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Variant Cathepsin L Activity from Gastric Cancer Tissue

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Cathepsin L activity was partially purified by S-Sepharose FF chromatography, concanavalin-A Sepharose chromatography, phenyl-Superose column chromatography, Mono S column chromatography, and TSK G3000SWXL column chromatography from gastric cancer tissue. The optimal pH of cathepsin L from gastric cancer tissue was 7.4, and the activity was retained even at alkaline pH. Heat stability tests showed that cathepsin L from gastric cancer tissue was heat stable; that is, 65% activity was retained after incubation at 56°C for 60 min. The molecular weight of cathepsin L from gastric cancer tissue was estimated as 115 kD by gel filtration or 110 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme showed a different affinity for wheat germ agglutinin-Sepharose than cathepsin L from gastric normal mucosa. These results suggest that cathepsin L from gastric cancer tissue may play an important role in gastric cancer invasion through the destruction of the surrounding extracellular matrix by its proteolytic activity.

Key words: Cathepsin L — Proteinase — Gastric cancer

Cathepsin L (EC 3.4.22.15) is a cysteine proteinase whose physiological role is considered to be degradation of proteins in lysozomes. Cathepsin B (EC 3.4.22.1), which also belongs to the class of cysteine proteinases, has been well studied in relation to cancer invasion and metastasis. Cathepsin B can degrade the components of the extracellular matrix, such as collagen, fibronectin, and proteoglycans.[1] Therefore, cathepsin B in cancer cell membranes or released from cancer cells may facilitate cancer cell invasion through the destruction of the surrounding extracellular matrix by its proteolytic activity. There is some experimental evidence supporting a role in cancer invasion and metastasis for cathepsin B.[1–5] However, there have been only a few studies on the relation of cathepsin L to cancer proliferation and evolution.[1] In this study, we purify and characterize the cathepsin L from gastric cancer tissue, and compare its properties with those of cathepsin L from normal gastric mucosa.

MATERIALS AND METHODS

Gastric tissue Gastric tissue was obtained from a patient with gastric cancer by surgical operation and stored at −80°C until use.

Abbreviations used: Con A, concanavalin A; kD, kilodalton; Z-Phe-Phe-CHN_2, carbobenzoxy-L-phenylalanyl-L-phenylalanine CHN_2; Z-Phe-Ala-CHN_2, carbobenzoxy-L-phenylalanyl-L-alanine CHN_2; Z-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide; Arg-MCA, L-arginine 4-methyl-Coumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; GlcNAc, N-acetylglucosamine.

Enzyme assay Cathepsin L activity was measured as described previously.[6] Z-Phe-Arg-MCA was purchased from Peptide Institute, Inc., Osaka. All enzyme activities are expressed in terms of the amount of liberated AMC per minute.

Purification of cathepsin L from gastric cancer tissue Cathepsin L of gastric cancer tissue was purified as described previously with slight modifications.[7] Gastric cancer tissue was homogenized in ice-cold 1,000 m*M* sodium acetate buffer, pH 5.0, containing 1 m*M* EDTA and 0.2% Triton X-100 in a Waring blender at 0°C following sonication for 20 s. The resulting homogenate was stirred overnight at 4°C, and the debris was removed by centrifugation at 15,000*g* for 20 min. The pooled supernatants were acidified to pH 4.2 with 2 *M* HCl, and precipitated protein was removed by centrifugation at 15,000*g* for 20 min. A 20–65% saturation ammonium sulfate fraction of the supernatant was taken and the precipitated protein was redissolved in 20 m*M* sodium phosphate buffer, pH 5.8, containing 1 m*M* EDTA. Ammonium sulfate was removed by filtration on a Sephadex G-25 column. The pooled active fractions were applied to an S-Sepharose FF column (5×25 cm) equilibrated with 20 m*M* sodium phosphate buffer, pH 5.8, containing 1 m*M* EDTA. The elution rate was 8 ml/min. A linear gradient of NaCl from 0 to 0.5 *M* was used and 20 ml fractions were collected. Enzyme active fractions were collected, concentrated by ultrafiltration using a Labo cassette (molecular weight cut-off 10 kD) and dialyzed against 20 m*M* sodium phosphate buffer, pH 5.8 containing 0.2 *M* NaCl, 1 m*M* $CaCl_2$, and 1 m*M* $MnCl_2$. Then the fractions were applied to a Con A column (1.6×20 cm) equilibrated with the same buffer. The column was

