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# ETHER-LINKED FERULIC ACID AMIDES IN NATURAL AND WOUND PERIDERMS OF POTATO TUBER

JONATHAN NEGREL, BRIGITTE POLLET* and CATHERINE LAPIERRE*

Laboratoire de Phytoparasitologie INRA-CNRS, Station de Génétique et d'Amélioration des Plantes, INRA BV 1540, 21034 Dijon Cédex, France; *Laboratoire de Chimie Biologique, INRA-CBAI, Institut National Agronomique, 78850 Thiverval-Grignon, France

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**Key Word Index**—*Solanum tuberosum*; Solanaceae; potato; periderm; wound-healing; hydroxycinnamic acid amides; feruloyltyramine; thioacidolysis.

**Abstract**—The occurrence of ether-linked ferulic acid amides (feruloyltyramine and/or feruloyloctopamine) in suberin-enriched samples of natural and wound periderms of potato tubers was established by thioacidolysis and desulphuration, followed by GC–mass spectrometry of diagnostic dihydroferuloyltyramine. Analysis of samples premethylated in the presence of diazomethane showed that the major part of the ether bonds involved the ferulic moiety of the amides; 20% of the amides released upon thioacidolysis were nevertheless attached to cell walls through the tyramine phenolic group. In addition, dicovalently linked ferulic acid amides were evidenced. These bridges were twice as abundant in the wound than in the natural periderm. Copyright © 1996 Published by Elsevier Science Ltd

## INTRODUCTION

The synthesis and integration into cell walls of hydroxycinnamic acid amides of tyramine and octopamine is a very early response of potato tubers to fungal attack [1] and to wounding [2]. These amides are postulated to contribute to the formation of a phenolic barrier, which makes cell walls more resistant to enzymic hydrolysis [1, 3]. In wound-healing tuber tissues, the synthesis of feruloyltyramine and feruloyloctopamine is triggered within 3 to 4 h [2], substantially before that of suberin [4, 5]. Both amides [2] and tyramine [2, 6] and octopamine [2] are released by alkaline hydrolysis of natural and wound periderms of potato tubers. As feruloyl amides are good substrates of peroxidase *in vitro* [7], they were speculated to participate, with other phenolics, in a peroxidase-mediated polymerization, yielding a polyaromatic domain [8, 9]. If so, a substantial portion of feruloyl amides could be attached to cell walls in suberized samples by alkali-resistant bonds, such as ether linkages. Recent solid state $^{13}C$ NMR studies suggested that the phenolic domain of potato suberin largely comprised a hydroxycinnamic acid-derived polymer [8].

To obtain an insight into the attachment mode of feruloyl amides to the cell walls of suberized tissues, we have used thioacidolysis, a method developed to investigate lignin structure and which specifically proceeds by cleavage of ether bonds [10]. By so doing, we aimed at recovering diagnostic compounds from putative feruloyl amides ether-linked to cell wall polymers. We applied this technique to natural and wound-healing potato periderms, which were previously subjected to enzymic and solvent treatments in order to remove part of the polysaccharides and, more importantly, soluble phenolics.

During the process of oxidative coupling, the ether bonds liable to link feruloyl amides to cell wall components involve either the phenolic groups of the ferulic and/or tyramine moieties or the $\beta$-position of the ferulic side-chains. To discriminate between these etherification sites, we performed further thioacidolysis experiments on diazomethane-permethylated samples [10], in order to methylate and thereby label the free phenolic groups not involved in ether bonding. The present study reports the identification and quantitative determination of ether-linked feruloyl amides by thioacidolysis of natural or wound-healing potato tuber periderms, as well as the relative quantitation of their ether-bonding sites.

