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Original article

Characterization of lucidin formation in *Rubia tinctorum* L.

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Abstract

In order to approach lucidin formation (a strong mutagen or a carcinogen) from a physiological standpoint, hairy roots of *Rubia tinctorum* L. were established by a transformation of *Agrobacterium rhizogenes* strain 15834 and cultured in a liquid woody plant medium without plant hormones. The anthraquinone pigment composition of the intact hairy roots was essentially the same as that of the intact non-transformed (normal) roots, in which lucidin *O*-β-D-primeveroside (LuP) was one of the major pigments. Lucidin was scarcely detected in the intact hairy roots, but was a main pigment after the squash treatment. The crude protein extract of intact hairy roots exhibited LuP-glycosidase activity (an activity converting LuP to lucidin). This activity was also detected in the roots of the normal plants at a high level, but slightly in the stems and not in the leaves. Methyl jasmonate enhanced the LuP production and LuP-glycosidase activity in the hairy roots. On the other hand, ethephon or salicylic acid had either no effect or rather an inhibitory effect on them. After partial purification of LuP glycosidase, the resultant active fraction producing a major band with an apparent Mr of 68 kDa exhibited the substrate specificity for both aglycon and sugar-moiety. The sugar released from LuP by this fraction was neither D-glucose nor D-xylose and was hydrolyzed into them. These results suggest that LuP specific β-primeverosidase (EC 3.2.1.149) exists in the roots of *R. tinctorum* and is involved in the systematic defense system.
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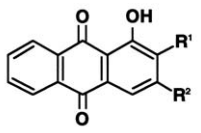
Keywords: Hydroxyanthraquinone; Lucidin; β-Primeveroside; *Rubia tinctorum*; Wounding

1. Introduction

Rubia tinctorum L. (Rubiaceae) is widely distributed in southern and southeastern Europe, in the Mediterranean area, and in central Asia [5]. Its reddish roots (madder roots) contain various secondary metabolites, mainly hydroxyanthraquinones, and these compounds are used for the dyeing of textiles and in the treatment of kidney and bladder stones. On the other hand, there are many reports on the genotoxicity of this plant with or without the addition of a metabolic-activating system [3,15,24,27]. Almost all these studies ascribed the genotoxic activity of this plant to lucidin (1,3-dihydroxy-2-hydroxymethyl-9,10-anthraquinone; Fig. 1) and its derivatives. Furthermore, lucidin exhibited cytotoxic activity as determined by the Ames test using *Salmonella typhimurium* TA100 [15]. In intact plants, the majority of lucidin is present in the form of 6-*O*-β-D-xylopyranosyl-β-D-



	R¹	R²
1	CH_2OH	O-glc^{6}→^{1}xyl
2	CH_2OH	OH
3	O-glc^{6}→^{1}xyl	H
4	OH	H

Fig. 1. Structures of lucidin-3-*O*-β-primeveroside (LuP, 1), alizalin-3-*O*-β-primeveroside (AlP, 3), and their aglycons (lucidin, 2; alizarin, 4). glc, D-glucose; xyl, D-xylose.

Abbreviations : AlP, alizarin 2-*O*-β-primeveroside ; ET, ethephon ; fw, fresh weight ; HPLC, high performance liquid chromatography ; LuP, lucidin 3-*O*-β-primeveroside ; MJ, methyl jasmonate ; NP, nitrophenyl ; SA, salicylic acid ; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis ; TLC, thin layer chromatography ; WP, Woody Plant.

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glucopyranoside, specifically lucidin 3-*O*-β-primeveroside (LuP; Fig. 1), which gets converted to lucidin by endogenous enzyme(s) [6,20]. In our preliminary experiment, an increase in lucidin and a decline in LuP were observed in the wounded regions of the roots of *R. tinctorum*. This analogy has been found in many other secondary plant metabolites. For example, naturally occurring glycosides, e.g. cycasin and rutin, are also inactive per se as determined by mutagenicity tests; however, when these are hydrolyzed by the action of glycosidases, stable or unstable aglycons with mutagenic properties are formed [26]. In some cases, the activation of such secondary metabolites is catalyzed by exogenous or endogenous general glycosidases. On the other hand, some plants prepare particular glycosidases, which exhibit aglycon specificity and/or sugar-moiety specificity for the activation of secondary-metabolite glycosides [13]. Although it was reported that LuP in a crude pigment solution was converted to lucidin by incubation with acetone powder prepared from fresh madder roots [6], most of the characteristics of this enzyme have not been clarified. In this study, we focused on the property of the wound-induced lucidin formation in fresh roots of *R. tinctorum* from a physiological viewpoint and its characterization, including partial purification of LuP glycosidase. We established a hairy root culture of this plant by an infection of *Agrobacterium rhizogenes*, which proliferated vigorously in the liquid culture medium without plant hormones. As a result, large quantities of intact roots were easily obtained and used as the main plant material in this study.

