Endogenous hydrolysis of glucotropaeolin to benzyl isothiocyanate in hairy root cultures of *Tropaeolum majus* L.

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Summary

The conditions of endogenous hydrolysis of glucotropaeolin to bioactive benzyl isothiocyanate for *T. majus* hairy root cultures giving high yields of glucotropaeolin and myrosinase were optimized. After *in vitro* glucotropaeolin hydrolysis at pH 5.5, optimal for myrosinase activity, 54% of glucotropaeolin were converted to benzyl isothiocyanate, 22% to benzyl cyanide, and 1.9% to benzyl thiocyanate. During endogenous glucotropaeolin hydrolysis, maximal benzyl isothiocyanate yield, 87% of tissue glucotropaeolin, was detected at pH 7.5, in the 60th min of incubation. In the presence of 100 μM ascorbic acid, the rate of hydrolysis increased and in the 60th min, 99% of glucotropaeolin was converted to benzyl isothiocyanate. The highest benzyl isothiocyanate yield, 0.746 mmol/10 g fresh weight, was obtained during endogenous hydrolysis at pH 7.5, in the presence of 100 μM ascorbic acid from the hairy roots with glucotropaeolin content enhanced by precursors (phenylalanine + cystein) and inhibitor of phenylalanine ammonia-lyase activity (L-1-amino-2-phenylethylphosphonic acid).

Key words:

ascorbic acid, benzyl cyanide, benzyl glucosinolate, benzyl isothiocyanate, benzyl thiocyanate, hairy root homogenates, myrosinase-catalyzed hydrolysis, nasturtium, pH.

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1. Introduction

Glucosinolates are anionic β-D-*S*-glucosides found in 16 families of dicots. They share a common structure but varying aliphatic, aromatic or indolyl aglycon chains. In their native form, glucosinolates show low biological activity but they always co-exist, in different compartments, with myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) which, following tissue damage, hydrolyses them to a group of bioactive compounds (for review: 1). These breakdown products exhibit toxic activity against herbivorous insects, plant and human pathogens and other microorganisms (2-5). Glucotropaeolin (benzyl glucosinolate) is an aromatic glucosinolate characteristic of *Tropaeolum majus* L. (nasturtium). Upon hydrolysis of glucotropaeolin by myrosinase, an unstable aglucon intermediate is formed, which spontaneously rearranges to form benzyl isothiocyanate (BITC), benzyl cyanide (phenylacetonitrile, BC), benzyl thiocyanate (BTC), benzyl carbonium ion, benzyl alcohol, benzaldehyde or ascorbigen (6-8) (fig. 1). The structures of glucosinolates and reaction conditions such as: temperature, hydration, pH, the presence of ascorbic acid, thiol compounds, ferrous ions have, the potential to influence the hydrolysis and type of hydrolysis products (9-12). During myrosinase–catalyzed hydrolysis of endogenous glucosinolates, in aqueous homogenates of disrupted tissues, pH was found to influence not only the activity of myrosinase and the ratio of isothiocyanates to nitriles, but also the further degradation of isothiocyanates (13,14).

In numerous studies BITC has been shown to inhibit phase I detoxification enzymes and induce mammalian phase II detoxification enzymes, thus preventing the initiation of cancer induced by chemical carcinogens. BITC has been also shown to induce apoptosis of malignant and putative malignant cells, exhibiting chemotherapeutic properties in addition to chemoprotection (15-18). Nowadays, plant cell and tissue cultures appear to be the promising approaches for both the search for new antitumor compounds and as an alternative, a precisely controlled technology of the efficient production of selected plant-derived drugs (19-21). Hairy root cultures in particular have become the focus of interest because of their infinite and rapid growth, metabolic stability and ability to synthesize some secondary metabolites at the level comparable to, or even higher than that in planta (22-24).

We have already reported the establishment of hairy root cultures of *T. majus* that are capable to produce glucotropaeolin and myrosinase in yields higher than that in *in vitro* plant cultures. Moreover, we obtained enhanced glucotropaeolin production from the cultures by: medium optimization, addition of precursors (Phe and Cys), elicitation (salicylates and methyl jasmonate), addition of inhibitor of L-phenylalanine ammonia-lyase (PheP), and combination of these techniques (25,26). The aim of this research was to establish optimal conditions for highly productive endogenous myrosinase-catalysed hydrolysis of glucotropaeolin to BITC in homogenates of *T. majus* hairy roots.

