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Determination of Cathepsins D and E in Various Tissues and Cells of Rat, Monkey, and Man by the Assay with β-Endorphin and Substance P as Substrates

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ABSTRACT—We developed a new method for the assay of cathepsins D and E. The method was based on the different hydrolytic activities of cathepsins D and E against β-endorphin and substance P. The method was applied to the determination of the levels of cathepsins D and E in various tissues and cells of rat, monkey, and man, and was clarified to be much more specific, sensitive, and quantitative than the ordinary hemoglobin-digestion method. The levels of cathepsin D were high in adrenal and spleen, and the levels of cathepsin E were high in gastrointestinal tissues, bone marrow, and lymph node. The variations in level were much wider in the case of cathepsin E than in the case of cathepsin D. This might reflect that cathepsin D is a house-keeping lysosomal enzyme in a variety of cells and cathepsin E is involved in the physiological activities of certain types of tissues and cells.

INTRODUCTION

Cathepsins D (EC 3. 4. 23. 5) and E (EC 3. 4. 23. 34) are intracellular endoproteinases belonging to members of the family of aspartic proteinase (Rawlings and Barrett, 1995). Cathepsin D is known to be localized in lysosomes (Barrett,1977), while cathepsin E has been shown to be localized in the endoplasmic reticulum and endosomes (Bennett *et al.*, 1992; Finley and Kornfeld, 1994). They have general proteolytic activities against protein substrates such as hemoglobin and albumin (Barrett, 1977; Kageyama, 1995), and are thought to be involved in the intracellular processing or degradation of proteins and peptides (Barrett, 1977; Lees *et al.*, 1990; Athauda *et al.*, 1991; Kageyama, 1993; Kageyama *et al.*, 1995, 1996). It is rather difficult to determine their levels in various tissues and cells by the conventional assay method with hemoglobin as substrate (Anson and Mirsky, 1932) because of their similar hydrolytic activities against hemoglobin. Quantitative determination of their levels in tissue or cell homogenates by the hemoglobin-digestion method was carried out after removing the counter cathepsin by immunoprecipitation with its specific antibody (Muto *et al.*, 1988; Sakai *et al.*, 1989; Yonezawa and Nakamura, 1991). The procedures are, however, rather exhaustive since specific antibodies are necessary. Therefore, it will be very much desired that a specific substrate for each cathepsin is available. Recently, we have found that cathepsins D and E have quite different hydrolytic activities against various peptide substrates (Kageyama, 1993, 1995; Kageyama *et al.*, 1995). Cathepsin D has been shown to degrade β-endorphin very rapidly and be almost inactive on substance P. Reverse relationship has been found in the case of cathepsin E. These differences are thought to be useful for the specific determination of cathepsins D and E in crude tissue or cell homogenates.

In this report, we describe a new method for the assay of cathepsins D and E and its application to the determination of the levels of both cathepsins in various mammalian tissues and cells.

MATERIALS AND METHODS

Materials

Rat (Yonezawa *et al.*, 1987, 1988) and monkey (Kageyama *et al.*, 1995) cathepsins D and E were purified as described previously. β-Endorphin, substance P, pepstatin, and E-64 were purchased from

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Peptide Institute, Inc. (Minoh-shi, Japan), bovine hemoglobin substrate powder from Worthington Diagnostic System Inc. (Freehold, NJ, USA).

Preparation of homogenates of mammalian tissues and cells

Tissues were removed from 1-month-old rats and young Japanese monkeys (*Macaca fuscata*) immediately after death by exsanguination under deep anesthesia in accordance with guidelines of Primate Research Institute, Kyoto University. Cells dispersed from lymph node and thymus were used as lymphocytes and thymocytes, respectively. Rat and bovine endothelial cells were gifts from Drs. J. Ando and T. Ueno, respectively, of Hokkaido University. Various cell lines were obtained commercially. Each tissue was homogenized in 10 volumes of 0.02 M sodium phosphate buffer, pH 7.0, containing 0.4% Tween 20 with a mechanical homogenizer. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was used as an enzyme solution. Cells harvested were homogenized in the same buffer with a sonicator and the crude homogenate was used as an enzyme solution. For long-term storage each supernatant or homogenate was mixed with an equal volume of glycerol and kept at -20°C. No significant loss of activities of cathepsins D and E was observed for several months. Protein concentration in each sample was determined by the method of Lowry *et al.* (1951).

Assay of hemoglobin-digesting activity

The proteolytic activity against hemoglobin was determined by the method of Anson and Mirsky (1932) with a modification (Kageyama, 1995).