## RESULTS AND DISCUSSION

### *Release of feruloyltyramine- and feruloyloctopamine-ethanethiol adducts by thioacidolysis of suberized periderms*

Upon thioacidolysis, natural and wound periderms of potato tubers release a series of monomeric and dimeric phenolics [6, 11]. Most of them are identical to those recovered from cell wall lignins. Their total amount established the occurrence of a lignin-like polymer in

the suberin-enriched samples, at concentrations ranging between 1 and 2% at the lowest [11]. Their relative proportions revealed that this lignin-like polymer has both similarities to and differences from xylem lignin [11]. Besides the adducts derived from the lignin-like domain of suberin, thioacidolysis of potato suberized samples repeatedly yielded an unknown compound, which eluted at a much higher GC retention time. Its mass spectrum was indicative of a feruloyltyramine derivative, formed from the addition of ethanethiol to the double bond of the ferulic moiety. To confirm this assignment, we subjected feruloyltyramine to thioacidolysis and recovered, with a 90% yield, the thiol adduct **1** (Fig. 1), identical to the unknown compound released by potato periderms. The mild desulphuration of compound **1** over Raney nickel quantitatively provided dihydroferuloyltyramine (DHFT, **3**, Fig. 1) and, more importantly, made GC quantitative determination possible. The amide bond of feruloyltyramine thus essentially survived both the thioacidolysis and the desulphuration steps.

When feruloyloctopamine was treated with the thioacidolysis reagent, addition of ethanethiol on the double bond of ferulic acid also occurred, but another ethanethiol molecule reacted with the $\beta$-hydroxyl group of octopamine, yielding the dithioethyl derivative **2** (Fig. 1). Compared with **1**, this derivative proved even more difficult to analyse by GC due to its high $M_r$. As expected, desulphuration of **2** over Raney nickel also afforded DHFT.

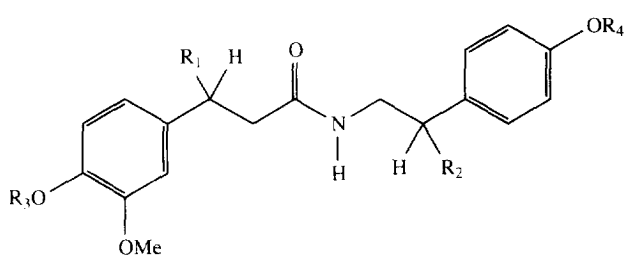
Thioacidolysis followed by desulphuration of natural and wound-healing potato periderms, previously subjected to enzymic and solvent extractions (see Experimental), liberated 6.05 and 16.38 $\mu$mol $g^{-1}$ of DHFT, respectively (Table 1). Due to the specificity of thioacidolysis towards ether cleavage and on the basis of the experiments with model compounds, this DHFT, representing 0.23 and 0.63% (by weight) of the natural and wound periderms, respectively, most likely originated from ether-linked feruloyltyramine and/or feruloyloctopamine. The desulphuration step aimed at a precise GC determination, and did not allow us to discriminate between these two sources. In the present state of our knowledge, it is not possible to know what proportion of the total cell wall-linked feruloyl amides is released by thioacidolysis. Other bonding patterns, such as those involving carbon–carbon linkages, cannot be characterized by the present method, which is only intended to cleave ether bonds.

Table 1. Yield of dihydrohydroxycinnamic acid amides recovered by thioacidolysis and Raney nickel desulphuration of natural and wound-healing potato tuber periderms ($\mu$mol $g^{-1}$ extractive-free suberin-enriched preparations). Values are means of duplicate experiments (3% standard error)

| Compound | Natural periderm | Wound periderm |
|---|---|---|
| Dihydroferuloyltyramine | 6.05 | 16.38 |
| Dihydroferuloyl-3-methoxytyramine | 0.67 | Tr |
| Dihydrocoumaroyltyramine | 0.16 | 0.49 |

Tr: trace.

Importantly, thioacidolysis liberates a considerably higher amount of amides than mild alkaline hydrolysis (1 M KOH, 37°, 4 hr) [2]. Moreover, the DHFT yield (Table 1) far exceeds the total yield of tyramine, octopamine and feruloyl amides released by this mild KOH treatment (16.38 versus 2.1 $\mu$mol $g^{-1}$ of wound periderms prepared under identical conditions) [2], as well as the amount of tyramine released by more severe alkaline hydrolyses (3% KOH for 2 hr or 2 N NaOH for 1 hr at reflux temperature) [6]. This DHFT yield is only




| | | | | |
|---|---|---|---|---|
| **1** | $R_1$ = EtS | $R_2$ = H | $R_3$ = H | $R_4$ = H |
| **2** | $R_1$ = EtS | $R_2$ = EtS | $R_3$ = H | $R_4$ = H |
| **3** | $R_1$ = H | $R_2$ = H | $R_3$ = H | $R_4$ = H |
| **4** | $R_1$ = H | $R_2$ = H | $R_3$ = Me | $R_4$ = Me |
| **5** | $R_1$ = H | $R_2$ = H | $R_3$ = H | $R_4$ = Me |
| **6** | $R_1$ = H | $R_2$ = H | $R_3$ = Me | $R_4$ = H |