successively washed with the same buffer until the absorbance at 280 nm reached the baseline. Then the column was eluted with the same buffer containing 0.2 *M* α-methyl-D-mannoside. Next, the active fractions were dialyzed against the phenyl-Superose column starting buffer (0.1 *M* phosphate buffer, pH 6.8, containing 1.7 *M* ammonium sulfate), and applied to the phenyl-Superose column (1×10 cm) with a linear gradient of ammonium sulfate from 1.7 to 0 *M*. The active fractions were then dialyzed against the Mono S column starting buffer (10 m*M* sodium phosphate buffer, pH 5.8), and applied to the Mono S column (0.5×5 cm) with a linear gradient of NaCl from 0 to 0.5 *M*. The active fractions were dialyzed against 20 m*M* sodium acetate buffer, pH 5.8, containing 0.2 *M* NaCl, applied to a TSK G3000SWXL column equilibrated with the same buffer and then eluted at a flow rate of 1.0 ml/min. The purified enzyme was dialyzed extensively against double-distilled water, and freeze-dried. The lyophilized sample was stored at −80°C until the enzyme characterization study.

Purification of cathepsin L from normal gastric tissue Purification of cathepsin L from normal gastric tissue was performed as described above. The purified enzyme was dialyzed extensively against double-distilled water, and freeze-dried. The lyophilized sample was stored at −80°C until the enzyme characterization study.

Substrate specificity test Substrate specificity was studied with Z-Phe-Arg-MCA and Arg-MCA (Peptide Institute, Inc.) and azocasein using highly purified samples. Measurement of hydrolysis of Z-Phe-Arg-MCA and Arg-MCA was performed as described previously.[6] All enzyme activities are expressed as the amount of AMC liberated per minute. Azocasein assay was also performed as described previously.[6]

Optimal pH and heat stability Highly purified samples from gastric cancer tissue and normal gastric mucosa were used to determine the optimal pH and heat stability. The optimal pH of cathepsin L was assayed using 0.2 *M* phosphate (pH 5–8.5) buffer. Heat stability was tested at 37°C and 56°C for 60 min at pH 6.0. The remaining enzyme activity after exposure to these temperatures for 5, 10, 20, 30, and 60 min was assayed.

Effect of inhibitors The inhibitory activities of several enzyme inhibitors such as soybean trypsin inhibitor (1 mg/ml), leupeptin (1 mg/ml), Z-Phe-Phe-CHN_2 (1 mg/ml), and Z-Phe-Ala-CHN_2 (1 mg/ml) were tested using highly purified samples.

Alkaline pH stability test The stability of purified cathepsin L from gastric cancer tissue and normal gastric tissue was tested at alkaline pH. Purified cathepsin L was preincubated at pH 7.0, 7.4, 8.0 and 8.5 for 5, 10, 20 and 30 min at 37°C and the remaining activity was assayed.

Molecular weight determination The apparent molecular weights of cathepsin L were determined by using a gel filtration column (TSK G3000SWXL) and SDS-PAGE. A highly purified sample was applied to the column equilibrated with 10 m*M* phosphate buffer, pH 6.0, containing 0.2 *M* NaCl. The elution rate was 1 ml/min. The column was calibrated with the following standard proteins: glutamate dehydrogenase, 290 kD; lactate dehydrogenase, 142 kD; enolase, 67 kD; adenylate kinase, 32 kD; and cytochrome *c*, 14.2 kD. SDS-PAGE was carried out in gradient gel from 10% (cathode) to 15% (anode) for 64 mV-h. After fixation with 20% trichloroacetic acid solution, the gel was stained with Coomassie blue.

Isoelectric point determination of cathepsin L Isoelectric points were determined by using a Mono P column (0.5×20 cm, Pharmacia). A highly purified sample was applied to the column equilibrated with 0.025 *M* imidazole-HCl buffer, pH 7.4. A pH gradient from 4 to 7 was made by elution with Polybuffer 74 (Pharmacia)-HCl, pH 4.0.

