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Assay and Biochemical Properties of the Proteinase Inhibitor-inducing Factor, a Wound Hormone[1]

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ABSTRACT

An assay has been developed for the proteinase inhibitor-inducing factor (PIIF), a wound hormone. PIIF is present in tomato (*Lycopersicum esculentum* var. Bonnie Best) leaf extracts and induces accumulation of proteinase Inhibitor I when the extracts are supplied briefly to excised leaves that are subsequently incubated in water under constant light. An active water-soluble crude PIIF solution was conveniently prepared from autoclaved and lyophilized tomato leaves. Accumulation of Inhibitor I, induced by crude PIIF, is linear, commencing at about 8 to 10 hours after feeding and continues for several hours. Evidence is presented that the PIIF-induced accumulation of Inhibitor I, determined immunologically, is accompanied by the accumulation of other trypsin and chymotrypsin inhibitors, determined enzymatically. The accumulation of Inhibitor I is inhibited by actinomycin D and cycloheximide but not by chloramphenicol or rifampin. PIIF cannot be replaced by traumatin, indoleacetic acid, gibberellic acid, kinetin, ethylene, or abscisic acid. PIIF activity was not destroyed by incubation with a number of proteolytic, carbohydrase, phosphatase, or pyrophosphatase enzymes. The active substance is insoluble in lipid solvents.

Wounding of tomato or potato leaves by insects results in the light-dependent accumulation of proteinase Inhibitor I in leaves throughout the wounded plants within a few hours following wounding (2, 3). The wounded leaf tissue releases a hormone-like substance, PIIF,[3] that is translocated from the wound to the other tissues of the plant where it induces a light-dependent accumulation of Inhibitor I (3).

In this communication a quantitative assay for PIIF in tomato leaf extracts is described. Utilizing this assay, several properties of the PIIF-induced proteinase inhibitor system have been studied.

MATERIALS AND METHODS

α-Chymotrypsin (crystallized three times) and trypsin (crystallized twice) were purchased from Worthington Biochemical Corp.; gibberellic acid, indoleacetic acid, and kinetin from Nutritional Biochemical Co.; traumatin and abscisic acid from K and K Laboratories; actinomycin D and chloramphenicol from Sigma Chemical Co.; and rifampin from Calbiochem. Ethryl was a gift of Dr. Max Patterson, Washington State University.

Tomato plants (*Lycopersicum esculentum* var. Bonnie Best) were germinated in Jiffy-7 peat pellets (Cascade Seed Co., Spokane, Wash.) in subdued light and transferred to growth chambers under 1000 ft-c at 31 C. Gro-lux fluorescent lights were utilized, supplemented with 60-w incandescent bulbs (one bulb per 6 ft of incandescent lamps). The Jiffy pellets were fertilized at planting and again after germination with an 0.06% solution of fertilizer, containing nitrogen, phosphorus and potassium (30-10-10), and 0.001% ferric iron. Unless otherwise mentioned, plants were used for assay when they were about 7.5 to 10 cm in height and having two well formed leaves and a third, smaller apical leaf. The assay for PIIF in excised leaves, described herein, requires that the young plants be grown pest-free and with optimal additions of fertilizers. We have found that leaves from plants supplied with low levels of nitrogen or iron do not respond well to PIIF, nor do plants grown under subdued light. On the other hand, if healthy young plants are transplanted after the root systems have become well established the roots tear and apparently release PIIF, resulting in high levels of inhibitors in the leaves. Inhibitor I was assayed immunologically by the method of Ryan (10). Purified Inhibitor I (9) from potatoes was used as a standard. Tomato and potato Inhibitor I are immunologically very similar (4), but the quantitation is relative to the potato Inhibitor I, since the tomato Inhibitor I has not been fully purified.

