Pergamon

PII: S0031-9422(97)00676-6

Phytochemistry, Vol. 47, No. 5, pp. 743–747, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

CONVERSION OF 3-DEMETHYLTHIOCOLCHICINE INTO THIOCOLCHICOSIDE BY *CENTELLA ASIATICA* SUSPENSION CULTURES

N. BOUHOUCHE, J. M. SOLET, A. SIMON-RAMIASA, J. BONALY* and L. COSSON†

Laboratoire de Botanique et Phytochimie, * Laboratoire de Biologie Cellulaire, Université Paris-Sud, Faculté de Pharmacie, 5 Rue J. B. Clément, 92296 Châtenay-Malabry Cedex, France

(*Received in revised form* 4 *June* 1997)

Key Word Index—*Centella asiatica*; Apiaceae; cell suspension culture; glucosylation; 3-de-methylthiocolchicine; thiocolchicoside; glucosyltransferase.

Abstract—Exogenously supplied 3-demethylthiocolchicine was converted into 3-*O*-glucosylthiocolchicine (thiocolchicoside) by a cell suspension culture of *Centella asiatica*. Around 30% of 3-demethylthiocolchicine (136 μM) was glucosylated after an 11-day incubation period. *In vitro* glucosylation by cell-free extracts demonstrated that the enzymatic reaction required specifically uridine diphosphate-D-glucose (UDPGlc) as a high energy glucose donor. Various endogenous phenolic compounds were assayed for their effect on the glucosyltransferase reaction.

INTRODUCTION

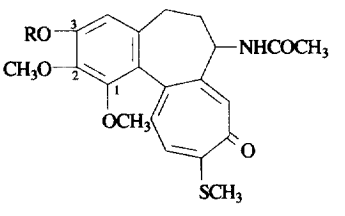
Plant cell cultures are able to glucosylate a variety of exogenously supplied compounds, such as simple phenols [1–3], coumarins [4], flavonoids [5] and cardenolides [6]. Glucosylation plays a key role in endogenous secondary metabolite storage because glucosylated metabolites show decreased chemical reactivity and increased water solubility. Glucosylation is also considered as a mechanism for xenobiotic detoxification, such as agrochemicals [7, 8] and as a method for synthesis or conversion of molecules with useful biological activities [9]. Previous studies have shown the ability of a cell suspension of *Centella asiatica* to metabolise thiocolchicine, a hemisynthetic compound [10]. A two-step metabolic process could involve hydroxylation of the 2-*O*- and 3-*O*-methoxyl group of the molecule leading to 2- and 3- demethylthiocolchicine, followed by glucosylation leading to 2-*O*- and 3-*O*-monoglucosylderivatives. 3-*O*-Glucosylthiocolchicine (thiocolchicoside) is of special interest due to its analgesic and myorelaxant properties [11]. It is commonly used (Coltramyl®, Roussel Laboratories) in rheumatology, orthopaedics and traumatology, for the treatment of muscular spasms of central origin.

This paper reports on the direct glucosylation of 3-demethylthiocolchicine (**1**) by *C. asiatica* cell suspensions. We also investigated the enzymatic system, i.e. glucosyltransferase, involved in this glucosylation process.

RESULTS AND DISCUSSION

3-*Demethylthiocolchicine supply and glucoside formation*

Our preliminary experiments [10] have shown that cell suspension cultures of *C. asiatica* could glucosylate exogenously supplied **1** into its corresponding *O*-glucoside, thiocolchicoside (**2**). The glucosylated product was identified by direct comparison with an authentic sample using HPLC analysis. Because of the toxicity of **1** [12, 13], the relationship between addition time and efficiency of glucosylation was investigated. **1** was added at 136 μM to cell suspension cultures at



3-Demethylthiocolchicine (1) R = H

3-O-Glucosylthiocolchicine (Thiocolchicoside) (2) R = ß-glucosyl

† Author to whom correspondence should be addressed.

Table 1. Effect of time of supply of **1** on **2** production; *C. asiatica* cell cultures were incubated with **1** (136 μM) for seven days

Day of administration	Glucoside concentration (μM)	Glucosylation efficiency (%)
0	2.5	1.8
7	24.0	17.6
14	10.5	7.7

Glucoside concentration indicates total amounts of thiocolchicoside in both cells and medium.