Fig. 1. Structure of ethanethiol adducts (**1** and **2**) formed upon thioacidolysis of feruloyltyramine and feruloyloctopamine, respectively. These adducts were also recovered from potato periderms. After thioacidolysis and desulphuration over Raney nickel of model compounds or potato periderms, both adducts were transformed into dihydroferuloyltyramine (DHFT, **3**). When periderm samples were permethylated by diazomethane prior to thioacidolysis and desulphuration, a mixture of DHFT and of three DHFT methyl ethers (**4–6**) was recovered.

slightly exceeded by that of tyramine released by a drastic alkaline treatment (2 N NaOH, 160°, 3 hr) [6]. As thioacidolysis and desulphuration of potato periderms did not provide any detectable free tyramine, but substantial amounts of DHFT, this protocol seems an efficient alternative to alkaline hydrolyses for the estimation of insoluble feruloyl amides in suberized samples.

In natural and wound potato periderms, the DHFT yields approximate those of the degradation products released by the lignin-like polymer [11]. Similar to the adducts derived from this polymer, DHFT was recovered in an amount twice as high from wound periderm than from natural periderm (Table 1). Taken together, these data confirm that wounding efficiently triggers not only the synthesis of ferulic acid amides, together with a lignin-like polymer, but also the covalent attachment of these amides to cell walls, possibly on the lignin-like polymer template. These results argue for the oxidative incorporation of a substantial portion of feruloyl amides into potato periderms through ether linkages, particularly in wound-healing samples. Accordingly, past compositional work based on mild alkaline treatments underestimated the importance of feruloyl amides in suberized cell walls. Such a conclusion is consistent with solid state $^{13}C$ NMR data indicative of the occurrence of ferulic acid associated with wound-healing suberized cell walls through 8-O-4′ ether and phenylcoumarin bonding patterns [8].

Besides DHFT, dihydroferuloyl-3-methoxytyramine could be quantified from natural potato periderm, being recovered in trace amounts from the wound sample (Table 1). The identity of this derivative was authenticated by the appropriate model. Accompanying the feruloyl derivatives, another compound, assigned to dihydrocoumaroyltyramine (DHCT) from its mass fragmentation pattern, was recovered as a minor component. Similar to DHFT, DHCT was released in higher amounts from wound than from natural periderms (Table 1).

### *Evaluation of relative proportion of different phenolic groups involved in etherification of ferulic acid amides*

To gain a more detailed insight into the way the ferulic amides are linked to cell walls, their free phenolic groups were premethylated by diazomethane, prior to thioacidolysis and desulphuration. The completeness of the methylation performed on solid samples was ensured by a prolonged and repeated exposure to the reagent. A good indication of this completeness was afforded by the recovery of a pyrazoline derivative of feruloyltyramine from permethylated periderms (data not shown). This derivative is formed from the 1,3-dipolar addition of diazomethane to the double bond of ferulic acid [12], which proceeds at a reaction rate much slower than the methylation of acidic groups [13]. The pyrazoline derivative did not survive the desulphuration step, which regenerated the dihydroferuloyl analogues, as shown by experiments with model compounds.

Table 2. Relative frequencies of various DHFT derivatives **3–6** (Fig. 1) recovered from thioacidolysis and desulphuration of permethylated natural and wound periderms of potato tubers. Results expressed as relative molar percentages of total amount of DHFT derivatives **3–6**

| DHFT derivative | Natural periderm | Wound periderm |
|---|---|---|
| 3 | 8 | 15 |
| 4 | 52 | 33 |
| 5 | 32 | 44 |
| 6 | 8 | 8 |

Four dihydroferuloyl amide derivatives (**3–6**, Fig. 1) were recovered from permethylated samples. Their relative frequencies are shown in Table 2 and reflect the relative involvement of the phenolic groups in the etherification. It should be kept in mind that the etherification of the phenolic groups, reflected by compounds **3**, **5** and **6**, does not exclude a prior additional etherification on the ferulic side-chain.