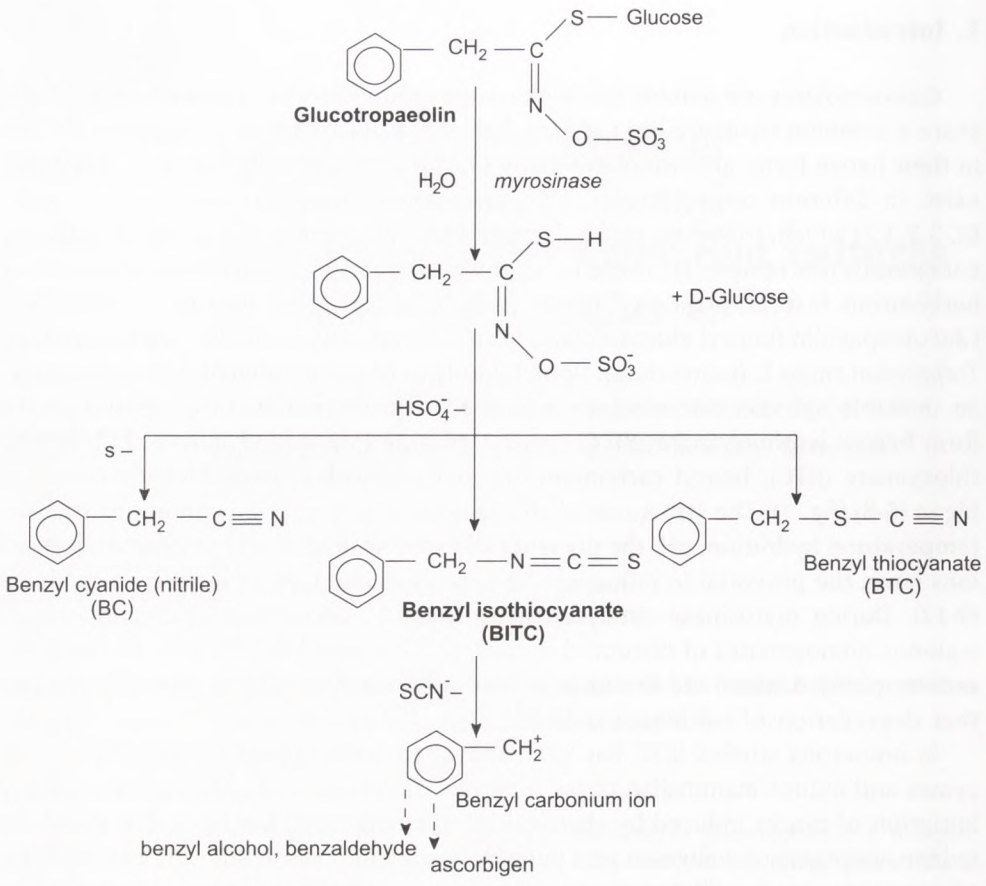

Fig. 1. Products of myrosinase-catalyzed hydrolysis of glucotropaeolin.

2. Material and methods

2.1. Hairy root cultures

Hairy roots were obtained after the transformation with *Agrobacterium rhizogenes* LBA 9402 (Ri 1855). The cultures were maintained in 300 ml Erlenmeyer flasks with 100 ml of liquid B5 (27) medium containing 3% sucrose and 0,02% peptone from casein (pH 5.8), on the orbital shaker (110 rpm), in the dark, at 22°C. Hairy roots were subcultured every 3 weeks. For stimulation of glucotropaeolin production, 9-day-old hairy roots were treated for 24 hours with the mixtures of: Phe (0.6 mM)+Cys (0.6 mM)+PheP (0.5 mM); Phe (0.6 mM)+Cys (0.6 mM)+ASA (0.2 mM); or PheP (0.5 mM)+ASA (0.2 mM). After these treatments, the cultures

were drained off the culture medium and transferred to the fresh B5 medium for 6 days. Control hairy roots were treated with water and then transferred and cultured in the same manner. In all experiments we used hairy roots from the 6th day after treatment.