different stages of growth, *viz*, subculture time (day 0), exponential phase (day 7) and stationary phase (day 14). After a 7-day incubation period, total glucoside concentrations in cells and media were measured (Table 1). When **1** was added at subculture time, efficiency of glucosylation was very low (1.8% of initial substrate added) due to the antimitotic effect of the substrate. Addition at exponential and stationary phase led to higher yields 17.6% and 7.7%, respectively. This increase can be explained by biomass increase during cell growth. Addition of **1** at stationary phase, rather than at exponential phase, did not give a better yield of **2** with a 7-day incubation period. Addition at day 14 led to nutrient exhaustion and cell lysis affecting glucosylation efficiency. Thus, in biotransformation experiments, addition of **1** at 7-day-old cells appeared to be a good compromise between sufficient biomass required for biotransformation and an incubation period that led to a maximum yield of glucoside. The relationship between **2** yield and **1** concentration up to 306 μM is shown in Fig. 1. No decrease in the dry weight (ca 10 g l^{-1}) of the cells was observed after administration of **1**. The amount of **2** increased with the increase of **1** up to 200 μM. At higher concentrations, glucoside production was nearly stable and independent of **1** concentration.

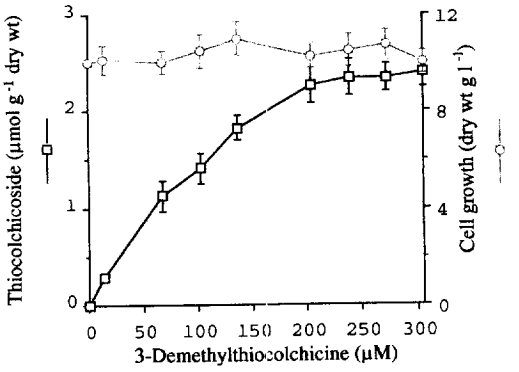


Fig. 1. Effect of **1** concentration on *C. asiatica* growth and thiocolchicoside production. **1** was administrated to 7-day-old cells with an incubation period of 7 days.

Moreover, intracellular concentration of **1** further increased (data not shown). This result could be explained by a saturation of the enzymatic system involved in this reaction.

Time-course of thiocolchicoside formation

1 was administered to *C. asiatica* cell suspension cultures at the exponential growth stage. Amounts of **1** and **2** were monitored in both cells and culture medium for 14 days (Fig. 2). The amount of **1** in the medium decreased gradually during the incubation period (Fig. 2A). This decrease was accompanied by a corresponding increase of glucoside in the cells (Fig. 2B) from 6.5 μM (5%) after 1 day to ca 40 μM (30%) after 11 days. There was evidence that during this time **1** is glucosylated to the corresponding *O*-glucoside. **2** accumulated mostly in the cells (89%) and negligible amounts were released in the medium. Furthermore, microscopic observation of the cells after 2 days incubation with **1** showed a substantial increase of vacuoles (1.5 fold). Accordingly, the glucoside most probably accumulated in these organelles. This is supported by the general assumption that the cell vacuole is the main storage site for glycosides [14], which could arise either from endogenous secondary metabolism [15, 16] or from xenobiotic detoxification, such as the β-D-glucoside conjugates of the herbicide 2,4-dichlorophenoxyacetic acid in soybean [17].

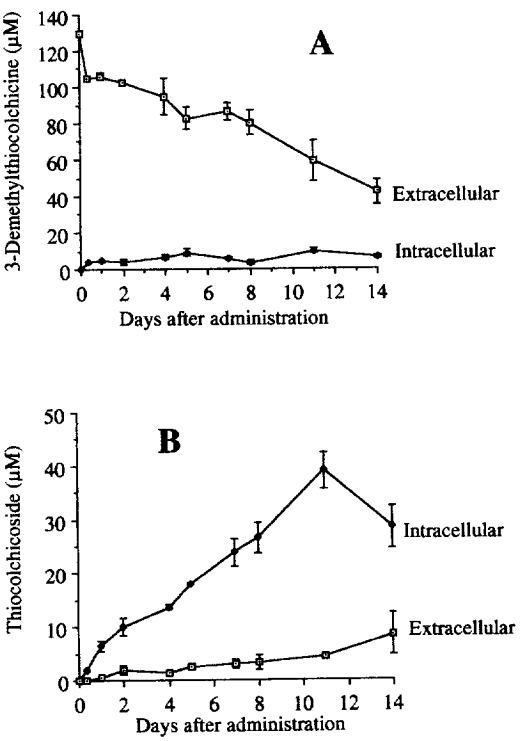


Fig. 2. Changes in amounts of substrate (A) and glucoside (B) in the cells and medium of *C. asiatica* suspension cultures after administration of **1** (136 μM) at exponential phase.