Assay of β-endorphin- and substance P-hydrolyzing activities

The procedures were based on those described in our previous reports (Kageyama, 1993, 1995). In brief, the reaction mixture contained 0.2 M buffer at an appropriate pH, 50 μM β-endorphin or substance P, and an appropriate amount of enzyme. The total volume was 20 μl. When a crude tissue homogenate or its supernatant was used as the source of enzyme, E-64 was added to a final concentration of 1 μM. The reaction mixture was incubated at 37°C for 2 hr, and the reaction was stopped by the addition of 60 μl of 3% perchloric acid. After removal of any precipitated materials by centrifugation, each reaction mixture was subjected to high pressure liquid chromatography on a column (0.46 cm, inner diameter, ×25 cm) of ODS-120T (Tosoh Corp., Tokyo, Japan) that had been equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of acetonitrile that contained 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The intact β-endorphin or substance P and the products of hydrolysis were eluted within 20-26 min.

RESULTS

Hydrolytic activities of purified cathepsins D and E against protein and peptide substrates

Hydrolytic activities of purified enzymes against hemoglobin were examined (Fig.1). Rat and monkey cathepsins D was maximally active at around pH 3. Rat cathepsin D retained nearly half of the maximal activity at pH 2 while monkey cathepsin D was almost inactive at pH 2. Rat and monkey cathepsins E was maximally active at around pH 3, with similar activity at pH 2. According to these results, the levels of cathepsins D and E in the monkey tissue homogenate might be determined based on the difference in hemoglobin-digestive activities at pH 2 and 3. However, since the hemoglobin-digestion method is not so sensitive, prolonged incubation time is necessary in the case of most tissue homogenates. The determination of the levels of cathepsins D and E in the crude homogenates of rat tissues by the hemoglobin-digestion method is actually impossible since both cathepsins have similar pH-dependent activities against hemoglobin. To determine the levels of cathepsins D and E in rat tissues, it has been reported that the removal of the counter cathepsin with its specific antibody was necessary (Muto *et*

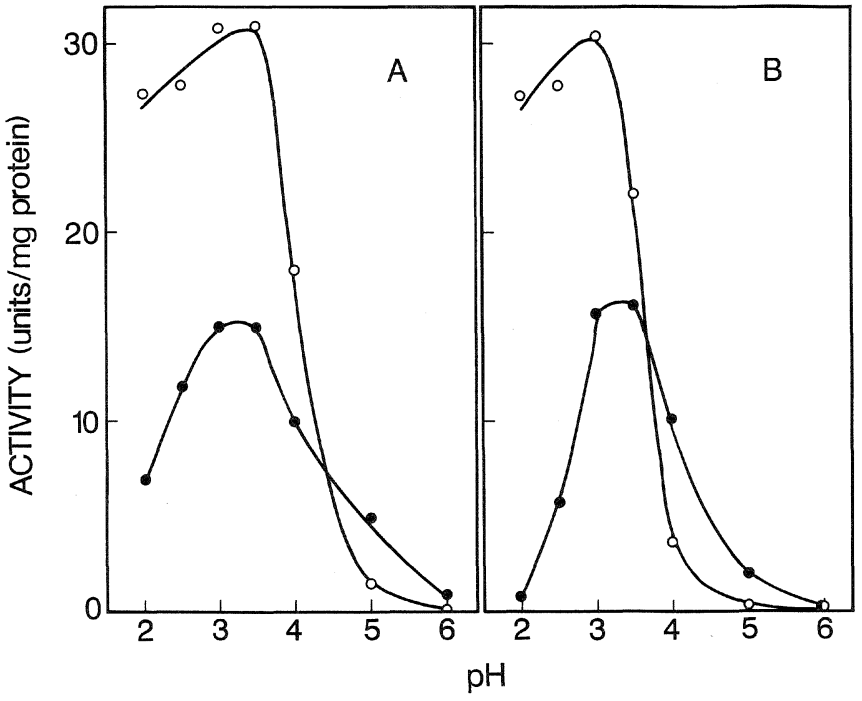

Fig. 1. Dependence on pH of the hydrolysis of hemoglobin by cathepsins D (●) and E (○) from rat (A) and monkey (B).

al., 1988; Sakai *et al.*, 1989; Yonezawa and Nakamura, 1991).