2.2. Glucotropaeolin content and myrosinase activity

Glucotropaeolin and myrosinase were extracted from fresh material (FW) and analyzed according to the method described elsewhere (25). One unit enzyme is defined as the amount of myrosinase hydrolyzing 1 μM of glucotropaeolin in 1 min.

2.3. *In vitro* myrosinase-catalyzed hydrolysis of glucotropaeolin

25 μl of enzyme extract from control cultures were mixed with 500 μl of citrate buffer (50 mM, pH 5.5) and 250 μl of 0.2% glucotropaeolin (Merck). Hydrolysis was conducted at 37°C for 30 min.

2.4. Endogenous myrosinase-catalyzed hydrolysis of glucotropaeolin

To study the influence of pH and L-ascorbic acid (Asc) on glucotropaeolin hydrolysis, hairy roots from control cultures were used. Hairy roots were drained off, dried with filter paper, and 2 g of roots (FW) were used for quantification of initial (t_0) glucotropaeolin content. For glucotropaeolin hydrolysis, roots (10 g FW) were homogenized in ice bath with 100 ml of 50 mM sodium phosphate buffer. To study the influence of pH the buffers with pH values 6.5, 7.0, 7.5, and 8.0 were used. To study the influence of Asc the buffers supplemented with Asc at concentrations 50 μM, 100 μM, 150 μM were used. The hydrolysis in roots with enhanced glucotropaeolin content was analysed using the buffer with pH 7.5 supplemented with 100 μM Asc. Hydrolysis was conducted at 37°C for 90 min, and the products of glucotropaeolin hydrolysis were analyzed in 15 min intervals. Hydrolysis was stopped with dichloromethane (1 ml after *in vitro* hydrolysis and 50 ml after endogenous hydrolysis) and cooling down the reaction mixture to 0°C.

2.5. Analysis of products of glucotropaeolin hydrolysis

Hydrolysis products were extracted at 8°C with dichloromethane (3 × 1 ml after *in vitro* hydrolysis and 3 × 50 ml after endogenous hydrolysis) while shaking. The dichloromethane phase was separated by centrifugation, collected, dried over anhy-

drous Na_2SO_4, and evaporated under vacuum in the rotary evaporator. The residue was dissolved in 5 ml acetonitrile, centrifuged, filtered, and analyzed. HPLC was performed on Hypersil RP C18 column (250 × 4 mm, 5 μm particle size) at 246 nm, with gradient elution 55-65% acetonitrile in 0.1% trifluoroacetic acid, at flow rate of 0.8 ml/min (28, modified). The isolated BITC, BTC and BC were identified using HPLC by comparison of their retention times with the retention times of standards BITC (Fluka), BTC (Fluka), and BC (Sigma), respectively. Quantification of the compounds was based on the calibration curve of standards. Yields of products of glucotropaeolin hydrolysis are expressed as percentage of initial (t_0) glucotropaeolin, and BITC yield as mmol/10 g FW.

All extractions were done in duplicates. The data presented in this work are expressed as means ± standard deviation (SD) of 3 independent experiments.

3. Results

To identify the types of compounds possibly formed from glucotropaeolin (gltr) by the action of *T. majus* hairy roots myrosinase *in vitro* myrosinase-catalyzed hydrolysis was performed. *In vitro* hydrolysis was conducted using commercial glucotropaeolin and myrosinase (activity 53.0 ± 6.73 U/g DW) isolated from hairy roots, at pH 5.5, optimal for its activity. Three products of glucotropaeolin hydrolysis: BITC, BTC and BC were identified by HPLC on the basis of retention times of standards. HPLC analysis showed that after 30 min of *in vitro* myrosinase-catalyzed hydrolysis at pH 5.5, over 93% of initial (t_0) glucotropaeolin disappeared (fig. 2). BITC was the dominant product and its yield was about 58% of products (54% of t_0 glucotropaeolin). BC was also formed to a considerable extent; its yield was about 23.6% of products (22% of t_0 glucotropaeolin). Small amount of BTC (1.9% of products) was also detected. About 15.3% of t_0 glucotropaeolin was converted into unidentified compounds (N.I.) (fig. 2).

