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Variant cathepsin B activity secreted from human pancreatic cancer cell lines into protein-free chemically defined medium

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Summary: Cathepsin B activity was found in serum-free spent media of human pancreatic cancer cell lines, Panc-1 and MiaPaca. Cathepsin B activity was partially purified by gel filtration on TSK G3000SW, Con-A Sepharose chromatography, Phenyl-Superose column chromatography, and Mono S column chromatography. The optimal pH of cathepsin B was 7.4, and the activity was retained even at alkaline pH. Heat stability test showed tha the enzyme was heat stable; that is, 50% activity was retained after incubation at 56°C for 60 min. These results suggest that cathepsin B secreted from human pancreatic cancer cell is a variant type and may play an important role in pancreatic cancer invasion or metastasis through destruction of the surrounding extracellular matrix by its proteolytic activity. *Gastroenterol Jpn 1989;24:699–706*

Key words: *cathepsin B; metastasis; pancreatic cancer; proteinase*

Introduction

Cathepsin B is a cysteine proteinase physiological role of which is considered to degrade proteins in lysosomes. In our laboratory, we found high cathepsin B activity in the serum-free spent media of many human cancer cell lines that can proliferate in protein-free chemically defined medium without any other supplements. In this study, we partially purified and characterized the cathepsin B secreted from human pancreatic cancer cell lines, Panc-1 and MiaPaca, into protein-free chemically defined media. Their properties are discussed and compared to the cathepsin B from human liver and pancreas.

Materials and Methods

Cell Lines: Human pancreatic cancer cell lines Panc-1 and MiaPaca were obtained from the NCC library. These cell lines have been maintained in protein-free chemically defined medium without any other supplements.

Human liver and pancreas: Human liver and pancreas were obtained at autopsy and stored at −80°C until use.

Concentration of Protein-free Spent Media of Panc-1 and MiaPaca: Protein-free spent media were collected and concentrated about 100-fold by Labo Cassette (molecular weight 10,000 cut-off, Millipore Japan Co., Ltd., Tokyo, Japan) after removing cell debris, and freeze-dried.

Enzyme Assay: Cathepsin B activity was

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Abbreviations used: Con A = concanavaline 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-Arg-Arg-MCA = Carbobenzoxy-L-Arginyl-L-Arginine 4-Methyl-Coumaryl-7-Amide; Z-Phe-Arg-MCA = Carbobenzoxy-L-Phenylalanyl-L-Arginine 4-Methyl-Coumaryl-7-Amide; Arg-MCA = L-Arginine 4-Methyl-Coumaryl-7-Amide; Bz-Arg-MCA = Benzoyl-L-Arginine 4-Methyl-Coumaryl-7-Amide; AMC = 7-Amino-4-Methyl-Coumarine; SDS-PAGE = Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis.

measured as described previously[1]. Z-Arg-Arg-MCA was purchased from Peptide Institute, Inc., Osaka, Japan. All enzyme activities are expressed as the amount of liverated AMC per minute.

Purification of cathepsin B from Panc-1 and MiaPaca: 20mg of freeze-dried, serum-free spent medium of Panc-1 or MiaPaca was suspended in 10ml of 10mM Tris-HCl buffer, pH7.4, containing 0.2M NaCl. Insoluble substances were removed by centrifugation and filtration. The sample was applied to a TSK G3000SWP gel filtration column equilibrated with a 10 mM Tris-HCl buffer, pH7.4, containing 0.2M NaCl, and then eluted with the same buffer by a flow rate of 4ml/min. 4ml fractions were collected. The enzyme active fractions were pooled, concentrated, and dialyzed against a 10mM Tris-HCl buffer, pH7.4, containing 0.2M NaCl, 1 mM $CaCl_2$, and 1mM $MnCl_2$. The sample was applied to a Con A-Sepharose column (1.6 × 20 cm) equilibrated with a 10mM Tris-HCl buffer, pH7.4, containing 0.2M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ by a flow rate of 1.0ml/min. The column was successively washed with the same buffer until the absorbance at 280nm reached baseline. Then the column was eluted with the same buffer containing 0.2M alpha-methyl-D-mannoside. In the next step, enzyme active fractions were concentrated and dialyzed against a 0.1M phosphate buffer, pH6.8, containing 1.7M ammonium sulfate, and then applied to a phenyl-Superose column (1 × 10 cm) with a linear gradient of ammonium sulfate from 1.7 to 0M. The active fractions were pooled, concentrated, and dialyzed against a 10mM sodium phosphate buffer, pH5.8. The sample was then applied to a Mono S column (0.5 × 5 cm) with a linear gradient of NaCl from 0 to 0.5M. The active fractions were collected, concentrated, dialyzed against double distilled water extensively, and freeze-dried. The lyophylized sample was reserved at −80°C until enzyme characterization study.

