Some Enzymatic Properties of Cathepsin A from Carp White Muscle^{*1}

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(Received August 5, 1976)

Some enzymatic properties of the highly purified cathepsin A from white muscle of carp were examined. The optimal pH of the cathepsin A was 4.6 in 0.2 M acetate buffer solution. Approximate K_m values of the cathepsin A for carbobenzoxy (CBZ)-Glu-Phe, CBZ-Glu-Tyr and CBZ-Gly-Phe were 0.42, 0.75 and 14 mM, respectively, in 0.2 M acetate buffer solution, pH 5.0. Acetyl-Phe-Tyr (I_2) was hardly hydrolyzed by the enzyme. The thermostability of the enzyme was increased in the presence of NaCl and sucrose. Hg^{2+} , pCMB and diisopropyl fluorophosphate were inhibitory on the enzymatic activity. The inhibition of the enzyme by antipain was competitive for CBZ-Glu-Tyr, with K_i value of 17 μM . The enzymatic activity was inhibited by all the thiol compounds tested at high concentrations. Some discussions were presented as to whether carp cathepsin A and catheptic carboxypeptidase among cathepsins are the same enzyme or not. In the enzyme assay, the fluorometric method using fluorescamine showed some advantages to the conventional ninhydrin method in simplicity, rapidity, and sensitivity.

During the course of experiments on the role of proteins which induce "Modori" phenomenon in Kamaboko production, cathepsin A was highly purified from the white muscle of carp¹⁾.

Several investigations have been published on the purification procedures and enzymatic properties of cathepsin A in bovine spleen^{2,3)}, chicken muscle^{2,4)}, rat liver⁵⁻⁹⁾ and pig kidney¹⁰⁻¹³⁾. It is of interest to compare with the enzymatic properties of cathepsin A from fish muscle and those from mammals.

In the present study, the highly purified cathepsin A from carp white muscle was examined on some enzymatic properties. The enzymatic activity was assayed by the usual ninhydrin colorimetric method as well as the fluorescence method using fluorescamine, a new reagent for the fluorometric detection of primary amine.¹⁴⁻¹⁵⁾

Experimental

Carp cathepsin A Cathepsin A was prepared from the white muscle of carp, *Cyprinus carpio*, as described previously¹⁾. The final specific activity was 2.0 μ mole of CBZ-Glu-

^{*1} This was presented at the 95th Annual Meeting of Pharmaceutical Society of Japan at Nishinomiya, April, 1975.

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Tyr cleaved per min per mg of protein at 37°C and pH 5.0 in 0.2 M acetate buffer solution.

Materials N-Carbobenzoyl-L-glutamyl-L-phenylalanine (CBZ-Glu-Phe), N-carbobenzoyl-L-glutamyl-L-tyrosine (CBZ-Glu-Tyr), N-carbobenzoyl-L-glycyl-L-phenylalanine (CBZ-Gly-Phe) were purchased from the Protein Research Foundation, Osaka. N-Acetyl-L-phenylalanyl-3, 5-diiodo-L-tyrosine [A-Phe-Tyr (I_2)] was obtained from E. Merck AG. Darmstadt. Diisopropyl fluorophosphate (DFP) and L-1-tosylamido-2-phenylethyl chloromethylketone (TPCK) were obtained from Sigma Chemical Co. and Seikagakukogyo, Tokyo, respectively. Antipain, [(S)-1-carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valyl-argininal¹⁶⁾, was generously provided from Dr. H. UMEZAWA of the Institute of Microbial Chemistry, Tokyo and fluorescamine, 4-phenylspiro[furan-2(3H), 1'-phthalan]-3, 3'-dione, was also obtained from the Roche Institute of Molecular Biology, New Jersey. p-Chloromercuribenzoic acid (pCMB) was used after purification according to the method of BOYER¹⁷⁾. Iodoacetic acid was recrystallized from chloroform. Other chemicals were of analytical grade. Solutions were prepared with deionized and distilled water obtained by all-glass apparatus.