Lectin affinities of cathepsin L from gastric cancer tissue and gastric normal mucosa Con A-Sepharose and WGA-Sepharose column chromatographies were performed. A WGA-Sepharose column (1.6×10 cm) was prepared in 20 m*M* sodium phosphate buffer, pH 6.0. The highly purified sample was applied to the column at a flow rate of 0.5 ml/min. The column was eluted successively with the same buffer containing 0.2 *M* NaCl, and then the same buffer containing 0.4 *M* GlcNAc. Con A-Sepharose column chromatography was performed as described above.

Protein determination The protein content was determined using the Bio-Rad microassay procedure.[8]

RESULTS

Purification of cathepsin L from gastric cancer tissue Typical elution profiles are shown in Figs. 1–5. Gastric cancer cathepsin L showed affinities for the S-Sepharose FF column, Con A-Sepharose column, phenyl-Superose column and Mono S column (Figs. 1–4). The result of the SDS-PAGE study was consistent with the purity of our prepared sample, showing a single protein band (Fig. 6). The result of purification of cathepsin L from gastric cancer tissue is summarized in Table I.

Purification of cathepsin L from normal gastric mucosa The result of purification of cathepsin L from gastric normal mucosa is summarized in Table I. Typical elution profiles are shown in Figs. 1–5. Normal gastric mucosa cathepsin L was purified in the same way as gastric cancer tissue cathepsin L and had affinities for the S-Sepharose column, Con A-Sepharose column, phenyl-Superose column, and Mono S column (Figs. 1–4). In the S-Sepharose, phenyl-Superose, and Mono S columns, cathepsin L from normal gastric mucosa was eluted at

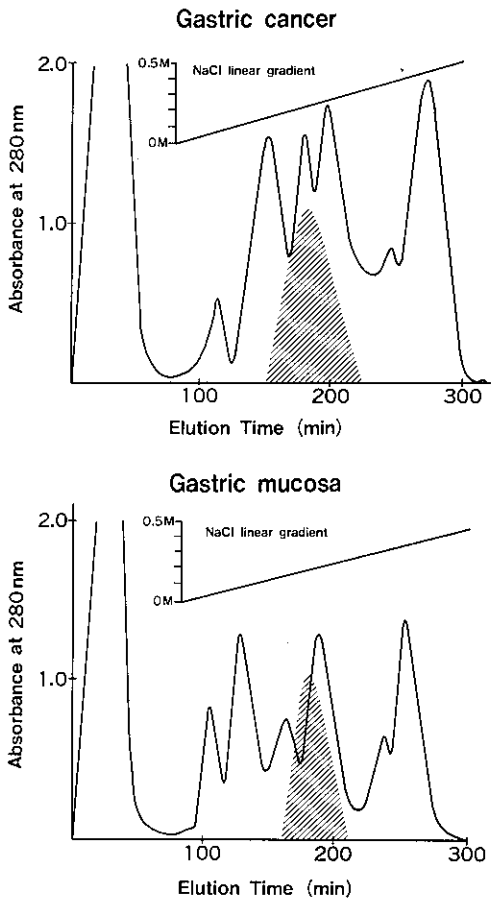

Fig. 1. Elution patterns of cathepsin L from gastric cancer tissue and normal gastric mucosa from an S-Sepharose FF column. ——, Absorbance at 280 nm; (▨), cathepsin L active fraction.

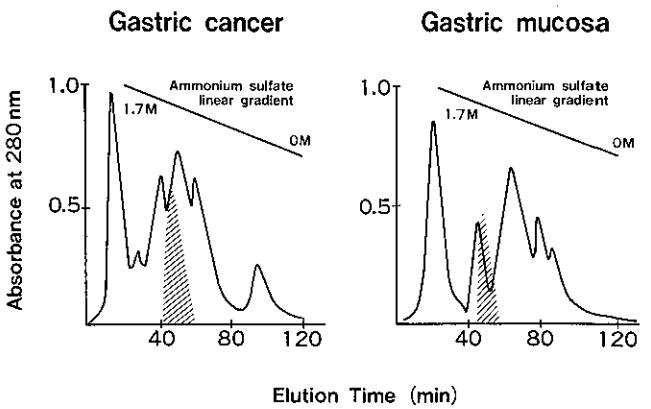

Fig. 3. Elution patterns of cathepsin L from gastric cancer tissue and normal gastric mucosa from a phenyl-Superose column. ——, Absorbance at 280 nm; (▨), cathepsin L active fraction.

