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Type and concentration of redox reagents influencing nitrile formation upon myrosinase (*Brassica carinata*)-catalyzed hydrolysis of glucosibarin

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ABSTRACT

The ratio of isothiocyanates (ITCs) to nitriles formed in the myrosinase-catalyzed hydrolysis of glucosinolates is a key factor determining the physiological effect of glucosinolate containing plants and materials. In this context, the mechanism by which nitrile formation occurs is not well understood. In the present paper we have studied the effect of three redox reagents – Fe^{2+}, glutathione (GSH) and ascorbic acid – on the profile of products obtained upon the hydrolysis of a model glucosinolate (glucosibarin ((2*R*)-2-hydroxy-2-phenylethylglucosinolate)) catalyzed by *Brassica carinata* myrosinase. A Micellar Electrokinetic Capillary Chromatography method that allows following on-line the hydrolysis of the glucosinolate, the formation of the degradation products and the oxidation of GSH was used. Increasing the concentration of Fe^{2+} and GSH (from 0.25- to 2-fold molar excess with respect to the glucosinolate) increased the ratio of nitrile ((2*R*)-2-hydroxy-2-phenylethylcyanide) to oxazolidine-2-thione ((5*S*)-5-phenyloxazolidine-2-thione), whereas increasing the concentration of ascorbic acid decreased this ratio. Low concentrations of ascorbic acid favored nitrile formation. A mechanism for nitrile formation involving a disulfide bond in the myrosinase complex is proposed.

1. Introduction

Glucosinolates are a group of allelochemicals present in all plants of the order Capparales and in few other plants [1–4] where they co-occur with myrosinase isoenzymes (EC 3.2.1.147) [5,6]. These isoenzymes, which form large molecular weight complexes with associated proteins, contain various thiol groups and are activated by ascorbic acid [5,7–9]. In the presence of water, myrosinases catalyze the hydrolysis of the β-D-thioglucopyranoside bond in glucosinolates giving rise to an unstable thiohydroxamate-O-sulfonate intermediate [3], which further rearranges to a variety of different products depending on the parent glucosinolate and the hydrolysis conditions (Fig. 1) [10–12].

Isothiocyanates (ITCs) have been the most frequently studied glucosinolate hydrolysis products [2,3,13]. Nevertheless, nitriles are also produced upon glucosinolate hydrolysis and their formation is favored by acidic pH and certain redox reagents, such as thiol groups and Fe^{2+} [14–19]. The presence of certain proteins in the myrosinase complex – e.g. epithiospecifier protein (ESP) – has been shown to promote nitrile formation; however, the specific mechanism(s) by which nitriles are formed remains largely unknown [20,21].

A wide range of biological activities has been documented for ITCs [22–27]. On the contrary, nitriles generally display a lower level of biological activity [4,28,29]. Further knowledge on the mechanisms by which certain factors influence the profile of glucosinolate transformation products is hence needed, since the ratio of ITCs to nitriles will determine the biological effects of glucosinolate containing plants and materials.

In the present study, we have evaluated the effect of different concentrations of Fe^{2+}, ascorbic acid and glutathione (GSH), individually and combined, on the profile of products formed upon the hydrolysis of glucosibarin ((2*R*)-2-hydroxy-2-phenylethylglucosinolate) by *Brassica carinata* myrosinase (Fig. 1). Glucosibarin was chosen because of two special features: (i) the phenyl group, which allows for the detection of the nitrile and any other potential degradation products and (ii) the hydroxyl group at C-2 in the side chain, which, in aqueous systems, makes the relatively insoluble and highly unstable ITC to spontaneously cyclise to the more soluble (5*S*)-5-phenyloxazolidine-2-thione (OZT). Hence, formation of OZT in our reaction prevents unspecific reaction of the ITC with other nucleophiles in the medium (e.g. thiol and amino groups in

