Degradation of the Isoflavone Biochanin A-7-O-glucoside-6″O-malonate and Phenylacetic Acids by *Fusarium javanicum*

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The isoflavone conjugate biochanin A-7-O-glucoside-6″-O-malonate is degraded by *Fusarium javanicum* with an esterase to yield biochanin A-7-O-glucoside which is further cleaved by a glucosidase to the aglycone. Biochanin A is funnelled into a known catabolic sequence (Z. Naturforsch. **37c**, 861 (1982)). Induction of the catabolism of *p*-methoxyphenylacetic acid is linked to biochanin A degradation, whereas *p*-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid degradation is substrate-induced.

Introduction

Phytopathogenic *Fusarium* fungi have been demonstrated as potent degraders for preinfectional isoflavones [1] and isoflavonoid phytoalexins [2]. Degradation of plant defense compounds by the invading fungi can be considered as one important mechanism for the expression of fungal pathogenicity [3].

In the course of our studies on the catabolism of the isoflavone biochanin A (**3** in Fig. 3) by *Fusarium javanicum* [4, 5] we have now determined with this fungus the degradation of biochanin A-7-O-glucoside-6″-O-malonate (**1**). Such malonylesters are the main isoflavone constituents in *Cicer arietinum* and several other Leguminosae [6]. This appears to be the first report on the disintegration of such malonate plant conjugates by a plant pathogen.

Furthermore, we describe some observations on the catabolism of substituted phenylacetic acids, **6–8**, which had previously been found as intermediates in the degradation of **3** by *F. javanicum* [4].

Methods

Fungus

Fusarium javanicum (Koord)

(Centraalbureau voor Schimmelcultures, CBS 203.32) was stored and grown as previously described [4]. When phenylacetic acids were used as carbon source (0.5%), glucose was omitted from the growth medium.

Compounds

Biochanin A-7-O-glucoside-6″-O-malonate was isolated from *Cicer arietinum* plants [6]. All other compounds were from previous studies [4, 6, 7].

Degradation experiments

Preparation of mycelia for degradative studies, standard incubation assays (26 °C; Gyrotory shaker, 170 rpm) with potassium phosphate buffer (0.05 M; pH 7.5) and preincubation of mycelia with various substrates have been carried out according to our earlier reports [4, 5]. Substrates were incubated at 10^{-4} M; if necessary, substrates were predissolved in small amounts of 2-methoxyethanol.

Aliquots (5 ml) from incubation assays were freed from mycelium by filtration, acidified with 10 µl 10 N H_2SO_4 and thoroughly extracted with 5 ml diethylether for 1 min. The ether phase was brought to dryness under vacuum at 20 °C and the residue was redissolved in acetone. These samples were stored at −20 °C until HPLC analysis.

HPLC analyses

The HPLC system for analytical separations of isoflavones and their conjugates has been described [6, 7]. Isoflavones were measured at 261 nm, dihydrobiochanin A was recorded at 291 nm and the 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4 H-py-

Abbreviations: HPLC, high performance liquid chromatography; TLC, thinlayer chromatography.

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ran was quantitated at 241 nm. External standardisation was used for quantitative determinations.

Chromatography

TLC was conducted on silica gel F 254 plates with the following solvent systems (V/V): S_1: CH_2Cl_2: methanol, 15:1. S_2: *n*-propanol: 25% NH_3: H_2O, 6:3:1; S_3: $CHCl_3$: acetic acid, 7:3.

Isoflavones and phenylacetic acids were detected by spraying with fast blue salt B.

Spectroscopic methods

The measurement of UV spectra has been described [6]. Phenylacetic acids were assayed at 275 nm.

Malonate ester stability

The biochanin A-7-O-glucoside-6′-O-malonate was assayed for pH-dependent hydrolysis by incubation at 10^{-4} M and 30 °C. The following buffers were used: pH 4.0–4.5: Tri-Na-citrate/HCl, 0.1 M; pH 5.0–7.0: KH_2PO_4/K_2HPO_4, 0.05 M; pH 7.0–8.5: Tris/HCl, 0.1 M.

