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Properties of a carboxypeptidase from aloe

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Aloe (*Aloe arborescens* Mill var. *natalensis* Berger) has been valued in Japan as family medicine for skin injury and burns. We have been studying this plant from pharmacological [1] and immunological [2] aspects to evaluate its therapeutic use as an anti-inflammatory agent. In a previous report we reported that aloe contains enzyme(s) hydrolyzing bradykinin, which might explain its anti-inflammatory effect and also that the enzyme seems to split bradykinin between Gly^4 and Phe^5 [1]. However, while attempting to purify the enzyme, we found that one of the hydrolysis products from bradykinin was arginine, the amino acid located at both terminals. We, therefore, reinvestigated the action of the enzyme on bradykinin and found that it may be regarded as a "serine carboxypeptidase" [3].

A crude enzyme preparation was obtained as follows. The exudate from fresh leaves of aloe was treated with 3 vol. of acetone and the precipitate was dissolved in 0.05 M NaOAc buffer, pH 6.0. The solution was brought to 70 per cent saturation of $(NH_4)_2SO_4$ and the resulting precipitate was dissolved in 0.05 M NaOAc buffer, pH 6.0. Precipitates were successively obtained by the addition of $(NH_4)_2SO_4$ to make 35, 55 and 75 per cent saturations and were assayed for bradykininase activity. The highest activity was found in the precipitate at 35–55 per cent saturation of $(NH_4)_2SO_4$ which was dissolved in 0.05 M NaOAc buffer, pH 6.0 and dialyzed against the same buffer.

Bradykininase activity was estimated by a semi-quantitative method. The enzyme was incubated with 1 mM bradykinin in 0.05 M NaOAc buffer, pH 6.0 at 37° for 1 hr and the amount of amino acid released (arginine) was determined by the ninhydrin method [4]. Effects of various reagents and metals on the bradykininase activity of the enzyme were examined as follows: the enzyme was preincubated with a reagent or a metal ion at a final concentration shown in Table 1 for 20 min at 37° in 0.05 M NaOAc buffer, pH 6.0. The substrate bradykinin was then added to make a final concentration of 1 mM and the mixture was incubated at 37° for 30 min. The reaction was terminated by heating the mixture in a boiling water bath. The amount of arginine liberated was determined with a Shimadzu Dual-Wavelength TLC Scanner CS-900 after high-voltage paper electrophoresis at 3,600 V in a pyridine–acetic acid buffer, pH 3.5 and ninhydrin staining. Optimum pH of the enzyme for bradykinin and Z-Gly-Pro-Leu-Gly were estimated by using 0.05 M acetate buffers, pH 4.0–6.5 and 0.05 M phosphate buffers, pH 6.0–7.5 with pH intervals of 0.5. Synthetic bradykinin, angiotensin I and Z-Gly-Pro-Leu-Gly were purchased from Protein Research Foundation, Osaka, Japan.

The hydrolysis of bradykinin by the crude enzyme preparation was followed by analyzing the amounts of free amino acids released after various incubation times. As shown in Fig. 1, the peptide was split sequentially from the COOH-terminal. The release of Arg, Phe, Pro and Ser was further confirmed by high-voltage paper electrophoresis and by paper chromatography. The enzyme also hydrolyzed a decapeptide angiotensin I, thus releasing the C-terminal amino acids Leu, His, Phe and Pro. Action of the enzyme on a smaller synthetic peptide Z-Gly-Pro-Leu-Gly was then studied and the products were found to be Gly and Leu. These results indicate that the enzyme from aloe is a carboxypeptidase rather than an endopeptidase.