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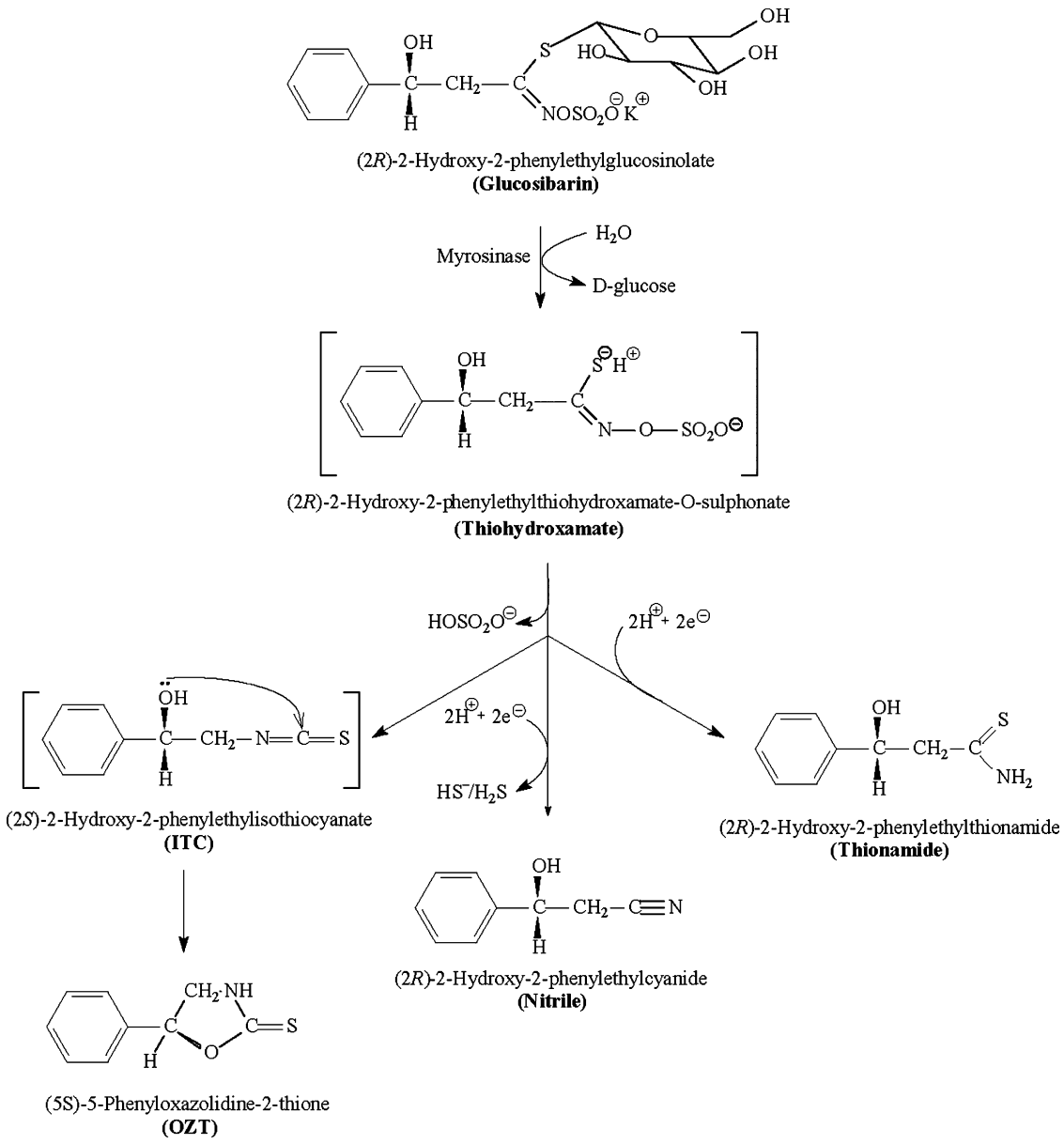

Fig. 1. Structures and names of the parent glucosinolate and the products produced in the myrosinase-catalyzed hydrolysis.

myrosinase), which would hinder the study of the hydrolysis reaction.

A Micellar Electrokinetic Capillary Chromatography method that allows following on-line the degradation of the glucosinolate and the simultaneous formation of the products was used [30]. The oxidation of GSH and Fe^{2+} was also estimated and this allows us to propose a mechanism for nitrile formation.

2. Experimental

2.1. Chemicals used

Disodium hydrogenphosphate was purchased from Riedel-de Häen (Seelze, Germany), ascorbic acid from Bie & Berntsen (Rødovre, Denmark), ferrous sulphate heptahydrate from Merck (Darmstadt, Germany) and oxidized glutathione (GSSG) from ICN Biomedicals Inc. Aurora, OH, USA. Sodium cholate, taurine, trigonellinamide (TNA), ferric chloride hexahydrate, HEPES buffer (N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid, sodium salt), trichloroacetic acid (TCA), chlorhydric acid, hydroxylamine monohydrochloride, ferrozine chromogen (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, disodium salt), reduced glutathione (GSH) and dehydroascorbic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Glucosinolates and myrosinase

Glucosibarin used in the experiment was from the laboratory collection [4,31]. Seeds of *B. carinata* cv. BRK-147-A were obtained from Koipesol (Spain) and used as source for the enzyme myrosinase (EC 3.2.1.147). Myrosinase isoenzymes were purified, characterized and assayed following the method used in our laboratories [5,32]. The enzyme preparation used in the experiments was a pool of the six most active aliquots eluted from the Concanavalin A affinity chromatography column (ca. 5 enzyme units

per mL (U/mL) obtained from 10 g of defatted seed material) diluted to 3 U/mL prior to use. One enzyme unit (U) was defined as the amount of enzyme that catalyzes the transformation of 1 μmol of substrate per minute.