Measurement of cathepsin A activity The enzymatic activity was measured by (1) the ninhydrin colorimetric method as described previously¹⁾ or (2) the fluorometric method in the following procedures. The reaction mixture consisted of 0.5 μ g of the enzyme protein, 1 mM substrate and 0.2 M acetate buffer, pH 4.7 or 5.0, and 0.1 M NaCl in the total volume of 0.5 ml, and was incubated at 37°C for 30 min. The reaction was stopped by adding 2.2 ml of 0.5 M borate buffer, pH 8.5. While mixing vigorously with a tube mixer at room temperature, 0.3 ml of 0.03% fluorescamine solution in acetone was added using an autoburette. The reaction occurred within seconds at room temperature. Fluorescence was determined with a HITACHI Model 204 spectrofluorometer at excitation wavelength of 390 nm and emission wavelength of 465 nm. Tyrosine or phenylalanine was used as a standard compound under the same assay conditions. A blank was run by incubating the reaction mixture without substrate, followed by the addition of the borate and substrate at the end of the incubation period. The fluorescence intensity of the assay mixture was corrected by subtracting the blank intensity.

Determination of protein The protein concentration was determined either by microbiuret method^{18,19)} or the method of LOWRY *et al.*²⁰⁾ standardized with bovine serum albumin (Fraction V).

Results and Discussion

Effect of pH on the activity of cathepsin A The pH-activity profile of cathepsin A for the hydrolysis of CBZ-Glu-Tyr was investigated using 0.2 M acetate, 0.2 M phosphate or MacIlvaine buffer (Fig. 1). This enzyme showed the optimal activity at pH 4.6 in acetate buffer and at pH 5.0 in MacIlvaine buffer. At a neutral pH range, little activity

Fig. 1.

Fig. 2.

Fig. 1. Effect of pH on the hydrolysis of CBZ-Glu-Tyr by carp cathepsin A. 0.7 μ g of the enzyme protein was incubated at 37°C for 30 min in 0.5 ml of the following buffer solutions containing 0.1 M NaCl and 1.0 mM CBZ-Glu-Tyr at various pH's. The activity was estimated by the fluorescence method. The relative fluorescence was expressed by percentage of the fluorescent intensity of 20 nmole of tyrosine. Buffer system: 0.2 M acetate-HCl buffer from pH 1.8 to 2.9; 0.2 M sodium acetate-acetic acid buffer from pH 3.8 to 5.7 (—○—); 0.2 M phosphate buffer from pH 6.6 to 7.7 (—●—) or MacIlvaine from pH 1.7 to 7.1 (—×—).

Fig. 2. Effect of pH on the hydrolysis of CBZ-Glu-Phe, CBZ-Gly-Phe or A-Phe-Tyr (I_2) by carp cathepsin A.

0.55 μ g of the enzyme protein was incubated at 37°C for 30 min in 0.5 ml of the following buffer solutions containing 0.1 M NaCl and 1.0 mM substrate at various pH's. The enzymatic activity was estimated by the fluorescence method. Buffer system: 0.2 M acetate-HCl buffer from pH 1.8 to 2.9; 0.2 M sodium acetate-acetic acid buffer from pH 3.8 to 5.7 or 0.2 M phosphate buffer from pH 6.6 to 8.1. The symbols were as follows: —●— CBZ-Glu-Phe, —○— CBZ-Gly-Phe, —×— A-Phe-Tyr (I_2).

was observed in both buffers. As shown in Fig. 1, the activity in acetate buffer was about 6 times higher than that in MacIlvaine buffer. This seems to indicate an inhibitory effect of citrate in the latter buffer. These observations accord substantially with the results by Dor¹⁰⁾ reporting that the activity of pig kidney cathepsin A was partially inhibited by citrate and also by phosphate. Consequently, acetate buffer was used hereafter. As shown in Fig. 2, the pH-activity curves of the enzyme for the hydrolysis of CBZ-Glu-Phe or CBZ-Gly-Phe were similar to that for CBZ-Glu-Tyr, showing optimal pH to be 4.6 for both substrates. The optimal pH of the carp enzyme was slightly acidic than that (pH 5.2–5.9) of the enzyme obtained from internal organs of mammals^{9, 12, 13)}. A-Phe-Tyr (I_2) which has been reported to be susceptible substrate for pepsin²¹⁾ was hardly hydrolyzed by the carp enzyme.

Cathepsin A was originally defined as the enzyme which hydrolyzes CBZ-Glu-Tyr, a synthetic substrate for pepsin and is not activated by the addition of thiol compounds. Therefore, this enzyme has been considered to be a pepsin-like endoproteinase²²⁻²⁵. However, recent studies revealed that it is also one of carboxypeptidases^{2,12,13}.

Approximate K_m values of the carp cathepsin A for CBZ-Glu-Phe, CBZ-Glu-Tyr and CBZ-Gly-Phe were determined to be 0.42, 0.75 and 14 mM, respectively in acetate buffer solution, pH 5.0. The affinity of the carp cathepsin A for CBZ-Gly-Phe was higher than that ($K_m=1.0-7.5$ mM) of the enzyme from rat liver lysosome^{6,7,9,26}. However, the affinity of the carp cathepsin A for CBZ-Gly-Phe was slightly lower than that ($K_m=5.4-10.6$ mM) of the latter^{6,7}.

