# Key Role of Fe$^{2+}$ in Epithiospecifier Protein Activity

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The chemical nature of the hydrolysis products from the glucosinolate−myrosinase system depends on the presence or absence of supplementary proteins such as epithiospecifier proteins (ESPs). ESPs promote the formation of epithionitriles from terminal alkenyl glucosinolates and, as recent evidence suggests, simple nitriles at the expense of isothiocyanates. From a human health perspective isothiocyanates are the most important because they are major inducers of carcinogen-detoxifying enzymes. Fe$^{2+}$ is an essential factor in ESP activity, although several recent studies have highlighted discrepancies in the understanding of the ESP−iron interaction. To investigate further the role iron species play in regulating ESP activity, four ESP-containing seedpowders were analyzed for ESP and myrosinase activities, endogenous iron content, and glucosinolate degradation products after the addition of iron species, specific chelators, and reducing agents. For the first time this paper shows the effect of these additions on the hydrolysis of individual glucosinolates that constitute the total pool. Aged seeds and 3-day seedlings were also tested to investigate the effects of seed storage and early plant development on iron levels and ESP activity. The four ESP-containing plant systems tested gave two distinctive responses, thus providing strong evidence that ESPs vary markedly in their Fe$^{2+}$ requirement for activity. The results also indicated that reduction of ferric to ferrous iron drives variations in ESP activity during early plant development. The reverse oxidation reaction provided a convincing explanation for the loss of ESP activity during seed storage. Aged seeds produced seedlings with substantially lower ESP activity, and there was a concomitant loss in germination rate. It was concluded that manipulation of endogenous iron levels of ESP-containing plants could increase the conversion of glucosinolates to isothiocyanates and enhance potential health benefits.

KEYWORDS: Epithiospecifier protein activity; total and ferrous iron; nitrile formation

## INTRODUCTION

Glucosinolates are sulfur-containing glycosides with alkyl, alkenyl, or indole side chains found in brassicaceous plants (*1*). These plants include economically important vegetables such as broccoli (*Brassica oleracea* var. *italica*), cabbage (*Brassica oleracea* var. *capitata*), and radish (*Raphanus sativus*) as well as oilseed crops such as crambe (*Crambe abyssinica*).

The bioactive properties of these plants are attributed to the hydrolysis products formed from glucosinolates upon tissue disruption by endogenous thioglucosidases, namely, myrosinases (EC 3.2.3.1). The myrosinase-mediated hydrolysis of glucosinolates generates an unstable aglycone intermediate, which is immediately converted to a wide range of products including the bioactive isothiocyanates, and the less effective epithionitriles and simple nitriles (reviewed in ref 1).

The potential chemoprotective benefits of dietary isothiocyanates have been extensively reviewed (*2*, *3*). One of the principal mechanisms of this protection involves the ability of isothiocyanates to induce a battery of xenobiotic metabolizing enzymes that detoxify carcinogens (*4*, *5*). By contrast, nitriles are ineffective as inducers of these detoxification enzymes (*6−8*) and less effective as antiproliferative agents (*9*). Because of the potential health benefits associated with isothiocyanates, together with an increasing consumption of raw *Brassica* seedlings (sprouted seeds) and vegetables (*10*, *11*), a detailed understanding of factors controlling isothiocyanates or alternative glucosinolate degradation products is necessary.

The extent of glucosinolate hydrolysis by myrosinase and the structure and concentration of the products so formed are influenced by various features of the hydrolysis environment such as pH and temperature or the presence or absence of certain protein factors, the best known being epithiospecifier proteins (ESPs) (*1*, *12*).

ESPs are found in many (broccoli, cabbage, crambe) but not all (radish) glucosinolate-containing plants (*12−14*). In the presence of ESP, isothiocyanate formation is prevented in favor of nitriles (*14−18*). Depending on the side chain structure of glucosinolates,

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the hydrolysis products can be epithionitriles (formed from glucosinolates with a terminal alkenyl group) or simple nitriles (formed from all other glucosinolates).

It is well-known that ESP requires Fe for its activity (*12,14,16,17*). An early study found that the amount of $Fe^{2+}$ required to yield predominantly nitriles was similar to the amount of total iron present in the seed (*19*). Later research showed that $Fe^{2+}$ levels may be more important than total iron as $Fe^{2+}$ is more readily available for participating in metabolic reactions (*20*). Several investigations (*19,21*) into the influence of iron on nitrile formation produced mixed results, and it was reported in the latter study that these anomalies could be explained by natural variations in endogenous $Fe^{2+}$ (*21*). Speculation continued with the suggestion that seeds with significant ESP activity contained sufficient endogenous $Fe^{2+}$ (*22*). Since these reports, only limited, if any, research has been conducted into the effects of endogenous iron levels on ESP activity, although these earlier studies highlighted inconsistencies in this area.

A study into the ESP activity of *Brassica* seeds confirmed that $Fe^{2+}$ is essential for ESP activity by showing a promotion of the effects of ESP, even at very low levels (*22*). The same study found seeds that exhibited significant ESP activity contained sufficient endogenous $Fe^{2+}$, whereas the other seeds tested did not. The addition of extra $Fe^{2+}$ enhanced ESP activity in these latter seeds to detectable levels. Several studies since have also highlighted various responses in ESP activity to $Fe^{2+}$ addition given by different plants, thereby casting doubt on the conserved nature (sequences of bases or amino acids in the ESP proteins of different species that show similarity greater than that due to chance alone) of ESP (*14−16, 23*).

Several other factors have been implicated in influencing ESP activity. We have previously found convincing evidence that the early stages of plant development had a profound effect on ESP activity in some *Brassica* species (*18*). To date, no explanation for these differing activity changes during early plant development has been offered.

Earlier studies (*19, 24−26*) indicated that prolonged storage or aging of seeds produced more isothiocyanates than nitriles on hydrolysis, prompting one researcher to speculate that this indicated a loss of ESP activity (*26*). These findings were not followed up nor was any explanation given for this phenomenon, which is surprising as there may be potential to grow seedlings with decreased ESP activity.

