture system is very important. Nishida et al[28] first isolated and purified TPI from the medium of a malignant melanoma cell line. In the present study, the author first purified TPI from a human breast cancer cell line and confirmed the existence of LMW-

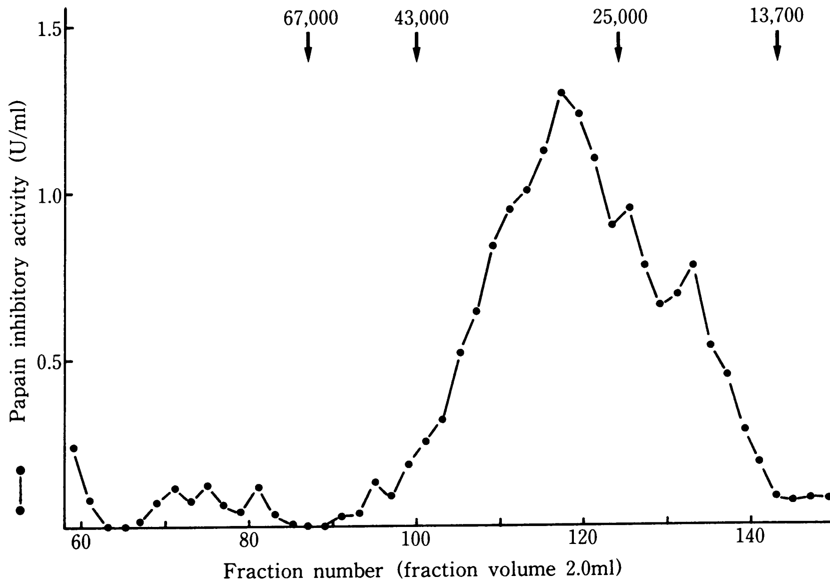

Fig. 12. Sephadex G-100 chromatography of the inhibitor derived from cultured cell after papain-Sepharose affinity chromatography. Molecular weight standards were following (albumine 67,000, ovalbumin 43,000, chymotrypsinogen 25,000, ribonuclease 13,700).

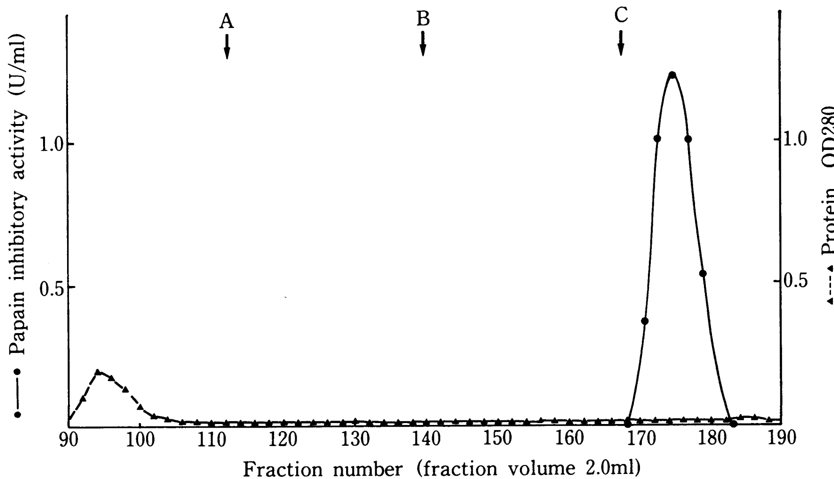

Fig. 13. Sephacryl S-200 rechromatography of TPI (MW 28,000) from cultured cells. A-C were MW standards (A=158,000, B=44,000, C=17,000).
After rechromatographies of papain-Sepharose and Sephacryl S-200, only TPI with MW of 14,000 was proven to exist.

TPI (MW 14,000) in the medium of a cultured cell line.

These results make us speculate strongly that LMW-TPI may be a specific inhibitor for breast cancer cells and that HMW-TPI may be a non-specific serum type inhibitor.

