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Stabilization of Pig Kidney Cathepsin A by Sucrose and Chloride Ion, and Inhibition of the Enzyme Activity by Diisopropyl Fluorophosphate and Sulfhydryl Reagents

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1. Cathepsin A, the enzyme which hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine, was obtained from pig kidney. The enzyme was labile after dialysis but was stabilized by the addition of sucrose or KCl. In the presence of both stabilizers, the enzyme was active between pH 3 and 6.
2. The enzyme activity was inhibited by diisoprophyl fluorophosphate and sulfhydryl reagents such as *p*-chloromercuribenzoic acid (PCMB). The enzyme was not activated by cysteine but enzyme which had been treated with PCMB was reactivated by cysteine.
3. The possible identity of cathepsin A and catheptic carboxypeptidase is discussed.

Cathepsin A was originally defined as the enzyme from bovine spleen splitting CGT (*1–3*). Later, enzymes which hydrolyze the same substrate were differentiated into cathepsin A and catheptic carboxypeptidase or cathepsin IV (*4*, *5*). Cathepsin A was characterized by its optimal action near pH 5, and the absence of a requirement for activation by cysteine or of inhibition by iodoacetic acid (*1*, *3*, *4*). Catheptic carboxypeptidase has been reported to be optimally active at pH 3.6 and requires SH compounds for activation (*5*, *6*).

However, recent results on both enzymes (*7–16*) have confused the two and it is difficult to differentiate the enzymes by the above criteria. For example, cathepsin A has been observed to have carboxypeptidase activity (*7*, *13*, *15*). Inhibition of cathepsin A by PCMB (*11*), and DFP (*10*, *13*, *15*), and inhibition of catheptic carboxypeptidase C by DFP (*16*) have been described.

The present communication demonstrates that the enzyme obtained from crude lysosomal particles of pig kidney was stabilized by the addition of sucrose and chloride ion. The pH dependences of the stability and activity of the enzyme were determined in the presence of the stabilizers. The conditions under which the enzyme was completely inhibited by DFP or sulfhydryl reagents are described and the possible identity of cathepsin A and catheptic carboxypeptidase is discussed.

The abbreviations used: CGT, carbobenzoxy-L-glutamyl-L-tyrosine; DFP, diisopropyl fluorophosphate; PCMB, *p*-chloromercuribenzoic acid.

MATERIALS AND METHODS

Substrate—CGT was obtained from the Protein Research Foundation, Minoh, Osaka.

Assay of Enzyme Activity—Cathepsin A activity was determined with CGT as a substrate by the colorimetric ninhydrin procedure

of Moore and Stein (*17*). The reaction mixtures (1 ml) containing 0.003 M substrate, 0.05 M sodium acetate buffer, pH 5.2, 0.5 M sucrose, 0.1 M KCl, and enzyme were incubated at 37°C for 10 to 30 min. The reaction was stopped by the addition of 2 ml of ninhydrin reagent, and was used for the colorimetric determination. Activity is expressed as μmoles tyrosine released per minute. Specific activity is expressed as the activity per mg protein. Protein content was determined according to Lowry *et al.* (*18*).

Enzyme Preparation—The enzyme solution was prepared from pig kidney as follows: Two volumes (v/w) of 0.45 M sucrose solution containing 0.001 M EDTA and 0.01 M Tris-HCl, pH 7.2, were added to minced pig kidneys. After homogenization of the tissue with a Waring blendor for 20 sec, lysosome-rich particles were obtained by differential centrifugation. The supernatant (1,000 g for 3 min) obtained from the homogenate was centrifuged for 5 min at 7,000 × *g*. The pellet was suspended in the starting sucrose solution and centrifuged for 5 min at 7,000 × *g*. The pellet was resuspended in the starting sucrose solution and was broken up with a Waring blendor for 5 min followed by sonication for 2 min. The sonicate was centrifuged for 60 min at 78,410 × *g*. The supernatant was used throughout the experiments as solubilyzed enzyme from lysosome-rich particles.