In the current study a series of experiments was conducted to investigate in greater detail the role iron plays in regulating ESP activity as well as provide evidence to clarify the discrepancies previous studies have highlighted. Here we investigated (1) the role of endogenous iron in regulating ESP activity, (2) whether the iron requirement for nitrile formation and therefore ESP activity varies between species, (3) whether changes in Fe levels, in particular, the $Fe^{2+}/Fe^{3+}$ ratio, drive ESP activity changes during early plant development, and (4) the effects of seed storage on ESP activity and provide an explanation for the changes in activity that occur.

Other investigations into ESP activity have focused on understanding nitrile formation using purified recombinant ESP in model systems (*14−17*). Information on ESP activity and its influencing factors based on natural systems is very rare, particularly in recent literature. Because the major interest in this study concerned the ESP activity in situ, measuring the activity in the defatted seeds was preferred over the activity of the purified or recombinant protein, which is more commonly reported (*14−17*). This study provides evidence of the essential role of the reduction of $Fe^{3+}$ to $Fe^{2+}$ driving changes in ESP activity and hence nitrile formation. These investigations were conducted by additions of Fe, chelators, and reducing agents to the natural hydrolysis extracts from selected seedpowders and monitoring fluctuations in the hydrolysis products from individual glucosinolates present in these seedpowders.

Recently, several papers (*27−29*) have identified a group of nitrile-specifier proteins (AtNSPs) in *Arabidopsis thaliana*, which in conjunction with myrosinase generated simple nitriles but not epithionitriles. The response of these seedpowders to the addition of Fe and chelators was used to evaluate the likelihood of AtNSP activity contributing to simple nitrile formation in these samples.

## MATERIALS AND METHODS

**Materials.** The broccoli seeds (cv. Saga) were a gift from OptiGrow Pty. Ltd. The cabbage cv. Red Dutch seeds were purchased from Eden Seeds (Lower Beechmont, Australia). Royston Seeds (Mudgee, Australia) provided the radish cv. Cherry Belle seeds. Crambe seeds were a gift from the Centre for Legumes in Mediterranean Agriculture, The University of Western Australia. Seeds of wild turnip (*Brassica tournefortii*) were collected by Chris Calverley, Launceston, Tasmania. Organic solvents (HPLC grade) and most general purpose reagents were purchased from Biolab (Australia) Pty. Ltd. Other chemical reagents, including phenyl and propyl isothiocyanates, purified *Sinapis alba* myrosinase, bathophenanthroline disulfonic acid (BPDS), deferoxamine, sinigrin, and stannous chloride, were purchased from Sigma Aldrich (Australia). (2*S*)-1-Cyano-2-hydroxy-3,4-epithiobutane (CHEB), (*S*)-1-cyano-2-hydroxy-3-butane (CHB), and epi-progoitrin for ESP activity measurements were purified from crambe seeds using previously described extraction and purification methods outlined in Williams et al. (*18*).

**Preparation of Seedpowders.** The method used was that described previously (*22*). Seeds (10 g) were ground to a fine powder and defatted by repeated extraction with dry hexane (5 × 100 mL). The powders were air-dried and together with remaining seed were stored in airtight containers in a desiccator.

**Preparation of Seedlings.** Seeds were germinated in accordance with the International Seed Testing Association recommendations (*30*). Seeds were positioned between paper sheets, wetted with water, rolled in a wet towel, and placed in a dark incubator at 25 °C. Preliminary experiments indicated a germination rate of > 85% or above for all of the seeds tested. Seedlings with no defect on the cotyledon's shoot and root system were classified as a normal seedlings and used for the analysis. If any defects (as in ISTA rules) were found, the seedlings were classified as abnormal and discarded.

**Measurement of ESP Activity.** The activity of ESP was measured by examining the products formed from the purified glucosinolate epi-progoitrin when hydrolyzed by a purified myrosinase enzyme in the presence of the extract under study. Epithiospecifier protein activity was defined as the ratio of epithionitrile CHEB to simple nitrile CHB formed in the presence of excess myrosinase and iron. For each seedling sample, 350 μL of 50 mM acetate buffer containing 1 mM ferrous sulfate and 1 mM dithiothreitol (DTT; pH 5.5) was combined with 50 μL of purified myrosinase solution (0.5 U/mL) and 50 μL of ESP-containing extract prepared as per Matusheski et al. (*31*). To initiate the reaction, 50 μL of 5 mg/mL epi-progoitrin solution was added, and the samples were allowed to incubate for 1 h in a 25 °C shaking water bath. After incubation, 20 μL of 0.5 mg/mL phenyl isothiocyanate in acetonitrile (internal standard) was added to each sample. After vortexing, the solution was transferred to a Teflon microcentrifuge tube and extracted with 1 mL of methylene chloride. The tubes were centrifuged to fully separate layers, and the organic phase was collected. The methylene chloride extracts were analyzed by GC as described previously (*31*) but with modifications to the column oven temperature program. An initial temperature of 60 °C was held for 4 min, then ramped to 95 °C at 10 °C/min. The temperature was then immediately increased from 95 to 110 °C at 2 °C/min and then from 110 °C to a final temperature of 200 °C at 10 °C/min. The final temperature was held for 10 min. The GC was calibrated using standard curves of 1− 100 mg/mL phenyl isothiocyanate, CHB, and CHEB purified from seeds of crambe in methylene chloride. The ESP activity assay described was based on the method outlined previously (*31*) and was designed for determining activity in seedlings or mature plants. Modifications to the assay were developed to

enhance the sensitivity for the analysis of seeds and seedpowders. Defatted seedpowders (0.1 g) were combined with 700 $\mu$L of $Fe^{2+}$-containing acetate buffer and 100 $\mu$L of purified myrosinase solution. Addition of 100 $\mu$L of 5 mg/mL epi-progoitrin solution initiated the reaction. Incubation conditions were the same as above, but after incubation, 40 $\mu$L of the internal standard was added to each sample. The methylene chloride extracts obtained from these samples were analyzed for ESP activity in the same manner. The seedpowder weight of 0.1 g increased the sensitivity of the assay over the original for all of the samples tested. However, increasing the weight to 0.2 g provided no sensitivity enhancement; in fact, most exhibited a reduction. Several other ESP activity assays utilize purified sinigrin (*17*) instead of purified epi-progoitrin; this was not used in this study as a previous paper voiced concerns about the high volatility of the simple nitrile hydrolysis product, allyl cyanide (*32*).