Aliquots of the incubation assays were analyzed for malonate ester hydrolysis by HPLC. Samples were either handled as described for the standard incubation assays or the buffer solutions were directly injected in the HPLC apparatus.

Enzyme assays

Mycelium of *F. javanicum* was thoroughly homogenized in a mortar with potassium phosphate buffer (0.05 M, pH 7.5, 1 ml/g) at 0 °C for 15 min. The homogenate was centrifuged (30000 × *g*, 40 min) and the supernatant solution was directly used for enzyme measurements.

The pellet obtained after centrifugation was 5-times resuspended in the same buffer and recentrifuged. The final pellet was suspended (1 ml/g) in potassium phosphate buffer (0.05 M, pH 7.5) and used as such for incubation purposes.

Enzyme assays contained in a total volume of 2000 µl: 500 µl crude protein preparations or resuspended cell debris. 1450 µl buffer (as mentioned under stability tests) and 50 µl substrate solution. Substrates were measured at a concentration of 10^{-4} M or 10^{-5} M. Enzyme assays were incubated at 30 °C for 60 min and stopped by the addition of 1000 µl methanol. After removal of protein by centrifugation, the supernatant solutions were assayed for substrates or products by HPLC as described above.

In order to determine the effect of glucono-1,5-lactone the enzyme assays contained in the 2 ml total volume 1250 µl of buffer solution, 500 µl protein preparations, 50 µl substrate and 200 µl 0.1 M glucono-1,5-lactone in buffer. Thus, the glucosidase inhibitor was routinely applied at a 0.01 M concentration. Protein was determined by the method of Bradford [17].

Results and Discussion

Metabolism of biochanin A conjugates

Isoflavone-7-O-glucoside-6″-O-malonate esters are comparatively labile compounds [6] which are readily decomposed to the isoflavone-7-O-glucosides when kept in alcoholic solutions. Stability tests of **1** in aqueous buffer solutions (10^{-4} M; 30 °C) between pH 4.0 and 8.5 have now been carried out. Aliquots of the incubation assays were analyzed by HPLC [7] which allows quantitative determinations of the malonate ester, the glucoside and the aglycone, respectively. Over an incubation period of 10 h the malonate ester, **1**, was found to be stable in a pH range of 4.5 to 8.0. An example is given in Fig. 1. Lower or higher values led to considerable hydrolysis of **1**.

In standard incubation assays with mycelial preparations of *F. javanicum* the metabolism of **1** (10^{-4} M) was quantitatively followed by measuring aliquots with our HPLC systems [7, 8]. As shown in Fig. 1, the isoflavone-7-O-glucoside-6″-O-malonate, was disintegrated within 2 h. Biochanin A-7-O-glucoside (**2**), biochanin A (**3**), and dihydrobiochanin A (**4**), respectively, were found as subsequent metabolites. The very low accumulation of the glucoside (**2**), indicates the presence of very active glucosidase activity in the mycelium.

Similar incubations with biochanin A-7-O-glucoside show (Fig. 2) that **2** is much more rapidly cleaved by the fungus than the malonate ester **1**. In these assays the subsequent transient accumulation of **3, 4**, and the previously determined 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran, (**5**), could be observed. Since the data in Figs. 1