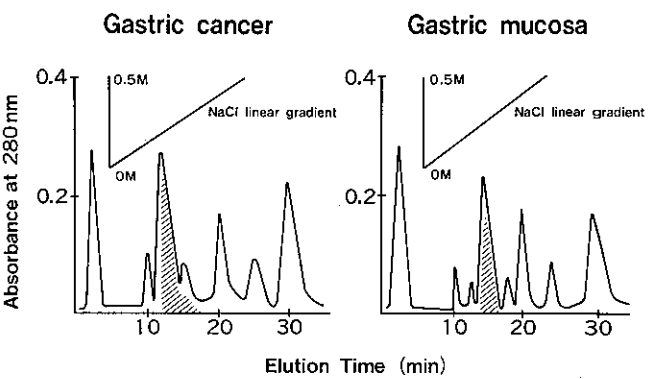

Fig. 4. Elution patterns of cathepsin L from gastric cancer tissue and normal gastric mucosa from a Mono S column. ——, Absorbance at 280 nm; (▨), cathepsin L active fraction.

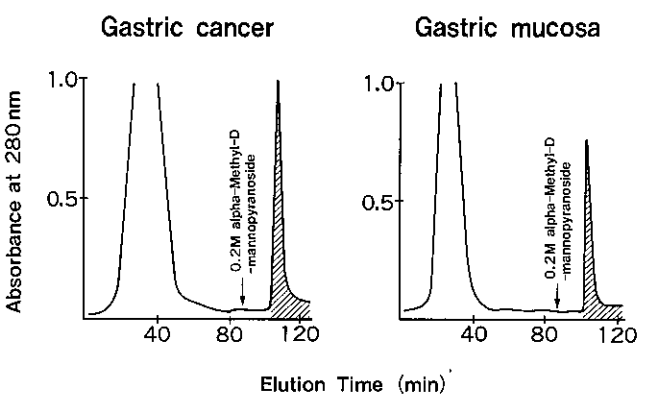

Fig. 2. Elution patterns of cathepsin L from gastric cancer tissue and normal gastric mucosa from a Con A-Sepharose column. ——, Absorbance at 280 nm; (▨), cathepsin L active fraction.

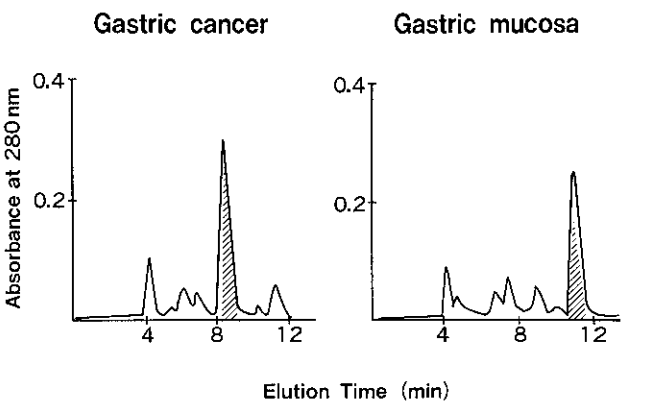

Fig. 5. Elution patterns of cathepsin L from gastric cancer tissue and normal gastric mucosa from a TSK G3000SWXL column. ——, Absorbance at 280 nm; (▨), cathepsin L active fraction.

slight different ammonium sulfate and NaCl concentrations from cathepsin L from gastric cancer tissue. The result of the SDS-PAGE study was also consistent with the purity of our prepared sample, showing a single protein band (Fig. 6).

Substrate specificity test Because of the similarity of cathepsins H (EC 3.4.22.16) and B to cathepsin L, it was necessary to rule out the presence of these enzymes in our preparations. Our enzymes did not hydrolyze Arg-MCA (Table II). This property is uncharacteristic of cathepsin H.[6] In azocasein assay, the azocaseinolytic activities of our prepared enzymes were all enhanced by adding 3 *M* urea (Table II). This is characteristic of cathepsin L and uncharacteristic of cathepsin B, since cathepsin B activity is depressed (more than 90%) by 3 *M* urea.[6] Furthermore, hydrolysis of Z-Phe-Arg-MCA by our enzymes was not depressed at all by adding anti-cathepsin B. Judging from this substrate specificity study, our prepared enzymes were considered to be cathepsin L, free of cathepsins H and B.