RESULTS

Enzyme Activity in the Preparation—The activity and yield of the enzyme during preparation are summarized in Table I. About a half of the total activity in the whole homogenate was concentrated in the lysosome-rich particles. Solubilization of the enzyme from the particles was incomplete in the present experiment, but about 70 to 80% of the particulate enzyme was solubilized after repetition of the treatment.

Stability of the Enzyme—The solubilized enzyme was stable for more than a week at 5°C, pH 7.2, but lost its activity during dialysis against buffers of pH 7 or 5, as shown in Table II. Acidic conditions were favorable for the enzyme but some decrease of the activity was observed during dialysis for 72 hr at 5°C, pH 5.2. The results indicate that some substance(s) which had a stabilizing effect on the enzyme was removed during dialysis. Several substances were added to the dialyzed enzyme and their protective effects against inactivation were investigated. EDTA, various divalent cations and reducing reagents in 0.01 M had no effect. Sucrose in relatively high concentrations and KCl stabilized the enzyme, as shown in Table III.

The dialyzed enzyme lost its activity almost completely within 60 min at 37°C, pH 7.2. Sucrose (0.5 M) or KCl (0.1 M) partly protected the enzyme from inactivation. The copresence of sucrose and KCl greatly enhanced the stabilitby. As regards stabilizing effect, KCl could be replaced by NaCl or $MnCl_2$, and sucrose could be replaced by other sugar alcohols. EDTA did not affect the stabilization by sucrose or KCl but phosphate lowered the stabilizing effects.

Sucrose and KCl were effective not only for dialyzed enzyme but also for undialyzed enzyme preparations. Undialyzed enzyme solution which contained sucrose was inactivated at 50°C, pH 5.2, but was protected from heat

TABLE I. Summary of enzyme preparation procedure. Eight fresh pig kidneys were used, as described in the text.

	Volume (ml)	Protein (mg/ml)	Enzyme activity		
			Activity/ml	Specific activity ($\times 10^3$)	Total activity
Tissue homogenate	2,200	40.0	0.641	16.0	1,410
Lysosome-rich particles	370	65.6	2.09	31.9	774
Solubilized enzyme	145	24.0	1.09	45.6	159

TABLE II. Inactivation of the enzyme during dialysis. *Five ml of the enzyme solution (24 mg protein/ml) containing 0.45 M sucrose, 0.01 M Tris-HCl, and 0.001 M EDTA at pH 7.2, was dialyzed against 500 ml each of 0.01 M acetate buffer, pH 5.2, and 0.01 M Tris-HCl buffer, pH 7.2, at 5°C. The buffer solutions were changed every 12 hr. Then, 10 μl aliquots were used for activity assay.*

	Activity remaining (%)			
	Dialyzed		Stored	
	at pH 7.2	at pH 5.2	at pH 7.2	at pH 5.2†
24 hr	53	96	100	100
72 hr	11	82	100	100

† The pH of the enzyme solution was adjusted with 1 M acetic acid.

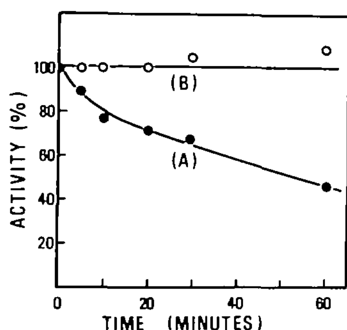

Fig. 1. Heat stability of undialyzed cathepsin A solution at 50°C, pH 5.2. The pH of enzyme solution (24 mg/ml) containing 0.45 M sucrose, 0.01 M Tris-HCl, and 0.001 M EDTA, was adjusted to pH 5.2 with 1 M acetic acid. The enzyme solutions were incubated at 50°C, in the absence (A) or in the presence (B) of 0.1 M KCl. Then, 10 μl aliquots were used for activity assay after appropriate time intervals, as indicated in the figure.

