# An Evaluation of Pea Histones as Disease Resistance Factors

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ABSTRACT

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We propose that basic pea proteins rich in lysine and arginine, primarily the histones LAK (slightly lysine-rich), KAS (moderately lysine-rich), and ARE (arginine-rich) are potentially more important than pisatin in the pea tissue's resistance to plant pathogenic fungi. These histones inhibit the growth of *Fusarium solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* in vitro at concentrations which approximate cellular levels. Synthetic basic proteins such as poly-L-lysine and basic proteins from other organisms also inhibit the growth of *F. solani* f. sp. *phaseoli*, as well as that of other *Fusarium* spp. The presence of such inhibitory compounds may prevent the proliferation of *F. solani* in nonhost tissue.

*Additional key words*: disease resistance, biochemistry of disease resistance.

Phytoalexin-type compounds have attracted much interest in recent years as determinants of disease resistance (6, 16, 24). The phytoalexin, pisatin, has been of interest to us because: (i) it is detected in inoculated tissue within 6 hr (10, 22); (ii) it reduces temporarily the percent germination and linear growth (3, 4) of *Fusarium solani* spp. at levels which approximate those produced in inoculated pea seedlings, (iii) fungal strains pathogenic to peas degrade pisatin more readily than closely-related strains nonpathogenic on peas (3); and (iv) the activation of the pathway of pisatin synthesis in pea plants provides a precise biochemical system for quantitating a portion of the tissue's response to a pathogenic organism (5, 9, 11, 12, 18). There is also an overlap of the period in which elicitor-induced pisatin production is dependent on RNA and protein synthesis and the period in which the disease-resistance response depends on RNA and protein synthesis as disignated by inhibitor studies (10). We have been reluctant to designate pisatin as the major component controlling disease resistance in peas because pathogenic and nonpathogenic strains of *F. solani* cannot always be distinguished on the basis of their sensitivity to pisatin (3). Also, pisatin levels are often very low or nondetectable in tissues at the time that resistance is observed cytologically (22). We have determined that *F. solani* f. sp. *phaseoli* will proliferate in pea pod tissues containing 427 μg pisatin/g fr. weight 15 days after inoculation. These results and others reported previously (10) are not compatible with the concept that pisatin is a major component of the resistance to *F. solani*. If pisatin, the major low-molecular-weight antifungal compound of peas (19, 20), is not the major determinant of disease resistance, the search must be extended to higher-molecular-weight compounds.

We have been interested (12) in the influence of basic proteins on regulatory processes in the cells of higher plants. Subsequently, we began testing the effect of basic proteins on the growth and development of plant pathogenic fungi. In this report, we present an assessment of the relative importance of low-molecular-weight antifungal compounds, such as pisatin, and higher-molecular-weight basic proteins, such as histones, in the resistance of pea tissue to *F. solani* f. sp. *phaseoli*. A preliminary report on the role of histones in disease resistance has been presented (17).

## MATERIALS AND METHODS

**Selective extraction of pea histones.**—Histones KAP (very lysine-rich), LAK (slightly lysine-rich), KAS (moderately lysine-rich), and ARE (arginine-rich) (13) were extracted using method I of Johns (14) to obtain histone fractions comparable to the commercially available calf thymus (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.) histones. Pea seeds were surface-sterilized and grown in the dark in sand for 1 wk. The seedling shoots were harvested and the chromatin was extracted according to Bonner et. al. (1). The pure chromatin, rather than the somewaht crude minced calf thymus, utilized by Johns (14) was utilized as the starting material for the histone extraction. An electrophoretic analysis of the histone fractions that were prepared showed minor cross-contamination of histone types in both the pea histone and the commercially prepared calf thymus histone fractions. Only the pea histone LAK was electrophoretically pure.

**Assay of the antifungal properties of histone.**—The antifungal properties of histone were assayed in vitro in sterile solutions of Vogel's medium (23), containing a

weak extract of pea pods (2 g intact pods in 1,000 ml medium) and varying the levels of the pea histones KAP, LAK, KAS, or ARE. The media were applied in 0.1-ml volumes to a microscope slide (pre-autoclaved in a petri plate). Five $\mu$liters of a spore suspension (< 100 spores pregerminated for 1-16 hr) of *F. solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli*, or other test organisms were applied to the droplet of medium with an automatic dispensing pipet. Fresh spores of the obligate parasites *Erysiphe graminis* f. sp. *tritici* and *Puccinia recondita* (produced on host plants grown in enclosed chambers) were dusted on the droplet. The growth of the fungi was recorded after 24 and 48 hr. Since the germination of spores and the hyphal growth of *Fusarium* spp. was essentially terminated in the presence of inhibiting concentration of most of the histones, a statistical calculation of linear growth or cell mass increase was not employed.