**Measurement of Myrosinase Activity.** Myrosinase activity present in the seeds was measured by the hydrolysis of a known amount of sinigrin added to the extracts as previously described (*33*). Before analysis, the extracts derived from cabbage seeds were incubated for 1 h at 40 °C in an oven to hydrolyze the endogenous glucosinolates (predominantly sinigrin) present in the cabbage.

The method was based on that reported by Verkerk and Dekker (*33*), which was developed primarily for measuring activity in juice. Various modifications were tested to adapt the protocol for the analysis of seedpowders. The method adopted in this study involved mixing 1.5 mL of distilled water with 0.2 g of seedpowders, which was incubated for 50 min at 20 °C. The resulting mixture was centrifuged at 5000*g* for 10 min at room temperature. Supernatant (0.5 mL) was mixed with 100 $\mu$L of 6 mM sinigrin solution and allowed to incubate at 40 °C for 20 min. The reaction was stopped by adding 12 mL of 100% hot methanol (10 min at 75 °C). The resulting mixture was centrifuged (5000*g*, 10 min, room temperature), and the remaining sinigrin was isolated from the collected supernatant and analyzed by HPLC. Myrosinase activity was expressed as micrograms of sinigrin consumed per minute per gram (fw).

To determine any non-myrosinase-related degradation of the sinigrin, a control sample of 6 mM sinigrin solution was tested under assay conditions.

**Analysis of Glucosinolates.** To 1 g of seedpowder was added 10 mL of boiling ultrapure water, and the mixture was boiled for 5 min. The resultant slurry was transferred to a 20 mL volumetric flask and sonicated for 5 min. The extract was filtered through Whatman no. 4 filter paper and then made to the mark with ultrapure water (*34*). The samples were stored at −20 °C until HPLC analysis.

The levels of glucosinolates were determined by HPLC as previously described (*35*) but with several important modifications. The HPLC instrument was an Alliance 2690 (Waters, Milford, MA) liquid chromatograph attached to a Gemini C$_{18}$, 5 $\mu$m, 250 × 4.6 mm (Phenomenex) column. The mobile phase was a linear gradient from 100% 50 mM ammonium acetate to 50 mM ammonium acetate/methanol (80:20) in 50 min. The column, which was protected with an appropriate guard column, was operated at ambient temperature with a flow rate of 1.0 mL/min. The injection volume was 10 $\mu$L. The wavelength for detecting glucosinolates was 235 nm, and data were processed using Delta (Digital Solutions, Australia) software. Peak identification and homogeneity were confirmed by having the HPLC system coupled to a Micromass ZMD quadrapole mass analyzer. Quantification of glucosinolates was determined using purified or commercially available standards or, if unavailable, based on commercially available high-purity sinigrin (99.3%) and converted to glucosinolate on the basis of the relative peak areas obtained with equimolar concentrations of sinigrin (*11*, *35*).

**Analysis of Glucosinolate Hydrolysis Products.** The analysis of hydrolysis products was performed by modifying the method reported previously (*31*). This method was changed to accommodate the analysis of seedpowders. To triplicate seedpowder samples (0.25 g) was added 0.75 mL of ultrapure water (or solutions of iron, specific chelators, or reducing agents). In the case of $Fe^{2+}/Fe^{3+}$ additions, iron solutions of 0.5 mM were added, consistent with the concentrations added by Burow et al. (*17*) as well as giving a 2 M excess of iron in the assay system, consistent with Bellostas et al. (*36*). The mixture was shaken at room temperature for 1 h to allow hydrolysis to occur, after which the appropriate internal standard (40 $\mu$L) was added. A 1 h incubation was used in this study to be consistent with previous studies (*13*, *22*, *26*). The hydrolysis products were extracted with 1 mL of methylene chloride and, after

centrifugation, the extract was dried (anhydrous sodium sulfate) and stored ready for GC analysis. For GC analysis, 5 $\mu$L of methylene chloride extract was injected into a 1:30 split Varian 3900 GC system using a Varian autosampler CB-8410. The flow path consisted of a 4 mm i.d. single-gooseneck liner, a 3 m J&W DB-5 guard column, and a 30 m J&W DB-5 capillary column (0.25 mm i.d., 0.25 $\mu$m film) with flame ionization detection. The injector was held at 200 °C, and the detector was set at 280 °C. Initial temperature was 40 °C for 2 min, ramped from 40 to 260 °C at 10 °C/min. The final temperature of 260 °C was held for 10 min. Nitrogen was used as the carrier gas. Quantification was determined by standard curves of purified hydrolysis products or based on the peak area relative to that of the internal standard, propyl isothiocyanate. Response factors (RF) relative to propyl isothiocyanate were calculated for compounds not commercially available by using the effective carbon number (ECN) concept (*37*).

To assess the likelihood of nonenzymatic generation of nitriles, any possible glucosinolate degradation products were extracted with methylene chloride direct from the defatted seedpowders without hydrolysis.

For product identification by MS, the column was coupled to an MSD 5975 mass spectrometric detector (Agilent Technologies, USA) with helium as the carrier gas at a linear velocity of 56 cm/min and at flow rate of 2.4 mL/min. Mass spectra in the electron impact (EI) mode were generated at 70 eV and collected over the range from *m/z* 35 to 350 for scan runs. Data analysis was carried out with the help of the MSD ChemStation Data Analysis (Agilent Technologies, USA) software. Products were identified using mass spectra and retention times of authentic standards and published MS spectra (*38*).

**Measurement of Total and Ferrous Iron Content.** Total iron content of seedpowder samples was determined in triplicate by inductively coupled plasma optical emission spectrophotometer (ICPOES) analysis following a digestion with a premixed combination of nitric and perchloric acids containing vanadium as a catalyst.

Ferrous iron was determined according to the method of Pierson and Clark (*20*) modified by utilizing aqueous solutions of the chelating agent, BPDS. Ferric ion was calculated by difference between total and ferrous ion.

**Measurement of Ascorbic Acid Content.** Ascorbic acid content was determined on the seedpowders and 3-day seedlings by the 2,6-dichloro-indophenol titrimetric method (*39*).