TABLE III. Effects of various substances on the stability of cathepsin A at 37°C, pH 7.2, for 1 hr. The enzyme (24 mg protein/ml) was dialyzed overnight against 0.01 M Tris-HCl buffer, pH 7.2. One milliliter of solution containing 100 μl of the dialyzate and various substances, was incubated for 1 hr at 37°C, pH 7.2. Then, 200 μl aliquots used for activity assay.

Exptl. No.	Additions	Residual activity (%)
1	0.01 M Tris-HCl	4.3
2	0.01 M Tris-HCl+0.1 M KCl	26.2
3	0.01 M Tris-HCl+0.5 M Sucrose	22.9
4	(3)+0.1 M KCl	62.7
5	(3)+0.1 M NaCl	59.2
6	(3)+0.1 M $NaNO_3$	46.3
7	(3)+0.1 M Na_2SO_4	17.7
8	(3)+0.1 M CH_3COONa	27.7
9	(3)+0.1 M $MnCl_2$	62.6
10	(3)+0.1 M $Mn(NO_3)_2$	37.2
11	(3)+0.1 M $MnSO_4$	17.3
12	(3)+0.1 M $(CH_3COO)_2Mn$	23.4
13	(2)+0.5 M Mannitol	57.5
14	(2)+0.5 M Sorbitol	51.9
15	(2)+0.5 M Glycerol	46.0
16	(4)+0.01 M EDTA	61.0
17	(4)+0.1 M Tris-HCl	56.4
18	(4)+0.1 M CH_3COONa	68.3
19	(4)+0.08 M Na_2HPO_4	37.0
20	(4)+0.08 M Tricine	79.2

inactivation by the addition of KCl, as shown in Fig. 1.

The pH Dependences of the Stability and Activity of the Enzyme—The stability of the enzyme in the presence or absence of sucrose and KCl was determined at various pH's as shown in Fig. 2, A. In the absence of sucrose and KCl, the enzyme was most stable at pH 5 and it was more stable in acetate buffer than in citrate buffer. In the presence of sucrose and KCl, the enzyme was stable in the pH region between 3 and 6 in acetate buffer.

The pH dependence of the activity was determined as shown in Fig. 2, B. The maximum activity was observed at pH 5, and 90% of the maximum activity was observad at pH 3.

Inhibitors of the Enzyme Activity—The inhibitory effects of various organic acids on cathepsin A were examined, as shown in Table IV. Phthalic acid and caproic acid greatly inhibited the activity and butyric acid and citric acid partly inhibited it at 0.1 M. The effects of metal-chelating reagents and divalent cations were studied, as shown in Table V. EDTA