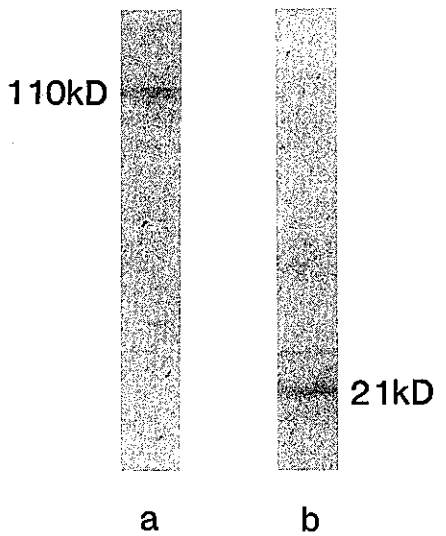

Fig. 6. SDS-PAGE pattern of cathepsin L from gastric cancer tissue (lane a) and normal gastric mucosa (lane b)

Table II. Substrate Specificity of Cathepsin L from Gastric Cancer Tissue and Normal Gastric Mucosa

	Activity (%)	
	Gastric cancer tissue	Normal gastric mucosa
Synthetic substrate[a]		
Z-Phe-Arg-MCA	100	100
Arg-MCA	0	0
Protein substrate[b]		
Azocasein	100	100
Azocasein + 3 *M* urea	254	238

a) Substrate concentration was 20 μM in all cases. Enzyme assays were performed for 10 min with a fixed amount of enzyme. Activities are expressed as percentage of Z-Phe-Arg-MCA hydrolysis.
b) Activities are expressed as percentage of azocaseinolysis without 3*M* urea.

Table I. Summary of Purification of Cathepsin L from Gastric Cancer Tissue and Normal Gastric Mucosa

	Protein (mg)	Total activity (μmol/min)	Specific activity (nmol/min/mg)
Gastric cancer			
Pooled active fractions[a]	1800	350	194.4
S-Sepharose FF column	480	275	572.9
Con A-Sepharose column	120	138	1150.0
Phenyl-Superose column	18.4	185	10054.3
Mono S column	4.3	102	23720.9
TSK G3000SW column	1.1	52	47272.7
Gastric mucosa			
Pooled active fractions[a]	1320	59	44.7
S-Sepharose FF column	348	38	109.2
Con A-Sepharose column	91	21	230.8
Phenyl-Superose column	14	38	2714.3
Mono S column	3.5	24	6857.1
TSK G3000SW column	0.9	11	12222.2

a) Pooled active fractions were prepared as described in "Materials and Methods."

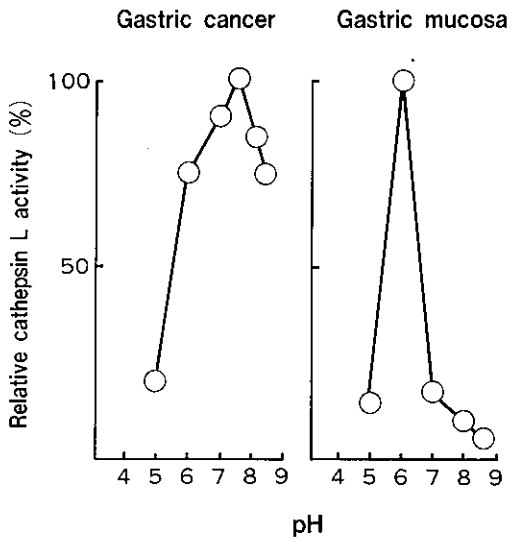

Fig. 7. pH optimum of cathepsin L. The optimal pH of cathepsin L was tested using 0.2 *M* phosphate buffer (pH 5–8.5).