In vitro *studies on the biosynthesis of* 3-O-*glucosyl-thiocolchicine*

Glucosylation of **1** into its *O*-glucosylderivative by *C. asiatica* cell suspension cultures suggested the presence of a glucosyltransferase with activity towards this compound. Such an enzyme has not yet been described. *In vitro* enzymatic activity was first investigated with crude extracts from 7-day-old cells. Boiling of the protein extract resulted in a loss of product formation showing the transferase reaction was dependent on the presence of active protein. An *in vitro* enzyme assay was successfully carried out with an ammonium sulphate (30–70%) precipitated protein fraction. The desalted crude enzyme was incubated with UDPGlc (uridine diphosphoglucose), AMP (adenosine monophosphate) and **1**. The specificity of this enzyme towards the glucose donor was investigated. UDPGlc was the most efficient co-substrate and TDPGlc (thymidine disphosphoglucose) showed a very slight activity (3% relative to UDPGlc). The other glucose nucleotides investigated [ADPGlc (adenosine diphosphoglucose), CDPGlc (cytidine diphosphoglucose) and GDPClc (guanosine diphosphoglucose)] exhibited no effect. This preference for UDPGlc as a sugar donor is quite typical for plant glucosyltransferases [18, 19].

The glucosylation reaction rate was proportional to protein concentration up to 2 mg per assay. Product formation was linear with time up to 15 h incubation at a protein concentration of 500 μg per assay. Enzyme activity was not enhanced by addition of mono- or divalent cations, like sodium, potassium, calcium and magnesium at 1 and 10 mM. Moreover, inhibition of this activity was observed after addition of metal ions at either 1 mM or 10 mM: inhibitions were, respectively, 16 and 65% for manganese, 57 and 100% for zinc, and 75 and 92% for copper. These results indicate that the glucosyltransferase does not require mono- or divalent ions for optimal activity. However, excess levels of metal can be inhibitory.

In order to study substrate specificity of this reaction, we carried out a series of enzymatic assays with various phenolic compounds. We also tested asiatic acid (Roche Nicholas Laboratories), a specific endogenous triterpenoid of *C. asiatica* [20], known for various biological activities, especially wound-healing [21]. These compounds were tested for their effect on **1** glucosylation. Table 2 presents the results of enzyme activity in the presence of both substrate **1** and the compound tested at 1 mM as compared to activity with **1** alone. Reference specific activity was ca 20 pmol min^{-1} mg^{-1} protein. Six of the eight compounds tested showed a significant inhibition of the activity. Phenol and catechin did not affect enzyme activity. For resorcinol, saligenin, naringenin, salicyclic acid and *O*-coumaric acid, inhibition ranged from 9% for salicyclic acid to 91% for naringenin. Inhibition with asiatic acid was 53%. When the test was performed at 2 mM, catechin exhibited significant inhibition (74%).

Table 2. Effect of various phenolic compounds on glucosyltransferase activity on crude enzyme extract

Substrate (1 mM)	Relative glucosyltransferase activity (%)	s.d. (%)
Control	100	7
Phenol	97	2
Resorcinol	76	4
Saligenin	83	1
Salicyclic acid	91	2
O-Coumaric acid	62	8
Catechin	100	8
Naringenin	9	11
Asiatic acid	53	7

Quantification of inhibition was based on enzyme inhibition by comparison with control containing **1** alone. s.d.: standard deviation based on 3–5 replicates (100% specific activity was 20 pmol min^{-1} mg^{-1}).

Our results demonstrate a clear inhibition of the enzymatic activity by a range of endogenous simple phenolics and flavonoids, thereby suggesting a broad substrate specificity. Many other plant glucosyltransferases show a broad substrate specificity towards endogenous substrates, such as the flavonoid transferases isolated from strawberry fruits [22] and grapefruit seedlings [2]. Also, enzymes isolated from soybean seedlings [24] exhibited a wide substrate specificity for quite different classes of endogenous secondary metabolites and moreover, were, associated with metabolism of the herbicide, bentazone. Thus, a broad specificity seems to be a characteristic of some plant enzymes involved in secondary metabolism.

We attempted to purify the glucosyltransferase from cell-free extracts prepared from cell suspensions. After ammonium sulphate fractionation, the enzyme preparation was passed through an anion-exchange (DEAE-Trisacryl) and gel filtration (Sephadex G-100) columns. In both chromatographic systems, a single peak of glucosyltransferase activity was observed (data not shown). The enzyme was purified 30-fold with a yield of 20%. Hydrophobic interaction- and affinity-chromatography (Phenyl Sepharose and UDPG-agarose, respectively) resulted in a complete loss of activity. Further purification of the enzyme is in progress.