TABLE 1. The effect of pea histones on growth of *Fusarium solani* f. sp. *pisi* and f. sp. *phaseoli* within 24 hr

| Histone | Histone concentration ($\mu$g/ml) inhibiting growth of *F. solani*[a] | |
|---|---|---|
| | Supplemented with complete[b] media in vitro | |
| | f. sp. *pisi* | f. sp. *phaseoli* |
| KAP (very lysine-rich) | (40-100) | (60-100) |
| LAK (slightly lysine-rich) | 60-100 | 50-80 |
| KAS (moderately lysine-rich) | 30-80 | 40-80 |
| ARE (arginine-rich) | 60-80 | 40-70 |
| Mixture of KAP, LAK, KAS, and ARE[c] | 50-60 | 50-80 |

[a]Histone solutions over a range of concentrations from 0-200 $\mu$g/ml were placed in slide wells and then inoculated with pregerminated *Fusarium* macroconidia. Growth was observed after 24 hr. Growth essentially stopped at the recorded concentration, except for those concentrations in parentheses which only partially inhibited growth.

[b]These values represent a summary of three experiments.

[c]These four histones were combined in equal concentrations. The total amount of histone represented the total protein content.

## RESULTS AND DISCUSSION

**Effect of histones on the in vitro growth of Fusaruim solani.**—Pea histones, calf thymus histones, and other basic proteins inhibit the growth of both *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* when included in a complete nutrient medium (Tables 1 and 2). The four classes of histone are inhibitory within comparable ranges of concentration. The content of histone contained within the nucleus of pea cells is approximately 50 $\mu$g/g fresh weight of the tissue (1). The histone-to-DNA ratio of pea chromatin varies as the tissue proceeds through differentiation (2), but the corresponding variation in the cytoplasmic level of histone is not known. Histone KAP, even though inhibitory, does not appear to terminate the growth of the fungus within 24 hr as do the other types of histones.

The mixing of histone types (Table 1) does not result in synergistic enhancement of inhibition within a given experiment. *Fusarium solani* f. sp. *pisi* and f. sp. *phaseoli* differ only slightly in tolerance to concentrations of histone.

Very high histone concentrations (500-1,000 $\mu$g) cause partial lysis of the macroconidia. The lysis is not accompanied by complete emptying of cell content, however. The termination of growth caused by physiological levels of histone is probably not due to a pH effect, since the histone solutions are approximately pH 6.0. Histones applied exogenously at concentrations 10 $\mu$g/ml below the inhibitory concentration sometimes cause a change in the morphology of the fungus, but growth often approximates that of the untreated controls. Within a given experiment, the zone of demarcation between concentrations of histones which allow growth and those which allow no growth seldom exceeds a 10 $\mu$g/ml interval. The variation in minimal histone concentrations which inhibit growth appears to be related to the length of time germination of the macroconidia has proceeded prior to being transferred to the treatment solution. The early stages of germination (1-2 hr) are more sensitive to lower concentrations of histones than later stages of spores which have been pre-germinated and grown in Vogel's medium for 16 hr.

TABLE 2. Effect of calf thymus histones and other basic proteins on the growth of *Fusarium solani*

| Basic protein | Protein conc. which inhibit growth of *F. solani*[a] f. sp.: | |
|---|---|---|
| | *phaseoli* ($\mu$g/ml) | *pisi* ($\mu$g/ml) |
| Protamine | 5-10 | 5-10 |
| KAP histone, calf thymus | 50 | (40-100) |
| ARE histone, calf thymus | 50 | (50-100) |
| Protamine, KAP, LAK, and ARE histone[b] | 30 | 40 |
| Nucleohistone, calf thymus | No inhibition[c] | No inhibition[c] |
| Nucleoprotamine, Salmon | No inhibition[c] | No inhibition[c] |
| Poly-L-lysine | 50 | 50 |
| RNase, calf | No inhibition[c] | No inhibition[c] |

[a]Basic protein solutions over a range of concentrations from 0-500 $\mu$g/ml were placed in the wells of microscope slides and then inoculated with *F. solani*. Growth was observed after 24 hr. Growth essentially stopped at the recorded concentration except for those concentrations in parentheses which only partially inhibited growth.

[b]These four basic proteins were combined in equal concentrations. The total $\mu$g/ml represent the total protein content.