2.3. Measurement of Fe^{2+} ions in the solutions

Prior to utilization in the MECC assay each new Fe^{2+} stock solution was analyzed for Fe^{2+} and total iron in order to determine the exact volume of solution needed in the assay. The method used is based on the reaction with the chromogene ferrozine [33] and it is modified from Kapsokefalou and Miller [34] and adapted for microscale use in 96-well microplates. Fe^{2+} was analyzed by adding 100 μL non-reducing protein precipitate solution (non-RPP; 1 g TCA, 1 mL 37% HCl adjusted to 10 mL with water), to 200 μL aliquots. Total iron was analyzed by adding 100 μL reducing protein precipitate solution (RPP; 1 g TCA, 1 mL 37% HCl, 0.5 g hydroxylamine monohydrochloride adjusted to 10 mL with water) to 200 μL aliquots of sample or to ferrous sulphate in twofold dilutions starting at 1 mM $FeSO_4$ for quantification. The aliquots containing either non-RPP or RPP were left overnight at room temperature, centrifuged (2575 × *g*; 10 min), and duplicates of 100 μL supernatants were placed in microtiter wells, then mixed with 200 μL HEPES buffer (0.3 M, pH 9.9), and 25 μL ferrozine chromogen solution (5 mg/mL in water). The absorbance was measured in microplate reader (Bio kinetic reader EL 340 Microplate; Bio-Tek™ Instruments; Software: KC3; KinetiCalc for Windows, Vers. 1.5) at 570 nm immediately after ferrozine addition for the quantification of Fe^{2+} and after 1 h for determination of total iron content.

2.4. MECC equipment and methodology

Analyses were performed using a Hewlett-Packard HP^{3D} CE capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with diode array detector. Data processing was carried out by use of a HP Vectra 5/100 mHz Pentium with HP ChemStation Rev. B. 01.03. The formation of the OZT, the nitrile, the thionamide and the complex from glucosibarin was followed on-line with the method developed by Bellostas et al. [30]. The run buffer was composed by 35 mM sodium cholate, 100 mM disodium phosphate, 500 mM taurine, 2% 1-propanol and the pH was kept at 8.2.

2.5. Reaction procedure

The reaction medium was composed by TNA (20 μL of a 100 mM solution) as an internal standard, glucosibarin (20 μL of a 50 mM solution), Milli-Q water (6.5 μL) and acetate buffer (3.5 μL of a 100 mM pH 5 solution). Different volumes of $FeSO_4$ and GSH solutions (see below) were added to this reaction medium in order to have 0.25-, 0.5-, 1- and 2-fold molar excess of each cofactor with respect to the glucosinolate. In the experiments with ascorbic acid, stock solutions containing the desired amount of ascorbic acid, Milli-Q water and acetate buffer were made in order to maintain pH and TNA and glucosibarin were added as indicated below. The final pH of the reaction medium was in no case lower than 4.5. The cofactors were added simultaneously with myrosinase (5 μL of the 3 U/mL solution (ca. 0.015 U in vial)) and the reaction mix was incubated for 24 h. Data is presented as the mean of at least two independent experiments.

2.5.1. Preparation of the Fe^{2+}-containing myrosinase assays

A solution of 0.8 M citric acid was adjusted to pH 5.5 and $FeSO_4$ was added to a final $FeSO_4$ concentration of 0.4 M. Final pH of this $FeSO_4$ stock solution ranged between 4.5 and 5. The content of Fe^{2+} in this solution was determined by the procedure described above, which allowed calculating the volume needed in order to have the desired concentration of Fe^{2+} ions in the reaction medium. This solution was prepared every time a new determination was to be conducted. Different volumes (μL) of this solution were added to the reaction mixture in order to have the Fe^{2+}:glucosinolate fold molar excess required, which corresponded to final Fe^{2+} concentrations of 4.9–34.5 mM.

2.5.2. Preparation of GSH-containing myrosinase assays

A stock solution of 0.2 M GSH in 100 mM acetate buffer pH 5 was prepared and kept frozen until use. Different volumes (μL) of this solution were added to the reaction mixture in order to have the GSH:glucosinolate fold molar excess required, which corresponded to final GSH concentrations of 4.9–33.3 mM. The oxidation of GSH to GSSG in the absence of glucosinolate and myrosinase was assessed over a 24 h period and the values were taken as a reference for the calculation of the oxidation of GSH to GSSG during the myrosinase-catalyzed hydrolysis of glucosibarin.

2.5.3. Preparation of ascorbic acid-containing myrosinase assays

Stock solutions corresponding to different final concentrations of ascorbic acid (0.3–28.6 mM) were prepared by mixing the needed volume of a 0.1 M ascorbic acid solution with fixed volumes of purified water and acetate buffer pH 5. The stock solutions were adjusted to pH 5 with NaOH and a certain volume from each stock solution was mixed with TNA (20 μL of a 100 mM solution) and glucosibarin (20 μL of a 50 mM solution) in order to have the ascorbic acid:glucosinolate fold molar excess required in the final reaction mixture (from 0.0156- to 2-fold molar excess). The stock solutions were prepared freshly every time a new determination was to be conducted.

2.5.4. Preparation of myrosinase assays containing different mixtures of Fe^{2+}, GSH and ascorbic acid

Four separate experiments combining the three co-factors were performed. Ascorbic acid (0.25–2-fold molar excess with respect to glucosibarin) was combined in two separate experiments with GSH (33.3 mM) and Fe^{2+} (34.5 mM). GSH (0.25–2-fold molar excess with respect to glucosibarin) was combined with Fe^{2+} (34.5 mM). A final experiment combining the three co-factors in twofold molar excess with respect to glucosibarin was performed.