It was of particular interest that the molecular weight of LMW-TPI from cultured cells was 28,000 and that papain-Sepharose rechromatography of this inhibitor produced only LMW-TPI with the molecular weight of 14,000 (Fig. 12 and 13). Recently, Tsushima et al[49] purified three different cystein protease inhibitors with the molecular weights of 10,000, 25,000 and 80,000 from malignant melanoma tissue, and found that cystein protease inhibitor with the molecular

weight of 80,000 disappeared after papain-Sepharose rechromatography. They speculated that inhibitor with molecular weight of 80,000 may be an artifact of the papain-Sepharose column, or it could be a dimer form of the MW 40,000 inhibitor. The author also assumes that TPI with MW 28,000 may be a dimer form of TPI with MW of 14,000. There is another possibility that the molecular weight of TPI may be 28,000 in cancer cell and may be changed to 14,000 when excreted from cell into the medium. This problem is under investigation in our laboratory.

It has been reported[15,19,28,40,49] that the TPIs derived from both serum and tissues were considerably stable for temperature and pH variation. Brzin et al[6] reported that "Stefin", TPI purified from cytosol of human polymorphonuclear granulocytes is stable above pH 3.0 and below 60°C. The present results are in accordance with these reports showing that LMW-TPI purified from breast cancer tissue extracts is stable for heat and pH variation. And LMW-TPI purified from normal mammary gland tissue extracts also showed the same tendency. Many investigations have reported that TPIs derived from tissue[15,22,44,45], serum[14,39,40,47,53] and urine[43,46] were likely to have almost the same inhibitory spectrum for proteases. Purified LMW-TPI in this report inhibited some thiol proteases such as ficin, papain and cathepsin B. This TPI, however, didn't inhibit serine proteases such as trypsin, thrombin, plasmin and urokinase. This indicates that this TPI is a specific and competitive inhibitor for thiol proteases.

Malignant neoplasms have been thought to contain and secrete more thiol proteases than the surrounding normal tissues[35-37,41,42]. TPIs have also been reported to be more abundant in neoplasms than in surrounding normal tissues[28,31,33]. In this study, the author found that breast cancer extracts had higher thiol protease inhibitory activities than normal mammary gland extracts. Waxler et al[55-57] isolated and purified LMW trypsin inhibitor (MW 17,000) from normal breast tissue and breast cancer tissue, and reported that normal breast tissue contained significantly greater net proteinase inhibitory activity. Blackwood et al[5] reported that there was reverse correlation between grading of tumor progress and trypsin inhibitory activity in cancer tissue. The author also measured trypsin inhibitory activities in breast cancer and normal mammary gland tissue extracts (Table 2). However, no remarkable trypsin inhibitory activities were detected in any samples. The cause for these discrepancies is still unclear.

Although the roles of TPI in breast cancer tissue is still unclear, TPI may play an important role in the growth and metastasis of breast cancer. These problems are under investigation in our laboratory.

Furthermore, there are two problems to be resolved in this study. 1. Why does cancer tissue have less HMW-TPI in than normal breast tissue? 2. Why doesn't breast cancer cell line have HMW-TPI? Breast cancer cells may have lower ability to produce HMW-TPI than normal mammary gland cells. This is currently under investigation. The difference of antigenicity against anti-UTPI IgG between HMW-TPI and LMW-TPI may be useful for diagnosis in near future.

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REFERENCE

1. **Andrews,P.** 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J.**96**:595—606.
2. **Astrup,T. and Albrechtsen,O.K.** 1957. Estimation of the plasminogen activator and the trypsin inhibitor in animal and human tissues. Scand. J. Clin, & Lab. Invest. **9**:233—243.