**Statistical Analysis.** Statistical analysis for each experiment was performed with ANOVA and Fisher's protected LSD ($\alpha = 0.05$) using Jump7 statistical analysis software (from SAS).

## RESULTS

**ESP, Myrosinase, and Fe in Seedpowders.** We analyzed five seedpowders (four containing ESP) for ESP and myrosinase activities and endogenous iron content. To highlight differences, measurements were also conducted on the seeds of non-ESP-containing radish cultivar Cherry Belle. Crambe and wild turnip seedpowders had similarly high ESP activity (**Table 1**). The seedpowders from broccoli and cabbage formed a second pair in that they had only relatively low levels of ESP activity. No ESP activity was detected in radish seeds. By contrast, the myrosinase activities of all seedpowders were almost identical (**Table 1**).

Endogenous iron (i.e., total, $Fe^{2+}$, and by subtraction $Fe^{3+}$) concentrations in the seedpowders were measured to determine whether the levels were sufficiently high to affect nitrile formation. There was no evidence of any relationship between total Fe or $Fe^{2+}$ levels and ESP activity, with the non-ESP-containing radish seeds possessing slightly higher total concentrations than the highly active crambe seeds. To a limited extent the $Fe^{2+}/Fe^{3+}$ ratio was associated with ESP activity with wild turnip the exception (**Table 1**).

**Glucosinolate Profiles of Seedpowders.** As an aid to the identification of the hydrolysis products formed by these seedpowders, their glucosinolate profiles were determined (**Table 2**). The principal glucosinolate present in crambe seeds was epi-progoitrin ((2*S*)-2-hydroxy-3-butenyl glucosinolate), comprising 94% of the total pool. Sinigrin (2-propenyl glucosinolate) contributed the

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**Table 1.** Distribution of ESP Activity,[a] Myrosinase Activity, and Total, Ferrous, and Ratio of Ferrous/Ferric[b] Iron Content in the Seeds of Five Brassicaceous Species[c]

| | | | | iron content (mg/kg, dw) | | |
| species name | common name | ESP activity[b] | myrosinase activity ($\mu$g of sinigrin consumed/min/g, fw) | total | $Fe^{2+}$ | $Fe^{2+}/Fe^{3+}$ |
|---|---|---|---|---|---|---|
| *Crambe abyssinica* | crambe | 3.49 ± 0.47 e | 175 ± 20 e | 68 ± 3 e | 50 ± 4 e | 2.8 |
| *Brassica tournefortii* | wild turnip | 2.51 ± 0.47 f | 175 ± 23 e | 102 ± 3 f | 37 ± 4 f | 0.6 |
| *Brassica oleracea* L. *capitata* | cabbage cv. Red Dutch | 0.25 ± 0.06 g | 182 ± 25 e | 89 ± 11 g | 58 ± 7 e | 1.8 |
| *Brassica oleracea* L. *italica* | broccoli cv. Saga | 0.20 ± 0.06 g | 174 ± 29 e | 88 ± 9 g | 45 ± 2 g | 1.0 |
| *Raphanus sativus* | radish cv. Cherry Belle | nd | 191 ± 20 e | 77 ± 6 eh | 31 ± 3 fi | 0.7 |

[a] Expressed as the ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed from purified epi-progotrin. [b] Derived by subtraction of $Fe^{+2}$ content from total iron content. [c] Data are the mean ± standard error of three replicates. Within each species means followed by a common letter are not significantly different at *p* = 0.050; nd, not detected.

**Table 2.** Percentages of Glucosinolate Content and Glucosinolate Degradation Products Formed on 1 h of Hydrolysis in (**a**) Crambe, (**b**) Wild Turnip, (**c**) Cabbage Cv. Red Dutch, (**d**) Broccoli Cv. Saga, and (**e**) Radish Cv. Cherry Belle Seeds[a]

| glucosinolates | | | 1 h hydrolysis products | | |
|---|---|---|---|---|---|
| common name | chemical name | % | common name | chemical name | % |
| **(a) crambe** | | | | | |
| 1. epi-progoitrin | (2S)-2-hydroxy-3-butenyl | 94 | goitrin | 2-hydroxy-3-butenyl OZT | 10 |
| | | | (S)-CHB | 1-cyano-2-hydroxy-3-butene nitrile (SN) | 20 |
| | | | (S)-CHEB | 1-cyano-2-hydroxy-3,4-epithiobutane (ETN) | 70 |
| 2. sinigrin | 2-propenyl | 6 | | nd | |
| total | | 100 | total | | 100 |
| **(b) wild turnip** | | | | | |
| 1. gluconapin | 3-butenyl | 87 | 3-butenyl ITC | 3-butenyl ITC | 14 |
| | | | P-NIT | pent-4-ene nitrile (SN) # | 2 |
| | | | EP-NIT | 4,5-epithiopentyl nitrile (ETN) # | 80 |
| 2. progoitrin | (2R)-2-hydroxy-3-butenyl | 9 | goitrin | 2-hydroxy-3-butenyl OZT | 2 |
| | | | (R)-CHB | 1-cyano-2-hydroxy-3-butene (SN) | 2 |
| 3. OH-glucobrassicin | 4-hydroxyindol-3-ylmethyl # | 4 | | nd | |
| total | | 100 | total | | 100 |
| **(c) cabbage cv. Red Dutch** | | | | | |
| 1. sinigrin | 2-propenyl | 38 | allyl ITC | 2-propenyl ITC | 19 |
| | | | EB-NIT | 3,4-epithiobutyl nitrile (ETN) | 2 |
| 2. glucoiberin | 3-methylsulfinylpropyl # | 32 | iberin | 3-methylsulfinylpropyl ITC # | 35 |
| | | | iberin nitrile | 4-methylsulfinylbutyl nitrile (SN) # | 5 |
| 3. progoitrin | (2R)-2-hydroxy-3-butenyl | 20 | goitrin | 2-hydroxy-3-butenyl OZT | 20 |
| | | | (R)-CHB | 1-cyano-2-hydroxy-3-butene (SN) | 6 |
| 4. glucoraphanin | 4-methylsulfinylbutyl | 5 | sulforaphane | 4-methylsulfinylbutyl ITC | 12 |
| 5. glucoiberverin | 3-methylthiopropyl # | 5 | iberverin | 3-methylthiopropyl ITC # | 1 |
| | | | iberverin nitrile | 4-methylthiobutyl nitrile (SN) # | 2 |
| total | | 100 | total | | 100 |
| **(e) broccoli cv. Saga** | | | | | |
| 1. glucoraphanin | 4-methylsulfinylbutyl | 79 | sulforaphane | 4-methylsulfinylbutyl ITC | 70 |
| | | | sulforaphane nitrile | 5-methylsulfinylpentyl nitrile (SN) | 25 |
| 2. glucoerucin | 4-methylthiobutyl # | 16 | erucin nitrile | 5-methylthiopentyl nitrile (SN) # | 5 |
| 3. OH-glucobrassicin | 4-hydroxyindol-3-ylmethyl # | 5 | | nd | |
| total | | 100 | total | | 100 |
| **(f) radish cv. Cherry Belle** | | | | | |
| 1. glucoraphenin | 4-methylsulfinyl-3-butenyl | 93 | sulforaphene | 4-methylsulfinyl-3-butenyl ITC | 97 |
| 2. glucoraphanin | 4-methylsulfinylbutyl | 5 | sulforaphane | 4-methylsulfinylbutyl ITC | 3 |
| 3. OH-glucobrassicin | 4-hydroxyindol-3-ylmethyl # | 2 | | nd | |
| total | | 100 | total | | 100 |