2.6. Measurement of the oxidation of Fe^{2+} in the presence of glucosibarin and myrosinase

Two sets of experiments were performed. In the first, the control sample was prepared of acetate buffer (1 mL 100 mM) and Milli-Q water (3 mL). Three reaction mixtures were then prepared: (a) glucosibarin (40 μL 10 mM), (b) glucosibarin (40 μL 10 mM) and myrosinase (0.015 U), and (c) glucosibarin (40 μL 10 mM) myrosinase (0.015 U) and GSH (4 μL 200 mM). Acetate buffer (1 mL 100 mM pH 5) was added to the three samples and the volume was adjusted to 4 mL with Milli-Q water. To all samples $FeSO_4$ (7.2 μL 100 mM) was added and pH was measured to 5. Aliquots (200 μL) were collected for measurements of Fe^{2+} in solution as described above at time points corresponding to 0, 10, 30, 60, 120, 180 min and 24 h. In the second set of experiments, a higher concentration of Fe^{2+} was used: the control sample (a) was prepared with acetate buffer (560 μL 100 mM pH 5); three reaction mixtures were then prepared: (b) glucosibarin (140 μL 50 mM), (c) glucosibarin (140 μL 50 mM) and myrosinase (0.015 U), and (d) myrosinase (0.03 U). The volume of the three reaction mixtures was adjusted to 700 μL with acetate buffer (100 mM pH 5). To all samples, $FeSO_4$ (140 μL 100 mM) was added and pH was measured to 5. Aliquots (200 μL) were collected for measurements of Fe^{2+} and total iron

content in solution as described above at time points corresponding to 0, 10, 30, 60, 120 and 180 min.

2.7. Calculations

Concentrations of glucosibarin and nitrile (benzylcyanide instead of (2*R*)-2-hydroxy-2-phenylethylcyanide was used as standard) were determined by the use of concentration–response curves of the pure compounds relative to TNA (40 mM) at 206 nm in MECC (cholate buffer, 30 °C). The ε value used for the glucosibarin derived OZT was the one previously obtained [30]. The molar response factor of the thionamide was assumed to be identical to that of the parent glucosinolate at 206 nm[35].

3. Results and discussion

3.1. Validity of the MECC method

The MECC method used [30] proved to be a valuable tool in the study of the myrosinase-catalyzed hydrolysis of glucosibarin in the presence of redox reagents. The method allowed detecting all potential glucosinolate hydrolysis products, even at low concentrations, which in turn permitted following the stoichiometry of the reaction at 206 nm. Moreover, the method was successful in separating all components in the reaction mixture: glucosibarin ((2*R*)-2-hydroxy-2-phenylethylglucosinolate), the degradation products expected – nitrile ((2*R*)-2-hydroxy-2-phenylethylcyanide), thionamide ((2*R*)-2-hydroxy-2-phenylethylthionamide) and the ITC-derived OZT ((5*S*)-5-phenyloxazolidine-2-thione) – the postulated glucosinolate–Fe^{2+} complex [35], GSH and GSSG and ascorbic acid. This allowed calculating the rates of glucosibarin degradation and formation of the different transformation products as well as estimating the oxidation of GSH. The choice of glucosibarin as a model has been explained above. Previous experiments showed that it represented well the behavior of other glucosinolates [30].

3.2. Effect of Fe^{2+} on the product profile of glucosibarin hydrolysis

Raising Fe^{2+} concentration in the presence of myrosinase enhanced glucosibarin hydrolysis and this resulted in the nitrile being the major degradation product at the expense of the OZT (Fig. 2). An increased formation of nitriles in the presence of Fe^{2+} has been previously observed both in the presence [16–20,36,37] and the absence of myrosinase [35,38–41]. Similarly, inhibition of ITC (in this case OZT) formation in the presence of myrosinase and Fe^{2+} has been observed as a consequence of enhanced production of nitriles [16–19,36,37]. Contrary to what was observed in the absence of myrosinase [38–41], formation of OZT was not inhibited in the present study.

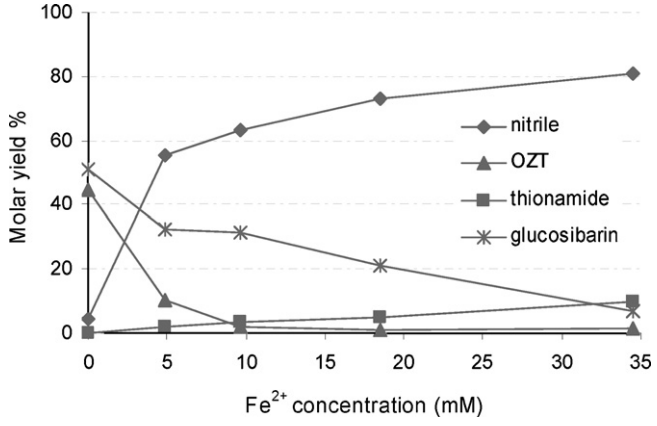

Fig. 2. Molar yield (in %) of glucosibarin and its hydrolysis products after 24 h incubation with myrosinase (0.015 U) and increasing Fe^{2+} concentration at pH 5.