3. **Bang,N.U. and Mattler,L.E.** 1978. Sensitivity and specificity of plasma serine protease chromogenic substrates. Haemostasis **7**:98–104.
4. **Barrett,A.J. and Kirschke,H.** 1981. Cathepsin B, cathepsin H and cathepsin L. p.535–561. *In* L. Lorand(ed.), Methods in enzymology vol.80. Academic Press, London.
5. **Blackwood,C.E., Mandl,I. and Long,M.E.** 1965. Proteolytic enzymes and their inhibitors in human gynecological tumors. Am. J. Obst. & Gynec. **91**:419–429.
6. **Brzin,J., Kopitar,M. and Turk,V.** 1983. Protein inhibitors of cystein proteinases 1. Isolation and characterization of stefin, a cytosolic protein inhibitor of cystein proteinases from human polymorphonuclear granulocytes. Hoppe-Seyler's Z. Physiol. Chem. **364**:1475–1480.
7. **Cuatrecasas,P.** 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. J. Biol. Chem. **245**:3059–3065.
8. **Dresden,M.H., Heilman,S.A. and Schmidt,J.D.** 1972. Collagenolytic enzymes in human neoplasms. Cancer Res. **32**:993–996.
9. **Geyer,H., Afting,E.G. and Toussi,P.** 1984. Activities of proteinases and of a proteinase B inhibitor in tumors of the human uterus. Arch Gynecol **234**:181–188.
10. **Green,G.D.J., Kembhavi,A.A., Davies,M.E. and Barrett,A.J.** 1984. Cystatinlike cysteine proteinase inhibitors from human liver. Biochem. J. **218**:939–946.
11. **Hestrin,S.** 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. **180**:249–261.
12. **Hirado,M., Iwata,D., Niinobe,M. and Fujii,S.** 1981. Purification and properties of thiol protease inhibitor from rat liver cytosol. Biochim. Biophys. Acta **669**:21–27.
13. **Järvinen,M.** 1976. Purification and properties of two protease inhibitors from rat skin inhibiting papain and other SH-proteases. Acta. Chem. Scand. **B30**:933–940.
14. **Järvinen,M.** 1979. Purification and some characteristics of two human serum proteins inhibiting papain and other thiol proteinases. FEBS Letters **108**:461–464.
15. **Järvinen,M. and Rinne,A.** 1982. Human spleen cysteineproteinase inhibitor: purification, fractionation into isoelectric variants and some properties of the variants. Biochim. Biophys. Acta **708**:210–217.
16. **Järvinen,M., Rinne,A. and Räsänen,O.** 1978. A protein reminiscent of epidermal SH-protease inhibitor occurs in squamous carcinoma of the lung. Acta. histochem. Bd. **63**: 300–303.
17. **Katunuma,N. and Kominami,E.** 1983. Structures and functions of lysosomal thiol proteinases and their endogeneous inhibitor. Curr. Top. Cell Regul. **22**:71–101.
18. **Kirschke,H., Langner,J., Riemann,S., Wiiedreanders,B. and Bohley,P.** 1980. Cysteine proteinases of lysosomes. p.69–79. *In* P. Sträuli, A.Barret and A. Baici (eds.), Proteinases and tumor invasion, Raven Press.
19. **Kominami,E., Wakamatsu,N. and Katunuma,N.** 1982. Purification and characterization of thiol proteinase inhibitor from rat liver. J. Biol Chem. **257**:14648–14652.
20. **Kopitar,M., Brzin,J., Zvonar,T., Löcnikar,P., Kregar,I. and Turk,V.** 1978. Inhibition studies of an intracellular inhibitor on thiol proteinases. FEBS Lett. **91**:355–359.
21. **Lenny,J.F.** 1980. Inhibitors associated with the proteinases of mammalian cells and tissues. Curr. Top. Cell. Regul. **17**:25–57.
22. **Lenny,J.F., Tolan,J.R., Sugai,W.J. and Lee,A.G.** 1979. Thermostable endogeneous inhibitors of cathepsin B and H. Eur. J. Biochem. **101**:153–161.
23. **Liotta,L.A., Tryggvason,K., Garbisa,S., Hart,I., Foltz,C.M. and Shafie,S.** 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature **284**:67–68.
24. **Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265–275.
25. **Markus,G., Takita,H., Camiolo,S.M., Corasanti,J.G., Evers,J.L. and Hobica,G.H.** 1980. Content and characterization of plasminogen activators in human lung tumors and normal lung tissue. Cancer Res. **40**:841–848.
26. **Mullins,D.E. and Rohrlich,S.T.** 1983. The role of proteinases in cellular invasiveness. Biochim. Biophys. Acta **695**:177–214.
27. **Muramatsu,M., Onishi,T., Makino,S., Fujii,S. and Yamamura,Y.** 1965. Inhibition of caseinolytic activity of plasmin by various synthetic inhibitors. J. Biochem. **57**:402–406.
28. **Nishida,Y., Sumi,H. and Mihara,H.** 1984. A thiol protease inhibitor released from cultured human malignant melanoma cells. Cancer Res. **44**:3324–3329.
29. **Ohkubo,I., Kurachi,K., Takasawa,T., Shiokawa,H. and Sasaki,M.** 1984. Isolation of a human cDNA for α_2-thiol proteinase inhibitor and its identity with low molecular weight kininogen. Biochemistry **23**:5691–5697.
30. **Okumichi,T.** 1985. Studies on the protease inhibitors in lung cancer tissue 1.Purification of urinary trypsin inhibitor-like inhibitor from human lung cancer tissue. Medical J. Hiroshima University **33**:1–16 (in Japanese).
31. **Okumichi,T.** 1985. Studies on the protease inhibitors in lung cancer tissue 2.Purification of thiol protease inhibitor from human lung cancer tissue. Medical J. Hiroshima University **33**:17–30 (in Japanese).
32. **Okumichi,T., Nishiki,M., Takasugi,S., Toki,N. and Ezaki,H.** 1984. Isolation of urinary trypsin inhibitor-like inhibitor from human lung cancer