Enzymatic determination of total chymotrypsin and trypsin inhibitors in tomato leaves was performed by the method of Hummel (7). Chymotrypsin was assayed with acetyl-L-tyrosine ethyl ester at 237 nm. Four μg of chymotrypsin in 50 μl of mM HCl were preincubated with 50 μl of 47 mM tris-HCl buffer, pH 8.1, with varying aliquots of water or clarified leaf juice, and then assayed. Trypsin was assayed with benzoyl-L-arginine ethyl ester at 247 nm. Two μg of trypsin in 50 μl of mM HCl were preincubated with 50 μl of 47 mM tris-HCl, pH 8.1, and either water or leaf extract before assaying. Leaf juice for each experiment was obtained from 15 leaves by grinding with a mortar and pestle, precooled to 2 C, and expressing the juice through a hand-operated garlic press. The juice was centrifuged at 40,000 rpm at 2 C for 25 min in a Spinco ultracentrifuge with a type 40 head. The clear supernatant (about 1.5 ml) was carefully removed with a Pasteur pipet and stored at 2 C.

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[3] Abbreviation: PIIF: proteinase inhibitor-inducing factor.

RESULTS

Effect of Wound Size and Location on the Accumulation of Inhibitor I. Previous work had shown that the wound-induced accumulation of Inhibitor I in leaves of young tomato plants was dependent upon the severity of wounding when the leaves were crushed between the flat end of a wooden dowel and a flat file (2) (to simulate insect wounding).

I have further investigated the effect of the wound size and location on the accumulation of Inhibitor I in leaves of young tomato plants. The plants had three well formed leaves and a small apical leaf. A wound was produced by crushing the lowest terminal leaflet between a wooden dowel (either 0.8 or 0.15 cm in diameter) and a rat tail file (2). The wound was made in various locations on the leaflet or its petiole (Fig. 1). The plants were incubated 24 hr under 800 ft-c at 31 C and the juice of the uppermost unwounded leaf was assayed for Inhibitor I (2).

The amount of inhibitor accumulated in adjacent, unwounded leaves was dependent upon both the size and location of the wounding (Fig. 1). The signal, PIIF, was released most effectively from large wounds on green tissues near the main vein and not at all from wounds on petiole tissue. These results further supported our earlier report (3) that a clean cut of the petiole with a razor blade did not in itself release enough PIIF to cause accumulation of Inhibitor I.

Development of an Assay for PIIF. The above experiments indicated that excised leaves could be employed to assay PIIF.

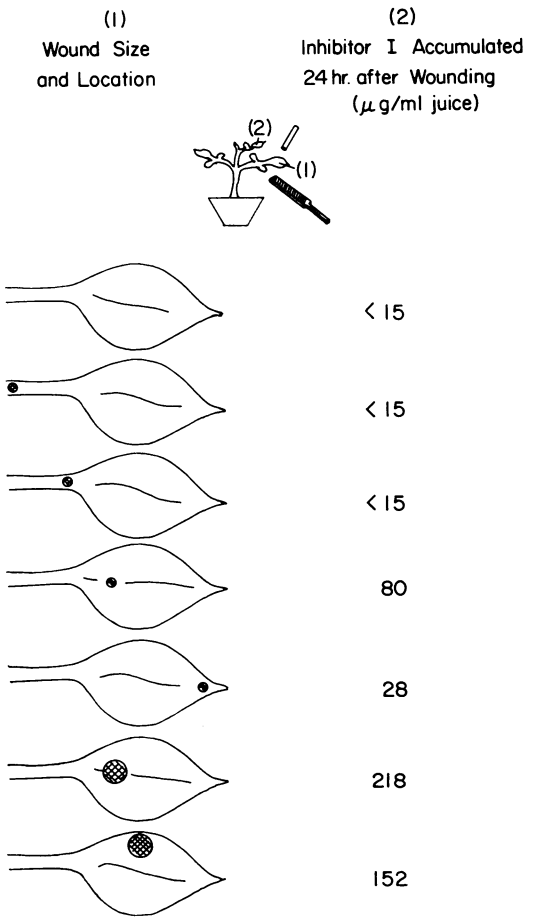

FIG. 1. Effects of wound size and location on the accumulation of Inhibitor I in unwounded intact leaves one petiole above wounded leaves. Wounding made by crushing leaves or petioles between wooden dowels and a flat file.

Table I. *Inhibitor I Accumulation in Excised Tomato Leaves in Response to PIIF in Tomato Leaf Juice*

Six leaves were supplied with leaf juice or water from small vials, through the cut ends of the petioles under normal laboratory lighting at 21 C, then transferred to vials containing water at 31 C and 800 ft-c for 24 hr, and assayed immunologically.