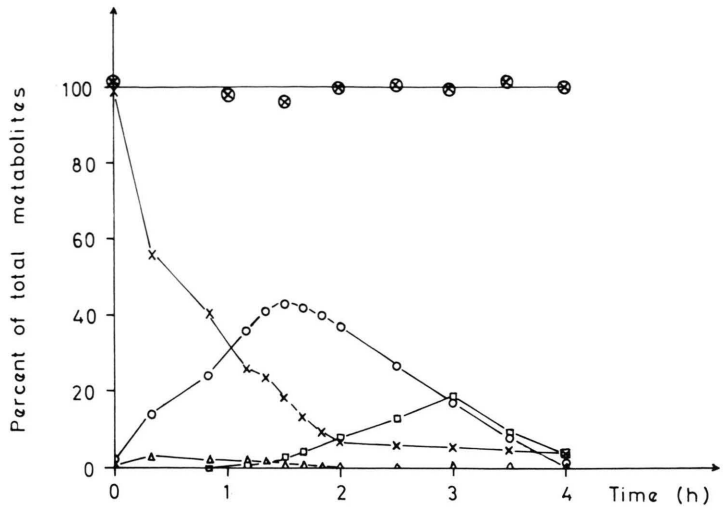

Fig. 1. Conversion of Biochanin A-7-O-glucoside-6″-O-malonate (×——×) into biochanin A-7-O-glucoside (△——△), biochanin A (○——○) and dihydrobiochanin A (□——□) by mycelial preparations of *Fusarium javanicum*. Isoflavone conjugate transformation reactions were quantitatively measured by HPLC separations. In control experiments without mycelium (⊗——⊗) the stability of the substrate malonate ester was tested under the incubation conditions (pH 7.5).

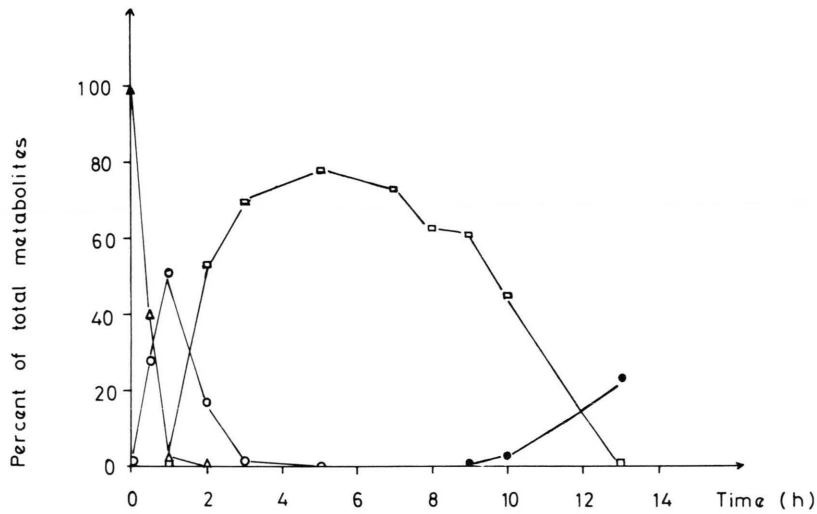

Fig. 2. Conversion of biochanin A-7-O-glucoside (△——△) into biochanin A (○——○), dihydrobiochanin A (△——△) and 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran (●——●) by mycelial preparations of *Fusarium javanicum*. Experimental procedure as given in the legend of Fig. 1.

and 2 are plotted as percent molar concentrations of metabolites in the incubation medium, inspection of the curves clearly shows that none of the various catabolites accumulates quantitatively. In contrast to other studies with *F. oxysporum* [9] substantial amounts of catabolites **2**, **3**, **4**, and **5** are metabolized without intermediate excretion into the medium.

Conversion of **1** to **2** results in the formation of malonic acid. This compound which is known for its inhibitory action on cellular metabolism in various organisms [10], does, however, not interfere with biochanin A catabolism by *F. javanicum*. Incubations of mycelial preparations of *F. javanicum* with both **1** and **2** in the presence of a 5-fold molar excess of malonic acid revealed essentially the same results as shown in Figs. 1 and 2. Slower rates of conversion of substrates or a decrease in the accumulation of any catabolites could not be observed. The metabolic fate of malonic acid itself was, however, not further investigated.

The formation of **2** and **3** from **1** by *F. javanicum* is best explained by the action of first an esterase and then a glucohydrolase. Since only a small amount of **2** is transiently accumulated during the disintegration of **1** (Fig. 1), the presence of a specific glucohydrolase which removes 6-O-malonylglucose from **1** cannot be excluded. In such a case the esterase reaction would occur after hydrolysis of the

glucoside. Application of a glucosidase inhibitor will allow to distingush between these two possibilities because **1** would not be degraded.

