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CHROMATOGRAPHIC ANALYSIS OF ISOFLAVONOID ACCUMULATION IN STRESSED *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; garden pea; stress metabolism; isoflavonoids; HPLC.

Abstract—High-performance liquid chromatography has been used to study isoflavonoid accumulation in copper(II) chloride stressed *Pisum sativum*. Liquiritigenin, isoliquiritigenin, formononetin, pseudobaptigenin, afrormosin and anhydropisatin have been identified in addition to the pterocarpan phytoalexin pisatin. The relationships of these metabolites to isoflavonoid biosynthesis and stress response in pea are discussed.

INTRODUCTION

The accumulation of phytoalexins has been demonstrated in many plant tissues in response to infection, chemical stress and a variety of other stimuli [1–3]. However, their precise function in disease resistance has not been proved. In fact, after two decades of research the views presented in the literature range from their possible implication [4] to a dismissal of their role in the determination of resistance or susceptibility [5]. Ward and Stoessl [5] have suggested that phytoalexin production "has been recognized because its products are fungitoxic." This implies that potentially interesting and informative additional metabolites may have been ignored. A major factor which has hampered a comprehensive study of the accumulation of phytoalexin-related metabolites has been the lack of a chromatographic procedure which can easily resolve and quantify complex metabolite mixtures. This report describes the application of a high-performance liquid chromatographic (HPLC) procedure to the analysis of the isoflavonoid stress response of *Pisum sativum* L. (garden pea). The relationships of the identified metabolites to isoflavonoid biosynthesis and stress response in pea are discussed.

RESULTS

The chromatographic fingerprint of the *de novo* accumulation of metabolites by copper(II) chloride stressed pea is illustrated in Fig. 1. Fig. 1A shows that the unstressed plant does not accumulate significant quantities (detection limit *ca* 0.3 μg/g) of isoflavonoid metabolites. Hydrolytic extraction for the detection of isoflavonoid glycosides as their aglycones showed that there was not a reservoir of isoflavones in the unstressed plant either as aglycones or as glycosides. Fig. 1B shows that copper(II) chloride stress will cause the accumulation of a number of compounds which were not present in the unstressed plant. Variation of the ethanol content of the HPLC solvent mixture from 0 to 10% confirmed the absence of additional isoflavonoid metabolites which may have been obscured by the solvent front peak or by long retention times.

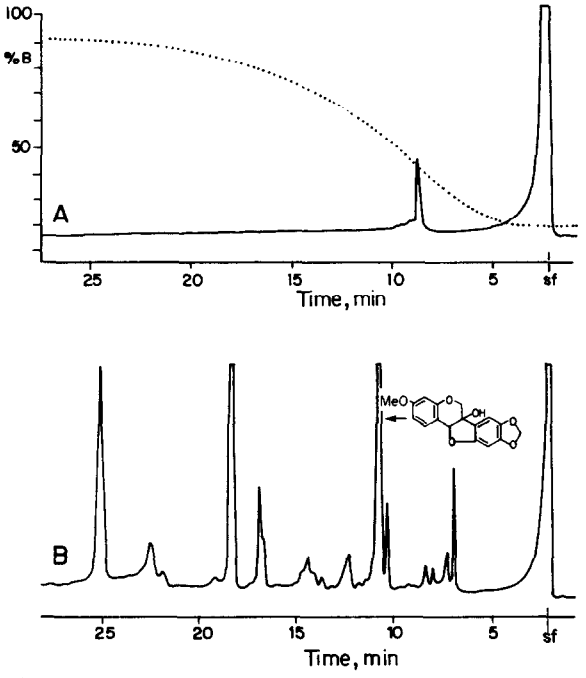

Fig. 1. Gradient chromatogram of unstressed (A) and stressed (B) *Pisum sativum*. Solvent A—hexanes; solvent B—CH_2Cl_2–EtOH–HOAc, 97:3.2. The gradient used is shown on chromatogram A. Sample B was taken 96 hr after stress. Both chromatograms had 50 μl of a 0.1 ml/g sample injected.

