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GLUCOSINOLATE CONTENTS OF REGENERATED PLANTLETS FROM EMBRYOIDS OF HORSERADISH

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Key Word Index—*Armoracia rusticana*; Cruciferae; horseradish; cell cultures; embryoids; regenerated plantlets; HPLC; glucosinolates; myrosinase.

Abstract—Glucosinolates are known to occur mainly in differentiated tissues of Cruciferae plants. In an effort to improve the production of these compounds, regenerated plantlets were induced from horseradish embryoids. Subsequently, glucosinolate contents were compared with those found in the whole plant, suspension cells, embryoids and calli. Qualitative production of glucosinolates was similar in both regenerated plantlets and wild plants with regard to 2-phenylethyl- and 2-propenyl-glucosinolates. In contrast, thioglucoside contents changed with cell differentiation, because indoleglucosinolates occur only in calli, suspension cells and embryoids. Furthermore, it has been shown that the specific activity of myrosinase, which is the enzyme responsible for glucosinolates hydrolysis, declines with tissue age. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Glucosinolates are a class of secondary compounds, widely distributed throughout the Cruciferae. These compounds are thioglucosides, linked to different side-chains. Myrosinase or thioglucoside glucohydrolase (EC 3.2.3.1), responsible for the enzymic hydrolysis of glucosinolates, would appear to occur in all plant species which produce glucosinolates [1]. The initial enzymic cleavage of the thioglucoside yields D-glucose and an unstable aglucone. The latter then undergoes rearrangements to form isothiocyanate, thiocyanate, nitrile, hydroxynitrile, epithionitrile or oxazolidine-2-thione moieties, depending on such factors as the glucosinolate structure, pH and the presence of compounds that modify the action of the enzyme [2].

The importance of glucosinolates and their breakdown products in plants used as food, feed and medicine has been known since ancient times. Recently, many plant products of commercial or medicinal importance have been obtained in large amounts, from cell cultures of higher plants [3, 4]. However, in many reports, the presence of glucosinolates could not be shown in callus tissues induced from various species [5, 6]. When thioglucosides were found in cell cultures, their contents remain very low compared with the whole plant [7]. Moreover, immunochemical labelling experiments on rapeseed plants have shown that only specialized cells, termed 'myrosin cells' contain myrosinase [8]. Thus, this specific compartmentation of the myrosinase-glucosinolate system in cruciferous plant tissues suggests that morphological or biochemical differentiation may be necessary in order to produce sufficient amounts of thioglucosides.

In the work presented here, the glucosinolate contents of regenerated plantlets from embryoids of *Armoracia rusticana* were compared with those of the whole plant, embryoids, suspension cells and calli. The occurrence of myrosinase was investigated in parallel.

RESULTS AND DISCUSSION

The occurrence of glucosinolates and their destructive enzymes, often called myrosinases, was investigated in various differentiated horseradish tissues (Table 1). HPLC analysis of purified extracts of regenerated plantlets revealed the presence of two alkelynic glucosinolates, 2-phenylethyl- and 2-propenyl-, which appear to be confined to roots and leaves, respectively. The composition of samples of both small and large leaves was similar except that an unidentified compound was detected in large leaves of regenerated plantlets. The effectiveness of plantlet regeneration requires careful evaluation in terms of comparing glucosinolate contents between *in vitro* and *in vivo* plants. It has been reported that the leaves of horseradish

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Table 1. Glucosinolate contents and myrosinase activity of various horseradish tissues

Materials	Medium†	Glucosinolate contents* ($\mu g\ g^{-1}$ dry wt) IMG	OH-IMG	PhG	PrG	Total contents	Myrosinase activity‡	Specific activity§
Callus (28 days)	MS	4.95 (0.3)	0.32 (0.03)	–	–	5.27	12.8 (0.7)	5.56
Suspension cells (21 days)	MS	0.09 (0.02)	–	–	–	0.09	–	–
Suspension cells (21 days)	D-MS	0.31 (0.04)	–	–	–	0.31	–	–
Embryoids (28 days)	D-MS	0.71 (0.02)	–	–	–	0.71	–	–
Roots of regenerated plantlets (3 months)	H-MS	–	–	2.35 (0.4)	–	2.35	7.15 (0.4)	3.19
Small leaves of regenerated plantlets (3 months)	H-MS	–	–	–	16.4 (4)	16.4	56.41 (3.7)	4.70
Large leaves of regenerated plantlets (3 months)	H-MS	–	–	–	28.3 (5)	28.3	102.37 (5.4)	3.57

Mean values of three independent extractions ± (S.D.).

*Indole-3-methylglucosinolate, IMG; 4-hydroxy-indole-3-methylglucosinolate, OH-IMG; 2-phenylethylglucosinolate, PhG; 2-propenylglucosinolate, PrG.

†Murashige and Skoog basal salts with organic substances, MS; 2,4-D-free-MS, D-MS; hormone-free-MS, H-MS.

‡Myrosinase activity expressed as units g^{-1} fr. wt.