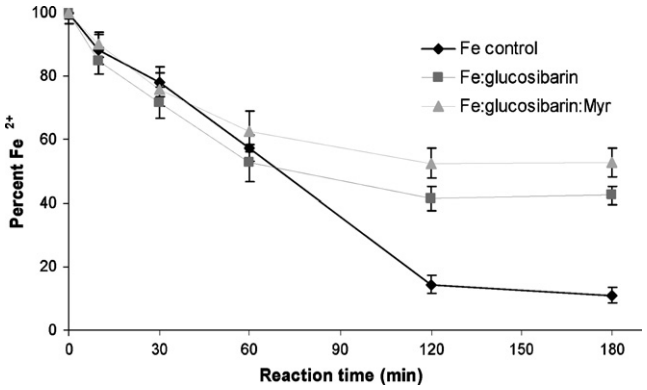

Fig. 3. Concentration of Fe^{2+} (in % relative to $t = 0$) in three reaction mixtures: (1) Fe-control; (2) Fe + glucosibarin; (3) Fe + glucosibarin + myrosinase.

The nitrile represented between 82 and 92% of the total pool of products at all Fe^{2+} concentrations tested (Fig. 2). Similar values of nitrile production have been reported at lower Fe^{2+} concentrations and fold molar excess in the presence of myrosinase [17,18,36,37], as well as when up to 50 mM [41] and eightfold molar excess Fe^{2+} [35] were used in the absence of myrosinase.

It has been suggested that nitrile formation by Fe^{2+} in the absence of myrosinase involves the formation of a glucosinolate–Fe^{2+} complex, which leads to the formation of the nitrile and, in the case of glucosinolates bearing a hydroxyl group at C-2 in the side chain, a thionamide [35,38–41]. In the present experiments, both the postulated complex and the thionamide were observed and their proportion in the product mixture increased with increasing concentrations of Fe^{2+}. Previous reports showing quantities of nitriles similar to those found in the present experiments discarded the non-enzymatic pathway of nitrile formation due to a low Fe^{2+} concentration used [16,36,37]. However, the presence of the glucosinolate–Fe^{2+} complex and the thionamide in our experiments may suggest that, even at the lowest Fe^{2+} concentration (5 mM), the non-enzymatic reaction may have taken place.

The effect of Fe^{2+} has been closely linked to the pH of the solution, and e.g. at pH higher than 7.5 no effect of the addition of Fe^{2+} on the production of nitriles was observed [18]. This is probably due to the oxidation of Fe^{2+} to Fe^{3+} by free oxygen in the neutral to alkaline solution [42], and it highlights the relevance of the method used in the present study for determination of iron species in the reaction medium.

The ratio Fe^{2+}:Fe^{3+} was followed during the first 3 h of reaction in different reaction mixtures (Fig. 3). Fe^{2+} was oxidized to the highest extent in the absence of glucosibarin and myrosinase (control) and in the presence of myrosinase alone (not shown). The addition of glucosibarin resulted in the protection of Fe^{2+} from oxidation as previously observed [35], whereas the presence of the glucosinolate and the enzyme resulted in a slightly greater protection of Fe^{2+} than in the presence of the glucosinolate alone. Oxidation of Fe^{2+} was prevented to even a higher extent in the presence of GSH (not shown), which is expected from the redox potentials (Table 1).

3.3. Effect of GSH on the product profile of glucosinolate hydrolysis

Increasing the concentration of GSH in the absence of any other cofactor favored the production of the nitrile from glucosibarin

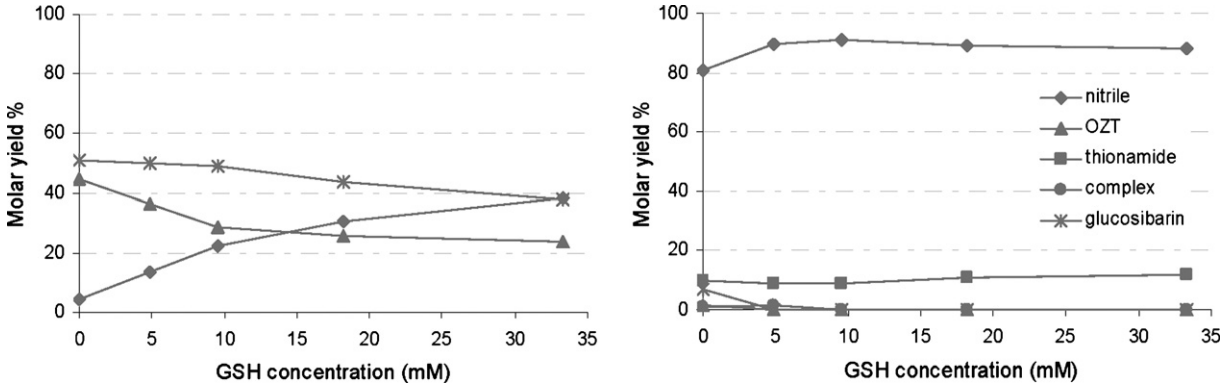

Fig. 4. Molar yield (in %) of glucosibarin and its hydrolysis products after 24 h incubation with myrosinase (0.015 U) and increasing GSH concentration at pH 5. Left: GSH alone; right: GSH + 34.5 mM Fe^{2+}.

almost in a linear fashion with respect to the concentration of GSH added (Fig. 4 left).