Purification of cathepsin B from human liver: Cathepsin B of human liver was purified as described previously with slight modifications[2]. Human liver tissue was homogenized in ice-cold 1000mM sodium acetate buffer, pH5.0, containing 1mM EDTA and 0.2% Triton X-100 in a Waring blender at 0°C following sonication for 20s. The resulting homogenate was stirred overnight at 4°C, and the debris was removed by centrifugation at 15,000g for 20min. The pooled supernatants were acidified to pH4.2 with 2M HCl, and precipitated protein was removed by centrifugation at 15,000g for 20min. A 20-65% saturation ammonium sulfate fraction of the supernatant was taken and the precipitated protein was redissolved in 20mM sodium phosphate buffer, pH5.8, containing 1mM EDTA. Ammonium sulfate was removed by filtration on a Sephadex G-25 column. The pooled active fractions were applied to the S-Sepharose FF column (5 × 25 cm) equilibrated with a 20 mM sodium phosphate buffer, pH5.8, containing 1 mM EDTA. The elution rate was 8ml/min. A linear gradient of NaCl from 0 to 0.5M was used and 20ml fractions were collected. Enzyme active fractions wre collected, concentrated by ultrafiltration using Labo cassette (molecular weight cut off 10kd) and dialyzed against a 20mM sodium phosphate buffer, pH5.8, containing 0.2M NaCl, 1mM $CaCl_2$, and 1mM $MnCl_2$. Then the fractions were applied to the Con A Column (1.6 × 20 cm) equilibrated with the same buffer. Con A column chromatography was performed as described above. Next the active fractions were dialyzed against the phenyl-Superose column starting buffer (0.1M phosphate buffer, pH6.8, containing 1.7M ammonium sulfate), and then applied to the phenyl-Superose column (1 × 10 cm). The phenyl-Superose column chromatography was performed as described above. The active fractions were then dialyzed against the Mono S column starting buffer (10mM sodium phosphate buffer, pH5.8), and applied to the Mono S column (0.5 × 5 cm). Mono S column chromatography was performed as described above. The active fractions were dialyzed against a 20mM sodium acetate buffer, pH5.8, containing 0.2M NaCl, and then applied

to the TSK G3000SWXL column equilibrated with the same buffer and then eluted by a flow rate of 1.0ml/min. The purified enzyme was dialyzed against double distilled water extensively, and freeze dried. The lyophilized sample was reserved at −80°C until enzyme characterization study.

Purification of cathepsin B from human pancreas: Cathepsin B of human pancreas was purified in the same manner as cathepsin B of human liver described above. Purified enzyme was lyophilized and reserved at −80°C until use.

Substrate specificity: Substrate specificity was studied about Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Arg-MCA, and Bz-Arg-MCA using highly purified samples. All these substrates were purchased from Peptide Institute, Inc., Osaka, Japan. Measurement of hydrolysis of Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Arg-MCA, and Bz-Arg-MCA was performed as described previously[1]. All enzyme activities are expressed as the amount of liverated AMC per minute. Azocasein assay was also performed as described previously[1].