Treatment	Inhibitor I Accumulated
	μg/ml juice
Water, 24 hr	27
Fresh leaf juice, 20 min; water, 24 hr	247
Steamed leaf juice, 20 min; water, 24 hr	271

Since little or no PIIF was released by the excision, it was possible to add extracts containing PIIF to the excised leaves and cause them to accumulate Inhibitor I.

Several leaves were excised cleanly with a razor blade, their petioles placed in water, incubated 24 hr at 31 C under 800 ft-c and assayed for Inhibitor I. No inhibitor accumulated. However, when similarly excised leaves were first incubated for 20 min with tomato leaf juice (obtained by mascerating fresh leaves and clarifying by centrifugation at 15,000 rpm) and then with water for 24 hr in 800 ft-c at 31 C, they accumulated significant quantities of Inhibitor I (Table I). This indicated that the leaf juice contained PIIF and it initiated accumulation of Inhibitor I when supplied briefly to excised leaves. Table I further shows that subjecting tomato leaves to live steam for several minutes did not destroy their PIIF content, but may have enhanced it.

A stock supply of leaf tissue was prepared by autoclaving several thousand grams of tomato leaves and freeze-drying them. PIIF was readily solubilized from the freeze-dried leaves. Dispersion of from 20 to 50 mg of the dried leaves per ml water and clarification by centrifugation at 15,000*g* resulted in a green liquid that, when supplied to excised leaves, causes appreciable induction of Inhibitor I when incubated 24 hr in light under 800 ft-c at 31 C. Figure 2 shows the effect of supplying excised leaves with a water extract of 37 mg of dry leaves per ml for periods of time ranging from 2 to 30 min. Even a 2-min incubation period gave nearly maximum induction of accumulation of Inhibitor I. It was determined with T_2O that in 2 min about 1 to 2 μl of crude juice was taken up by the leaves. It is assumed that the uptake was effected by transpiration of the leaf. The distance from the cut petiole to the leaf tip that PIIF had to travel was usually about 5 to 7 cm.

An estimate of the potency of PIIF in the lyophilized tomato leaves was obtained by dispersing different quantities of autoclaved, dry tomato leaves in water, centrifuging, and allowing the extracts to be taken up by excised tomato leaves for 15 min, then incubated in light as before. Figure 3 shows that the accumulation of Inhibitor I is dependent upon the concentration of dried leaf solubles below about 30 mg of dry leaf per ml water. At very high concentrations (100 mg if dry leaf tissue per ml water), accumulation was apparently inhibited. At this concentration, the leaves wilted somewhat while supplying the solution. Some of the soluble leaf materials may have been toxic to the leaves. It is also noted that the amount of inhibitor I that accumulated differed with different lots of plants. This is apparently a fertilizer effect and is under study.

For further studies of the physiological properties of PIIF a standard solution of dry leaf solubles of 50 mg per ml was routinely prepared. This solution is called "crude PIIF" and produced maximum accumulation of Inhibitor I when supplied

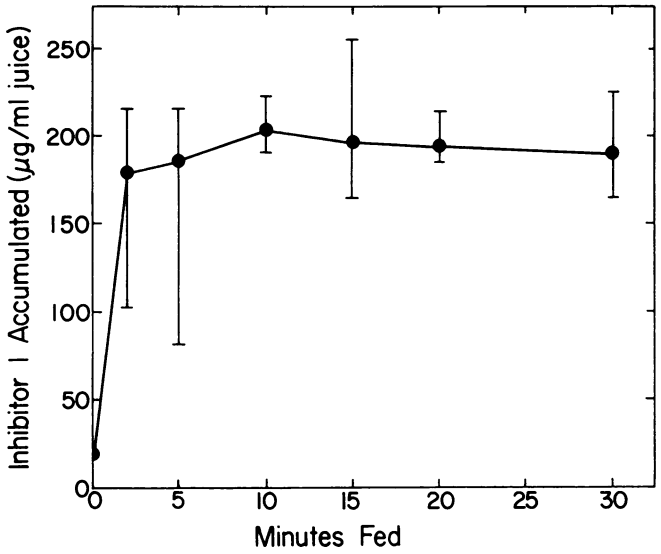

FIG. 2. Accumulation of Inhibitor I in excised tomato leaves as a function of time of uptake of crude PIIF. After imbibing juice for the times indicated the leaves were transferred to water for 24 hr at 800 ft-c and 31 C and assayed for Inhibitor I. The average accumulation of Inhibitor I in four leaves is plotted with the variability shown by bars.

