WOUND-INDUCED PEPTIDASE ACTIVITY IN TOMATO LEAVES [1]

M. Walker-Simmons and C. A. Ryan
Department of Agricultural Chemistry and
Program in Biochemistry and Biophysics
Washington State University
Pullman, Washington 99164

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SUMMARY: Severe wounding of leaves of young tomato plants induced an increase in peptidase activity in wounded, as well as non-wounded, leaves. This increase paralleled the large accumulation of inhibitors of the serine endopeptidases that is induced by wounding. However, endopeptidase activity of the leaves of wounded plants did not increase, nor did the activities of two non-proteolytic enzymes, catalase and acid phosphatase. The substrate specificity of the peptidase and its susceptibility to the inhibitors DFP and PCMB were similar to those of the plant carboxypeptidases. This wound-induced accumulation of peptidase activity appears to be mediated by the wound hormone PIIF and may be involved in the shift in protein metabolism of leaves that leads to proteinase inhibitor accumulation.

INTRODUCTION

Severe wounding of even single leaves of tomato or potato plants causes the release of the wound hormone PIIF (proteinase inhibitor inducing factor) that rapidly travels throughout the plant to unwounded leaves where it initiates the accumulation of proteinase inhibitors I and II (1). We have suggested that this may be an immune-like response directed toward digestive proteinases of invading pests to arrest their capacity to utilize the proteins of the plant as food (2, 3).

In this communication we report that a peptidase activity resembling the plant carboxypeptidases (4) significantly increases in wounded tomato plants in unwounded leaves. The increase parallels that of the proteinase inhibitors and is apparently responding to the wound hormone PIIF, or a similar signal produced by wounding, that induces the peptidase activity.

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MATERIALS AND METHODS

Young tomato plants (*Lycopersicum esculentum* var. Bonnie Best) were grown in a growth chamber at 1,000 ft-c for a 17-hr day. The plants were utilized when they were about three weeks old, 10 to 15 cm tall, and had three developing leaves. Plants were wounded by crushing the lower leaf between a flat file and a rubber stopper. Only unwounded upper leaflets were used for enzyme or inhibitor assays.

The concentration of the proteinase inhibitors I and II in leaf juice was determined by the immuno-radial diffusion method described by Ryan (5). Purified inhibitors I and II from tomato leaves (6) were used as standards.

Crude leaf extracts for peptidase, acid phosphatase, and catalase assays were prepared by macerating leaves with a mortar and pestle with sand and Tris buffer (1 g leaves per 3.0 ml 0.2 M Tris buffer, pH 8, containing 0.2 M sucrose and 1.5 mM EDTA*). β-Mercaptoethanol (0.1% v/v) was added to the extracts for peptidase and acid phosphatase assays. After maceration the leaf extracts were centrifuged at 100,000*g* for one hour at 0°C. The clear supernatant was assayed immediately after centrifugation. Peptidase was assayed with the substrate BTPA (7). Fifty to 150 µl of extract was incubated with 1.35 ml of 0.1 M sodium phosphate buffer, pH 6.2, for 5 minutes at 35°C. Two-tenths ml of 3 mM BTPA in N,N-dimethyl formamide was added and the samples were incubated for 0 to 120 minutes at 35°C. The assay was terminated by adding 1.0 ml of 30% acetic acid. A unit of activity was defined as the change in absorbance of 0.01 per minute at 405 nm. Catalase (8) was assayed by adding 10 to 50 µl of extract to 3.0 ml of H_2O_2 substrate (0.30 ml of 30% H_2O_2 in 50 ml of 0.05 M potassium phosphate, pH 7.0), and measuring the change in absorbance at 240 nm. One unit is defined as one µmole of H_2O_2 decomposed per minute at 21°C. Acid phosphatase (9) was analyzed by adding 10 to 50 µl of extract to 1.2 ml sodium acetate buffer, pH 5.0, and incubating for 10 minutes at 35°C. Then 0.25 ml of 32 mM PNPP was added and incubated at 35°C for 0 to 60 minutes. The reaction was ended by adding 3 ml cold Tris-phosphate, pH 8.5, and the absorbance at 420 nm was measured immediately. Activity is reported in units where a unit is defined as a µmole PNPP released per minute.