Optimal pH and Heat Stability: Highly purified samples from Panc-1, MiaPaca, human liver, and human pancreas were used to determine the optimal pH and heat stability. The optimal pH of cathepsin B was assayed using 0.2 M phosphate (pH5-8.5) buffer. Heat stability was tested at 37°C and 56°C for 60min. The enzyme activity after exposure to these temperatures for 5, 10, 20, 30, and 60min was assayed.

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

Alkaline stability test: The stability of purified cathepsin B from Panc-1, MiaPaca, human liver and human pancreas was tested at alkaline pH. Purified cathepsin B was preincubated at pH7.0, 7.4, 8.0 and 8.5 for 5, 10, 20 amd 30 min and its remaining activity was assayed.

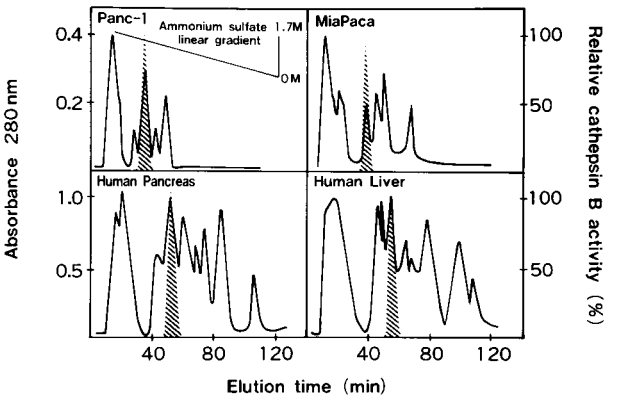

Fig. 1 Elution patterns of the cathepsin B by phenyl-Superose column chromatography; (—), absorbance at 280nm; (////), enzyme active fractions.

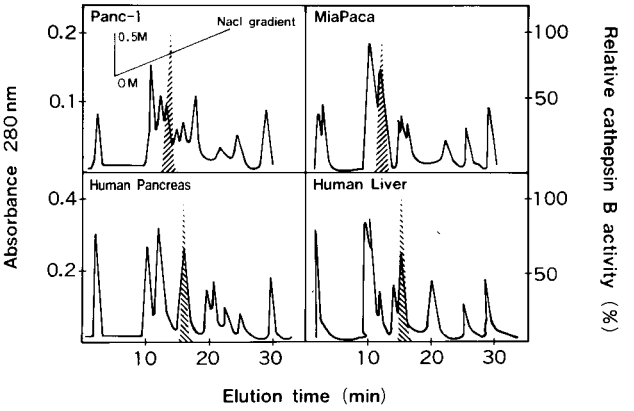

Fig. 2 Elution patterns of the cathepsin B by Mono S column chromatography; (—), absorbance at 280nm; (////), enzyme active fractions.

Molecular weight determination by gel filtration: Molecular weights of cathepsin B were determined using gel filtration column TSK G3000SWXL. A highly purified sample was applied to the column equilibrated with a 10mM phosphate buffer, pH6.8, containing 0.2M NaCl. The elution rate was 1ml/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.

Protein determination: The protein content was determined using the Bio-Rad microassay procedure[3].

Table 1 Summary of purification of cathepsin B from Panc-1

	protein (mg)	total activity (nmol/min)	specific activity (nmol/min/mg)
Freeze-dried spent medium	20	1257	62.85
TSK G3000SW gel filtration	4.2	1200	285.7
Con A-Sepharose column	1.76	1097	623.3
Phenyl-Superose column	0.224	1497	6683.1
Mono S column	0.056	857	15303.6

Table 2 Summary of purification of cathepsin B from MiaPaca

	protein (mg)	total activity (nmol/min)	specific activity (nmol/min/mg)
Freeze-dried spent medium	20	1057	52.85
TSK G3000SW gel filtration	5.2	961	184.8
Con A-Sepharose column	2.19	834	380.8
Phenyl-Superose column	0.418	1097	2624.2
Mono S column	0.108	724	6703.7

Isoelectric point determination of cathepsin B: Isoelectric points were determined by a Mono P column (0.5 × 20 cm, Pharmacia). A highly purified sample was applied to the column equilibrated with the 0.025M imidazole-HCl buffer, pH7.4. A pH gradient from 4 to 7 was made by elution with Polybuffer 74 (Pharmacia, Uppsala, Sweden)-HCl, pH4.0.