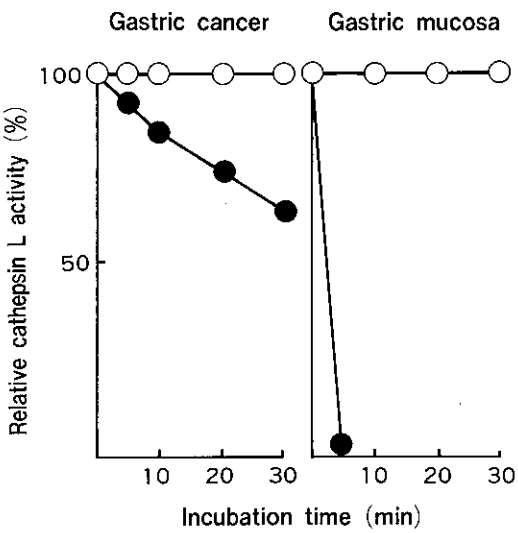

Fig. 8. Heat stability of cathepsin L. ○; 37°C, ●; 56°C.

Optimal pH The optimal pH for cathepsin L of gastric cancer tissue was 7.4. Its activity remained stable at alkaline pH. The optimal pH for cathepsin L of normal gastric mucosa was 6.0 (Fig. 7).

Heat stability The enzyme activity of cathepsin L of gastric cancer tissue was retained with no significant loss at 37°C. Furthermore, 65% of the initial activity was retained at 56°C after 60 min. On the other hand, the activity of cathepsin L of normal gastric mucosa was stable at 37°C, but was lost within 5 min at 56°C (Fig. 8).

Effect of inhibitors The effects of various compounds on the activity of cathepsin L of gastric cancer tissue and

Table III. The Effect of Various Compounds on the Activity of Cathepsin L from Gastric Cancer Tissue and Normal Gastric Mucosa

Compound	Concentration (mg/ml)	Remaining activity(%)	
		Gastric cancer tissue	Normal gastric mucosa
Soybean trypsin Inhibitor	1	100	100
Leupeptin	1	0	0
Z-Phe-Phe-CHN_2	1	0	0
Z-Phe-Ala-CHN_2	1	0	0

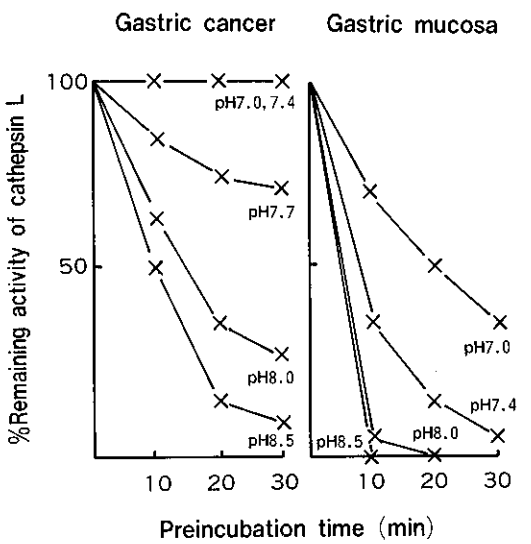

Fig. 9. Alkaline pH stability test of cathepsin L. The stability at alkaline pH of cathepsin L was tested. Cathepsin L was preincubated at pH 7.0, 7.4, 8.0, 8.5 for 5, 10, 20, and 30 min and its remaining activity was assayed.

Table IV. Lectin Affinities of Cathepsin L from Gastric Cancer Tissue and Normal Gastric Mucosa

	Binding percentage to lectin columns	
	Con A	WGA
Gastric cancer tissue	68	43
Gastric normal mucosa	65	73

normal gastric mucosa are summarized in Table III. The enzymes showed almost the same sensitivities to various inhibitors.

Alkaline pH stability test Cathepsin L from gastric cancer tissue and normal gastric mucosa was tested for stability at alkaline pH. Cathepsin L from normal gastric mucosa was inactivated at pH 7.4 for 30 min. On the other hand, cathepsin L from gastric cancer tissue was stable at pH 7.4 for 30 min (Fig. 9).

Molecular weight determination The molecular weight of cathepsin L from gastric cancer was estimated as 115 kD by gel filtration (Fig. 5) or 110 kD by SDS-PAGE (Fig. 6). On the other hand, the apparent molecular weight of cathepsin L from normal gastric mucosa was 25 kD by gel filtration (Fig. 5) or 21 kD by SDS-PAGE (Fig. 6).

Isoelectric point determination of cathepsin L Isoelectric points of cathepsin L from gastric cancer tissue and normal gastric mucosa were estimated as 4.0, 5.2, respectively. Cathepsin L from gastric cancer tissue had a rather acidic isoelectric point compared to the cathepsin L of normal gastric mucosa.