[a] Data are the mean of three replicates; nd, not detected. In the comparison of the nomenclature of glucosinolates and isothiocyanates with their corresponding nitriles, the numbering system must be adjusted because the nitrile carbon atom is counted as part of the alkyl chain. ETN, epithionitrile; ITC, isothiocyanate; OZT, oxazolidine-2-thione; SN, simple nitrile. # denotes use of sinigrin equivalents in quantification of glucosinolates or carbon number in quantification of hydrolysis products. Nondesignated values were quantified by use of standards.

remaining 6%. Wild turnip (*B. tournefortii*) seeds had a similar glucosinolate profile, with a terminal alkenyl glucosinolate, gluconapin (3-butenyl glucosinolate), comprising 87% of the total glucosinolate pool. Progoitrin ((2R)-2-hydroxy-3-butenyl glucosinolate) and 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl glucosinolate) were also present. Cabbage (cv. Red Dutch) seeds contained

sinigrin and glucoiberin (3-methylsulfinylpropyl glucosinolate), contributing equally to 70% of the total glucosinolates. There were also substantial amounts of progoitrin and glucoraphanin (4-methylsulfinylbutyl glucosinolate) and glucoiberverin (3-methylthiopropyl) present. In broccoli (cv. Saga) seeds glucoraphanin constituted 80% of the total glucosinolate pool. Glucoerucin (4-methylthiobutyl

**Table 3.** Normalized Percentage of Total Amount of Glucosinolate Degradation Products Formed after the Addition of $Fe^{2+}$, $Fe^{3+}$, and Chelators (EDTA, BPDS, and Deferoxamine) to the Hydrolysis Mixtures of (**a**) Crambe, (**b**) Wild Turnip, (**c**) Cabbage Cv. Red Dutch, and (**d**) Broccoli Cv. Saga Seeds[a]

| glucosinolate common name | hydrolysis product common name | std hydr | 0.5 mM Fe(II) | 0.5 mM Fe(III) | 20 mM EDTA | 10 mM BPDS | 10 mM deferoxamine |
|---|---|---|---|---|---|---|---|
| | | | | **(a) Crambe** | | | |
| 1. epi-progoitrin | (*S*)-CHEB (ETN) | 1.0 | 0.9 | 1.0 | 0.7 | 0.4 | 0.6 |
| | (*S*)-CHB (SN) | 1.0 | 1.0 | 1.0 | 0.8 | 0.5 | 0.6 |
| | goitrin (OZT) | 1.0 | 0.2 | 0.8 | 3.0 | 3.1 | 1.5 |
| | | | | **(b) Wild Turnip** | | | |
| 1. gluconapin | EP-NIT (ETN) | 1.0 | 0.9 | 0.7 | 0.3 | 0.2 | 0.4 |
| | P-NIT (SN) | 1.0 | 0.9 | 0.8 | 0.4 | 0.2 | 0.4 |
| | 3-butenyl (ITC) | 1.0 | 0.5 | 0.7 | 1.1 | 1.0 | 1.0 |
| 2. progoitrin | (*R*)-CHB (SN) | 1.0 | 0.8 | 1.0 | nd | nd | 0.3 |
| | goitrin (OZT) | 1.0 | nd | nd | 3.4 | 1.7 | 1.2 |
| | | | | **(c) Cabbage Cv. Red Dutch** | | | |
| 1. sinigrin | EB-NIT (ETN) | 1.0 | 10.5 | 1.4 | nd | nd | nd |
| | allyl (ITC) | 1.0 | 0.6 | 0.9 | 1.5 | 1.5 | 1.3 |
| 2. glucoiberin | iberin nitrile (SN) | 1.0 | 2.2 | 1 | nd | nd | nd |
| | iberin (ITC) | 1.0 | 0.2 | 0.4 | 1.1 | 1.0 | 0.9 |
| 3. progoitrin | (*R*)-CHB (SN) | 1.0 | 2.5 | 1.2 | nd | nd | nd |
| | goitrin (OZT) | 1.0 | 0.2 | 0.7 | 1.0 | 1.0 | 0.9 |
| | | | | **(d) Broccoli Cv. Saga** | | | |
| 1. glucoraphanin | SPHN (SN) | 1.0 | 2.9 | 1.0 | 0.5 | 0.4 | 0.7 |
| | SPH (ITC) | 1.0 | 0.2 | 0.7 | 1.5 | 1.2 | 1.1 |
| 2. glucoerucin | erucin nitrile (SN) | 1.0 | 2.2 | 0.9 | 0.4 | 0.4 | 0.7 |

[a] Data are the mean of three replicates. ETN, epithionitrile; ITC, isothiocyanate; OZT, oxazolidine-2-thione; SN, simple nitrile with SPHN, sulforaphane nitrile; SPH, sulforaphane (hydrolysis products of glucoraphanin).

glucosinolate) and 4-hydroxyglucobrassicin were also present in all of the cultivars tested. Predominant in radish seeds (cv. Cherry Belle) was glucoraphenin (4-methylsulfinyl-3-butenyl glucosinolate), which comprised 93% of the total glucosinolates, with small amounts of glucoraphanin and 4-hydroxyglucobrassicin making up the remainder.