SDS-PAGE analysis: 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.

Results

Purification of cathepsin B from Panc-1 and MiaPaca: The active fractions of cathepsin B from Panc-1 and MiaPaca after gel filtration which were applied to a Con A column passed the column completely. On the other hand cathepsin B from Panc-1 and MiaPaca showed affinities for the phenyl-Superose column and the Mono S column. Both cathepsin Bs were eluted at the same ammonium sulfate and NaCl fractions (**Figs. 1 and 2**). Purification of cathepsin B fron Panc-1 and MiaPaca is summarized in **Tables 1 and 2**.

Table 3 Summary of purification of cathepsin B from Human liver

	protein (mg)	total activity (μmol/min)	specific activity (nmol/min/mg)
Pooled active fractions[a]	3000	820	273.3
S-Sepharose FF column	658	620	942.2
Con A-Sepharose column	427	500	1170.9
Phenyl-Superose column	14	880	7719.2
Mono S column	23.5	724	30808.5
TSK G3000SW gel filtration	4.2	561	133571.4

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

Table 4 Summary of purification of cathepsin B from human pancraes

	protein (mg)	total activity (μmol/min)	specific activity (nmol/min/mg)
Pooled active fractions[a]	1500	250	166.7
S-Sepharose FF column	380	125	328.9
Con A-Sepharose column	220	100	454.5
Phenyl-Superose column	38.3	138	3603.1
Mono S column	9.6	98	10208.3
TSK G3000SW gel filtration	1.8	67	37222.2

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

Purification of cathepsin B from human liver: Human liver cathepsin B showed affinities for S-Sepharose FF column, phenyl-Superose column and Mono S column. However, human liver cathepsin B was eluted at different ammonium sulfate and NaCl fractions from the cathepsin B of Panc-1 and MiaPaca (**Figs. 1 and 2**). Purification of cathepsin B from human liver is summarized in **Table 3**.

Purification of cathepsin B from human pancreas: The purification of cathepsin B from human pancreas is summarized in **Table 4**. Human pancreas cathepsin B was purified in the same way as human liver cathepsin B and had the same affinities for S-Sepharose column, phenyl-Superose column, and Mono S column (**Figs. 1 and 2**).

Table 5 Substrate specificity of cathepsin B of Panc-1, MiaPaca, human liver, and human pancreas

Substrate	Activity (%)			
	Panc-1	MiaPaca	human liver	human pancreas
Z-Arg-Arg-MCA	60	62	58	67
Z-Phe-Arg-MCA	100	100	100	100
Arg-MCA	0	0	0	0
Bz-Arg-MCA	49	50	62	58

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.

Table 6 The effect of a number of compounds on the activity of cathepsin B of Panc-1, MiaPaca, human liver, and human pancreas

Compounds	Concentration	Remaining activity (%)			
		Panc-1	MiaPaca	Human liver	Human pancreas
soybean trypsin inhibitor	1 mg/ml	100	100	100	100
leupeptin	1 mg/ml	0	0	0	0
Z-Phe-Phe-CHN_2	1 mg/ml	0	0	0	0
Z-Phe-Ala-CHN_2	1 mg/ml	0	0	0	0

Substrate specificity test: The substrate specificity study of highly purified samples using synthetic MCA substrates is summarized in **Table 5**. Because of the similarity of cathepsin H and L with cathepsin B, it was necessary to rule out the presence of these enzymes in our preparation. Oue prepared enzymes did not hydrolyze Arg-MCA. This property is uncharacteristic of cathepsin H[1]. And with azocasein assay, the azocasinolytic activities of our prepared enzymes were all depressed (more than 95%) by adding 3M urea solutions. This is uncharacteristic of cathepsin L, since cathepsin L activity is enhanced by 3M urea[1]. Judging from this substrate specificity study, our prepared enzymes were considered to be cathepsin B, free of cathepsin H and L.