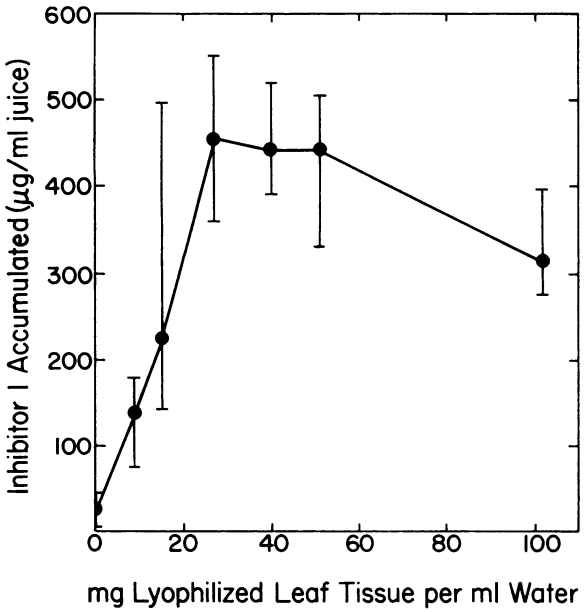

FIG. 3. Accumulation of Inhibitor I in excised tomato leaves as a function of the concentration of crude PIIF. Varying quantities of lyophilized leaf tissue was dispersed in water and centrifuged at 15,000*g*. The clarified leaf juice was taken up by the excised young tomato leaves through the cut petiole for 15 min. The leaves were transferred to water and incubated and assayed as in Figure 2. The average accumulation of Inhibitor I in four leaves is plotted with the variability shown by bars.

to the leaves for 15 min and incubated for 24 hr as described above.

Time Course of PIIF-induced Inhibitor I Accumulation. Crude PIIF was supplied to 42 young excised tomato leaves for 15 min, followed by water for 24 hr at 800 ft-c and 31 C. The accumulation of Inhibitor I in the leaf juice was determined at various times after the addition of crude PIIF (Fig. 4). Inhibitor I could not be found in the leaf juice for several hours following the uptake of crude PIIF. At about 10 hr the inhibitor began to accumulate and continued to do so at a steady rate for the duration of the experiment (31 hr). In several repetitions of this experiment the lag period of accumulation was consistently about 8 to 10 hr.

Total PIIF-induced Proteinase Inhibitor Activity in Tomato Leaves. Total inhibitory activity against chymotrypsin and trypsin in the juice of induced tomato leaves was determined spectrophotometrically. The clarified juice from very young tomato plants was not suitable for these assays. When this juice was added to either the chymotrypsin or trypsin assay system a colloidal precipitate formed that prevented measurement of enzyme rates. However, such interference was not observed in the juice of young leaves from older plants. Table II shows a comparison of inhibitors in leaves (*a*) induced with crude PIIF, (*b*) assayed directly after excision from the plants, and (*c*) excised and supplied only with water. Immunological assays of Inhibitor I show that PIIF caused about a 6-fold increase over intact leaves and a 3-fold increase over water controls. In contrast, PIIF caused total inhibitory activity against chymotrypsin to increase 12-fold over intact leaves and 6-fold over water controls. PIIF-induced trypsin inhibitor capacity increased about 8-fold over the intact leaves and 4-fold over water controls.

Effects of Protein-synthesis Inhibitors on the PIIF-induced Accumulation of Inhibitor I. Solutions of actinomycin D, cycloheximide, rifampin, and chloramphenicol were supplied to

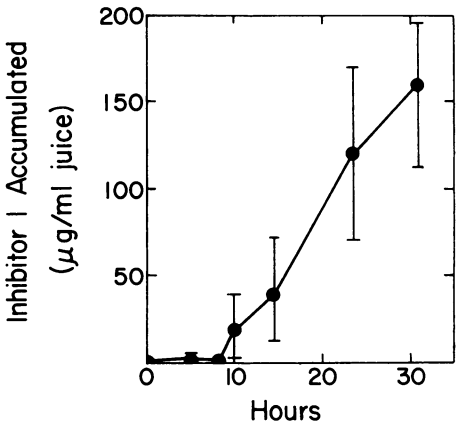

FIG. 4. Time course accumulation of Inhibitor I in detached leaves supplied with crude PIIF for 15 min followed by water under 800 ft-c at 31 C and assayed for Inhibitor I at the times indicated. The average accumulation of Inhibitor I in six leaves is plotted with the variability shown by bars.