Lectin affinities of cathepsin L from gastric cancer tissue and normal gastric mucosa The percentages of cathepsin L from gastric cancer tissue and gastric normal mucosa bound to Con A- and WGA-Sepharose columns are shown in Table IV.

DISCUSSION

It has been suggested that proteases produced and secreted by cancer cells play an important role in cancer invasion and metastasis.[1, 2, 9, 10] Cathepsin B and cathepsin L are well-known lysosomal cysteine proteases which are considered to play an important role physiologically in the general catabolism of proteins in lysosomal systems.[11] Cathepsin B has been well studied in relation to cancer invasion and metastasis.[1-5] On the other hand, there have been very few reports about cathepsin L and cancer invasion.[1]

Recently we have assayed the activities of cathepsin B, cathepsin L, and plasminogen activators (urinary-type plasminogen activator and tissue-type plasminogen activator) in homogenates of cancer tissue, normal tissue adjacent to the cancer tissue, and normal tissue distant from the cancer tissue from 30 patients undergoing surgery for gastric cancers, and we found that in the cancer tissue homogenates, the activities of cathepsin B, cathepsin L and tissue-type plasminogen activator were significantly higher than in normal tissues, although the activities of urinary-type plasminogen activator in cancer tissues were significantly lower than in normal tissues (unpublished results). Gastric cancer tissue used in this report also had higher activities of cathepsin L (total activity, 350 μmol/min; specific activity, 194.4 nmol/min/mg, as indicated in Table I) and cathepsin B (total activity, 120 μmol/min; specific activity, 66.7 nmol/min/mg) than those in normal gastric mucosa (cathepsin L total activity, 59 μmol/min; specific activity, 44.7 nmol/min/mg, as indicated in Table I, and cathepsin B total activity, 35 μmol/min; specific activity, 26.5 nmol/min/mg). From these results, we suggest that not only cathepsin B but also cathepsin L may be implicated in gastric cancer invasion.

In order to clarify the contribution of cathepsin L to gastric cancer proliferation and evolution, it is important to determine whether and how the cathepsin L produced by cancer cells differs from the cathepsin L of normal cells. From this viewpoint, we purified and characterized the cathepsin L from gastric cancer tissue in this study.

Cathepsin L separated from gastric cancer tissue in this experiment showed different molecular weight, different isoelectric point, different affinity to a lectin column, different heat stability, and different optimal pH and alkaline stability from cathepsin L separated from normal gastric mucosa, although they showed the same sensitivities to various inhibitors.

It has been suggested that cathepsin B from cancer may contribute to cancer invasion and metastasis because it is stable at alkaline pH.[12-16] Cathepsin L separated from gastric cancer tissue also showed this alkaline stability compared with cathepsin L from normal gastric mucosa. Heat stability of cathepsin L from gastric cancer tissue may be also a characteristic property, compared with cathepsin L from normal gastric mucosa. Probably, gastric cancer tissue-derived cathepsin L has an enzymatically more stable structure than cathepsin L from gastric normal mucosa, which would favor its accumulation at extracellular sites.

We also demonstrated that cathepsin L from gastric cancer tissue existed in high-molecular-weight form. The result of the SDS-PAGE study under reducing conditions demonstrated that this high-molecular-weight type cathepsin L existed in a single-chain form, showing a single protein band. It is possible that the existence of this high-molecular-weight cathepsin L may reflect a modification in the structure of cathepsin L associated with cellular malignant transformation. Cathepsin L is a glycoprotein.[6] It has been proposed that a structural change in the sugar chains of a glycoprotein produced by cells is associated with malignant transformation. Indeed, gastric cancer tissue-derived cathepsin L showed a different affinity for WGA-Sepharose than normal gastric mucosa-derived cathepsin L. Moreover, it is possible that this modification in the sugar chain structure of gastric cancer tissue-derived cathepsin L also contributes to its enzymatically more stable structure. The activity of cathepsin L in gastric cancer tissue and its properties of

alkaline stability and heat stability therefore suggest a contribution of cathepsin L to gastric cancer invasion.

We are now attempting further analysis of the sugar chain modification as well as the preparation of a monoclonal antibody for this variant type of cathepsin L.

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