**Additions of Fe and Chelators.** Control samples of the five seedpowders contained no nitriles or isothiocyanates. Gas chromatography−mass spectrometry (GC-MS) analysis detected only the hydrolysis products of the predominant glucosinolate, epi-progoitrin, in crambe seeds. **Tables 2** and **3** show that $Fe^{2+}$ addition to the crambe seedpowder produced no significant changes to either epithionitrile (ETN) or simple nitrile (SN) (ETN $p = 0.1866$, $R^2 = 0.387$; SN $p = 0.1777$, $R^2 = 0.377$) levels, but a significant decrease in the isothiocyanate derivate, goitrin ($p < 0.0001$, $R^2 = 0.997$). The addition of $Fe^{3+}$ to the crambe samples produced no significant change in the levels of either nitrile (ETN $p = 0.9262$, $R^2 = 0.002$; SN $p = 0.1029$, $R^2 = 0.477$) and an insignificant decrease in goitrin levels ($p = 0.0629$, $R^2 = 0.620$). Adding EDTA or BPDS to the crambe samples significantly decreased nitrile production (ETN $p = 0.0096$, $R^2 = 0.928$; SN $p = < 0.0005$, $R^2 = 0.984$) and significantly increased goitrin production ($p = 0.0066$, $R^2 = 0.992$). Deferoxamine addition had a smaller but still significant reducing effect on nitrile formation (ETN $p = 0.096$, $R^2 = 0.844$; SN $p = 0.109$, $R^2 = 0.822$) and caused a small but significant increase in goitrin levels ($p = 0.0066$, $R^2 = 0.870$).

Wild turnip seeds on hydrolysis of the major terminal alkenyl glucosinolate, gluconapin, and the minor terminal alkenyl glucosinolate, progoitrin, produced predominately epithionitrile from gluconapin but, surprisingly, only the simple nitrile from progoitrin (**Tables 2** and **3**). Addition of $Fe^{2+}$ and $Fe^{3+}$ had no discernible effect on the predominant species of nitrile hydrolysis

product from the two glucosinolates or for that matter the levels. Following a pattern similar to that for crambe, the amounts of isothiocyanate (ITC) or goitrin formed were significantly decreased (ITC $p = 0.0096$, $R^2 = 0.928$; goitrin $p = < 0.0005$, $R^2 = 0.984$). The use of all three chelators substantially decreased all nitrile formation while increasing isothiocyanate and goitrin production.

Cabbage cv. Red Dutch seeds on hydrolysis of the major terminal alkenyl glucosinolate, sinigrin, and the minor terminal alkenyl glucosinolate, progoitrin, produced predominately isothiocyanate with some epithionitrile from sinigrin, but again only the simple nitrile from progoitrin (**Tables 2** and **3**). The other major glucosinolate, the non-alkenyl glucoiberin, produced mainly the corresponding isothiocyanate with only moderate amounts of the simple nitrile. The addition of $Fe^{2+}$ to the cabbage seed assays caused a very large and significant increase ($p < 0.0001$, $R^2 = 0.998$) in epithionitrile formation and a smaller but still significant increase in both simple nitriles at the expense of isothiocyanate production, which significantly decreased ($p < 0.0001$, $R^2 = 0.997$). No significant change in either nitrile was found in cabbage (ETN $p = 0.9262$, $R^2 = 0.002$; SN $p = 0.7053$, $R^2 = 0.054$) seedpowders following $Fe^{3+}$ addition. Addition of EDTA or BPDS to the cabbage samples significantly decreased both types of nitrile (ETN, $p = 0.0096$, $R^2 = 0.928$; SN, $p = < 0.0005$, $R^2 = 0.984$) and significantly increased isothiocyanates ($p = 0.0066$, $R^2 = 0.992$). Deferoxamine addition had a smaller but still significant reducing effect on nitrile formation (ETN $p = 0.096$, $R^2 = 0.844$; SN $p = 0.109$, $R^2 = 0.822$).

Hydrolysis of the major non-alkenyl glucosinolate in broccoli seeds, glucoraphanin, produced predominately isothiocyanate with substantial amounts of the simple nitrile. Hydrolysis of the other glucosinolate, glucoerucin, formed only simple nitrile (**Tables 2** and **3**).

Article

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## (a) Crambe
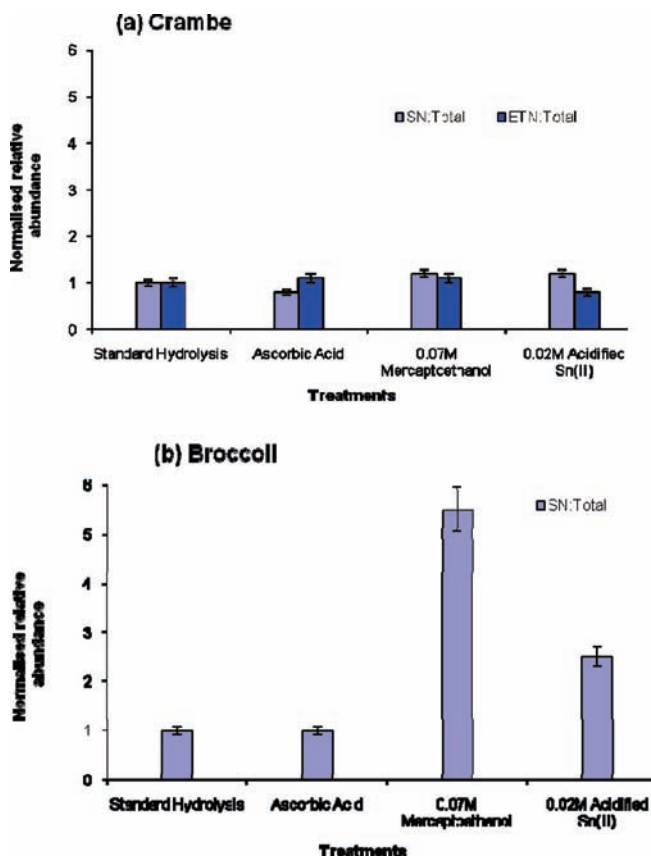


## (b) Broccoli



**Figure 1.** Normalized percentage of the proportion of nitriles/total amount of glucosinolate degradation products formed after the addition of 0.05 M ascorbic acid, 0.07 mM mercaptoethanol, and 0.02 M acidified $Sn^{2+}$ solutions to the hydrolysis mixtures of freshly prepared (**a**) crambe and (**b**) broccoli cv. Saga seedpowders. Error bars represent $\pm$ SEM (where $n = 3$).