Compound **3** (DHFT), without any methyl groups introduced by diazomethane, probably stems from feruloyl amides at least dicovalently bound to potato cell walls, through both of their phenolic groups. These etherified cross-links represent a few per cent of the total amount of the feruloyl amides released upon thioacidolysis. Interestingly, their relative concentration is twice as much in wound-healing than in natural periderm. This result suggests a higher stimulation of peroxidase activity and a more efficient strengthening of the cell walls in wound periderm.

Compound **4**, with two additional methyl groups, originates from feruloyl amides with free phenolic groups. This compound accounts for 52 and 33%, respectively, of the total DHFT derivatives (Table 2) in natural and wound periderms. As this compound was liberated from samples previously subjected to exhaustive extractions aiming at the removal of free amides, the most plausible source of **4** seems to be the amides ether-linked to cell walls at the $\beta$-position of ferulic side-chains. Such an etherification site leaves the two phenolic groups of the feruloyl amides free for methylation. One cannot completely rule out, however, the possibility of some residual free amides strongly associated with cell walls.

Compound **5**, methylated on the phenylethylamine ring, logically stems from feruloyl amides ether-linked through the ferulic phenolic group. The high proportion of **5** and **4** indicates that a significant proportion, *ca* 80%, of ether-linked feruloyl amides appears to be attached to cell walls only through the ferulic unit, leaving the phenolic group of tyramine free. This result is in agreement with electrochemical studies, which showed that the addition of a methoxy group *ortho* to the phenolic function facilitates the oxidation of the phenolic substrate [14]. The susceptibility of the ferulic moiety towards peroxidasic oxidation is also supported

by the repeated isolation of lignanamides comprising two feruloyltyramine molecules covalently linked by their ferulic moieties, from the roots of *Capsicum annum* [15] or from the fruits of *Cannabis sativa* [16–18]. Some of these dimers of feruloyltyramine, cannabisin E and cannabisin F [18], are built through an ether linkage involving both the phenolic group and the side-chain of the ferulic moiety. Other dimers show the involvement of the ferulic unit into arylnaphthalene [16, 17] or phenylcoumaran [15] bonding patterns. These various dimers were obtained *in vitro* by peroxidasic or chemical condensation of feruloyltyramine [15–18].

Symmetrically to **5**, compound **6**, methylated on the feruloyl phenolic group, probably originates from feruloyl amides ether-linked by the amine moiety. The relative proportion of **3** and **6** indicates that the etherification of the amides involves the phenolic group of tyramine or octopamine to a *ca* 20% extent. It is noteworthy that the ferulic unit is already attached through the phenolic group in **3** and possibly also through the side-chains in **3** and **6**. These results highlight the fact that ferulic acid amides of hydroxyphenylethylamines are excellent candidates for the peroxidasic cross-linking of polymers in the cell walls of potato periderms. These bridges could, however, also imply bonding patterns other than the alkyl aryl ether ones characterized herein.

In conclusion, this work has evidenced feruloyl amides etherified to the cell walls of natural or wound periderms of potato tuber and has confirmed the usefulness of thioacidolysis for the estimation of ether-linked phenolics. Interesting, our results also demonstrated the occurrence of dicovalently ether-linked feruloyl amides, particularly in wound-healing potato tuber periderm. Our results could not, however, give any indication on the localization of these amides inside the periderm. Cytochemical analysis of wound-healing reactions in potato tuber has shown that the incrustation of a lignin-like polymer in the middle lamella and primary wall precedes the deposition of suberin on the inner surfaces of cell walls in wound-healing tissues [19]. The fact that the amides are released from suberized periderms does not necessarily imply that they are directly bound to suberin. In this respect, thioacidolysis has the same limitation as other chemical [20, 21] or spectroscopic [8, 22] methods which cannot differentiate, in suberin-enriched preparations, the phenolics directly associated with suberin from those occurring in the non-suberized walls or wall layers. This study has, nevertheless, some interesting biochemical implications, since the formation of ferulic acid amide bridges further exemplifies a common strategy to reinforce plant cell walls which relies on the constitutive or stress-induced peroxidasic cross-linking of wall polymers [23, 24].

