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Biochemical Properties of Carboxypeptidase in Aloe Arborescens Miller Var. Natalensis Berger

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ABSTRACT

A carboxypeptidase was partially purified from **Aloe arborescens** Miller **var. Natalensis** Berger in a scale suitable for pharmacological studies. The enzyme was most active and stable at pH 5.0. The enzyme had a broad specificity against various synthetic peptides, being capable of splitting C-terminal proline. Its activity was inhibited almost completely by diisopropylfluorophosphate, strongly by transition metals such as Fe^{3+}, Hg^{2+} and Cu^{2+} and moderately by sulfhydryl reagents. These results indicate that aloe enzyme is a serine carboxypeptidase and appears to contain a sulfhydryl group that may be involved in its inactivation.

Key words: aloe arborescens; serine carboxypeptidase; optimum pH; substrate specificity; inhibitors of enzyme activity.

INTRODUCTION

Aloe arborescens Miller **var. natalensis** Berger, Japanese name "Kidachi Aloe", has been valued in Japan as a family medicine for skin injury and burns. We have been studying this plant from pharmacological (Fujita, **et al.**, 1976, 1979) and immunological (Fujita, **et al.**, 1978) points of view to rationalize its therapeutic use as anti-inflammatory agent.

In 1976, we found that the high-molecular weight fraction of aloe leaves contains an enzyme hydrolyzing bradykinin, a peptide that mediates inflammation and allergy (Fujita, et al., 1976). Subsequently, we showed that the enzyme is a carboxypeptidase, capable of hydrolyzing bradykinin from the C-terminal (Fujita, et al., 1979). In order to pursue pharmacological studies, it was necessary to establish a method for a large scale purification of the enzyme from aloe and characterize its biochemical properties. It was found that the enzyme is a serine carboxypeptidase with a broad specificity and is inhibited by transition metals.

MATERIALS AND METHODS

Plant material. The plants of **Aloe arborescens** ("Kidachi Aloe") have been cultivated in our herb garden at the Institute of Pharmacognosy, Hisai, Japan. Leaves of aloe can be obtained throughout the year.

Chemicals. Carbobenzoxy (Z-) and benzoyl (Bz-) derivatives of peptides were purchased from Protein Research Foundation, Osaka, and most of other chemicals from Wako Pure Chemical Industries, Osaka.

Activity assay. Carboxypeptidase activity was routinely assayed with Z-Gly-Pro-Leu-Gly as a substrate. The assay mixture consisted of 1.6 mM substrate and 0.05 M acetate buffer, pH 5.0. After incubation at 37℃ for 60 min., the reaction was stopped by heating in a boiling water bath for 5 min. and the amount of glycine released was determined by the ninhydrin method (Moore, 1968). One unit was defined as the amount of enzyme necessary to release one μmol of glycine per min. at 37℃. Protein content was determined by the method of Lowry et al. (1951).

Partial purification of aloe carboxypeptidase

Twenty kg of fresh leaves of aloe were homogenized with Polytron and the homogenate was filtered through Whatman GF/A paper. The filtrate (18 l) was concentrated in a cold room with Amicon DC-2 ultrafiltration apparatus (hollow fiber: HIDPIO). The concentrate consisting of components with molecular weights higher than 10kD was dialyzed against 0.05 M acetate buffer, pH 5.0 containing 0.5 M NaCl until no more fluorescent materials were dialyzed out.

The dialyzate (1680 ml) was cleared by centrifugation, and applied on a large column (20x75cm) of Ultrogel AcA-34. Elution with 0.05 M acetate buffer, pH 5.0 containing 0.5 M NaCl gave the carboxypeptidase fraction between low and high molecular weight fractions. The active fraction (8000ml) was concentrated to 2000 ml by ultrafiltration and to the concentrate was added ammonium sulfate (70% saturation). The precipitate was collected by centrifugation, dissolved in 0.02 M acetate buffer, pH 5.0 containing 10 mM EDTA and dialyzed against the same buffer.