Table II. *Accumulation of Inhibitor I and Total Inhibitory Activity in Detached Tomato Leaves in Response to Crude PIIF*

Fifteen leaves were combined for each treatment at 1000 ft-c at 31 C.

Treatment	Inhibitor I	Enzymes Inhibited[1]	
		Chymotrypsin	Trypsin
		μg/ml juice	
Control leaves	55	150	77
H_2O, 40 hr	90	380	163
Crude PIIF, 20 min; H_2O, 40 hr	305	1810	641

[1] Calculated at 50% inhibition in spectrophotometric assay.

Table III. *Effect of Protein Synthesis Inhibitors on PIIF-induced Inhibitor I Accumulation in Excised Tomato Leaves*

Inhibitor supplied for 20 min followed by crude PIIF for 15 min. Incubated at 1000 ft-c at 30 C.

Additions	Inhibitor I Accumulation
0.1 mM	*μg/ml juice*
None	180
Actinomycin D	48
Cycloheximide	0
Rifampin	189
Chloramphenicol	209

young leaves for 20 min followed by crude PIIF for 15 min and finally water for 24 hr at 31 C under 1000 ft-c. Table III shows that actinomycin D and cycloheximide are potent inhibitors of the PIIF-induced accumulation of Inhibitor I, whereas rifampin and chloramphenicol are not. Chloramphenicol consistently exhibited a slight stimulation of the response.

Comparison of PIIF with Other Plant Hormones. The hormone-like behavior of PIIF led to the assay of several available plant hormones for their possible roles in the wound-induced accumulation of Inhibitor I. None of the six hormones tested including GA, IAA, traumatin, abscisic acid, kinetin or ethylene, exhibited any capacity to induce accumulation of Inhibitor I when applied to excised leaves. Of the six hormones, only GA showed an antagonism of the (crude) PIIF-induced accumulation, and this was neither severe nor consistent.

Effect of Various Hydrolytic Enzymes on PIIF Activity in Crude Tomato Juice. Several enzymes were incubated for 2 hr at 31 C with crude PIIF (100 μg enzymes/ml crude PIIF) that had been adjusted to the pH region of maximal activity for each enzyme. The following enzymes, with the pH of incubation, were employed: chymotrypsin (pH 7.8); trypsin (pH 7.8); cellulase (pH 5.0; β-amylase (pH 4.5); β-glucuronidase (pH 3.8); phosphodiesterase (pH 9.3); ribonuclease (pH 7.3); pectinase (pH 4.5); and α-amylase (pH 6.9). None of the enzymes caused any significant decrease in the PIIF activity of the leaf extract, nor did any of the enzymes at the concentrations used contain PIIF activity.

DISCUSSION

An assay for the quantitation of PIIF in extracts of tomato leaves was developed from the observations that excised tomato leaves accumulated large quantities of Inhibitor I when supplied briefly with tomato leaf extracts through their cut petioles and transferred to water under constant light for 24 hr (Table I). Excised leaves supplied only with water did not accumulate Inhibitor I.

Autoclaved, freeze-dried tomato leaves provided a convenient source of crude PIIF with which to study the properties of the PIIF-induced accumulation of Inhibitor I in excised young tomato leaves (Figs. 2 and 3). A water extract of 50 mg dry leaves/ml water was routinely prepared and was clarified by centrifugation at 15,000 rpm. This solution, when supplied to young excised tomato leaves for 15 to 20 min, maximally induced accumulation of Inhibitor I after the leaves were transferred to water under 800 ft-c at 31 C for 24 hr (Fig. 2).