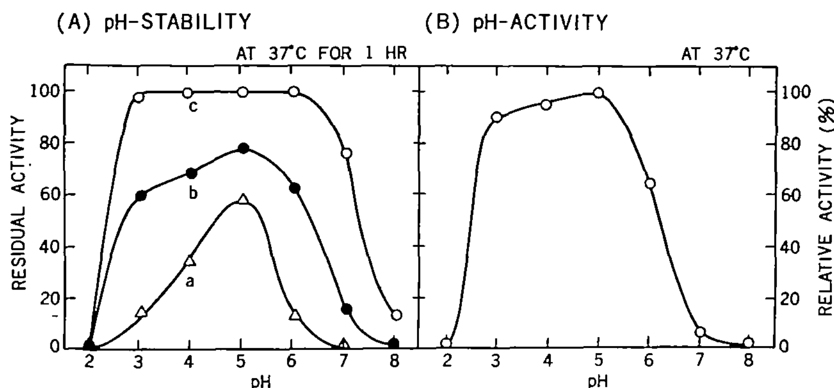

Fig. 2. A: The pH dependence of the stability of cathepsin A in the presence or absence of sucrose and KCl. The enzyme (2.4 mg protein/ml) was preincubated for 60 min at 37°C in 1 ml of the following solutions; solution containing 0.1 M sodium citrate buffer (curve a), solution containing 0.1 M sodium acetate, and 0.01 M Tris-HCl buffer (curve b), and solution containing the same buffer as curve b, plus 0.5 M sucrose and 0.1 M KCl (curve c). After preincubation, 100 μl aliquots were used for activity assay for 20 min at 37°C. B: The pH dependence of the activity in the presence of sucrose and KCl. The reaction mixture contained the enzyme (240 μg protein/ml), 0.5 M sucrose, 0.1 M KCl, 0.05 M acetic acid, and 0.01 M Tris at various pH's, which were adjusted with HCl or NaOH.

TABLE IV. Inhibition of cathepsin A by various organic acids. Various organic acids were contained in the activity assay mixture (1 ml) together with the enzyme (0.24 mg protein), 0.003 M CGT, 0.5 M sucrose, 0.1 M KCl, and 0.05 M acetate buffer (pH 5.2).

Additions	Concentrations† (M)	Activity (%)
None	—	100
Tartaric acid	0.10	65.3
Phthalic acid	0.02	27.0
	0.10	6.1
Citric acid	0.02	83.9
	0.10	30.6
Maleic acid	0.10	100
Fumaric acid	0.10	100
Oxalic acid	0.10	100
Butylic acid	0.02	70.5
	0.10	48.5
Caproic acid	0.02	14.0
	0.10	6.0

† Concentrations are those in the assay mixture. The pH of organic acids was previously adjusted to 5.2 with 1 N NaOH.

TABLE V. Effects of metal-chelating reagents and divalent cations on cathepsin A. The enzyme (0.24 mg protein) was preincubated in 0.7 ml of a mixture containing 0.71 M sucrose, 0.14 M KCl, 0.07 M acetate buffer, pH 5.2, and various reagents for 10 min at 37°C. Then, the activity was assayed after the addition of 0.3 ml of 0.01 M CGT.

Additions	Concentrations† (M)	Activity (%)
None	—	100
EDTA	3×10^{-3}	96.2
o-Phenanthroline	3×10^{-3}	76.3
$CaCl_2$	1×10^{-2}	100
$MgCl_2$	1×10^{-2}	100
$MnCl_2$	1×10^{-2}	100
$CoCl_2$	3×10^{-3}	100
$ZnCl_2$	3×10^{-3}	89.8

† Concentrations are those in the assay mixtures.

and *o*-phenanthroline slightly inhibited the enzyme but various divalent cations except for zinc, did not affect the activity. A 10% decrease of the activity was observed in the presence of 3×10^{-3} M $ZnCl_2$.

TABLE VI. Inhibition or activation of cathepsin A by sulfhydryl reagents or reducing reagents. The enzyme (2.4 mg protein/ml) was preincubated in 1 ml of a mixture containing 0.5 M sucrose, 0.1 M KCl, 0.01 M Tris-HCl buffer, pH 7.2, and various reagents for 20 min at room temperature (20–22°C). Then, 100 μl aliquots were used for activity assay.

Additions	Concentrations[1] (M)	Activity (%)
None	—	100
Iodoacetic acid	3×10^{-3}	97.6
	1×10^{-2}	91.8
	3×10^{-2}	78.1
	3×10^{-2} [2]	33.7
Iodoacetamide	3×10^{-3}	90.6
	1×10^{-2}	73.8
	3×10^{-2}	43.0
	3×10^{-2} [2]	7.0
PCMB	6×10^{-5}	76.0
	3×10^{-4}	6.0
	3×10^{-4} [2]	2.3
Cysteine	1×10^{-2}	100
Glutathione (red)	1×10^{-2}	61.7
Dithiothreitol	1×10^{-2}	45.5
PCMB+Cysteine[3]	$3\times10^{-4}+1\times10^{-2}$	44.7

[1] Concentrations are those in the preincubation mixture. [2] The enzyme was preincubated with the reagent for 60 min at room temperature. [3] PCMB was incubated with the enzyme for 20 min, then cysteine was added to the mixture, which was then further incubated for 20 min.