The dialyzate (60 ml) was applied to a column (5.8x22 cm) of DEAE Sephadex A-50 and eluted with 0.02 M acetate buffer, pH 5.0 containing 10 mM EDTA using a linear gradient from 0.0 to 0.5M NaCl. The enzyme fraction which appeared in the later part of the gradient was brought to a 70% saturation of ammonium sulfate.

The precipitate was dissolved in 0.05 M acetate buffer, pH 5.0 containing 5mM EDTA and 0.5 M NaCl and applied to a column (2.6 x 95 cm) of Ultrogel AcA-34 and eluted with the same buffer. Two peaks of protein fractions appeared from the column, the former of which contained the carboxypeptidase activity. This fraction was kept frozen and used for biochemical characterization.

RESULTS

Purification of carboxypeptidase from aloe leaves

A homogenate from 20 kg of aloe leaves was concentrated by ultrafiltration and subjected to gel chromatography on Ultrogel AcA-34 and ion exchange chromatography on DEAE Sephadex A-50 (Table 1).

Table 1. Purification of aloe carboxypeptidase from aloe leaves [a]

Procedure	Volume	Protein	Carboxypeptidase [b]		
	(ml)	(mg)	U total	U/mg	%Yield
Ultrafiltration	1680	3192	521	0.16	100
Ultrogel AcA-34	8000	1122	466	0.42	89
DEAE-Sephadex A-50	553	138	243	1.76	47
Ultrogel AcA-334	81	69	224	3.18	44

[a] Starting from 20 kg of aloe leaves.
[b] The activity was assayed with Z-Gly-Pro-Leu-Gly.

About 20-fold purification was achieved with 44% recovery of the activity. The isolated enzyme was almost homogeneous by polyacrylamide gel electrophoresis. However, the specific activity of 3.18 U/mg and the 20-fold purification appeared to be not high enough. Attempts to further purify the enzyme were unsuccessful because of instability of the enzyme. The enzyme preparation thus obtained was used for the following experiments.

Hydrolysis of Z-Gly-Pro-Leu-Gly

The synthetic tetra peptide was hyrolyzed by aloe carboxypeptidase and the reaction was followed by analyzing the amounts of amino acids released after various incubation times. As shown in Fig.1, the peptide was split from the C-terminal in a sequence of glycine, leucine, and proline. The release of glycine and leucine were rapid while the third residue proline was liberated rather slowly.