Optimal pH: The optimal pH for cathepsin B of Panc 1 and MiaPaca was 7.4, their activities remained stable at alkaline pH. The optimal pH

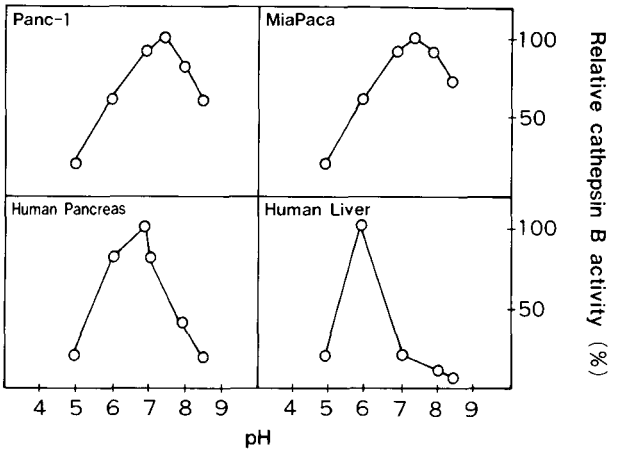

Fig. 3 pH optimum of cathepsin B. The optimal pH of cathepsin B was tested using 0.2M phosphate buffer (pH5-8.5).

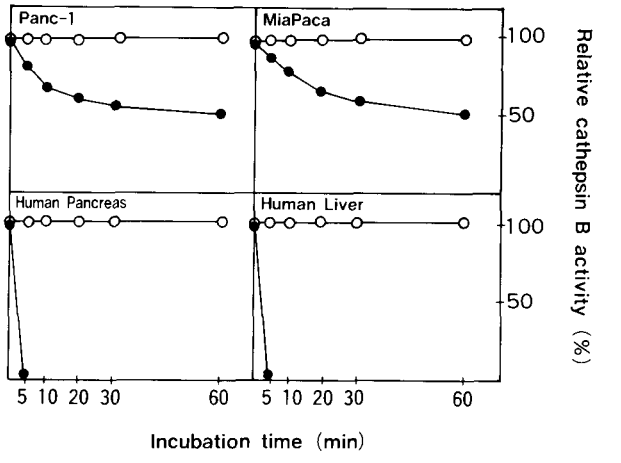

Fig. 4 Heat stability of cathepsin B. ○; 37°C, ●; 56°C.

for cathepsin B of human liver was 6.0 and for human pancreas cathepsin B was 6.8 (**Fig. 3**).

Heat stability: Enzyme activities of cathepsin B of Panc 1 and MiaPaca were retained with no significant loss at 37°C. Furthermore, 50% activity was still retained after treatment at 56°C for 60 min. On the other hand, activities of cathepsin B of human liver and pancreas were stable at 37°C, but were inactivated after treatment at 56°C for 5 min. (**Fig. 4**).

Effect of Inhibitors: The effect of a number of compounds on the activity of cathepsin B of Panc-1, MiaPaca, human liver, and human pan-

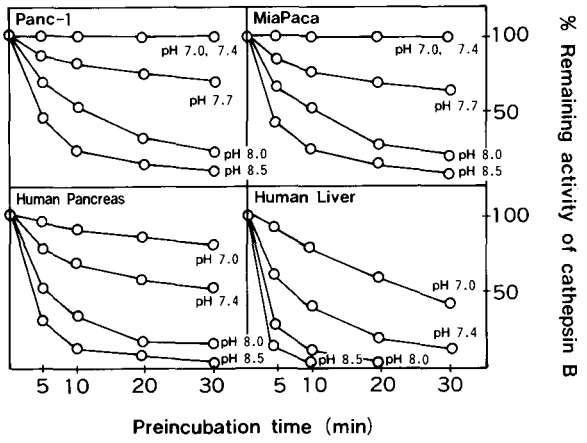

Fig. 5 Alkaline stability test of cathepsin B. The stability at alkaline pH of cathepsin B was tested. Cathepsin B was preincubated at pH 7.0, 7.4, 8.0, 8.5 for 5, 10, 20, and 30 min and its remaining activity was assayed.