Addition of $Fe^{2+}$ to the broccoli seed assays caused a significant increase ($p < 0.0001$, $R^2 = 0.998$) in both simple nitriles at the expense of isothiocyanates, which significantly decreased ($p < 0.0001$, $R^2 = 0.997$). Addition of $Fe^{3+}$ to the samples produced a small but insignificant decrease in isothiocyanate levels ($p = 0.2728$, $R^2 = 0.287$). No significant change in nitrile production was found in the broccoli ($p = 0.7053$, $R^2 = 0.054$) seedpowders following $Fe^{3+}$ addition. Addition of EDTA or BPDS significantly decreased nitrile production ($p = <0.0005$, $R^2 = 0.984$) and significantly increased isothiocyanate production ($p = 0.0307$, $R^2 = 0.728$). Deferoxamine addition had a smaller but still significant reducing effect on nitrile formation (SN $p = 0.0003$, $R^2 = 0.973$) but no effect on isothiocyanate levels ($p = 0.0987$, $R^2 = 0.534$).

Only the isothiocyanate hydrolysis product of the major glucosinolate, glucoraphenin, was detected in the radish seedpowder (**Table 2**), and thus no effect on nitrile formation by additions was monitored.

**Addition of Reducing Agents.** To establish whether reduction from $Fe^{3+}$ to $Fe^{2+}$ effects ESP activity variations, three reducing agents were added to the hydrolysis product assays of freshly prepared seedpowders. The proportions of nitriles to total hydrolysis products are presented in **Figure 1**. **Figure 1a** shows the normalized changes upon reducing agent addition for crambe, which was typical of both the crambe and wild turnip; in fact, changes for both seedpowders were almost identical. Broccoli results are shown in **Figure 1b** and were typical of both broccoli and cabbage seedpowders; again, the changes for both were almost identical.

Addition of ascorbic acid to the ESP-containing seedpowders increased the formation of nitriles and isothiocyanates (data not shown), although the nitriles to total products ratio did not change (crambe, $p = 0.5341$, $R^2 = 0.103$; broccoli, $p = 0.0.8993$, $R^2 = 0.004$).

Addition of mercaptoethanol highlighted the pairing of the ESP-containing seedpowders. Crambe (**Figure 1a**) and wild radish seeds showed a small, insignificant decrease (crambe, $p = 0.5675$, $R^2 = 0.088$) in epithionitrile ratios and a small, insignificant increase (crambe, $p = 0.7068$, $R^2 = 0.039$) in simple nitrile ratios. There was also a significant decrease in isothiocyanate production (data not shown). Mercaptoethanol addition to the other pair of seedpowders (broccoli shown in **Figure 1b** and cabbage) significantly enhanced (broccoli, $p = <0.0001$, $R^2 = 0.993$) nitrile production and decreased isothiocyanate formation (data not shown).

The powerful iron-reducing solution (*40*) acidified $SnCl_2$ (0.02 M HCl was added dropwise to the $SnCl_2$ suspension until the solid $SnCl_2$ dissolved, giving a final concentration of 0.02 M $Sn^{2+}$) caused changes that were similar to those seen with mercaptoethanol. Preliminary experiments indicated that acidification of pure water with 0.02 M HCl solutions decreased the levels of all hydrolysis products, although the proportion of nitriles remained the same as the proportion for standard hydrolysis. The $Sn^{2+}$ addition had no effect on the simple nitrile levels in crambe seedpowder (**Figure 1a**), but there was an insignificant decrease ($p = 0.3006$, $R^2 = 0.260$) in the epithionitrile proportion. Contrasting results were found for the broccoli samples, with a significant increase ($p = 0.0007$, $R^2 = 0.956$) in the nitrile/total products ratio on $Sn^{2+}$ addition (**Figure 1b**).

**Early Plant Development and ESP Activity, Endogenous Ascorbic Acid, and Fe.** ESP activity, ascorbic acid content, and $Fe^{2+}/Fe^{3+}$ ratios in the four nitrile-forming seedpowders and the corresponding day 3 seedlings (or, to use the commercial term for germinated seeds, sprouts) are shown in **Table 4**. The changes in ESP activity in the seedpowders and day 3 seedlings were consistent with the pairing of ESP-containing seedpowders. Crambe and wild turnip seeds showed a steady decrease in activity, whereas broccoli and cabbage showed a marked increase. Ascorbic acid levels and total Fe content (expressed on a dry weight basis) were consistently higher in all of the seedlings than in the seedpowders. However, the $Fe^{2+}/Fe^{3+}$ ratios paired crambe with wild turnip, showing no change ($p = 0.2879$, $R^2 = 0.272$) with plant development, and broccoli and cabbage, with a small but significant increase ($p = 0.0075$, $R^2 = 0.862$) (**Table 4**).

**Seed Aging and ESP Activity.** Changes of ESP activity together with the $Fe^{2+}/Fe^{3+}$ ratio in the four ESP-containing seedpowders with and without storage are given in **Table 5**. All seedpowders showed a decrease in ESP activity with storage and a corresponding decrease in $Fe^{2+}/Fe^{3+}$ ratio. Total iron content did not change during storage.

To investigate whether the decreased ESP activity of aged seeds can be translated to seedlings, crambe and broccoli cv. Saga seeds were germinated and harvested at 3 days. Seedlings grown from fresh and aged crambe seeds produced 3-day seedlings that showed only a slight insignificant decrease in ESP activity and a decrease in germination rate from 86 to 72%. Aged broccoli seeds had significantly decreased ESP activity and a reduction in germination rate from 89 to 68% (**Figure 2**).