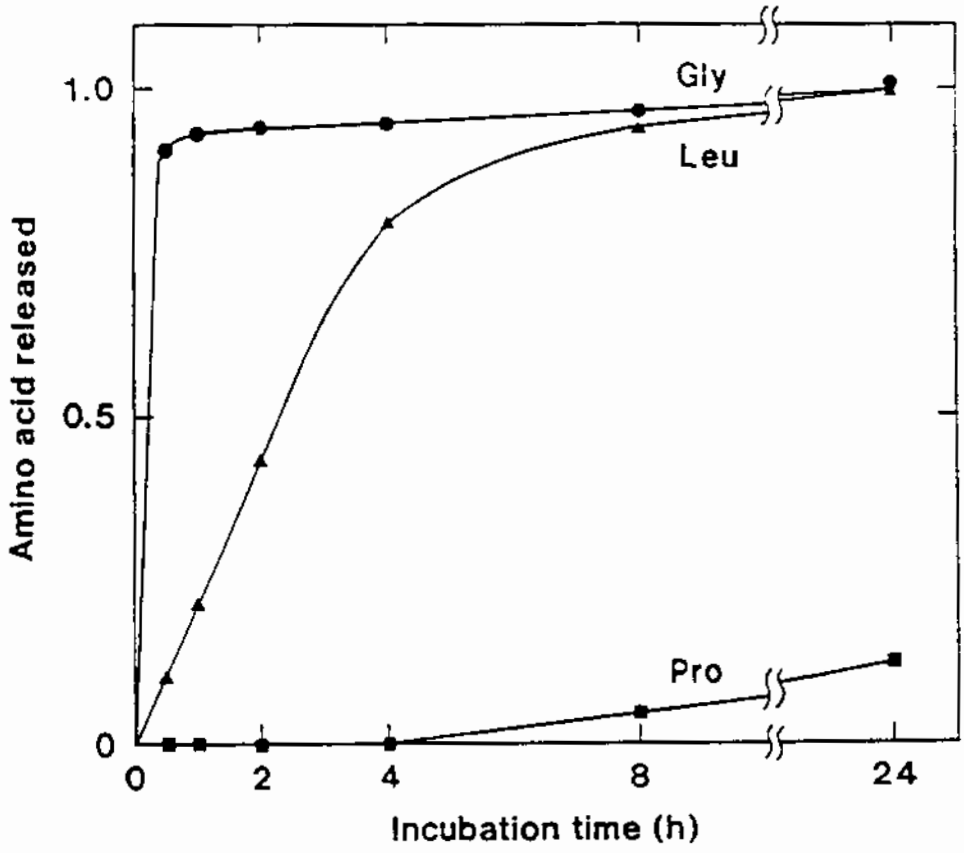

Fig 1. Hydrolysis of Z-Gly-Pro-Leu-Gly by aloe carboxypeptidase. One μmol of the peptide was incubated with 10 μg of the enzyme in 1.0 ml of 0.05 M acetate buffer, pH 5.0 at 37℃. 0.15 ml of the incubation mixture was withdrawn after 0.5, 1, 2, 4, 8 and 24 h and the reaction stopped by heating the mixture in a boiling water bath for 5min. Each mixture was evaporated to dryness in a vacuum desiccator, dissolved in a pH 2.2 buffer containing 0.15 μmol of norleucine and analyzed on a JEOL JLC-6AH amino acid analyzer.

Effects of pH and other factors on the activity and stability

The optimum pH of aloe carboxypeptidase for the hydrolysis of the tetra peptide was found to be about pH 5.0 (Fig. 2a). The enzyme was most stable at pH 5.0, but it was less stable at higher

pH (Fig. 2b). At pH 5.0 the enzyme remained fairly stable below 35℃ while at higher temperature it lost the activity relatively fast (Fig. 3). This heat inactivation was suppressed by high concentration of sucrose. Thus, after heating at 60℃ for 60 min, the activity was reduced to 30% while the addition of 1 M sucrose protected the enzyme with only 20% loss of the activity.

Dialysis and lyophilization resulted in considerable loss of activity. After dialysis against water the enzyme activity was reduced to 33% and the following lyophilization further decreased the activity to 21% of the original value. Again, the addition of 1 M sucrose protected the enzyme; the remaining activities were 90% and 84%, respectively.