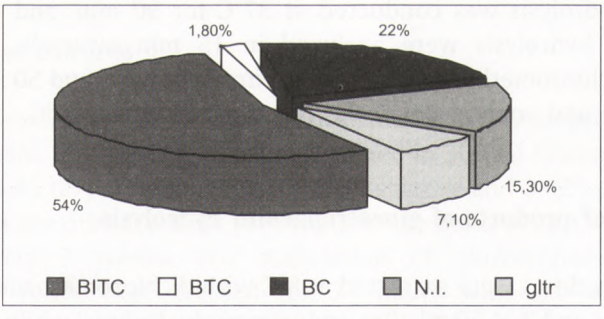

Fig. 2. HPLC profile of compounds produced after *in vitro* hydrolysis of glucotropaeolin, the conditions: pH 5.5, incubation time 30 min (n = 6).

Endogenous myrosinase-catalyzed hydrolysis of glucotropaeolin was conducted in hairy root homogenates (glucotropaeolin content 57.3 ± 6.07 mg/g DW). The influence of pH on BITC yield was studied for the pH range 6.5-8.0. At all studied pH values (6.5, 7.0, 7.5, 8.0), BITC was the dominant product of glucotropaeolin hydrolysis. It was found that the higher pH of incubated homogenates, the lower rate of glucotropaeolin hydrolysis, but more BITC and less BC were formed (fig. 3). At