## EXPERIMENTAL

*Plant material.* Potato tubers (*Solanum tuberosum* L. cv. Bintje) were purchased locally; they were stored at 4° and equilibrated at room temp. before use. Suberin-enriched periderm residues were prepd essentially as described in ref. [25]. Tubers were surface-sterilized with $NaOCl_3$ soln for 30 min and washed with sterile $H_2O$. Tubers were then peeled and natural periderms isolated by incubation for 24 hr at room temp. on a rotary shaker in a mixt. of cellulase (10 mg $ml^{-1}$, from *Trichoderma*) and pectinase (1 mg $ml^{-1}$, from *Penicillium occitanis*), in 0.1 M K-Pi buffer (pH 6). The suberized sheets were collected from the incubation medium and ground in a mortar in liquid $N_2$. The residue was then treated again with the cellulase–pectinase mixt., thoroughly washed with $H_2O$ and then extracted successively with MeOH and $CHCl_3$ for 24 hr in a Soxhlet extractor. For the prepn of wound periderm, tuber discs (5 × 10 mm) were cut with a cork borer and allowed to wound-heal in plastic Petri dishes at 25°, in darkness, under sterile conditions. After 14 days of suberization, tissue cylinders were cut in half and slices treated as described above for natural periderm.

*Permethylation.* Natural and wound-healing potato periderms were thoroughly permethylated by repeated addition of an $Et_2O$ soln of $CH_2N_2$. Between each addition of reagent, solvent was evapd under a $N_2$ stream. Once the yellow colour of $CH_2N_2$ had been observed to stay for 24 hr, methylation was considered to be complete and the solvent was evapd.

*Thioacidolysis and subsequent desulphuration.* The thioacidolysis reagent was prepd as previously described [26]. Suberin-enriched periderms (*ca* 20 mg) were added to 10 ml of reagent in a glass tube closed with a Teflon-lined screwcap. Thioacidolysis and extraction of the thioacidolysis phenolics were then performed as previously described [11, 26]. Desulphuration over Raney Ni of the thioacidolysis mixt. and GC and GC-MS analyses of the recovered degradation products were also performed as described [11, 26].

*GC-MS identification of TMSi reaction products.* Positive EIMS (70 eV). N-[2(4-hydroxyphenyl)ethylamine]-3(4-hydroxy-3-methoxy-phenyl)-3(thioethyl)-propionamide, **1** (Fig. 1). MS [TMSi] *m/z* (rel. int.): 519 (5) $[M]^+$, 459 (11), 458 (11), 281 (8), 267 (15), 266 (13), 236 (52), 223 (33), 207 (32), 192 (58), 179 (7), 177 (6), 73 (100). **2** (Fig. 1). MS [TMSi] *m/z* (rel. int.): 579 (4) $[M]^+$, 517 (4.5), 456 (8.5), 327 (8), 266 (29), 239 (94), 234 (20), 209 (16), 207 (16), 192 (30), 179 (23), 177 (13), 73 (100). *Dihydroferuloyltyramine* **3** (Fig. 1). MS [TMSi] *m/z* (rel. int.): 459 (23) $[M]^+$, 444 (7), 267 (62), 252 (15), 237 (9), 209 (31), 207 (8), 193 (25), 192 (100), 179 (29), 177 (16), 73 (89). **4** (Fig. 1). MS [TMSi] *m/z* (rel. int.): 343 (14) $[M]^+$, 267 (3), 209 (81), 164 (19), 151 (84), 134 (100), 121 (35), 107 (9), 91 (14), 91 (16), 77 (18). **5** (Fig. 1). MS [TMSi] *m/z* (rel. int.): 401 (23) $[M]^+$, 267 (79), 252 (28), 237 (23), 222 (5), 209 (58), 192 (11), 179 (19), 149 (10), 134 (82), 121 (33), 91 (9), 73 (100). **6** (Fig. 1). MS. [TMSi] *m/z* (rel. int.): 401 (5) $[M]^+$, 264 (5), 209 (51), 192 (100), 179 (14), 177 (16), 164 (13), 151 (48), 91 (5), 73 (54). *Dihydroferuloyl-3-methoxytyramine*. MS [TMSi] *m/z* (rel. int.): 489 (13) $[M]^+$,

474 (4), 267 (15), 237 (10), 222 (100), 209 (17), 207 (15), 192 (29), 179 (14), 73 (80).

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