TABLE VII. Inhibition of cathepsin A by DFP. The experimental conditions were the same as those in Table VI.

Additions	Concentrations[1] (M)	Activity (%)
None	—	100
DFP	10^{-3}	6.3
$CoCl_2$	10^{-3}	100
$CoCl_2$+DFP[2]	$10^{-3}+10^{-3}$	100
EDTA	10^{-3}	100
EDTA+DFP[2]	$10^{-3}+10^{-3}$	0
EDTA+Cysteine+DFP[2]	$10^{-3}+10^{-2}+10^{-3}$	0

[1] Concentrations are those in the preincubation mixture. [2] The substances were added to the enzyme in this order at 5 min intervals, and the mixture was further incubated for 20 min at room temperature.

The inhibitory effects of sulfhydryl reagents and reducing reagents on the enzyme activity were examined, as shown in Table VI. Iodoacetic acid, iodoacetamide, and PCMB partly inhibited the activity at low concentrations, but in the presence of higher concentrations of the inhibitors and after longer incubation periods with the enzyme, the activity was completely inhibited by iodoacetamide or PCMB. Cysteine had no effect on the activity and the other reducing reagent reduced the activity. Enzyme which had been inhibited by PCMB partly recovered its activity on the addition of cysteine.

The inhibitory effect of DFP was examined with or without EDTA or cobaltous ion, as shown in Table VII. The enzyme activity greatly decreased on the addition of DFP and was completely inhibited with the further addition of EDTA. The inhibitory effect of DFP was not observed when it was used together with $CoCl_2$. The results suggest that a metal dependent dialkylfluorophosphatase (*19*) in the enzyme preparation might be hydrolyzing DFP. Cysteine did not affect the inhibition by DFP.

DISCUSSION

The localization of cathepsin A in lysosomal particles has been reported in rat kidney (*20*) and liver (*21*). Catheptic carboxypeptidase (cathepsin IV) has also been found in rat liver lysosome (*12*). Since both enzymes act on the same substrate, CGT, it is difficult to differentiate them. In the present experiments, cathepsin A activity means a CGT-hydrolyzing activity assayed without the addition of cysteine at pH 5.2. This activity was found in lysosomal-rich particles of pig kidney in the present experiments.

Inactivation of cathepsin A during dialysis at pH 8 has been reported for the beef spleen and liver enzymes (*7*). Mellors reported that catheptic carboxypeptidase (cathepsin IV) of rat liver lysosome was most stable at pH 5.0

but lost its activity rapidly during chromatography on ion exchange and Sephadex columns (*12*). The present enzyme preparation was also labile after dialysis. However, it was discovered that the addition of both sucrose and KCl to the dialyzed enzyme protected the enzyme from inactivation. The copresence of sucrose (0.5 M) and KCl (0.1 M) is necessary for maximum stability of the enzyme at 37°C, pH 7.2. A higher concentration of sucrose (1 M) was more effective, but 0.4 M KCl had the same effect as 0.1 M. Lower concentrations of sucrose (0.1 M) and KCl (0.02 M) were not sufficient to stabilize the enzyme at 37°C, pH 7.2, but were effective at 5°C, pH 5.2. As regards stabilizing effect, sucrose could be replaced by other sugar alcohols. Similar protective effects of glycerol and other polyols have been reported for several enzymes (*e.g.* *22*, *23*).