tissue. Cancer Res. **44**:2011—2015.

33. **Okumichi,T., Nishiki,M., Takasugi,S., Yamane,M. and Ezaki,H.** 1984. Purification of thiol protease inhibitors in lung cancer tissue. Hiroshima J. Med. Sci. **33**:801—808.
34. **Ouchterlony,O.** 1958. Diffusion in gel methods from immunological analysis. Prog. Allergy **5**:1—78.
35. **Pietras,R.J., Szego,C.M., Mangan,C.E., Seeler,B.J., Burtnett,M.M. and Orevi,M.** 1978. Elevated serum cathepsin Bl and vaginal pathology after prenatal DES exposure. Obstet Gynecol **52**:321—327.
36. **Poole,A.R., Recklius,A.D. and Mort,J.S.** 1980. Secretion of proteinases from human breast tumors:Excessive release from carcinomas of a thiol proteinase. p.81—95. **In** P.Strauli, A.Barrett and A.Baici(eds.), Proteinases and Tumor Invasion, Raven Press.
37. **Poole,A.R., Tiltman,K.J., Recklius,A.D. and Stoker,T.A.M.** 1978. Differences in secretion of the proteinase cathepsin B at the edges of human breast carcinomas and fibroadenomas. Nature **273**:545—547.
38. **Roberts,P.S.** 1958. Measurement of the rate of plasmin action on synthetic substrates. J. Biol. Chem. **232**:285—291.
39. **Ryley,H.C.** 1979. Isolation and partial characterization of a thiol proteinase inhibitor from human plasma. Biochem. Biophys. Res. Commun. **89**:871—878.
40. **Sasaki,M., Taniguchi,K. and Minakata,K.** 1981. Multimolecular forms of thiol proteinase inhibitor in human plasma. J. Biochem. **89**:169—177.
41. **Sloane,B.F., Dunn,J.R. and Honn,K.V.** 1981. Lysosomal cathepsin B:Correlation with metastatic potential. Science **212**:1151—1153.
42. **Sloane,B.F., Honn,K.V., Sadler,J.G., Turner,W.A., Kimpson,J.J. and Taylor,J.D.** 1982. Cathepsin B activity in B 16 melanoma cells: A possible marker for metastatic potential. Cancer Res. **42**:980—986.
43. **Sumi,H. and Toki,N.** 1981. Inhibitors of acrosin and SH-protease in normal human urine(41209). Proc. Soc. Exp. Biol. Med. **167**:530—535.
44. **Takio,K., Kominami,E., Bando,Y., Katunuma,N. and Titani,K.** 1984. Amino acid sequence of rat epidermal thiol proteinase inhibitor. Biochem. Biophy. Res. Commun. **121**:149—154.
45. **Takio,K., Kominami,E., Wakamatu,N., Katunuma,N. and Titani,K.** 1983. Amino acid sequence of rat liver thiol proteinase inhibitor. Biochem. Biophy. Res. Commun. **115**:902—908.
46. **Taniguchi,K., Ito,J. and Sasaki,M.** 1981. Partial purification and properties of urinary thiol proteinase inhibitors. J. Biochem. **89**:179—184.