Peptidase activity was partially purified for esterase assays with a modified method of Matoba and Doi (10). One gram of leaves was macerated in 2.0 ml of 0.01 M acetate buffer, pH 5.2, containing 0.1 M sucrose and 1.5 mM EDTA, and centrifuged at 10,000*g* for 15 minutes. A 50 to 80% $(NH_4)_2SO_4$ precipitate was prepared from the clear supernatant, resuspended in the acetate buffer, and dialyzed against 0.01 M acetate buffer, pH 5.2, containing 0.1 M sucrose. Esterase activity was measured with the substrate ATEE (11). The rate of change in absorbance was measured at 230 nm.

Endopeptidase activity was measured on 1.5% (w/v) gelatin agar plates by the radial diffusion assay of Santarius and Ryan (12). Extracts for assay were prepared from 1 g leaves per 2.0 ml of 1.0 M Tris buffer, pH 7.4, containing 2×10^{-3} M chloramphenicol and 0.5×10^{-3} M polyvinyl pyrrolidone. The mixture was centrifuged for 20 minutes at 10,000*g* and the clear supernatant was used for assays. Activity is reported by $(D - D_o)$, where D is the diameter (cm) of the sample's clear radial diffusion zone and D_o is the diameter (cm) of the sample well. Chlorophyll was estimated in 80% acetone extracts of tomato leaves according to the procedure of Strain *et al.* (13).

*Abbreviations: EDTA, ethylenediaminetetraacetic acid; BTPA, N-benzoyl-L-tyrosine p-nitroanilide; PNPP, p-nitrophenyl phosphate; ATEE, N-acetyl-L-tyrosine ethyl ester; DFP, diisopropylflurophosphate; PCMB, p-chloromercuribenzoic acid.

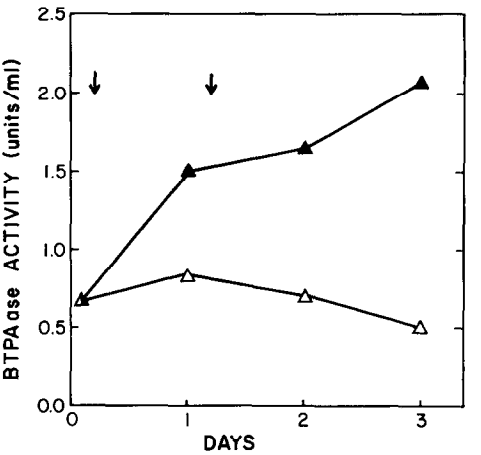

Figure 1. The accumulation of peptidase activity in undamaged upper leaves (-▲-▲-) of tomato plants whose lower leaves were severely wounded at times shown by the arrows. Peptidase activity in identical leaves as above from unwounded control plants are also shown (-△-△-).

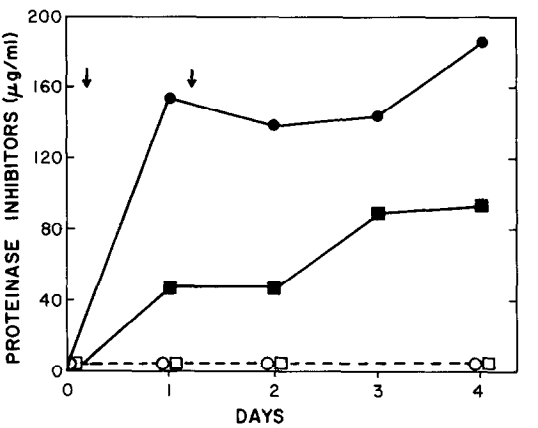

Figure 2. The accumulation of proteinase inhibitors in identical leaves as described in Figure 1. Inhibitor I in leaves from wounded (-●-●-) and unwounded (-○-○-) plants. Inhibitor II in leaves from wounded (-■-■-) and unwounded (-□-□-) plants.