Determination of both esterase and glucosidase activities for the hydrolysis of **1** and **2** were carried out with crude protein preparations from *F. javanicum* cells at pH 5 and 6. In preliminary measurements these pH values were found to give best results. The data in the table were obtained by measuring decrease of either **1** (esterase activity) and **2** (glucosidase reactions) or formation of **3** (glucosidase) both in the absence and the presence of the well-known glucosidase inhibitor glucono-1,5-lactone [12]. Transformation of the malonate ester,

Table I. Specific enzyme activities of esterase and glucosidase measured with crude protein preparations from *F. javanicum*. Symbols for substrates and products as given in Fig. 3. Formation of **3** in experiment B) occurred at a very low rate, only. Experiments in A) were carried out in the absence of glucono-1,5-lactone and in B) in the presence of the inhibitor.

	Substrate	pH	Products formed	Esterase Activity a) $\left\{\frac{\text{n Kat}}{\text{mg Protein}}\right\}$	Glucosidase Activity b) $\left\{\frac{\text{n Kat}}{\text{mg Protein}}\right\}$
A)	**1**	5	**2, 3**	0.102	0.071
		6	**2, 3**	0.119	0.082
	2	5	**3**	–	0.089
B)	**1**	5	**2, (3)**	0.084	0.006
		6	**2, (3)**	0.101	0.023
	2	5	**(3)**	–	0.018

a) Values obtained by measuring decrease of malonate ester **1**.
b) Values obtained by measuring formation of aglycone **3**.

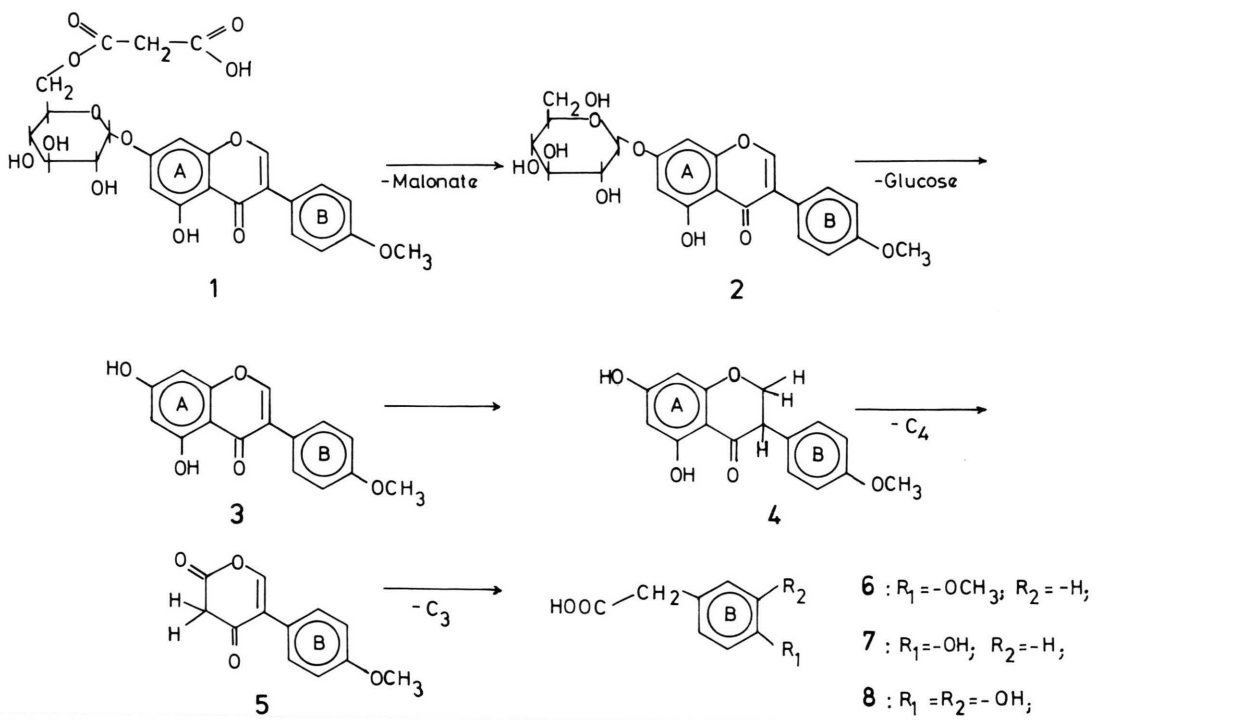

Fig. 3. Catabolic sequence for biochanin A-7-O-glucoside 6″-O-malonate by *Fusarium javanicum*. Structures of intermediates **4**–**8** were previously reported [4].