47. **Tokaji,G.** 1971. The chemical pathology of thermal injury, with special reference to burns SH-dependent protease and its inhibitor. Kumamoto Med. J. **24**:68—86.
48. **Towatari,T., Kawabata,Y. and Katunuma,N.** 1979. Crystallization and properties of cathepsin B from rat liver. Eur. J. Biochem. **102**:279—289.
49. **Tsushima,H., Sumi,H., Hamanaka,K., Toki,N., Sato,H. and Mihara,H.** 1985. Cystein protease inhibitors isolated from human malignant melanoma tissue. J. Lab. Clin. Med. **106**:712—717.
50. **Turk,V., Brzin,J., Ritonja,A., Eropkin,M., Borchart,U. and Machleidt,W.** 1983. Protein inhibitors of cystein proteinases 3. Amino-acid sequence of cystatin from chicken egg white. Hoppe-Seyler's Z. Physiol. Chem. **364**:1487—1496.
51. **Udaka,K. and Hayashi,H.** 1965. Further purification of a protease inhibitor from rabbit skin with healing inflammation. Biochim. Biophys. Acta **97**:251—261.
52. **Vasishta,A., Baker,P.R., Preece,P.E., Wood,R.A.B. and Cuschieri,A.** 1984. Serum proteinase-like peptidase activities and proteinase inhibitors in women with breast disease. Eur. J. Cancer Clin. Oncol. **20**:197—202.
53. **Wakamatsu,N., Kominami,E. and Katunuma,N.** 1982. Comparison of properties of thiol proteinase inhibitors from rat serum and liver. J. Biol. Chem. **257**:14653—14656.
54. **Wang,B.S., Mcloughlin,G.A., Richie,J.P. and Mannick,J.A.** 1980. Correlation of the production of plasminogen activator with tumor metastasis in B 16 mouse melanoma cell lines. Cancer Res. **40**:288—292.
55. **Waxler,B., Corey,J. and Wezeman,F.H.** 1984. Low molecunar weight proteinase inhibitors. 3. Quantification and isolation of activity from benign and malignant human breast tissue sample. J. Lab Clin. Med. **104**:528—537.
56. **Waxler,B. and Wezeman,F.H.** 1983. Low molecular weight proteinase inhibitors 1. Extraction and identification of activity from normal and malignant human breast tissues. Br. J. Exp. Path. **64**:252—260.
57. **Waxler,B. and Wezeman,F.H.** 1983. Low molecular weight proteinase inhibitors 2. Extraction and identification of activity from infiltrating ductal carcinoma during lactation. Br. J. Exp. Path. **64**:626—632.
58. **Wirl.G.** 1977. Extractable collagenase and carcinogenesis of mouse skin. Connective Tissue Res. **5**:171—178.
59. **Yamane,M., Nishiki,M., Kataoka,T., Kishi,N., Amano,K., Nakagawa,K., Okumichi,T., Naito,M., Ito,A. and Ezaki,H.** 1984. Establishment and characterization of new cell line (YMB-1) derived from human breast carcinoma. Hiroshima J. Med. Sci. **33**:715—720.