Hydrolytic activities of cathepsins D and E against peptide substrates have been known to be largely different. Monkey cathepsin D has been shown to have high hydrolytic activity at around pH 4 against β-endorphin but quite low activity against substance P, and the reverse relationship has been found in the case of cathepsin E (Kageyama *et al.*, 1995). These differences in catalytic properties between cathepsins D and E were also obvious in rat (Fig. 2). The rate of hydrolysis of β-endorphin by rat cathepsin D was 50-fold faster than that by rat cathepsin E. While, the rate of hydrolysis of substance P by rat cathepsin E was 500-fold faster than that by rat cathepsin D. Intracellular aspartic proteinases that hydrolyze β-endorphin and substance P have not been known except for cathepsins D and E, it is very much appropriate to determine these cathepsins in crude tissue or cell homogenates by assaying with β-endorphin and substance P as substrates.

Determination of cathepsins D and E in various tissues and cells

Rat tissues: Endopeptidase activities at pH 4 against β-endorphin and substance P were determined in various rat tissues (Table 1). These activities were largely different between tissues. The differences in substance P-hydrolyzing activities were more particular than those in β-endorphin-hydrolyzing activities. The hydrolytic activities against β-endorphin and substance P were suppressed completely by pepstatin and were not affected in the presence of other inhibitors such as E-64 and leupeptin (data not shown), showing that these activities were due to cathepsins D and E. The levels of cathepsins D and E in various tissues were calculated based on the differences in their specific activities against β-endorphin and substance P (Table 1). The level of cathepsin D was highest in adrenal followed by spleen and lung. The levels of cathepsin E were high in stomach, urinary bladder, thymus, and spleen, followed by bone marrow, lymph node, and intestine, and were low in nervous tissues, heart, liver, and kidney. The differences in the levels of cathepsin D between tissues were not so large as compared to the differences in the levels of cathepsin E.

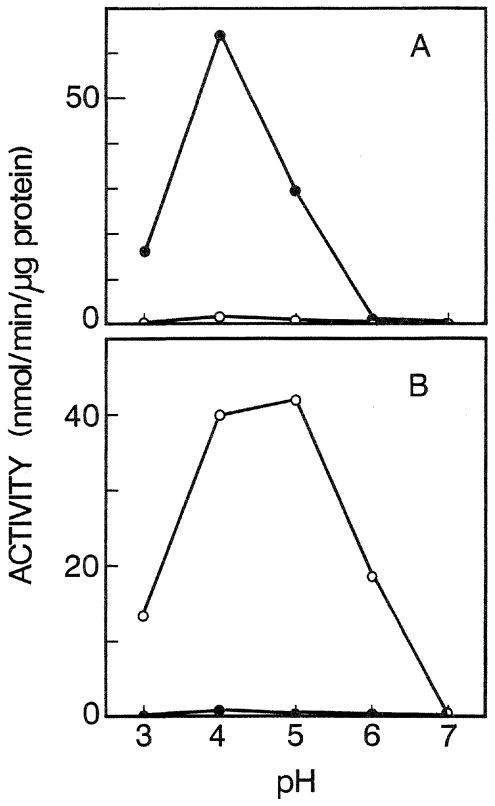

Fig. 2. Dependence on pH of the hydrolysis of β-endorphin (A) and substance P (B) by rat cathepsins D (●) and E (○). The buffers used were sodium formate (pH 3-4), sodium acetate (pH 5), and Tris/MES (pH 6-7).

Monkey tissues: The levels of cathepsins D and E in monkey tissues are shown in Table 2. The level of cathepsin D in each monkey tissue was largely similar to that in the corresponding rat tissue. High levels were found in adrenal, urinary bladder, and intestine. The levels of cathepsin E in monkey tissues were generally low as compared with those in rat tissues. Exceptionally, intestinal tissues such as duodenum contained high levels of cathepsin E. The levels of cathepsin E in bone marrow and lymph node were relatively higher than those in most of other monkey tissues although the levels were lower than those in the corresponding rat tissues.

Cells and Cell lines: The levels of cathepsins D and E in various cells and cell lines are shown in Table 3. The levels of cathepsin D were similar in most cells and cell lines, and were much higher than those of cathepsin E. Exceptionally, rat thymocytes and lymphocytes contained high levels of cathepsin E, being consistent with the results of the levels in thymus and lymph node.