RESULTS AND DISCUSSION

The initial observations that a peptidase activity accumulated in both damaged and undamaged leaves of severely wounded tomato plants resulted from a study of proteolytic enzymes in tomato leaves that might be associated with, or inhibited by, the proteinase inhibitors that accumulate in large quantities

TABLE I

Comparison of Activities in Wounded and Control Plants*

Measurement	Unwounded Plants	Wounded Plants
BTPAase (units/ml crude leaf extract)	0.69	1.67
Endopeptidase ($D - D_o$)**	0.98	0.82
Proteinase Inhibitor I (µg/ml leaf juice)	0	138
Proteinase Inhibitor II (µg/ml leaf juice)	0	47
Acid Phosphatase (units/ml crude leaf extract)	0.51	0.57
Catalase (units/ml crude leaf extract)	1460	1220
Chlorophyll (µg/g leaf tissue)	3980	3520

* Lower leaves of 3-week old tomato plants were wounded on two consecutive days. On the third day, non-damaged leaves from the wounded plants and leaves from the control plants were assayed as described in Materials and Methods.

** See Materials and Methods.

in wounded tomato plants. Although no leaf proteolytic enzyme activity was found to be inhibited by the tomato inhibitors, a striking increase in BTPAase activity was found in leaves of wounded tomato plants. In Figure 1, it is shown that peptidase activity accumulated in upper, unwounded tomato leaves of small plants whose lower leaves had been severely wounded by crushing at day 0 and day 1, as indicated by the arrows. This increase in peptidase activity parallels a large increase in proteinase inhibitors (Fig. 2) that is known to be mediated by the wound hormone PIIF (proteinase inhibitor inducing factor). By day 4, the leaves have usually accumulated the maximal amounts possible of both proteinase inhibitors and BTPAase activity. Thereafter the levels of inhibitors are known to slowly decline over several days. The increase in inhibitor I and

TABLE II

Effects of Inhibitors on Wound-Induced Peptidase Activity from Tomato Leaves

Inhibitors	Concentration	Remaining Activity (%) Peptidase
Control		100
Iodoacetamide	100 mM	93
$HgCl_2$	2 mM	25
DFP	1 mM	28
PCMB	0.3 mM	12
DFP + PCMB	1 mM + 0.3 mM	4

Partially purified peptidase activity (prepared from plants wounded on two consecutive days) in 0.01 M acetate buffer, pH 5.2, containing 0.1 M sucrose, was incubated with the inhibitors at the above concentration for 90 minutes at 25°C. After the incubation peptidase activity was measured with BTPA as described in Materials and Methods.

II and BTPAase activity was not accompanied by an increase in the endopeptidase activity, catalase, or acid phosphatase of the leaves, or in total chlorophyll, indicating that all proteins (enzymes) of the leaf are not equally affected by severe wounding (Table I).

The absence of an increase in endopeptidase activity indicates that the BTPAase activity may be a specific exopeptidase, possibly plant carboxypeptidase, or a dipeptidase. The accumulated BTPAase activity appears to be sensitive to both DFP and sulfhydryl reagents (Table II), similar to the known plant carboxypeptidases (4). Several plant carboxypeptidases have been reported to be strongly inhibited by DFP (10, 14, 15, 16), and the carboxypeptidase from barley (14) is also strongly inhibited by PCMB. In addition, both peptidase and esterase activities accumulated at the same rate as would be expected for a plant carboxypeptidase.

The induction of peptidase activity appears to be related to the large accumulation of proteinase inhibitors induced by wounding and mediated by the plant wound hormone PIIF. To our knowledge this is the first report of a wound

regulated peptidase activity in a plant leaf. It is possible that the peptidase activity is part of a regulated increase in intracellular protein turnover designed to supply free amino acids for synthesis of proteinase inhibitors as part of an immune-like process to help protect the plant from digestive proteinases of plant pests.

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