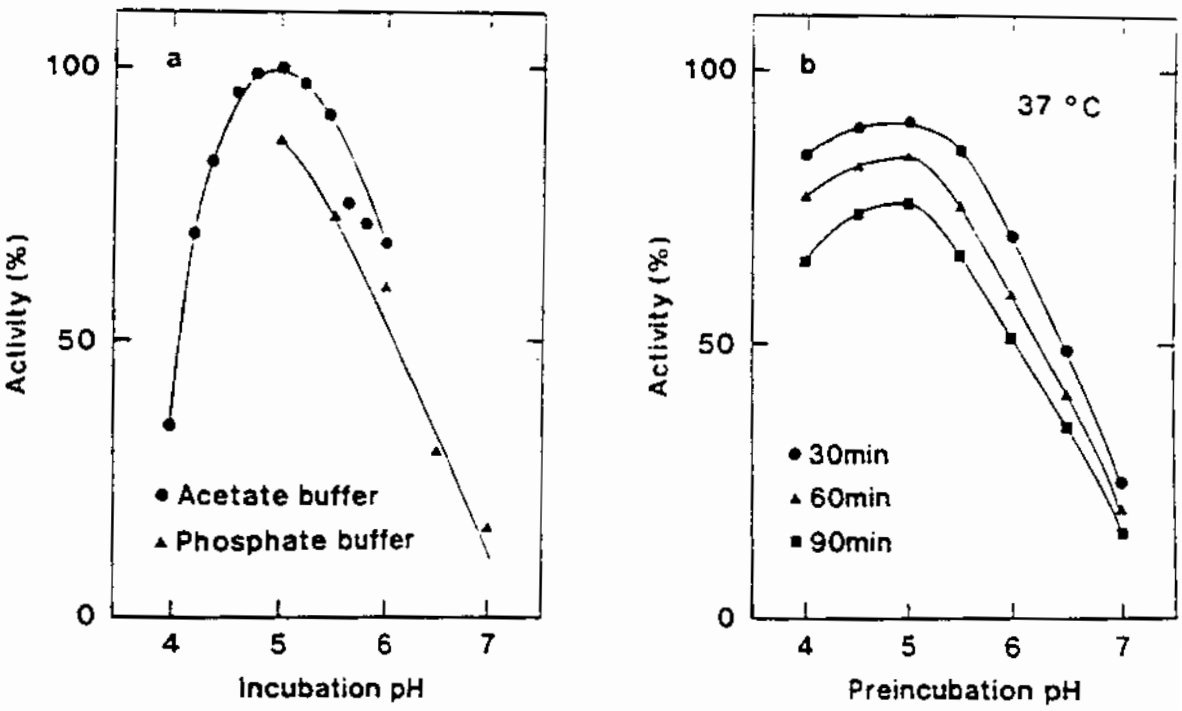

Fig 2. Effects of pH on the activity and stability of aloe carboxypeptidase. (a) Activity of the enzyme (1 μg/ml) in 0.05 M buffer at various pH. The activity was assayed with 1.6 mMZ-Gly-Pro-Leu-Gly. The activity values were those relative to the activity in 0.05 M acetate buffer, pH 5.0 (b) Stability of the enzyme (5 μg/ml) at 37℃ in 0.05 M acetate buffer, pH 4.0 - 6.0 or 0.05 M phosphate buffer, pH 6.5 and 7.0. After the preincubation for the indicated times, the mixture was diluted 20-fold with 0.05M acetate buffer, pH 5.0 containing 1.6mMZ-Gly-Pro-Leu-Gly and the enzyme activity remaining was assayed. The activity values were those relative to the activity in 0.05 M acetate buffer, pH 5.0 without the preincubation.