DISCUSSION

The specific, sensitive, and quantitative assay method was developed to determine cathepsins D and E in a variety of tissues and cells. The method was based on the different activities of cathepsins D and E against β-endorphin and substance P. The determination of the levels of cathepsins D and E in various tissues is rather difficult with the conventional assay method with hemoglobin since both cathepsins have similar general proteolytic activities against hemoglobin. To date, tissue distributions of cathepsins D and E have been reported only in rat (Muto *et al.*, 1988; Sakai *et al.*, 1989; Yonezawa *et al.*, 1993). In these reports, cathepsins D and E were assayed by the hemoglobin-digestion method after removing the counter cathepsin by immunoprecipitation with its specific antibody. Since our method with peptide substrates is much more specific than the hemoglobin-digestion method, the exhaustive process of immunoprecipitation is excluded. We compared the present results of the levels of cathepsins D and E in rat tissues with those of other authors. The levels of cathepsin D gave similar values to those reported by Sakai *et al.* (1989) but were different from those reported by Muto *et*

Table 1. Levels of cathepsins D and E in rat tissues

Tissue	Hydrolytic activity		Cathepsin D*			Cathepsin E*		
	βE	SP	This study	Sakai *et al.* (1989)	Muto *et al.* (1988)	This study	Sakai *et al.* (1988)	Muto *et al.* (1989)
	nmol/min /mg tissue protein		ng/mg tissue protein					
Cerebrum	14	0.34	220	150	940	5.5	nd	<10
Cerebellum	8.2	0.44	130			8.9		
Thymus	14	19	190	340	1890	450	190	220
Lung	17	1.9	270	390		42	30	<10
Heart	4.4	0.44	69	130	790	9.7	nd	<10
Liver	9.3	0.23	150	290	420	3.7	nd	<10
Stomach	12	56	99	810	1210	1330	1050	4730
Duodenum	3.5	3.9	49	550		92	30	
Jejunum	4.2	3.8	61	150		90	30	
Ileum	4.2	4.7	59	580		110	40	
Colon	4.3	2.3	65	240		54	100	
Spleen	23	20	330	630	1100	470	180	170
Kidney	12	0.29	190	220	730	4.6	5	<10
Adrenal	69	0.74	1090	790	2030	4.6	nd	<10
Pancreas	5.6	0.32	88			6.6		
Urinary bladder	12	22	150	310	2260	520	190	300
Muscle	2.5	0.16	39	40		3.3	nd	
Lymph node	12	9.0	180	220		210	180	
Bone marrow	7.8	16	98	210		380	130	
Blood cells	<0.01	0.11	<1			2.6		
Serum	0.05	<0.01	<1			<1		

*The levels of cathepsins D and E were calculated based on the β-endorphin(βE)- and substance P(SP)-hydrolyzing activities in the tissue homogenates and the specific activities of cathepsins D and E against these peptides (Fig. 2). nd, not detectable.

al. (1988) (Table 1). The relatively high levels of cathepsin D reported by Muto *et al.* (1988) might be due to the underestimation of the specific activity of cathepsin D. The levels of cathepsin E are in good agreement with those reported by Muto *et al.* (1988) and Sakai *et al.* (1989) (Table 1). It should be noted that our method enabled quantitative determination of cathepins D and E in some tissues such as nervous tissues where the levels of cathepsins D and E were very low. This high sensitivity is due to high hydrolytic activities of cathepsins D and E against β-endorphin and substance P, respectively.

The levels of cathepsins D and E in monkey tissues and various cell lines of rat and man were clarified for the first time. The level of cathepsin D in each tissue was largely similar between rat and monkey. The levels of cathepsin E were very different between two species. However, relatively high levels of cathepsin E were found in intestine, lymph node, and bone marrow in each species. These results suggest that cathepsin D is a lysosomal enzyme and has a 'house-keeping' role in a cell, and that cathepsin E is involved in the physiological activities of certain types of tissues such as gastrointestinal tissues, immune-associated tissues, and tissues associated with hematopoiesis. The preferential cleavage of substance P by cathepsin E (this study; Kageyama, 1993) may modulate the activity of gastrointestinal smooth muscles, since substance P is known to evoke the contraction of smooth muscles (Maggio, 1988). Preferential cleavages of invariant chain (Kageyama *et al.*, 1996) and neurotensin precursor (Kageyama *et al.*, 1995) by cathepsin E might be responsible for antigen processing (Bennett *et al.*, 1992; Finzi *et al.*, 1993) and the modulation of signal transduction (Kageyama, 1993), respectively.