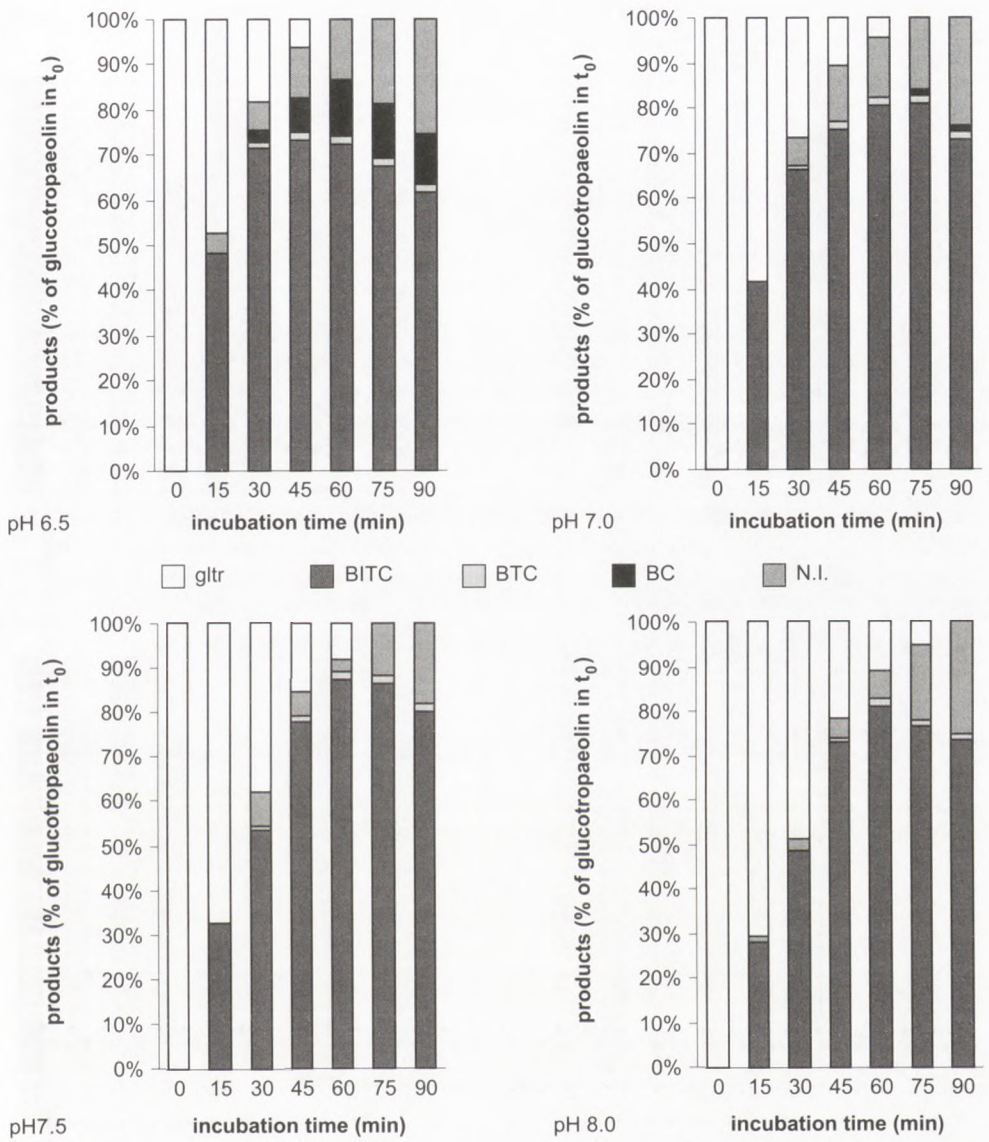

Fig. 3. Influence of pH and incubation time on the type and ratio of compounds produced during endogenous hydrolysis of glucotropaeolin (n = 6).

pH 7.5 no BC formation was detected. The highest yield of BITC, accounting for 87% of t_0 glucotropaeolin, was obtained after 60 min of incubation, at pH 7.5. Longer incubation led to the total hydrolysis of glucotropaeolin, but simultaneously BITC decreased and formation of N.I. increased (fig. 3).

The pH 7.5 was chosen to study the influence of ascorbic acid (Asc) (50-150 μM) on BITC yield. Asc was found to enhance the rate of glucotropaeolin hydrolysis and its optimal concentration for BITC formation was 100 μM (fig. 4). Only in the pres-