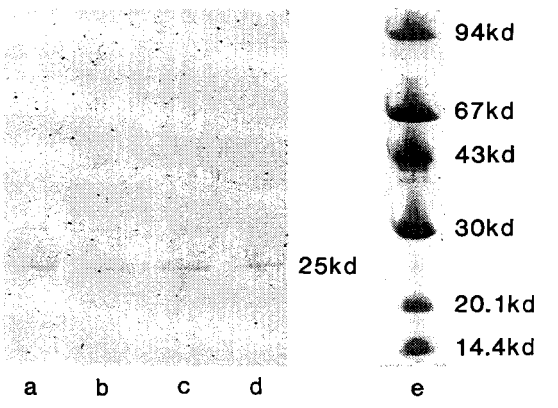

Fig. 6 SDS-PAGE pattern of cathepsin B of pancreatic cancer and cathepsin B of human liver and pancreas. SDS-PAGE was carried out on the gradient polyacrylamide gel (10-15%) and stained with Brilliant Coomasie blue. (a), Panc-1; (b), MiaPaca; (c), human liver; (d), human pancreas; (e), marker proteins-phosphorylase b (94kd), albumin (67kd), ovalbumin (43kd), carbonic anhydrase (30kd), trypsin inhibitor (20.1kd), and alpha-lactalbumin (14.4kd).

Table 7 Molecular weights and isoelectric points of cathepsin B of Panc-1, MiaPaca, human liver, and human pancreas

	Molecular weight	isoelectric point
Panc-1	25000	4.0
MiaPaca	25000	4.1
human liver	25000	5.5
human pancreas	25000	5.3

creas is summarized in **Table 6**. They showed almost the same sensitivities to various inhibitors.

Alkaline stability test: Cathepsin B from Panc-1, MiaPaca, human liver, and human pancreas was tested for stability at an alkaline pH. Cathepsin B from human liver was inactivated with 30min preincubation at pH7.4. On the other hand, cathepsin B from MiaPaca and Panc-1 was stable with 30min preincubation at pH7.4. Cathepsin B from human pancreas retained 50% activity with 30min preincubation at pH7.4 (**Fig. 5**).

Molecular weight determination by gel filtration: Molecular weights of cathepsin B from Panc-1, MiaPaca, human liver, and human pancreas are summarized in **Table 7**. All cathepsin B have the same estimated molecular weights of 25 kd.

Isoelectric point determination of cathepsin B: Isoelectric points of cathepsin B from Panc-1, MiaPaca, human liver, and human pancreas are summarized in **Table 7**. Cathepsin B from pancreatic cancer cell lines had rather acidic isoelectric points compared to the cathepsin B of human liver and pancreas.

SDS-PAGE analysis: The result of SDS-PAGE under reducing conditions were consistent with the purity of our prepared samples, showing a single protein band (**Fig. 6**) corresponding to a molecular weight of 25 kd. Cathepsin B purified from Panc-1, MiaPaca, human liver, and human pancreas were therefore considered to exist in a single-chain form.

Discussion

Cathepsin B is a well-known cysteine protease which degrades proteins in the lysosomal system. Cathepsin B is considered to contribute to the process of cancer invasion by destroying the surrounding matrix[4-8]. Cathepsin B has been separated from normal human tissues[9] and malignant human tissues[10,11]. However, in the process of the extraction of cathepsin B from malignant tissues, there may be contamination

derived from other cell components, such as white cells, fibroblasts, etc. The cell culture is the most useful method of clarifying the relationship between cathepsin B and the cancer because it can eliminate such contamination.