§Specific activity of myrosinase expressed as units mg^{-1} protein.

grown *in vivo* contain 2-propenyl-glucosinolate [9], while 30 glucosinolates were identified in roots [10]; however, in the absence of comparisons with authentic or synthetic material, the structures of some of the minor components must be regarded as tentative [2]. In this way, 2-propenyl-glucosinolate (240 $\mu g\ g^{-1}$ fr. wt) is by far the predominant thioglucoside in roots, followed by 2-phenylethyl-glucosinolate (55 $\mu g\ g^{-1}$ fr. wt). These results clearly demonstrate that regenerated plantlets were similar to horseradish plants grown *in vivo* with respect to qualitative composition of thioglucoside compounds. However, quantitative data of each glucosinolate were different from those found in literature [10]. The reason for the inconsistency may lie in the different age of the tissues compared since glucosinolate concentrations in various part of a single species change throughout the whole life cycle [11].

The individual glucosinolates present in regenerated plantlets were compared with those of embryoids, suspension cells and calli in order to investigate whether the distribution of these compounds resulted from cellular differentiation. Our data clearly demonstrate that the occurrence of indoleglucosinolates in embryoids, suspension cells and calli relates to their age. Thus, the highest content of indole-3-methylglucosinolate (glucobrassicin) was found in calli. This result is unsurprising as the chemical gradients, cell-to-cell contact and any incipient differentiation present appears to favour the synthesis of secondary compounds [12]. Because suspension cells and calli were grown on the same medium, the presence of glucobrassicin in calli may be the result of biochemical differentiations rather than medium composition. It has also been reported that younger and growing tissues synthesize more indoleglucosinolates than older and senescent tissues [13, 14]; these results raise the question of the role of glucosinolates in healthy plants, although several studies have confirmed that infestation of Cruciferae plants by specific insects results in the accumulation of thioglucoside in damaged tissues [15, 16].

Individual glucosinolate contents were determined by HPLC analysis of desulphoglucosinolates. This technique and HPLC analysis of intact glucosinolates are well established in many laboratories. Desulphoglucosinolate analysis is more time-consuming than HPLC of intact glucosinolates but the separation is more efficient for both glucosinolates identification and quantification.

Furthermore, attempts have been made to determine myrosinase activity in relation to the presence of their substracts in various differentiated cells of horseradish. Myrosinase activity was highly correlated with the occurrence of glucosinolates in sufficient amounts. Specific activities of the enzyme

decline with differentiation of tissues, which could signify that myrosinases might be considered as markers for tissue differentiation.

EXPERIMENTAL

Plant material. Horseradish plants (A. *rusticana* Gaertn.) were obtained from Germany in 1984 and cultivated under greenhouse conditions.

Culture methods. Callus stock material was isolated from horseradish leaves as previously described [17]. Cells were grown on solid culture medium under continous light at 25° and subcultured every 30 days in fresh medium. The latter consisted of Murashige and Skoog salf base [18] supplemented with sucrose (30 g l^{-1}), casein hydrolysate (500 mg l^{-1}), myoinositol (100 mg l^{-1}), thiamine (10 mg l^{-1}), pyridoxine (1 mg l^{-1}), nicotinic acid (1 mg l^{-1}), kinetin (0.2 mg l^{-1}) and 2,4-D (0.2 mg l^{-1}); 28-day-old calli were used.

Suspension cultures of cells were initiated by transferring 3 g of callus tissues into 100 ml of liquid culture medium contained in 250-ml flask and placed on an orbital shaker (150 rpm) under the above conditions of light and temp. Suspension cells were subcultured at intervals of 21 days for 6 months. The resulting mother soln of fine suspension cells was used to induce embryoids.

Liquid culture medium without of 2,4-D (100 ml) were inoculated with 10 ml of fine suspension cells then cultured as described above. Embryoid tissues were harvested then subcultured in the hormone-free medium in order to regenerate plantlets; 3-month-old regenerated plantlets were used for extractions.

HPLC analysis. Lyophilized plant material (200 mg) was treated in 2 ml of boiling MeOH–H_2O (7:3) for 5 min. Glucosinolates were extracted, purified on DEAE Sephadex A-25 and desulphated after reaction with arylsulphatase. The resulting desulphoglucosinolates were sepd on a RP column of Spherisorb ODS 2 (250 × 4.6 mm, 5 μm particle size) with an elution gradient (from 0 to 25% of MeCN in H_2O) then quantified by UV spectrophotometry at 229 nm according to the EEC method [19]. Desulphoglucosinolates were identified from their UV spectra and capacity factors [20]. Glucosinolate contents are expressed in $\mu g\ g^{-1}$ dry wt of material.

Determination of myrosinase activity. Tissues were homogenized with a pestle and mortar with sand and 0.01 M imidazole–HCl buffer pH 6.2 at 4°. The crude homogenate was centrifuged at 10 000 *g* for 15 min at 4° and the supernatant, dialysed through a Spectra/Por tubing (M_r cut-off 25 000) against the same buffer for 24 hr. From this crude enzyme extract, myrosinase activity was determined at 30° by coupled enzyme assay, measuring the amount of glucose liberated on hydrolysis of sinigrin [21]. One unit of myrosinase activity is defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol substrate min^{-1} at 30°.

Protein determination. Proteins were determined by the method of Bradford [22] using BSA as standard.

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