P. sativum accumulates five phytoalexins (**1**–**5**) [6–8]. The most extensively studied of these is pisatin (**1**) [9]. Fig. 1B shows that pisatin accumulates in the stressed plant while additional chromatographic analysis for the remaining four phytoalexins (Fig. 1B elution time *ca* 7–10 min) shows that these compounds do not accumulate to an appreciable extent until at least 8 days after stress. The identification of the remainder of the major peaks shown

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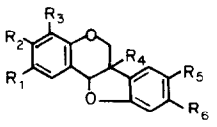

	R_1	R_2	R_3	R_4	R_5	R_6
1	-H	-OMe	-H	-OH	$-OCH_2O-$	
2	-H	-OH	-H	-H	$-OCH_2O-$	
3	-OMe	-OH	-H	-H	-H	-OMe
4	-OMe	-OMe	-H	-H	-H	-OMe
5	-OMe	-OMe	-OH	-H	-H	-OMe

in Fig. 1B is given in Fig. 2 with the exception of three non-isoflavonoid phenolics which will be described elsewhere. These compounds have all been observed to occur in other plants (liquiritigenin (**6**) [10], isoliquiritigenin (**7**) [11], formononetin (**8**) [12], pseudobaptigenin (**9**) [12], afrormosin (**10**) [12] and anhydropisatin (**11**) [13]) but have not been previously identified from pea.

DISCUSSION

The pterocarpans have been recognized as natural products for many years, mainly as heartwood constituents of various leguminous plants [12]. It was not until their observation as *de novo* metabolites produced by growing tissues in response to stress that a route was opened for studies of their biosynthesis [12]. Because the phytoalexins of *P. sativum* are pterocarpans it is not surprising that many of the observed metabolites of pea are related to known pathways of pterocarpan biosynthesis (Fig. 3) [12, 14–16]. It seems probable that the sequence of reactions **7** → **8** → **9** → **1** will produce the main pea phytoalexin pisatin (**1**) while formononetin (**8**) serves as the branch point to afrormosin (**10**) and pterocarpans **3**–**5**. The biosynthesis of pseudobaptigenin (**9**) from the chalcone (**7**)/flavanone (**6**) has been well documented [14, 15]. However, the steps which immediately preceed pisatin formation have not been as clearly defined. It seems likely by analogy to studies on the biosynthesis of demethylhomopterocarpin [15] that pseudobaptigenin (**9**) serves as a precursor of maackiain (**2**) but that anhydropisatin (**11**) does not. However, there is currently insufficient evidence to determine which of the sequences, **9** → **2** → **1** or **9** → **11** → **1**, is correct. Pterocarpans **3**–**5** are presumably derived from afrormosin (**10**). However, this pathway and the interconnections of **3**–**5** have not been proved.

Rate of accumulation data (Fig. 4) indicates that the isoflavonoid metabolites can be divided into two accumulation types. In the first case, both pisatin, which is a major end product of isoflavonoid stress metabolism, and formononetin (**8**), which is a key isoflavone intermediate in pterocarpan biosynthesis [12, 15], show similar accumulation profiles. The isoflavone afrormosin (**10**), however, which is presumably an intermediate in the biosynthesis of the three late-forming pterocarpans (**3**–**5**) only begins to accumulate at *ca* 48 hr after stress. These data support the concept that pisatin is a potentially significant stress metabolite [17] while pterocarpans **3**–**5** are part of a minor, latent pathway. These results indicate that the ability to determine the fingerprint of isoflavonoid accumulation can yield information which is of value to the biochemical and phytopathological interpretation of *P. sativum* stress metabolism.

EXPERIMENTAL

The HPLC system used in this study has been described previously [18].

Seedling culture and extraction. The pea seed used in these studies (*Pisum sativum* cv Melting sugar) was obtained from Buckerfields Ltd., Vancouver, B.C., Canada.