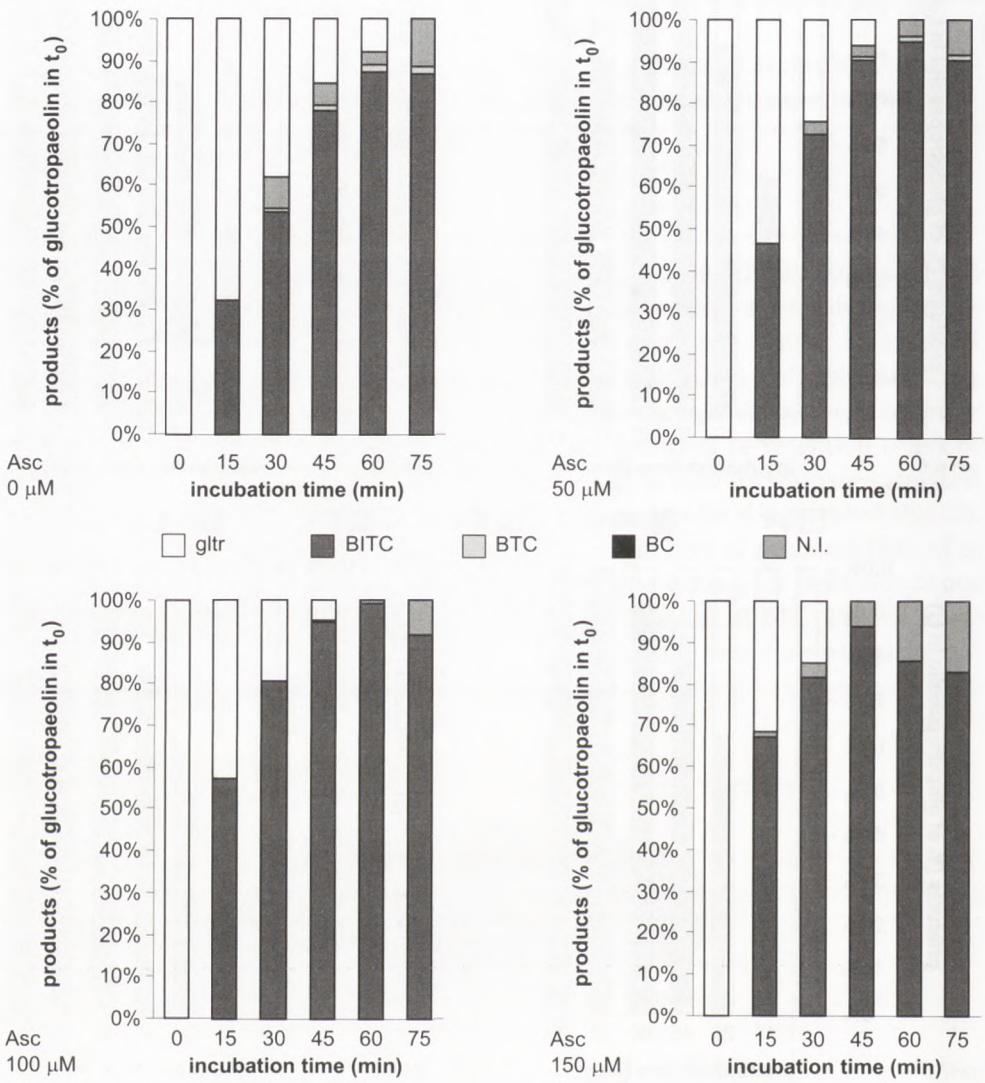

Fig. 4. Influence of Asc and incubation time on the type and ratio of compounds produced during endogenous hydrolysis of glucotropaeolin (n = 6).

ence of 50 μM Asc the formation of BTC was the same as in control (Asc 0 μM). In the presence of 100 μM Asc, almost total t_0 glucotropaeolin was converted into BITC; at the 60th min of incubation its yield was 99% of products and prolonged incubation led to BITC disappearance and an increase in formation of N.I. In the presence of 150 μM Asc, N.I. could be detected as early as at the 15th min of incubation (fig. 4). HPLC chromatograms of products formed during hydrolysis in the presence of 150 μM Asc and after 60th min of hydrolysis in the presence of 100 μM Asc showed increase in one fraction of N.I. (data not shown).

The yield of BITC obtained after endogenous hydrolysis of glucotropaeolin in homogenates of hairy roots with enhanced glucotropaeolin content was also studied. On the 6th day after the treatment with Phe+Cys+PheP, Phe+Cys+ASA, or PheP+ASA glucotropaeolin content in hairy roots reached 480, 423 and 420% of control, respectively (tab.). On the basis of the earlier results, endogenous hydrolysis was conducted for 60 min at pH 7.5 and in the presence of 100 μM Asc. It was found that in homogenates of hairy roots with enhanced glucotropaeolin content the effectiveness of glucotropaeolin conversion to BITC was the same as in control roots; after 60 min of incubation BITC yield was about 99% of products. The highest yield of BITC, about 0.746 mmol/10 g FW, was obtained from endogenous hydrolysis in homogenates of hairy roots with enhanced glucotropaeolin content by the treatment with Phe+Cys+PheP (tab.).

Table

Yield of BITC from endogenous hydrolysis of glucotropaeolin in homogenates of 10 g (FW) of hairy roots with enhanced glucotropaeolin content. Incubation time 60 min, pH 7.5, in the presence of 100 μM Asc (n = 6)

Treatments enhancing glucotropaeolin production in hairy root cultures	Glucotropaeolin content (mmol/10 g FW ± SD)	Percentage ± SD of glucotropaeolin converted to BITC	BITC yields (mmol/10 g FW ± SD)
Control (no treatment)	0.158 ± 0.0124	97.95 ± 6.63	0.155 ± 0.0105
Treatment with Phe+Cys+PheP	0.761 ± 0.0648	98.07 ± 5.77	0.746 ± 0.0439
Treatment with Phe+Cys+ASA	0.671 ± 0.0563	98.78 ± 5.27	0.662 ± 0.0342
Treatment with PheP+ASA	0.679 ± 0.0495	98.37 ± 4.62	0.668 ± 0.0314

4. Discussion

The major goal of this study was to establish the conditions for endogenous myrosinase-catalyzed hydrolysis of glucotropaeolin to bioactive BITC utilizing *T. majus* hairy root cultures giving high yields of glucotropaeolin and myrosinase. During *in vitro* hydrolysis of exogenous glucotropaeolin at pH 5.5, optimal for hairy root-derived myrosinase, we detected BITC, BTC, BC, and several N.I. Although BITC was the dominant product, more than 20% of initial glucotropaeolin was converted to BC. The studies on endogenous glucotropaeolin hydrolysis conducted at pH 6.0