It is important to purify and characterize the cathepsin B secreted from cancer cells and to determine whether and how it differs from the cathepsin B of normal cells. In our laboratory we have cultured many human cancer cell lines for a long time in a protein-free chemically-defined medium. With this protein-free cultivation method, the spent medium can be easily concentrated and freeze-dried after dialysis to distilled water. Therefore, cell-derived substances can be easily analyzed in this way.

With this protein-free cultivation method, we measured cathepsin B activity from cancer cell lines in serum-free spent medium, and obtained the result that many cancer cell lines secreted cathepsin B into protein-free chemically difined medium with high activity.

In this study we tried to purify and characterize the cathepsin B secreted from pancreatic cancer cell lines since the isolation and properties of cathepsin B secreted from pancreatic cancer cell line have not been reported.

Cathepsin B separated from 2 human cancer cell lines in this way showed the same molecular weight, the same affinity for cation-exchange column, the same affinity for phenyl-Superose colum, almost the same isoelectric point, almost the same heat stability, and the same optimal pH and alkaline stability. They also showed the same sensitivity to various inhibitors.

Cathepsin B separated from human liver and pancreas showed the same molecular weight, the same affinity for cation-exchange column, the same affinity for phenyl-Superose column, almost the same isoelectric point, the same heat stability, and the same sensitivity to various inhibitors. However, there were slight differences concerning optimal pH and alkaline stability. Namely, cathepsin B of human pancreas has an optimal pH of 6.8, which is more neutral than cathepsin B of the human liver. Moreover, cathepsin B of the human pancreas showed more stability at an alkaline pH than cathepsin B of the human liver. Optimal pH, alkaline stability, molecular weight, isoelectric point, heat stability, and sensitivity to various inhibitors of separated cathepsin B from the human liver agreed with previous reports[1,8,10]. On the other hand, there have been no reports about purification and characterization of cathepsin B of human pancreas. It is well known that lysosomal proteinases are unstable at a pH above neutral. Therefore, the greater alkaline stability of human pancreatic cathepsin B than human liver cathepsin B is rather curious. However, rabbit liver cathepsin L retained 70% of its activity after incubation at pH7.0 at 30°C for 1 hour[12]. Because the pH of pancreatic juice is alkaline, pancreatic cathepsin B may show such alkaline stability.

It has been said that cathepsin B from cancer may contribute to cancer invasion and metastasis because it is stalbe at alkaline pH[10,11,13-15]. However, it is considered that the environment around cancer cells may be rather acidic due to the substances derived from the matrix destroyed by cancer cells. Thus, the alkaline stability of cathepsin B from Panc-1 and MiaPaca, namely, alkaline stability of pancreatic cancer cathepsin B, may not be such a relatively unique characteristic property of cancer.

On the other hand, the heat stability of human pancreatic cancer cathepsin B may be a characteristic property, compared with the cathepsin B of the human liver and pancreas. Probably, pancreatic cancer cathepsin B has an enzymatically more stable structure than cathepsin B of normal tissue, which would favor its accumulation at extracellular sites. Furthermore, pancreatic cancer cathepsin B showed differentd affinities for phenyl-Superose and Mono S column and different isoelectric points from cathepsin B of human liver and pancreas. These findings suggested the existence of variant cathepsin B that might be unique to pancreatic cancer. An SDS-PAGE study showed that both cathepsin B of pancreatic cancer and cathepsin B of human liver and pancreas had

the single chain molecular form. Further characterization of the pancreatic cancer cathepsin B will require larger quantities of protease. We are now attempting the preparation of a monoclonal antibody for this variant type of cathepsin B.

Evidence of secretion of cathepsin B from human pancreatic cancer cell lines into protein-free chemically-defined medium and its property of heat stability are therefore probable proof of the contribution of human pancreatic cancer cathepsin B to cancer invasion and metastasis.

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