Seedling culture. In a typical experiment 10 g of seed was rinsed twice with water and the seed culled for cracked seed coats, etc. The seed was then placed in a 10 × 30 cm glass dish which had 1 cm of water-soaked filter paper placed in the bottom. A sheet of filter paper was placed over the seed and the container was covered loosely with a glass plate. The container was placed in a dark cabinet at room temp. (*ca* 23°). The seedlings were allowed to grow for *ca* 6 days with the addition of water to the paper mat as necessary. For large quantities of material, 100 g of seed was placed in a liter beaker and soaked overnight in water. The excess

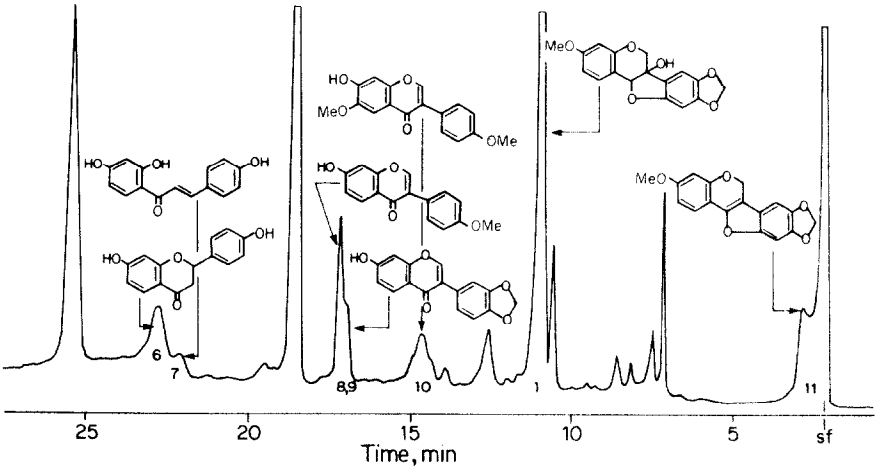

Fig. 2. The identified metabolites of *Pisum sativum*. The peaks are labelled by structure and compound number. Chromatographic conditions are the same as in Fig. 1.

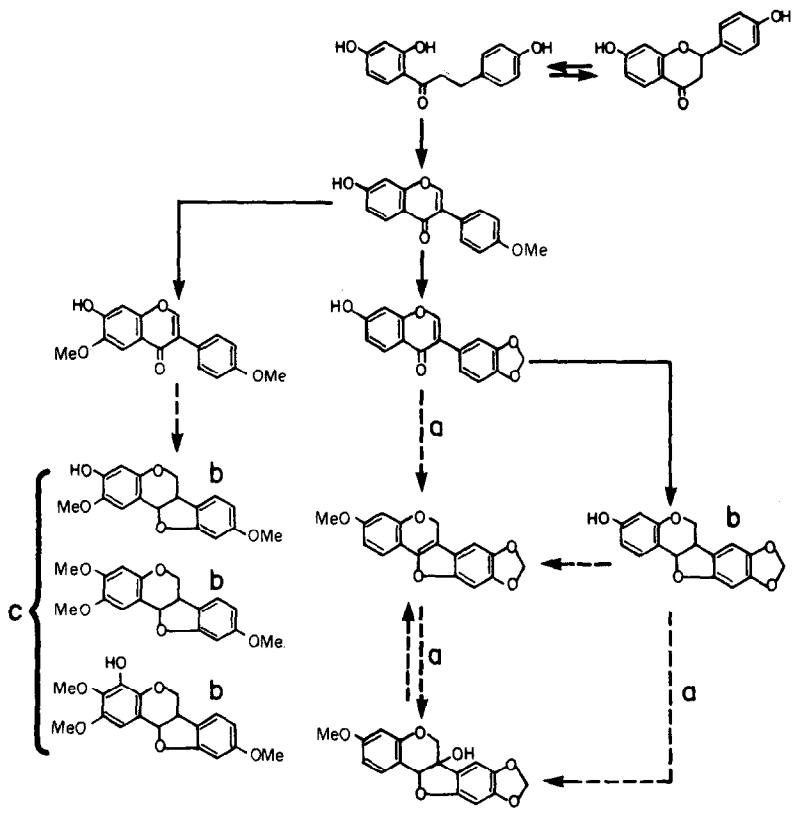

Fig. 3. Biosynthetic relationships of the observed isoflavonoid metabolites of *Pisum sativum*. Alternate possible routes to pisatin (a; [16]). Metabolites which were characterized in previous studies (b; [7, 8]). The biosynthetic relationships of pterocarpans **3**–**5** have not been conclusively determined (c).