It should be noted that cathepsins D and E are known to be synthesized as preproforms and processed to respective active forms on their way to final targeting organelles such as lysosomes and endosomes. The precursor forms of cathepsins D and E, namely, procathepsins D and E, have been found in some tissues (Kageyama and Takahashi, 1980; Hasilik *et al.*, 1982; Muto *et al.*, 1983; Yonezawa *et al.*, 1993; Fusek and Vetvicka, 1994). The ratio of the precursor form to the active form has been shown to be higher in the case of cathepsin E than in the case of cathepsin D (Yonezawa *et al.*, 1993; Kageyama, 1995). By the present assay method, we could determine the summed level of the precursor form and the active form of each cathepsin in each tissue or cell since procathepsins D and E were easily converted to the active cathepsins D and E, respectively, at pH 4 (Hasilik *et al.*, 1982; Kageyama *et al.*, 1992; Okamoto *et al.*, 1995). The maximal level of the active form can be estimated from this summed level. The assay conditions to determine the precursor form and the active form separately are under examination.

The involvement of another proteinases and amino/carboxypeptidases in the present assay method could be

Table 2. Levels of cathepsins D and E in monkey tissues

Tissue	Hydrolytic activity		Cathepsin D*	Cathepsin E*
	βE	SP		
	nmol/min /mg tissue protein		ng/mg tissue protein	
Frontal cortex	8.1	0.19	140	4.8
Hypothalamus	6.3	0.22	110	6.0
Pituitary	11	0.12	190	2.4
Cerebellum	9.3	0.26	160	6.8
Thymus	6.4	0.13	110	3.2
Lung	5.6	0.18	98	4.8
Heart	2.5	0.08	44	2.1
Liver	3.9	0.06	68	1.4
Duodenum	15	6.7	250	200
Jejunum	40	2.4	700	68
Ileum	20	1.6	350	46
Colon	23	0.57	400	15
Spleen	36	0.49	630	11
Kidney	4.0	0.14	70	3.8
Adrenal	45	0.37	790	6.2
Pancreas	8.1	0.12	140	2.7
Urinary bladder	30	0.50	530	12
Muscle	0.62	<0.01	11	<1
Lymph node	43	0.76	750	18
Bone marrow	5.6	0.75	97	22
Erythrocyte	0.01	0.12	<1	3.6
Leucocyte	5.3	0.19	93	5.2
Serum	<0.01	<0.01	<1	<1

*The levels of cathepsins D and E were calculated based on the β-endorphin(βE)- and substance P(SP)-hydrolyzing activities in the tissue homogenates and the specific activities of cathepsins D and E against these peptides (Kageyama *et al.*, 1995).

Table 3. Levels of cathepsins E and D in various cells and cell lines

Cell/Cell line	Hydrolytic activity		Cathepsin D*	Cathepsin E*
	βE	SP		
	nmol/min /mg protein		ng/mg protein	
Rat				
Lymphocytes	19	37	240	880
Thymocytes	17	12	250	280
Vein endothelial cells	15	0.40	240	6.7
Pheochromocytoma PC12(–)**	17	1.1	270	23
Pheochromocytoma PC12(+)**	23	1.3	360	27
Bovine				
Endothelial cells	23	0.48	400	12
Human				
Glioblastoma T98G	32	0.23	560	3.4
Astrocytoma NAC6	19	0.19	330	3.6
Neuroblastoma GOTO	11	0.09	190	1.5
Stomach cell line MKN45	5.2	0.84	90	25
Prostate cell line PC3	4.8	0.05	84	1.0
Uterus cell line A431	30	0.23	530	3.6
Endothelial cell line KIT-U	15	0.06	260	0.1
Osteosarcoma KHOS	11	0.05	190	0.3

*The levels of cathepsins D and E were calculated based on the β-endorphin(βE)- and substance P(SP)-hydrolyzing activities in the cell homogenates and the specific activities of cathepsins D and E against these peptides. The specific activities of human or bovine cathepsins D and E were assumed to be similar to those of monkey cathepsins D and E, respectively.
**PC12 cells were cultured in the absence (–) or presence (+) of dexamethasone, forskolin, and nerve growth factor (Sano and Kitajima, 1992).

excluded with various inhibitors. The addition of E-64 in the assay mixture was satisfactory to inhibit proteinases that are active on β-endorphin and substance P. However, although the possibility will be quite low, if some novel aspartic proteinases except for cathepsins D and E that could hydrolyze β-endorphin and substance P would exist in some tissues or cells, they might partly interfere with the present assay.

Since the present assay method is based on the different activities of cathepsins D and E against peptide substrates, it is necessary to know the specific activities of purified cathepsins D and E against these peptides to determine of the levels of both cathepsins in various tissues. When specific hydrolytic activities of cathepsins D and E are not known in some interested animals, the relative levels of cathepsins D and E could be estimated from the relative activities against β-endorphin and substance P in each tissue since the catalytic properties of cathepsins D and E were well conserved in different kinds of animals (Kageyama, 1993, 1995; Kageyama *et al.*, 1995).

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