The concentration of amino acid liberated from synthetic substrates has been generally determined by the ninhydrin colorimetric method²⁷ for the assay of cathepsin A or other peptidases. Fluorometric method using phthalaldehyde was presented by ROTH²⁸ and TAYLOR *et al.*²⁶. Sensitive fluorometric method with fluorescamine on assay of trypsin-like protease was also reported by BROWN *et al.*²⁹ using herring protamine sulfate, as a substrate, which was blocked at the terminal amino group with dinitrofluorobenzene. Both fluorometries are known to be about one hundreds times more sensitive than the conventional ninhydrin colorimetry. However, the sensitivity of the fluorescamine procedure in our present study was much higher than that of phthalaldehyde procedure. Besides, fluorescamine procedure has advantages over the phthalaldehyde technique in lower background fluorescence of the reagents and comparatively little fluorescence as caused by ammonia.

Effect of temperature on the reaction and thermostability of the cathepsin A The effect of the reaction temperature on the activity of the enzyme is shown in Fig. 3. The amount of product from CBZ-Glu-Phe incubated for 60 min was the highest at 50°C. However, in presence of 0.5 M NaCl and 0.2 M sucrose, as in the production of Kamaboko, the amount was the highest at 60°C. When the enzyme solution was incubated without a substrate at 65°C for 40 min, the activity was a little lost in the presence of NaCl and sucrose, but was remarkably lost in their absence (Fig. 4). These facts suggest that NaCl and sucrose protect the enzyme from heat inactivation.

Effect of various reagents on the activity of the cathepsin A Various metal ions such as Mn^{2+} , Ca^{2+} , Mg^{2+} and Zn^{2+} did not affect the activity, but Hg^{2+} and pCMB markedly inhibited the activity at 1 mM, as shown in Table 1. Metal chelators tested did not affect the activity either. The enzyme was partially inactivated by TPCK at 1 mM and by antipain at 0.25 mM. DFP completely inhibited the activity at 1 mM. It has been reported by LOGUNOV *et al.*³, MATSUDA *et al.*⁹ and DOI¹⁰ that the activity of cathepsin A from bovine spleen, rat liver lysosome and pig kidney was markedly inhibited by 1 mM DFP. However, TAYLOR *et al.*⁷ reported that DFP at a final concentration of 0.2 mM activated the

Fig. 3.

Fig. 3. Effect of temperature on the activity of carp cathepsin A.

72 μ g of the enzyme protein in 1.0 ml of 0.2 M acetate buffer solution, pH 5.0 containing 7.5 mM CBZ-Glu-Phe was incubated with (—○—) or without (—×—) 0.5 M NaCl and 0.2 M sucrose at the indicated temperature for 60 min. The activity was estimated by the ninhydrin colorimetric method¹⁾. The relative activities were expressed by percentage of the activity in the presence of 0.5 M NaCl and 0.2 M sucrose at 58°C.

Fig. 4.

Fig. 4. Effect of addition of NaCl and sucrose on the thermostability of carp cathepsin A at 65°C.

200 μ g of the enzyme protein was preincubated in 0.5 ml of 0.01 M potassium phosphate-0.005 M sodium borate buffer, pH 6.6 containing 0.1 mM EDTA in the presence of 0.5 M NaCl and 0.2 M sucrose (—○—) or in their absence (—×—). After preincubation the former mixture was diluted with 0.75 ml of the same buffer solution and the latter with 0.75 ml of the same buffer solution containing 0.333 M NaCl and 0.133 M sucrose. Thereafter, 0.5 ml-aliqouts were taken, followed by 120 min-incubation at 37°C with 0.2 ml of 1 M sodium acetate-acetic acid buffer, pH 5.0 and 0.3 ml of 25 mM CBZ-Glu-Phe. The phenylalanine liberated was estimated by the ninhydrin colorimetric method.¹⁾

cathepsin A from rat liver lysosome to 120% of the control. The inhibition of the carp enzyme by antipain was shown to be competitive for CBZ-Glu-Tyr used as a substrate by Lineweaver-Burk plotting (Fig. 5), from which the K_i value was found to be 17 μ M under the conditions as described in the legend of Fig. 5. MATSUDA *et al.*⁹⁾ reported that 100 μ g/ml of antipain was not inhibitory on cathepsin A purified from the lysosome fraction of rat liver. On the other hand, IKEZAWA *et al.*³⁰⁾ reported that ID_{50} value of antipain was 1.19 μ g/ml (2.0 μ M) on cathepsin A activity in the acetone powder from lysosomal-mitochondrial pellet of swine liver. The K_i value of antipain for the carp enzyme was about 10 times larger than ID_{50} value reported by IKEZAWA *et al.*³⁰⁾. The effects of DFP and antipain on the cathepsin A so far reported were different from one another, probably due to different enzyme sources and purification (isolation) procedures.