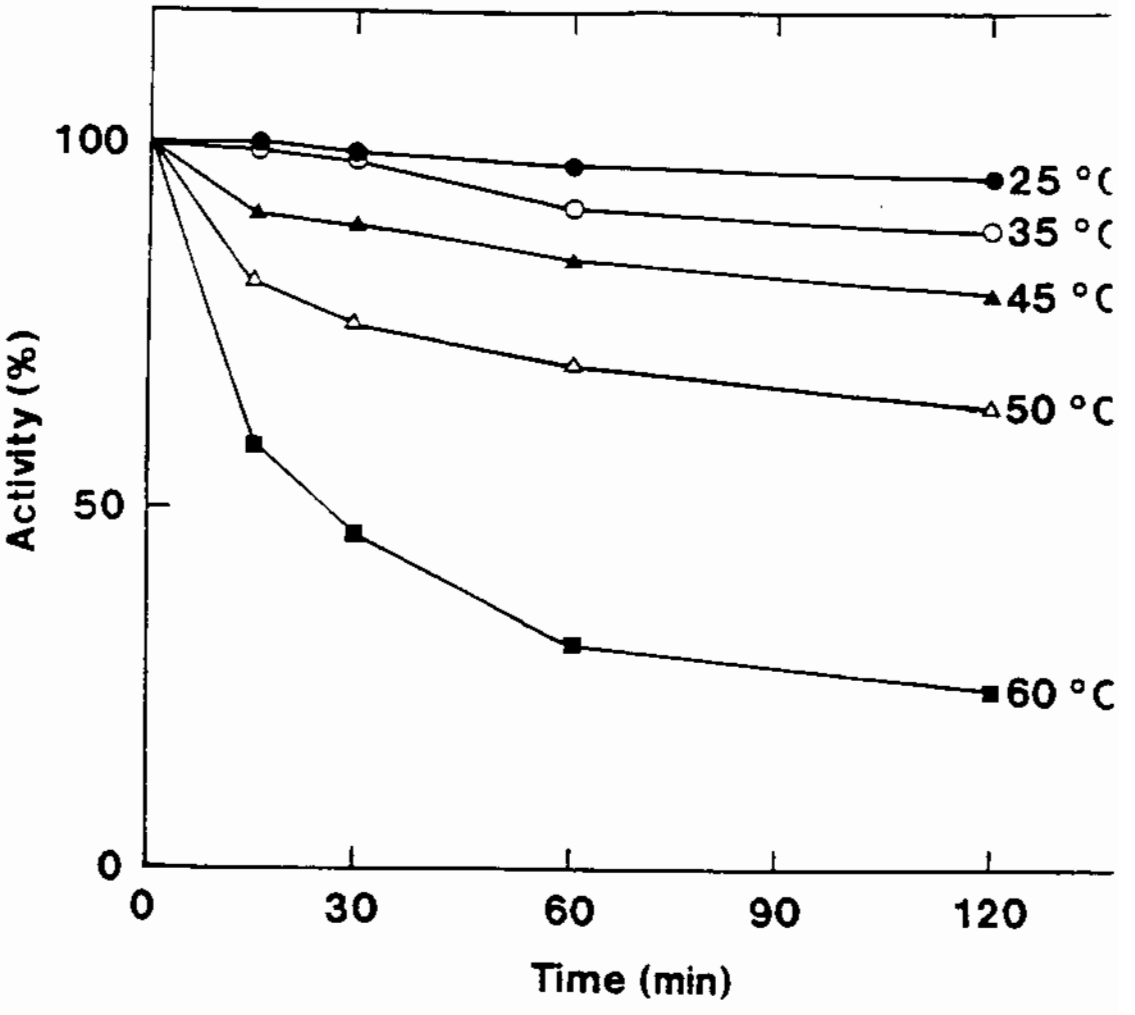

Fig 3. **Stability of aloe carboxypeptidase at pH 5.0 and various temperatures.** T
enzyme (5 μg/ml) was preincubated in 0.05M acetate buffer, pH 5.0 at t
indicated temperature. After the preincubation, the enzyme activity was assay
with Z-Gly-Pro-Leu-Gly.

Substrate specificity

The enzyme had the highest activity against Z-Phe-Tyr among the peptides studied (Table
But it also showed a rather broad specificity including the ability to split proline from Z-Gly-Pr
Among the peptides having glycine as the penultimate amino acid (Nos. 5-7), Z-Gly-Pro w
split at the fastest rate.

Table 2. Substrate specificity of aloe carboxypeptidase [a]

NO.	Substrate	Specific activity (U/mg)	Relative activity (%)
1	Z-Gly-Pro-Leu-Gly	3.86	100
2	Z-Phe-Tyr	7.45	193
3	Z-Glu-Tyr	1.19	31
4	Z-Glu-Phe	0.48	12
5	Z-Gly-Pro	0.38	9.8
6	Z-Gly-Leu	0.33	8.5
7	Z-Gly-Phe	0.14	3.6
8	Bz-Gly-His-Leu	0.41	11
9	Bz-Gly-Phe	0.19	4.9
10	Bz-Gly-Arg	0.17	4.4
11	Bz-Arg-p-nitroanilide	0.00	0.0
12	Leucine-p-nitroanilide	0.00	0.0

[a] 1.6 mM substrate was incubated with 0.25 - 2.0 μg enzyme at 37℃ for 60min. in 1.0ml of 0.05 M acetate buffer, pH 5.0.

The enzyme preparation did not contain a trypsin-type endoprotease and an aminopeptidase, as shown in the inability to split p-nitroaniline from Bz-Arg-p-nitroanilide and leucine pnitroanilide.