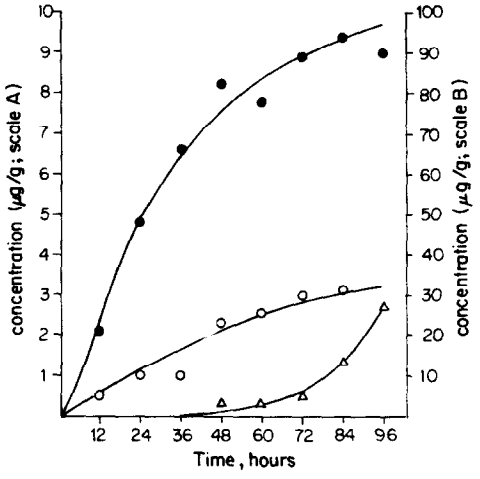

Fig. 4. Time course of metabolite accumulation. Scale A: Formononetin (○: **8**) and afrormosin (△; **10**). Scale B: Pisatin (●; **1**). Concentration based on wet wt.

water was decanted and the seed rinsed with water. The beaker was covered loosely with foil and placed in a dark cabinet. The water rinses were repeated twice daily for *ca* 6 days.

Stress procedure. The seedlings were stressed by soaking them for 30–45 min in a copper(II) chloride soln (3 or 9 × 10^3 M). After decanting the soln the samples were left loosely covered in the dark and moistened with water as necessary.

Extraction. After an appropriate growth period the plants were weighed (wet wt) and extracted as described previously [18]. The addition of pisatin to extraction blanks showed that it would not be degraded to anhydropisatin by the extraction procedure.

Metabolite isolation and identification. The metabolites **1**, **6**–**11** were isolated for identification by HPLC using the conditions given in Fig. 1. After HPLC isolation the compounds were identified by a comparison of their UV (with added reagents) [19], MS, TLC and HPLC properties with authentic samples. The pterocarpan region of the chromatogram (Fig. 1) was expanded by an isocratic HPLC run ([CH_2Cl_2–EtOH–HOAc (97:3:0.2)]/hexanes (15:85)) for further comparison with authentic samples. The retention times for pterocarpans **1**–**5** and **11** were 17.8, 12.0, 16.4, 5.7, 14.2 and 2.5 min respectively. The authentic samples were obtained as gifts (4-hydroxy-2,3,9-trimethoxy- (**5**), 3-hydroxy-2,9-dimethoxy- (**3**) and 2,3,9-trimethoxypterocarpan (**4**)), by isolation (pisatin (**1**) [6]) or by synthesis (liquiritigenin (**6**) [20], isoliquiritigenin (**7**) [21], formonoetin (**8**) [22], pseudobaptigenin (**9**) [22], afrormosin (**10**) [23], anhydropisatin (**11**) [6] and 3-hydroxy-8,9-methylenedioxypterocarpan (**2**) [24]).

Several of the isolates required additional HPLC purification steps prior to identification. Formononetin (**8**) and pseudobaptigenin (**9**) were separated using an HPLC procedure which was described previously [18]. The afrormosin peak was further resolved into two components by HPLC (gradient as in Fig. 1A; solvent A: hexanes; solvent B: CH_2Cl_2–EtOH–HOAc (99:1:0.2). Afrormosin eluted at 19.5 min on this system while the other component eluted at 19.0 min. The second component was an isoflavone which was not conclusively identified. The

anhydropisatin peak was further resolved from the solvent front by isocratic HPLC (solvent: CH_2Cl_2–EtOH–HOAc, 99:1:0.2). Anhydropisatin eluted at 3.5 min on this system.

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