1, in the absence of glucono-1,5-lactone led to both **2** and **3** whereas in the presence of the glucosidase inhibitor the glucoside, **2**, accumulated at high rates. Under these conditions very little **3** was formed. Comparison of the values obtained for the glucosidase activity without and with glucono-1,5-lactone shows that glucosidase activity became inhibited to more than 80%. Esterase activity leading to the accumulation of **2** was shown to be essentially uneffected by glucono-1,5-lactone.

The data in the table together with the results from Figs. 1 and 2 lead to a catabolic pathway as shown in Fig. 3. This first report on the fungal degradation of a malonyl glucoside plant conjugate demonstrates that fungi such as *F. javanicum* are able to disintegrate the genuine plant constituents which may accumulate in the living tissue at very high concentrations [6]. The data in Figs. 1 and 2 also demonstrate that hydrolysis of the conjugates and conversion of biochanin A appear to be carried out by constitutive enzymes.

Our results demonstrate that *F. javanicum* cells possess both soluble esterase and glucosidase activities. In addition, essentially the same results were obtained with thoroughly extracted and washed cell debris after extraction of soluble protein. Thus, bound forms of esterase and glucosidase can also be expected.

Future studies with purified enzymes from *F. javanicum* will have to show whether the esterase and the glucosidase involved in the metabolism of **1** and **2** are enzymes with narrow substrate specificity [13, 14]. Numerous reports, however, have shown that fungi often produce esterases and glucosidases with rather broad specificity [15, 16].

Metabolism of phenylacetic acids

Previous investigations on biochanin A catabolism by *F. javanicum* had shown that the substituted phenylacetic acids **6–8** (Fig. 3) were formed as metabolites of **3** [4]. It has also been argued [4, 11] that the catabolism of phenylacetic acids by *F. javanicum* is presumably only induced as part of isoflavone metabolism and not by the acids when given as sole substrates.

Comparative investigations on the degradation of the acids **6**, **7**, and **8**, respectively, both by mycelial preparations pretreated with **3** [10^{-4} M] for 16 and 22 h and by untreated cells have now been carried out. In standard incubation assays disappearance of substrates was followed by UV-spectroscopy and TLC assays of suitable aliquots.

When non-induced cells were incubated with **6**, **7** or **8**, respectively, degradation of both *p*-hydroxy- and 3,4-dihydroxyphenylacetic acid commenced after a 10–12 h lag phase and was completed after additional 4 h. In degradation assays with **7** the intermediate formation of **8** could clearly be demonstrated by TLC. In fact, **7** and **8** were shown to sustain growth of *F. javanicum* cells when given as sole carbon substrates.

In contrast to the hydroxyderivatives **7** and **8**, transformation or degradation of *p*-methoxyphenylacetic acid (**6**), by non-induced mycelial preparations could not be measured regardless of incubation period (up to 60 h) and amount of cells applied. In mycelial preparations of *F. javanicum* preincubated with biochanin A rapid degradation of **6**, **7**, and **8** with no or rather short lag-phases (1–3 h) was observed. Thus degradation of *p*-methoxyphenylacetic acid by *F. javanicum* has so far only been found in biochanin A-degrading cells, though the subsequent formation of **7** and **8** from **6** could not be demonstrated.

Quite surprisingly preincubation of cells with biochanin A led to mycelial preparations which appeared to be also induced for the degradation of 2,5-dihydroxyphenylacetic acid. Since this acid has not yet been found as a catabolite of **3**, the significance of this observation is presently unclear.

Future studies on the catabolism of **6** and the intermediates involved therein combined with suitable enzymic investigations on biochanin A and phenylacetic acids degradation in *F. javanicum* will provide a means to determine how the induction of catabolic enzymes for **6** is linked to biochanin A degradation.

Acknowledgement

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