Inhibition studies

Effects of various reagents and metal ions on the enzyme activity were studied (Table. 3). The enzyme was inhibited to some extents by the sulfhydryl reagents, iodoacetamide and p-chloromercuriphenylsulfonic acid. Diisopropylfluorophosphate, which is known to inhibit serine carboxypeptidase (Oosterban, et al., 1955), almost completely inhibited the activity. Most of the metal ions examined exhibited inhibitory effects (Nos 9 - 16). Among them. Fe^{3+}, Hg^{2+} and Cu^{2+} ions showed the strongest effects. The activating effect observed with EDTA seemed to be ascribed to the removal of trace amounts of inhibitory metal ions in the reaction medium. This was proved by an experiment in which inhibitory effect of 10 mM Fe^{3+} was completely suppressed by 20 mM EDTA.

Table 3. Effects of various reagents and metal salts on the activity of aloe carboxypeptidase [a]

No.	Reagent or metal salt	Concentration (mM)	Relative activity (%)
1	None	-	100
2	Iodoacetamide	1.0	85
3	p-Chloromercuriphenylsulfonic acid	1.0	36
4	o-Phenanthroline	1.0	99
5	EDTA. 2Na	10	104
6	Diisopropylfluorophosphate	1.0	18
		5.0	5
7	NaH_2PO_4	1.0	100
8	Nacl	10	100
9	$HgCl_2$	0.1	58
		1.0	33
10	$FeCl_3$	0.1	16
		1.0	2
11	$CdCl_3$	1.0	41
12	$ZnCl_2$	1.0	51
13	$CuCl_2$	0.1	44
14	$CoCl_2$	1.0	64
15	$MgCl_2$	1.0	59
16	$CaCl_2$	1.0	78

[a] The enzyme (5 ㎍) was preincubated in 0.6 ml of 0.05 M acetate buffer, pH 5.0 containing one of the reagent or metal salt at the indicated concentration for 20 min. at 37℃. 0.4 ml of 2 mM of Z-Gly-Pro-Leu-Gly were then added to the mixure and the activity was assayed.

DISCUSSION

A carboxypeptidase was purified from 20kg of leaves of **Aloe arborescens** and its biochemical properties were examined. Inhibition by diisopropylfluorophosphate indicates that aloe enzyme is a seine carboxypeptidase. Serine carboxypeptidases are widely distributed in fungi and higher plants and are also common in animal tissues (Breddam, 1986). It is presumed that as

intracellular enzymes, they participate in the general turnover of proteins and in providing free amino acids from the storage proteins. Most of these carboxypeptidases have pH optimum around 5.0 to 5.5 (Doi, **et al.**, 1980). Aloe carboxypeptidase was also found to be most active at pH 5.0.

Plant carboxypeptidases are known to have broad substrate specificity (Breddam, 1986). Aloe carboxypeptidase appears to be unique among them in that it shows a higher activity against Z-Gly-Pro than Z-Gly-Leu and Z-Gly-Phe. Carboxypeptidases from malted barley (Breddam, et al., 1985) and watermelon (Matoba & Doi, 1975) were shown to have the opposite specificity.

Aloe carboxypeptidase was inhibited by metal ions, especially Fe^{3+}, Hg^{2+} and Cu^{2+} and was also inhibited moderately by sulfhydryl reagents. Similar results have been reported for carboxypeptidase from rice leaves (Doi, **et al.**, 1980). The enzyme was inhibited by the sulfhydryl reagents, iodoacetamide, p-chloromercuriphenylsulfonic acid and $HgCl_2$. Bai and Hayashi (1979) examined the role of a single sulfhydryl group in carboxypeptidase Y from bakers' yeast and found that although the sulfhydryl group is not essential for the hydrolytic function, it is located at the site where the penultimate residue of the substrate interacts and the enzyme is thus inactive by sulfhydryl reagents. The present results suggest that aloe carboxypeptidase also contains a sulfhydryl group which may be located in the vicinity of the active site and involved in the inactivation of the enzyme. However, the exact role of the sulfhydryl group remains to be studied.

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