The time course of the PIIF-induced accumulation of Inhibitor I in excised leaves exhibited a lag period of about 10 hr during which no Inhibitor I accumulated. Thereafter Inhibitor I accumulated linearly at a rapid rate for several hours (Fig. 4). In the absence of crude PIIF no Inhibitor I accumulated.

The accumulation induced in leaves by crude PIIF includes other proteinase inhibitors besides Inhibitor I. Table II demonstrates that considerably more inhibitory activity against chymotrypsin and trypsin is present in induced leaves than can be accounted for by Inhibitor I estimated immunologically. Inhibitor I is known to inhibit three times its weight of chymotrypsin (9). The total inhibitory capacity increased far beyond this capacity. Based on a mol wt of 25,000 for chymotrypsin (6) and 9500 equivalent weight for inhibitors (9), a rough estimate of 725 μg of inhibitor proteins accumulated per ml leaf juice in PIIF-treated leaves, more than double the concentration of Inhibitor I. The differences are, in fact, much greater than shown, since the immunological quantitation of Inhibitor I is probably an overestimate because potato Inhibitor I was used as a standard and tomato Inhibitor I has been shown to be lacking some determinants. The purification of tomato Inhibitor I, now underway, hopefully will provide both the antiserum and standard for the absolute quantitation of tomato Inhibitor I in future studies.

The PIIF-induced accumulation of Inhibitor I in excised leaves was inhibited by both actinomycin D and cycloheximide but not by chloramphenicol or rifampin. This indicates that RNA synthesis and cytoplasmic ribosomes are somehow involved in the induction of inhibitors. The lack of inhibition by chloramphenicol or rifampin suggests that mitochondrial or chloroplast protein synthesis may not be directly involved in the accumulation. It is not clear at this time how to interpret these data. PIIF could be doing a variety of things, *e.g.*, it could be inhibiting the turnover of Inhibitor I. Alternatively, it might be reacting at the gene level, activating RNA synthesis resulting in inhibitor production.

The lag period of 8 to 10 hr before Inhibitor I accumulated is similar to that observed by Jacobson and Varner (8) for the GA-induced synthesis of amylase and proteinase production in barley half seeds. In barley GA added to isolated aleurone layers evokes cytological changes during the lag period similar to those that take place during normal germination; that is, vacuolation, breakdown of aleurone granules and an increase in the number of polysomes, mitochondria, and microbodies (17). The metabolism is shifted by GA from a dormant or low metabolic rate to a very active metabolism. This contrasts with the tomato leaf cells in which induction by PIIF is taking place in a vigorously metabolizing expanding leaf. The protein metabolism of the leaf is apparently redirected to the production of large quantities of proteinase inhibitors.

Evidently PIIF is turned over rapidly in the intact plant, since a single severe wound to a leaf of a growing plant results in inhibitor accumulation for only a day or two (Ryan, unpublished observation). Repeated woundings reinforce the accumulation, but within a couple of days after cessation of wounding, the total inhibitor levels peak and slowly decrease over several weeks.

We had studied the accumulation of Inhibitor I in excised leaflets for several years (11–15) before discovering that it was a wound hormone-dependent response. In those experiments, older terminal leaflets were induced by excising the leaf and removing the four lateral leaflets from the petioles. Thus, these leaves suffered a total of five wounds along the petiole, which evidently released enough PIIF into the terminal leaflet to induce the accumulation of Inhibitor I. Those studies established that Inhibitor I accumulation was a result of new synthesis of Inhibitor I and that the newly accumulated Inhibitor I protein could account for over 15% of the new proteins of the leaflets (13). It was also shown that the inhibitor accumulated in the central vacuole of the leaf cells as large, membraneless, protein bodies (15).

The PIIF factor behaves as a hormone in the traditional sense (16) as being a signal formed and released from a tissue and carried to another tissue where it has a specific effect. Thus, only minute quantities of PIIF could maximally induce inhibitor I accumulation in detached tomato leaves. Its specificity in producing an accumulation of proteinase inhibitors, apparently uncomplicated by growth effects, is novel among plant hormones. In will be of interest to learn the nature of the PIIF receptors and the sequence of biochemical events that lead to inhibitor accumulation.