[c]No inhibition at concentrations up to 1,000 $\mu$g/ml.

TABLE 3. Effect of calf thymus histones and other basic proteins on the growth of *Fusarium solani* f. sp. *pisi* and other fungal species

| Basic protein | Protein conc.[a] affecting germinating spores of: | | | |
|---|---|---|---|---|
| | *Fusarium solani* f. sp. *pisi*[b] (μg/ml) | *Fusarium oxysporum* f. sp. *asparagi* (μg/ml) | *Erysiphe graminis* f. sp. *tritici* (μg/ml) | *Puccinia recondita* (μg/ml) |
| Protamine | 25 | 25 | (50)[c] | (100)[c] |
| KAP histone, calf thymus | 50 | 100 | (50)[c] | (75)[c] |
| ARE histone, calf thymus | 75 | 75 | (75)[c] | (1,000)[c] |
| Poly-L-lysine (2,860 MW) | 25-75 | 75 | (75)[c] | (1,000)[c] |
| RNase, bovine pancreas | 1,000 | No. inhib. | (100)[c] | (75)[c] |

[a]Basic protein solutions in conc. of 0, 25, 50, 75, 100, 200, and 1,000 μg/ml were placed in the wells of microscope slides and then inoculated with spores of the various fungi. Growth was observed after 24 hr. Growth essentially stopped at the recorded concentration, except for those concentrations in parentheses which only partially inhibited growth.

[b]This culture of *F. solani f. sp. pisi* was isolated from the legume sanfoin.

[c]The concentrations of protein in parentheses cause a noticeable increase in lysis of the spores and a definite detrimental effect on the developing germ tube, but in no case was there complete termination of growth of the low percentage of spores that actually germinated in the test solutions.

It is believed that histones are synthesized in the cytoplasm and transported to the nucleus in synchronization with DNA synthesis within the cell (21). Once they have become localized in nucleoprotein they may be unavailable to the fungus. For example, nucleoproteins (Table 2), even at levels higher than those histone levels which inhibit *F. solani*, do not inhibit the in vitro growth of either of the formae speciales.

The alteration of the host cell which occurs at early stages of the host-parasite interaction may enable the newly synthesized, cytoplasmic histone to be translocated to the fungus in lieu of its normal translocation to the host nucleus. The work of Gurdon (8) established that although the histones in nucleoprotein do not readily migrate from one nucleus to another, histones introduced into the cytoplasm can freely migrate to nuclei, even to foreign nuclei. Basic proteins can readily permeate plant cells (7); thus, it is possible that newly synthesized histones can localize in cells other than the one in which they were synthesized. The levels of histone crucial for disease resistance would likely be those of each type of histone within the cytoplasm. The rate at which the histones are synthesized following inoculation would influence the level within the cytoplasm.

Pea histones appear to inhibit in vitro growth of *F. solani* in a manner cytologically similar to the inhibition of fungal growth by intact pea tissue. Further growth of *Fusarium solani* f. sp. *pisi* on excised pod tissue is inhibited for up to 7 days. When the lesion is transferred to potato-dextrose agar, the germinated macroconidia will resume growth. Similarly, the spores inhibited by physiological levels of histone will resume growth when transferred to potato-dextrose agar.

Histones extracted from calf thymus and basic proteins such as protamine from other animal cells can inhibit the growth of *F. solani* f. sp. *phaseoli* (Table 2), and other fungal pathogens (Table 3). Presumably, all tissues that do not serve as a host for *F. solani* have some potential to resist that fungus.

Our preliminary data implicating basic proteins as resistance components in plants may warrant further consideration for the following reasons: (i) resistance is the common reaction of nonhost organisms to the presence of plant pathogenic fungi such as *F. solani* f. sp. *phaseoli*; (ii) in the cells of higher organisms histones are major components that are synthesized in the cytoplasm (21) and their potential for movement across membranes (13) and association with foreign nuclei (8) has been established; and (iii) cytologically, the inhibited growth of *F. solani*, caused by histone concentrations comparable to those found in the plant cell resembles the cessation of its growth in intact pea tissue. The coding of histones is thought to involve multiple genes and these genes reportedly occur in clusters (15) within the genome. Therefore, changes in arrangement, or number, or potential for activation of histone genes could influence the potential for disease resistance. There is some variation in the effectiveness of the histones from different sources in inhibiting growth of *F. solani*, but little difference in the sensitivity of the formae speciales of that fungus to a given histone. Therefore, the proposed resistance mechanism may relate to the rate at which histone is synthesized following inoculations and to the subsequent rate of translocation of these proteins to the fungal cell.

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