Addition of thiol compounds has shown both negative [17] and positive results [17,19,20] in enhancing nitrile formation. In the latter case, the type of thiol group used was an important factor determining the total amount of nitriles formed. The oxidation capacity of thiol groups was rejected as the driving force in the formation of nitriles [17,19]. Oxidation of GSH was observed in the present experiments, although it was not proportional to the amount of nitriles formed (data not shown).

In the presence of twofold molar excess Fe^{2+} (34.5 mM), complete glucosibarin hydrolysis was observed at all GSH concentrations tested, with the nitrile accounting for approximately 90% of the total molar yield of products in all cases (Fig. 4 right). Increasing GSH concentration in the presence of Fe^{2+} resulted in a decreased proportion of OZT in the final product mixture, whereas the proportion of thionamide increased slightly when increasing GSH concentration (Fig. 4). GSH reduces Fe^{3+} to Fe^{2+} (Table 1), which can contribute to a stabilized concentration of Fe^{2+} in the solution, resulting eventually in an increased formation of thionamide through the non-enzymatic reaction [35].

3.4. Effect of ascorbic acid on myrosinase activity and the product profile of glucosinolate hydrolysis

An increased rate of glucosibarin degradation was observed already at the lowest concentration of ascorbic acid tested (0.3 mM; Fig. 5 left). Higher concentrations of ascorbic acid increased the speed of the reaction even further and at 9.1 mM (Fig. 5 right) only 3% of the initial glucosibarin concentration remained after 4 h incubation (data not shown).

Ascorbic acid has been reported to activate myrosinase to different extents depending on the species and tissue source of the enzyme [43–47]. In the present study, activation of myrosinase by ascorbic acid seemed to achieve a maximum between the two upper concentrations used (16.7–28.6 mM). The ascorbic acid concentration needed for the activation of seed myrosinase from different species ranges between 0.01 and 3 mM [44–50], although values as high as 50 mM have been reported for seedling myrosinase [51]. The differences in the degrees of activation reported in the literature have been described to be caused by minor differences in the active site of myrosinase isoforms that could affect the relative affinities for substrate and activator [52]. However, the degree to which myrosinase is purified and the assay used for determination of myrosinase activity are also likely sources of differences among the values obtained for the degree of activation.

In the presence of the lowest concentration of ascorbic acid tested (0.3 mM), nitrile production was already increased by 11-fold with respect to the absence of ascorbic acid, and this led the nitrile to overcome the OZT in molar yield percentage (Fig. 5 left). The proportion nitrile:OZT remained constant until the addition of 9.1 mM of ascorbic acid (0.5 molar excess; Fig. 5 right): from this concentration on, the percentage molar yield of OZT started increasing until it reached 80% of the molar yield of products. According to these results, ascorbic acid displayed a dual behavior depending on its concentration: while it enhanced nitrile compared to OZT formation at low concentrations, it increased the velocity of the reaction favoring OZT formation at high concentrations. Decreased nitrile formation as a consequence of increasing ascorbic acid concentrations has also been reported in the presence of ESP [20]. This has been explained by the increased velocity of the reaction, which leads to too high concentrations of the thiohydroxamate intermediate (Fig. 1) and hence to the Lossen rearrangement overriding the capacity of ESP to form nitriles [20].

Increasing the ascorbic acid concentration in the presence of GSH and Fe^{2+} (33.3 and 34.5 mM, respectively) had similar effects (Fig. 6). In both cases, all glucosibarin was degraded already at the lowest concentration of ascorbic acid. Similarly, the percentage molar yield of the OZT increased and that of the nitrile decreased with increasing concentration of ascorbic acid, although these changes were more marked and the final proportion between the products more even in the presence of GSH (Fig. 6 left). Despite there was a higher production of nitriles in the presence than in the absence of GSH, the effect of ascorbic acid and GSH was not additive. Increasing the concentration of ascorbic acid in the presence of Fe^{2+} increased the proportion of thionamide in the pool of degradation products (Fig. 6 right), which could be explained by the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid (Table 1). The addition of ascorbic acid to a glucosinolate–myrosinase solution containing Fe^{2+} and thiol groups has been previously shown to increase nitrile formation [19].

Table 1
Redox potential of the possible redox reactions between the factors used in our experiments.

Redox reaction	Redox potential ($\Delta E^{\circ\prime}_{pH7}$, V)
Ascorbic acid + $2Fe^{3+}$ → dehydroascorbic acid + 2Fe(II) + $2H^+$	0.713
Dehydroascorbic acid + 2GSH → ascorbic acid + GSSG + $2H^+$	0.158
2GSH + $2Fe^{3+}$ → GSSG + 2Fe(II) + $2H^+$	0.871

These reactions were found to take place at the conditions used in the MECC system.