The enzyme was stabilized specifically by chloride anion. Acetate ion had no effect but sulfate, phosphate (Table III), and citrate ions (Fig. 2, A) reduced the stability. The enzyme lost 40 percent of its activity in citrate buffer in 1 hr at 37°C, even in its most stable pH region at pH 5. The pH dependence of the stability of the present enzyme in citrate buffer is similar to that of catheptic carboxypeptidase (*12*) and that of cathepsin A activity (*7*). In the presence of both sucrose and KCl, the optimum pH of the activity was between pH 3 and 5, which is wider than that reported for cathepsin A and includes the pH optimum of catheptic carboxypeptidase. The results indicate that the narrow pH range known for cathepsin A activity may be due to the instability of the enzyme in other pH regions.

Cathepsin A activity was inhibited by various organic acids including citrate and caproate as in the case of pancreatic carboxypeptidase A [EC 3.4.2.1] (*24*). Inhibition of catheptic carboxypeptidase by indole acetic acid has been described (*5*). The results indicate that the use of citrate and phosphate buffers in the activity assay medium is unfavourable for cathepsin A because of inhibition and instability of the enzyme caused by these substances.

Cathepsin A was characterized by the absence of a requirement for activation by cysteine or of inhibition by iodoacetamide (*1–4*). Recently, inhibitions of cathepsin A by PCMB (*11*), Hg, and Ag (*11*, *13*, *15*) have been reported. The present experiments (Table V) show that cathepsin A is relatively insensitive to such sulfhydryl reagents, but is inhibited by them in the presence of high concentrations of the reagents and after long incubation periods with them. Catheptic carboxypeptidase is known to be an SH-enzyme requirinng cysteine for activity (*4*, *5*). However, the activation of catheptic carboxypeptidase by cysteine has been observed by Greenbaum and Sherman (*5*) in a purified extract of beef spleen known as the "Hg-ethanol" fraction of cathepsin B. In the present experiments, enzyme which had been inhibited by PCMB showed restored activity after incubation with cysteine. Thus, the enzyme treated with Hg was probably activated by cysteine.

Partial inhibition (*10*) and complete inhibition (*13*, *15*) of cathepsin A by DFP have been reported. The present results show that the activity in the crude enzyme preparation was completely inhibited by DFP in the presence of 10^{-3} M EDTA, but it was not inhibited in the presence of DFP with cobaltous ion. Mounter *et al.* (*19*) described the presence of a dialkylfluorophosphatase in pig kidney which is activated by Mn^{2+} or Co^{2+} ions. The absence of inhibition of cathepsin A by DFP in the presence of Co^{2+} might be accounted for by the action of this enzyme.

As described above, complete inhibition of the crude enzyme preparation by DFP or PCMB indicates the presence of a single type of enzyme which hydrolyzes CGT in pig kidney. Enzymes which are inhibited by both PCMB and DFP have been described among carboxypeptidases of plants and microorganisms (*e.g.*, *25–28*). If catheptic carboxypeptidase was contained in the present enzyme preparation, it was also inhibited by both PCMB and DFP. However, the presence of an enzyme which hydrolyzed CGT but had a different substrate specificity from cathepsin A was not detected during further purification of the crude enzyme preparation, as described in the succeeding paper (*29*). Similar hydrolytic rates for CGT,

CPP, and CPA observed with the present preparations (L and S) and various tissue homogenates indicate the absence of two types of enzyme hydrolyzing CGT (*29*).

The requirement for cysteine for activity cannot be used as a criterion to distinguish cathepsin A and catheptic carboxypeptidase, as discussed above.

The pH optimum of cathepsin A is known to be pH 5–5.6 (*3, 7, 13*), and that of catheptic carboxypeptidase to be pH 3–4 (*3, 5*). The present enzyme preparation was optimally active between pH 3 and 5 in the presence of both sucrose and KCl. The same pH dependence of the activity is observed in partially purified pig kidney cathepsin A, as will be described in the succeeding paper (*29*). All these data suggest that cathepsin A and catheptic carboxypeptidase are the same enzyme.

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