2. Results

2.1. Lucidin formation in fresh roots

The pigment composition of the roots of non-transformed (normal) plants and the hairy roots of *R. tinctorum* was analyzed by high performance liquid chromatography (HPLC) (System I) (Fig. 2). The HPLC profiles of the two intact (not squashed) materials were essentially the same, in which LuP and higher hydrophilic pigments were the main pigments. After the hairy roots were squashed with a pestle, their pigment composition changed drastically in quality. Hydrophilic pigments were observed to diminish, while lucidin and other lyophilic pigments were found to increase. Alizarin 2-*O*-β-primeveroside (ruberythric acid, AlP) (Fig. 1), which was the major anthraquinone glycoside in mature madder roots [25], was not detected in the materials used in this study (Fig. 2). Minor peaks detected around 18.5 min (the retention time of AlP in System I) in Figs. 2A and 2B, did not correspond to that of AlP, because their UV–Vis spectra were apparently different from that of AlP (data not shown).

Lucidin was scarcely detected in the intact hairy roots (Fig. 2B). In the time course experiment, the increase of lucidin was distinguishable at 1.5 min after the squash treatment and held for 10 min at least, but the long incubation period caused a decrease in the lucidin content (Fig. 3).

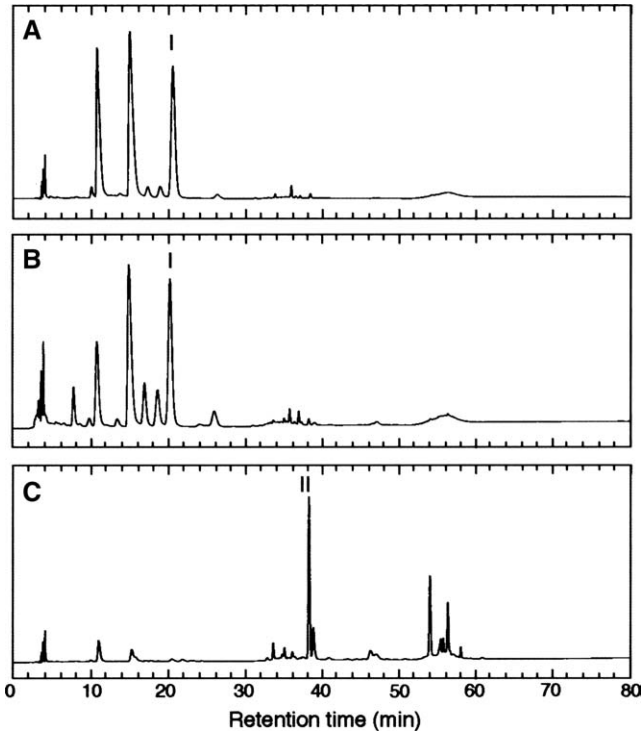

Fig. 2. HPLC profiles of the roots of normal plants (A) and the hairy roots (B and C) at 450 nm. Intact materials (A and B) and a squashed material (C), left for 10 min after the squash treatment, were extracted with 90% (v/v) ethanol at 80 °C for 10 min and at room temperature overnight. Each extract was concentrated and subjected to HPLC analysis (System I). Peaks obtained were designated as follows: I, LuP; II, lucidin.

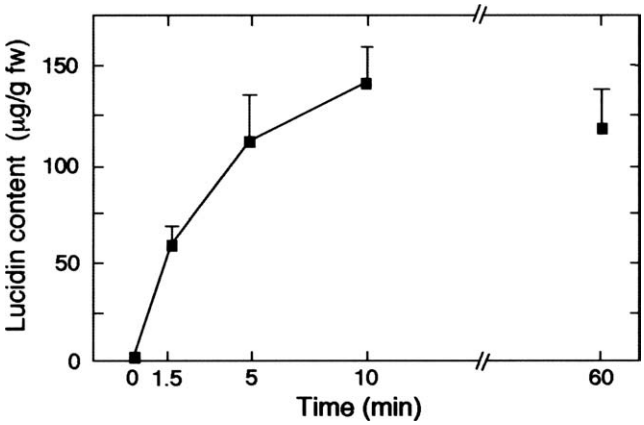

Fig. 3. Time course of lucidin formation after the squash treatment of the hairy roots. Hairy roots squashed with a pestle were periodically harvested and extracted with 90% (v/v) ethanol. Lucidin content was quantified from the peak area of the HPLC profile at 450 nm. Each point represents the mean and standard deviation (S.D.) of independent samples. ($N = 4$).

2.2. Distribution of LuP and LuP-glycosidase activity

Crude protein extract prepared from the intact (such as fresh and not wounded) hairy roots exhibited the activity converting LuP to lucidin (hereafter designated as 'LuP-glycosidase activity'). We examined the distribution of LuP-glycosidase activity and LuP in the normal plants (Fig. 4). LuP was found to be abundantly accumulated in plant roots but not in stems and leaves. LuP content in the plant roots

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