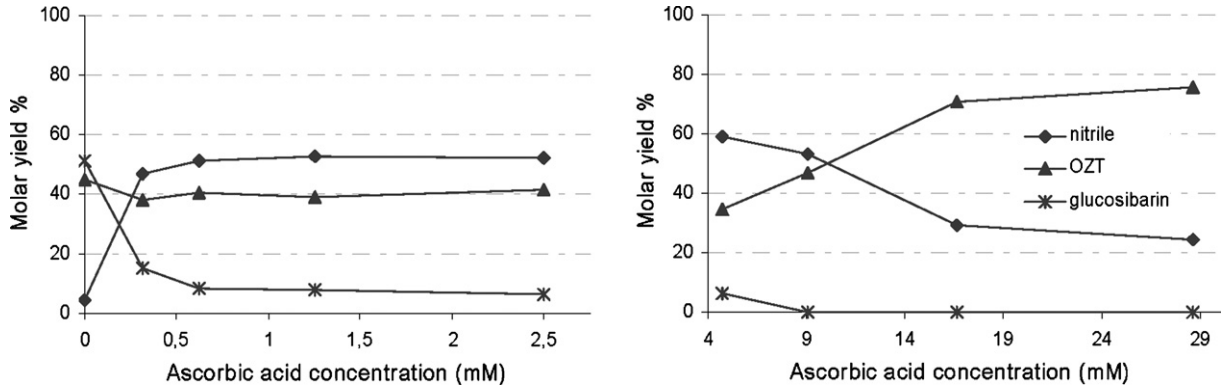

Fig. 5. Molar yield (in %) of glucosibarin and its hydrolysis products after 24 h incubation with myrosinase (0.015 U) and increasing ascorbic acid concentration at pH 5.

3.5. Physiological importance of Fe^{2+}, glutathione and ascorbic acid

Iron species, Fe^{2+} and Fe^{3+} are important micronutrients for all living organisms acting as co-factors for various oxidoreductases, and essential for the structural and bioactive formation of iron–protein complexes [42,53]. Iron can also form complexes with different biomolecules, e.g. glucosinolates, as considered in the present study.

GSH is an ubiquitous biomolecule and, together with ascorbic acid (vitamin C), they have specific biochemical functions in a large number of different redox systems. GSH is the primary substrate for glutathione-S-tranferases in the mercapturic pathway of the xenobiotica metabolism. In the present study, both GSH and ascorbic acid are considered in relation to their role as nucleophiles in combination with myrosinase, defining the types of products produced in glucosinolate transformation [10–13,35–37].

3.6. Proposed reaction mechanism

The myrosinase preparation used in the present experiments was obtained with the use of a gentle method that allows preserving myrosinase in its complex form [5]. A large number of subunits of molecular weight ranging between 20 and 70 kDa were detected by use of reducing SDS-PAGE [5], which may indicate the presence in the complex of proteins known to be associated with myrosinase, such as ESP, myrosinase-binding proteins (MBPs) and myrosinase-associated protein (MyAP) [9,14,54,55].

Myrosinase complexes contain a number of cysteine (Cys) residues: the MBPs and MyAP identified up-to-date contain at least four Cys residues each and the 150 kDa myrosinase complex contains at least seven [8,14,56]. The possibility for the Cys residues of MyAP to form disulfide bridges between them and/or with other Cys residues in other protein subunits within the myrosinase complex has already been suggested [56]. This may be supported by the lack of protection of Fe^{2+} from oxidation in the presence of myrosinase alone. Had the enzyme contained Cys residues in the form of free thiol groups, Fe^{2+} would have been protected from oxidation to Fe^{3+}, as it is in the presence of GSH (Table 1).

A redox-active disulfide bond in the myrosinase complex could be involved in nitrile formation by catalyzing the removal of the S from the unstable thiohydroxamate-O-sulfonate intermediate (Fig. 7). For epithionitrile formation, the S could then be swiftly transferred to the unsaturated side chain, whereas the formation of simple nitriles would require the release of S, e.g. in the form of HS^- [57], which would require two redox equivalents. In the present experiments, the redox equivalents could be provided by the interplay between Fe^{2+}, GSH, ascorbic acid and/or other thiol groups in the myrosinase complex. Apart from increasing the speed of the reaction, high concentrations of ascorbic acid could result in the reduction of the disulfide bond, suppressing nitrile formation.

Fe^{2+} has been reported to be especially effective in forming nitriles (Fig. 2; [20,37]). ESP possesses a series of β-sheets known as Kelch motifs [14], which are capable of binding metallic cations [58]. In this respect, it has already been suggested that this motif may serve a functional role by binding to Fe^{2+} at the active site of ESP [21].