in homogenates of tissues of *Alliaria petiolata* and extracts of *Carica papaya* seeds showed that BITC was the only derivative of glucotropaeolin (7,8). Although BITC was also the major product of endogenous glucotropaeolin hydrolysis at pH 6.75 in homogenates of seeds and seedlings of *Lepidium sativum* BC, BTC, benzyl alcohol, and benzaldehyde were detected as well (6). Numerous reports indicated that the formation of isothiocyanates and nitriles depended strictly on pH. It was found that a low, acidic pH, favored the formation of nitriles and that a higher, close to neutral pH favored the formation of isothiocyanates (9,10,14). It is possible that the high amount of BC obtained in our experiments was due to the effect of low pH (pH 5.5). Based on that, the pH values in the range of 6.5-8.0 were examined for endogenous myrosinase-catalyzed hydrolysis of glucotropaeolin. As expected, at pH above that optimal for hairy root-myrosinase activity, the rate of glucotropaeolin hydrolysis decreased. On the other hand, together with increasing pH values BITC yield increased. pH 7.5 was optimal for BITC formation and eliminated conversion of glucotropaeolin to BC. Maximal yield of BITC (87% of initial glucotropaeolin) was found for 60^{th} min of hydrolysis. Prolonged incubation resulted in disappearance of all glucotropaeolin, but instead of increase in BITC yield formation of N.I. was observed. Numerous studies showed that the duration of incubation affects the type of derivatives of glucosinolate hydrolysis, e.g. during endogenous hydrolysis in homogenates of seeds and seedlings of *L. sativum* and seeds of *C. papaya* reduced time of incubation did not allow sufficient glucotropaeolin decomposition, while during prolonged incubation secondary reaction occurred and BITC yield decreased (6,8). Some authors reported the spontaneous release of thiocyanate anions from isothiocyanates in aqueous media. This resulted in the formation of reactive carbonium ions which reacted non-enzymatically with water to form carbinols or/and with ascorbic acid to form ascorbigen (6,11,12). It is very likely that in the experiments reported here, lack of increase in BITC yield after 60 min of incubation was the result of simultaneous hydrolysis of glucotropaeolin to BITC and conversion of BITC to its derivatives such as benzyl carbonium ions and then benzyl alcohol, benzaldehyde, and ascorbigen.

Available data show that Asc is an "uncompetitive activator" of myrosinase which enhances simultaneously the V_{max} and K_m values (29,30). We found that after 60 min of endogenous glucotropaeolin hydrolysis in the presence of 100 μM Asc BITC yield reached 99% of products and afterward it started to decrease slowly. It is possible that Asc enhanced the rate of glucotropaeolin hydrolysis to BITC and thus suppressed overlapping of glucotropaeolin hydrolysis and BITC conversion to its derivatives. The ascorbigen formed in reaction of benzyl carbonium ion with ascorbic acid could be the fraction of N.I. observed on HPLC chromatograms after 60^{th} min of hydrolysis, in the presence of 100 μM Asc and after 15^{th} min in the presence of 150 μM Asc.

Since in our previous work we obtained hairy roots with glucotropaeolin content enhanced after treatment with the combination of precursors (Phe+Cys) with

elicitor (ASA) or phenylalanine ammonia-lyase inhibitor (PheP), we decided to examine whether this stimulation during root cultures affects subsequent endogenous hydrolysis of glucotropaeolin. Hydrolysis was conducted at pH 7.5, for 60 min, in the presence of 100 μM Asc, the conditions determined as optimal for glucotropaeolin conversion to BITC in homogenates of not stimulated hairy roots. We found that, similarly to not stimulated hairy roots, glucotropaeolin hydrolysis in homogenates of stimulated ones led to 99% conversion of glucotropaeolin to BITC. It enabled obtaining BITC yields 4-fold higher than that from endogenous glucotropaeolin hydrolysis in homogenates of not stimulated roots.

The results obtained indicate that *T. majus* hairy roots can be a rich source of glucotropaeolin and its bioactive derivative, BITC. The optimal conditions for endogenous myrosinase-catalyzed hydrolysis of glucotropaeolin in *T. majus* hairy root homogenates are pH 7.5 in the presence of 100 μM ascorbic acid.

Abbreviations: ASA – acetylsalicylic acid; Asc – L-ascorbic acid; BC – benzyl cyanide; BITC – benzyl isothiocyanate, BTC – benzyl thiocyanate; Cys – L-cystein; DW – dry weight; FW – fresh weight; gltr – glucotropaeolin; HPLC – high performance liquid chromatography; N.I. – unidentified compounds; Phe – L-phenylalanine; PheP – L-1-amino-2-phenylethylphosphonic acid.

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