EXPERIMENTAL

Cell cultures

The culture strain used was originally established from stem segments of *C. asiatica* L. [25] and maintained for 13 years in liquid Murashige and Skoog medium [26], supplemented with 2,4-D (10^{-6} M), 6-benzylaminopurine (10^{-6} M), sucrose (30 g l^{-1}) and casein hydrolysate (500 mg l^{-1}). Suspensions were subcultured every two weeks by inoculation of 3.5 ml

of cell suspension into 250 ml conical flasks containing 70 ml of fr. medium. Flasks were maintained on a gyratory shaker (89 rpm) at 26° with 16 h photoperiod (2500 erg cm^{-2} sec^{-1}). Under these culture conditions, a latent phase was found between 0 and 3 days, an exponential growth phase from 3 to 12 days and a stationary phase from 12 to 14 days.

Glucosylation experiments

3-Demethylthiocolchicine (**1**) (Roussel Laboratories) dissolved in 70% EtOH (20 mg ml^{-1}) was aseptically administered to cell suspension cultures seven days after subculture. Concn in the medium was adjusted to 136 μM.

Metabolite extraction

Cells were harvested on a filter, immediately frozen at −20°, then extracted by ultrasonic treatment in MeOH–H_2O (1:1). After centrifugation at 7500 *g*, the supernatant was isolated and the pellet extracted 2 more times. Supernatants were pooled and evapd to dryness under red. pres. The culture medium was also evapd. Both residues were resuspended in filtered MeOH–H_2O (2:3). After centrifugation, extracts were filtered through a 0.45 μm membrane before HPLC analysis. Concns ranging from 13 to 306 μM of **1** were added to 250 ml flasks containing 7-day-old cells in fr. medium, reaction taking place during seven days in the above described cell culture conditions. Glucoside amounts were measured by HPLC.

Reverse-phase HPLC

Amounts of **1** and **2** in the cells and medium were separately analysed by RP-HPLC using a LichroCart column C18 (125 × 4 mm, 5 μm particle size, Merck). Isocratic elution was performed under the following conditions: mobile phase: MeOH–H_2O (2:3); flow rate: 1 ml min^{-1}; detection by A at 360 nm. R_t (min): **1**, 12.7 and **2**, 3.3. The glucosylated product isolated from the cells was previously identified as thiocolchicoside [10] and confirmed in this study by direct comparison with an authentic hemisynthetic sample (Roussel Laboratories). Efficiency of glucosylation is expressed as a percent of initial substrate concn. The data (mean ± s.d.) were obtained from 3–5 replicated cultures.

Enzyme extraction

All procedures were carried out at 0–4°. Fr. cultured cells were resuspended in 1.5 ml g^{-1} fr. wt of 0.2 M Tris-HCl pH 7, containing 15 mM 2-ME and 2 mM EDTA (Buffer A). After adding 20 g l^{-1} insol. PVP (Polyclar AT), cells were homogenized with an Ultra-Turrax homogenizer, then extracted by ultrasonic treatment. The mixt. was filtered through cheesecloth and centrifuged for 15 min at 8000 *g*. Proteins in the supernatant were fractionated with $(NH_4)_2SO_4$ and the protein fr. between 30 and 70% satn, which contained most of the UDPGlc: 3-demethylthiocolchicine-*O*-glucosyltransferase activity, was collected by centrifugation for 30 min at 10000 *g*. The pellet was dissolved in 50 mM Tris-HCl pH 7.5, containing 10 mM 2-ME and 1 mM EDTA (Buffer B), and desalted by dialysis, stirring overnight against the same buffer. The desalted prepn was centrifuged and is referred to as enzyme prepn.

Glucosyltransferase assay

A standard incubation mixt. (1 ml) contained in buffer B, 1.5 mM **1**, 3 mM UDPGlc and 3 mM AMP. Reaction was initiated by addition of enzyme soln (500 μg protein), carried out at 37° for 3 h (the reaction being linear up to 15 h) and stopped by the addition of 100 μl 20% TCA. Denatured proteins were pptd by centrifugation for 10 min at 4000 *g* and glucoside content in the supernatant determined by RP-HPLC. Assays with phenolic compounds were carried out using the same conditions as described above, in the presence of **1** at 1 mM. Estimation of the relative enzyme activity is referred to reaction with **1** alone. Due to the water-insolubility, some phenolic compounds were dissolved in 70% EtOH and the percentage of EtOH in the assay mixture was adjusted to 7% for all assays. All cations tested were in the Cl form.

Protein determination

Protein content was determined using Bio-Rad dye-binding reagent with BSA as a standard [27].

Acknowledgements—We are grateful to Mr R. Spagnoli (Roussel Laboratories) and to Mr A. Loiseau (Roche Nicholas Laboratories) for the generous gifts of thiocolchicine derivatives and asiatic acid, respectively.

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