Properties of the enzyme were then investigated in order to compare with those of other carboxypeptidases. The optimum pH of the enzyme for bradykinin and Z-Gly-Pro-Leu-Gly was approx. 6 and 5, respectively. The effects of various reagents and metal ions on the enzyme activity are summarized in Table 1. Enzyme activity was little affected by L-cysteine or 2-mercaptoethanol which can activate plant thiol proteases such as papain [5] and ficin [6]. Although inhibited to some extent by *p*-chloromercuriphenylsulfonic acid, a potent SH reagent, the enzyme was not inhibited by iodoacetamide, an inhibitor of thiol proteases [5, 6]. Most of the metal ions examined exhibited more or less inhibitory effects on the enzyme, but chelating agents such as EDTA and 1,10-phenanthroline did not affect much the enzyme activity. Diisopropyl phosphofluoridate which is known to inhibit "serine carboxypeptidase" [3] completely inactivated the enzyme. From these data the enzyme from aloe should be classified as a serine protease, but neither a metal nor an acid protease according to the classification of proteases by Hart-

Table 1. Effects of various reagents and metal ions on the bradykininase activity of the enzyme from aloe

Reagent or metal	Concentrations (mM)	Relative degree of inhibition *
L-Cysteine	5	0/+
2-Mercaptoethanol	5	0/+
Iodoacetamide	1	0
p-Chloromercuriphenylsulfonic acid	1	+
EDTA	5	a/0
1,10-Phenanthroline	1	0/+
Diisopropyl phosphofluoridate	1	++
$HgCl_2$	1	++
$CuSO_4$	1	++
$CdSO_4$	1	++
$ZnCl_2$	1	++
$FeCl_3$	1	++
$CoCl_2$	1	+
$MgCl_2$	1	0/+
$CaCl_2$	1	0/+

* Compared to a control (without a reagent or a metal). Symbols are: a, activation; 0, no inhibition; +, partial inhibition; ++, complete or almost complete inhibition.

ley [7]. It is not possible at present to conclude whether sulfhydryl groups are directly involved in the catalytic action of the enzyme or not.

Several carboxypeptidases have been isolated from plant sources including bean leaves [8], citrus peel [9, 10] or citrus leaves [11] and cotton seedlings [12]. The carboxypeptidase from aloe has some features common with these plant carboxypeptidases; it is inhibited by diisopropyl phosphofluoridate, has optimum pH in a slightly acidic region and seems to have a broad specificity for C-terminal amino acids. On the other hand, the enzyme differs from pancreatic carboxypeptidase A [13] or B [14] in its ability to release proline and its inhibition by zinc and cobalt ions.

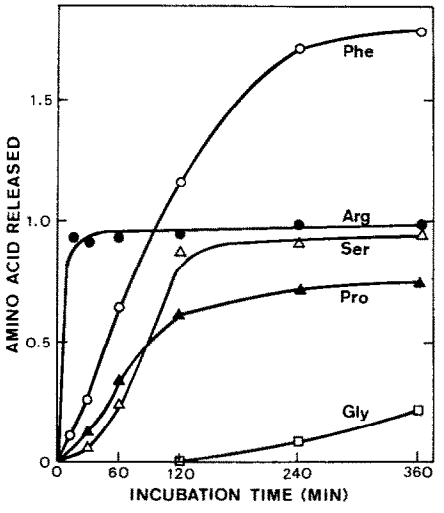

Fig. 1. Action of the enzyme from aloe on bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. One μmole of the peptide was incubated with approx. 50 μg of the enzyme in 1 ml of 0.05 M NaOAc buffer, pH 6.0 at 37°. 0.15 ml of the incubation mixture were withdrawn after 15, 30, 60, 120, 240 and 360 min and the reactions stopped by heating the mixtures in a boiling water bath for 5 min. Each mixture was evaporated to dryness in a vacuum desiccator, dissolved in pH 2.2 buffer containing 0.15 μmole of norleucine as internal standard and analyzed on a JEOL JLC-6AH amino acid analyzer.

In summary, aloe contains a carboxypeptidase capable of hydrolyzing bradykinin, angiotensin I and Z-Gly-Pro-Leu-Gly from their C-terminals. The optimum pH for bradykinin and Z-Gly-Pro-Leu-Gly were approx. 6 and 5, respectively and it was inhibited by diisopropyl phosphofluoridate and by heavy metals.

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