Such sulfhydryl reagents as iodoacetamide and iodoacetate inhibited the activity at high concentrations, but the activity was not inhibited by N-ethylmaleimide at 10 mM. It has been reported that cathepsin A from mammalian internal organs was not affected by

Table 1. Effect of various reagents on the activity of cathepsin A. 0.5–0.6 μg of the enzyme protein was preincubated in 0.45 ml of 0.22 M acetate buffer solution, pH 4.7 containing 0.11 M NaCl and various reagents in an indicated final concentration at 37°C for 30 min. The enzymatic activity was assayed by adding 50 μl of 10 mM CBZ–Glu–Tyr to the preincubation mixture. The tyrosine liberated was estimated by the fluorescence method.

Compounds	Concentrations	
	1 mM	10 mM
None	100	100
MnCl ₂	125	108
CaCl ₂	118	104
MgCl ₂	106	107
ZnCl ₂	91	72
HgCl ₂	15	0
EDTA	97	83
<i>o</i> -Phenanthroline	95	97
K ₃ Fe(CN) ₆	80	21
TPCK	66	35
DFP	0	0
Antipain	50 (0.25 mM)	—
Dithiothreitol	82	9
Mercaptopropionic acid	77	17
Thiomalic acid	76	11
2-Mercaptoethanol	71	11
Thioglycolic acid	69	26
Thiosalicylic acid	60	7
N-Ethylmaleimide	92	92
Iodoacetamide	83	46
Iodoacetic acid	68	55
pCMB	21	—

Fig. 5. Lineweaver-Burk plotting of CBZ–Glu–Tyr hydrolysis by the cathepsin A.

0.7 μg of the enzyme protein in 0.5 ml of 0.2 M sodium acetate-acetic acid buffer, pH 5.0, containing 0.1 M NaCl and the substrate was incubated with (—○—) without (—●—) 0.05 mM antipain at 37°C for 30 min. The enzymatic activities were determined by the fluorescence method.

thiol compounds^{7,9,10,12,13}, although the activity of catheptic carboxypeptidase from beef spleen^{31,32} and rat liver lysosome^{8,33} were accelerated by such compounds. The activity of carp cathepsin A was inhibited by all of the thiol compounds tested at high concentrations, as shown in Table 1. These observations accord substantially with the results by Doi *et al.*^{10,13} reporting that the activity of pig kidney cathepsin A was partially inhibited by thiol compounds at 10 mM.

Catheptic carboxypeptidase^{31,33} is defined as the enzyme which

hydrolyzes CBZ-Glu-Tyr at the optimal pH of 3.5, being activated by thiol compounds and inhibited by pCMB and iodoacetate. The catheptic carboxypeptidase has been considered to be distinguished from cathepsin A on the requirement of thiol compounds on activation and the optimal pH, as described above. However, DOI *et al.*^{10,11)} discussed the possible identification of cathepsin A as catheptic carboxypeptidase by the reasons that catheptic carboxypeptidase could not be separated from cathepsin A during the purification of proteolytic enzymes from pig kidney and that optimal pH of pig kidney cathepsin A was between 3 and 5 in the presence of sucrose and KCl in which that of catheptic carboxypeptidase is included. The activity of the carp enzyme was observed a little at pH 3.5 in the presence of 0.1 M NaCl, and was not affected by the addition of 10 mM 2-mercaptoethanol. These observations suggest that carp cathepsin A is unequivocally discriminated from catheptic carboxypeptidase which is defined as described above. However, further examination has to be made as to whether cathepsin A and catheptic carboxypeptidase are the same enzyme or not.

Acknowledgements

We are indebted to Dr. H. UMEZAWA, Director of the Institute of Microbial Chemistry, Tokyo, for his kind supply of antipain and the Roche Institute of Molecular Biology, New Jersey, for kind gift of fluorescamine. Thanks are also due to Mr. K. MITSUI for his helpful suggestions.

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