Of several plant hormones tested for PIIF activity, none was effective. This included traumatin (the wound hormone reported by Haberlandt [5] and later isolated by English *et al.* [1]), kinetin, IAA, abscisic acid, ethylene, and GA. Of this group, GA did exhibit an antagonism of PIIF action. However, this antagonism was usually not severe and was sometimes not found at all during several repetitions of the experiment with different batches of plants. The inconsistency of the GA response suggests that this hormone is not directly involved in the PIIF response.

Little is known of the chemical nature of PIIF. It is insoluble in lipid solvents such as chloroform-methanol (2:1), alcohols, acetone, or ether. Its activity in crude PIIF solutions was not destroyed by incubation with any of several hydrolytic enzymes. Preliminary indications are that it is only slowly dialyzable and elutes from Bio-Gel P2 and is retarded by ultrafiltration membranes with an apparent mol wt above 10,000.

With the assay system described herein, the purification of the PIIF factor is underway. It is anticipated that the purification of this substance will facilitate the study of the underlying mechanism of the wound-induced accumulation of proteinase inhibitors in plants. The rapid, specific response of excised leaves to this substance provides a simple, uncomplicated system for the study of protein synthesis and its regulation in plant leaves. The purification of PIIF should also bring us closer to understanding the ability of plants to respond to insect attack by producing proteinase inhibitors, and its possible role in plant protection as a primitive immune response.

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LITERATURE CITED

1. English, J., Jr., J. Bonner, and A. J. Hagen-Smit. 1939. The wound hormones of plants. II. The isolation of a crystalline active substance. Proc. Nat. Acad. Sci. U.S.A. 25: 323–329.
2. Green, T. R. and C. A. Ryan. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777.
3. Green, T. R. and C. A. Ryan. 1972. Wound-induced proteinase inhibitor in tomato leaves: some effect of light and temperature on the wound response. Plant Physiol. 51: 19–21.
4. Gurusiddaiah, S., T. Kuo, and C. A. Ryan. 1972. Immunological comparison of chymotrypsin inhibitor I among several genera of the Solanaceae. Plant Physiol. 50: 627–631.
5. Haberlandt, G. 1922. The cell-division hormone and its relations to wound healing, fertilization, parthenogenesis and to adventitious embryonic development. Biol. Zentralbl. 42: 145–171.
6. Hess, G. P. 1971. Chymotrypsin-chemical properties and catalysis. *In:* P. Boyer, ed., The Enzymes, Vol. III. pp. 213–248.
7. Hummel, B. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. Can. J. Biochem. 37: 1392–1399.
8. Jacobsen, J. V. and J. E. Varner. 1967. Gibberellic acid-induced synthesis of protease by isolated aleurone layers of barley. Plant Physiol. 42: 1596–1600.
9. Melville, J. C. and C. A. Ryan. Chymotrypsin inhibitor I from potatoes: large scale preparation and characterization of the subunit components. J. Biol. Chem. 247: 3445–3453.
10. Ryan, C. A. 1967. Quantitative determination of soluble cellular proteins in agar gels containing antibodies. Anal. Biochem. 19: 430–440.
11. Ryan, C. A. 1968. An inducible protein in potato and tomato leaflets. Plant Physiol. 43: 1880–1881.
12. Ryan, C. A. 1968. Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. Plant Physiol. 43: 1859–1865.
13. Ryan, C. A. and W. Huisman. 1970. The regulation of synthesis and storage of chymotrypsin inhibitor I in leaves of potato and tomato plants. Plant Physiol. 45: 484–489.
14. Ryan, C. A. and L. K. Shumway. 1970. Studies on the structure and function of chymotrypsin inhibitior I in the Solanaceae family. *In:* H. Fritz and H. Tschesche, eds., Proc. Int. Res. Conf. Protein Inhibitors, De Gruyter, Berlin. pp. 175–188.
15. Shumway, L. K., J. Rancour, and C. A. Ryan. 1970. Vacuolar protein bodies in tomato leaf cells and their relationship to storage of chymotrypsin inhibitor I protein. Planta 93: 1–14.
16. Webster's New World Dictionary. 1958. The World Publishing Company, New York, p. 700.
17. Yomo, H. and J. E. Varner. 1971. *In:* A. A. Moscona and A. Monroy, eds., Current Topics in Developmental Biology, Vol. 6. Academic Press, New York. pp. 111–144.