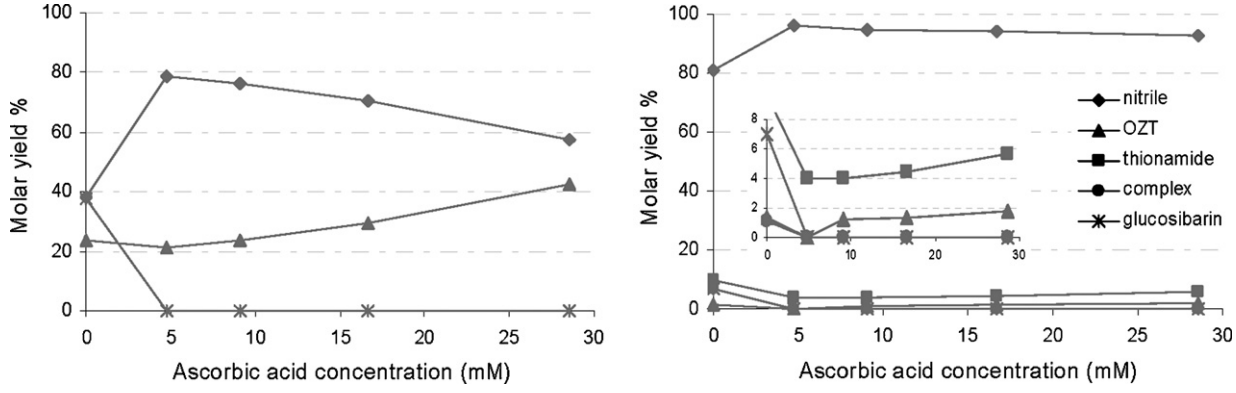

Fig. 6. Molar yield (in %) of glucosibarin and its hydrolysis products after 24 h incubation with myrosinase (0.015 U) and increasing ascorbic acid concentration at pH 5. Left: in the presence of 33.3 mM GSH; right: in the presence of 34.5 mM Fe^{2+}. The *y*-axis has been zoomed in.

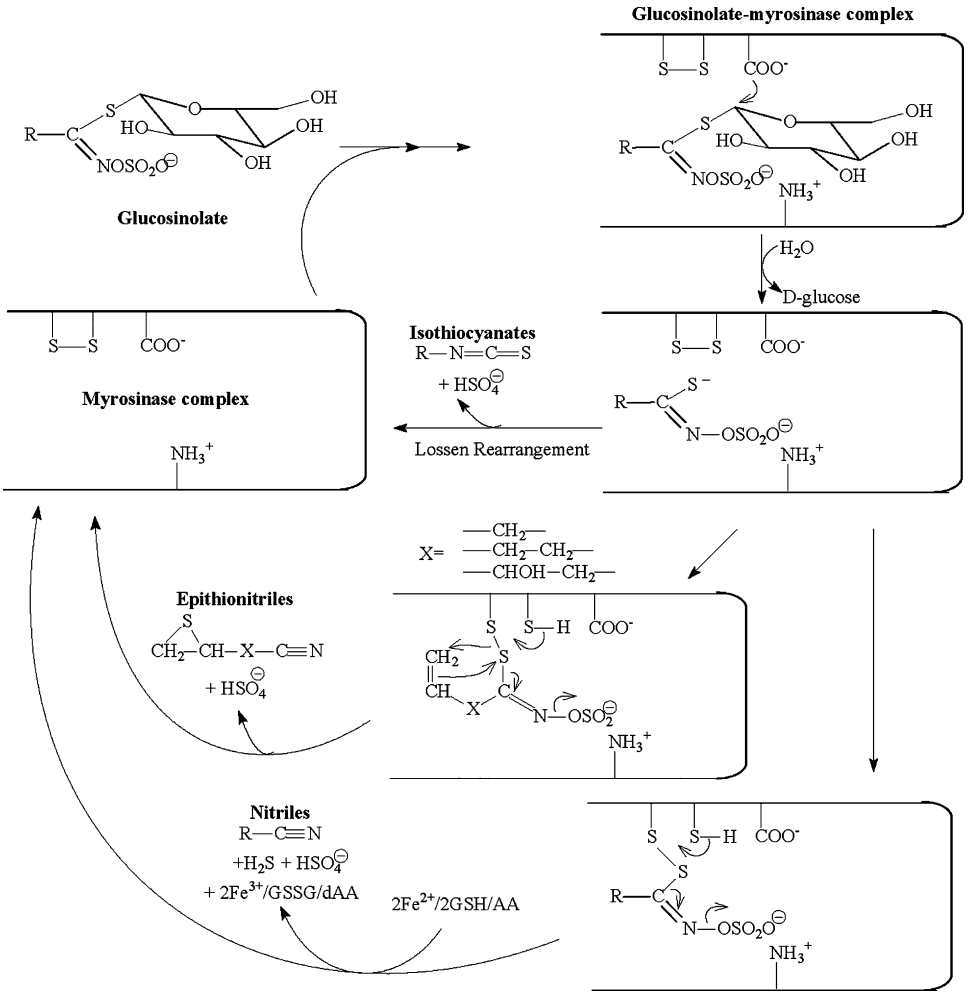

Fig. 7. Proposed reaction mechanism for myrosinase-catalyzed formation of nitriles and epithionitriles. Legend: R = any glucosinolate side chain; X = as shown in the figure.

4. Conclusion

The present experiments showed that increasing Fe^{2+} and GSH concentrations favoured nitrile formation at the expense of OZT upon the myrosinase-catalyzed hydrolysis of glucosibarin. Ascorbic acid showed a dual effect, with low concentrations favouring nitrile formation and high concentrations resulting in increased OZT formation. The proposed mechanism for nitrile formation involves a disulfide bond in the myrosinase complex and requires two redox equivalents, releasing nitrile and S in the oxidation state of minus two, e.g. in the form of HS^-.

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