## DISCUSSION

It was shown early that the amount of $Fe^{2+}$ required to form predominantly nitriles in crambe was the same order of magnitude as the amount of total iron found in the crambe powder, that is,

**Table 4.** Distribution of ESP Activity,[a] Ascorbic Acid Content, and Total, Ferrous, and Ratio of Ferrous/Ferric[b] Iron Content in the Seeds and Day 3 Seedlings of Four ESP-Containing Brassicaceous Species[c]

| | | | | iron content (mg/kg, dw) | | |
|---|---|---|---|---|---|---|
| plant name | plant age | ESP activity | ascorbic acid level (mg/kg, dw) | total | $Fe^{2+}$ | $Fe^{2+}/Fe^{3+}$ |
| crambe | seed | $2.56 \pm 0.46$ | $1.01 \pm 0.3$ | $77 \pm 5$ | $28 \pm 4$ | 0.6 |
| | 3-day seedling | $1.05 \pm 0.31$ | $8.04 \pm 0.8$ | $117 \pm 8$ | $40 \pm 6$ | 0.5 |
| wild turnip | seed | $2.20 \pm 0.39$ | $0.35 \pm 0.2$ | $91 \pm 5$ | $25 \pm 4$ | 0.4 |
| | 3-day seedling | $2.02 \pm 0.45$ | $10.11 \pm 1.2$ | $107 \pm 9$ | $38 \pm 4$ | 0.6 |
| cabbage cv. Red Dutch | seed | $0.16 \pm 0.08$ | $1.12 \pm 0.25$ | $75 \pm 10$ | $15 \pm 3$ | 0.3 |
| | 3-day seedling | $1.58 \pm 0.28$ | $7.74 \pm 1.5$ | $108 \pm 12$ | $53 \pm 6$ | 0.9 |
| broccoli cv. Saga | seed | $0.05 \pm 0.04$ | $2.31 \pm 0.9$ | $87 \pm 9$ | $21 \pm 2$ | 0.3 |
| | 3-day seedling | $1.94 \pm 0.37$ | $8.07 \pm 1.9$ | $128 \pm 12$ | $49 \pm 2$ | 0.6 |

[a] Expressed as the ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed from purifed epi-progotrin. [b] Derived by subtraction of $Fe^{+2}$ content from total iron content. [c] Data are the mean ± standard error of three replicates.
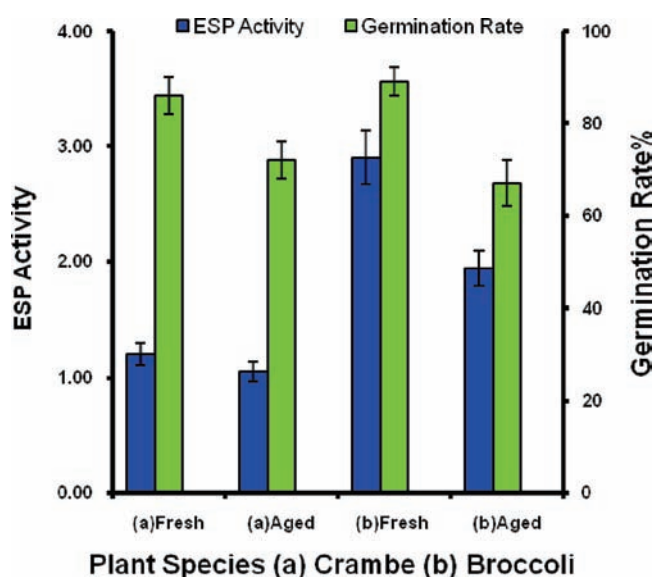
**Table 5.** Distribution of ESP Activity[a] and Total, Ferrous, and Ratio of Ferrous/Ferric[b] Iron Content in Freshly Prepared and Stored Seedpowders (15 Months at Room Temperature) of Four ESP-Containing Brassicaceous Species[c]

| | | | iron content (mg/kg, dw) | | |
|---|---|---|---|---|---|
| plant name | seed type | ESP activity (mg/kg, dw) | total | $Fe^{2+}$ | $Fe^{2+}/Fe^{3+}$ |
| crambe | fresh | $3.49 \pm 0.47$ | $68 \pm 3$ | $50 \pm 4$ | 2.8 |
| | stored | $2.56 \pm 0.46$ | $77 \pm 5$ | $28 \pm 4$ | 0.6 |
| wild turnip | fresh | $2.51 \pm 0.47$ | $102 \pm 3$ | $37 \pm 4$ | 0.6 |
| | stored | $2.20 \pm 0.39$ | $91 \pm 5$ | $25 \pm 4$ | 0.4 |
| cabbage cv. Red Dutch | fresh | $0.25 \pm 0.06$ | $89 \pm 11$ | $58 \pm 7$ | 1.8 |
| | stored | $0.16 \pm 0.08$ | $75 \pm 10$ | $15 \pm 3$ | 0.3 |
| broccoli cv. Saga | fresh | $0.20 \pm 0.06$ | $88 \pm 9$ | $45 \pm 2$ | 1.0 |
| | stored | $0.05 \pm 0.04$ | $87 \pm 9$ | $21 \pm 2$ | 0.3 |

[a] Expressed as the ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed from purified epi-progotrin. [b] Derived by subtraction of $Fe^{+2}$ content from total iron content. [c] Data are the mean ± standard error of three replicates.



**Figure 2.** Effect of seed storage on ESP activity (defined as the ratio of CHEB/CHB) and germination rate (%) in 3-day-old seedlings for (**a**) crambe and (**b**) broccoli cv. Saga. Error bars represent mean ± SEM (where *n* = 3).

126−213 ppm (*19*). The total iron levels in our study (68 ppm) were lower than this. Another point of difference was that radish seeds without ESP had higher total iron concentrations than the highly ESP-active crambe seeds. The authors (*20*) suggested that $Fe^{2+}$ levels were more important than total iron. They argued that $Fe^{2+}$ was available to participate in metabolic reactions and was more readily incorporated into molecular structures. Other authors (*21*) suggested that natural variations in endogenous $Fe^{2+}$ cause different levels of nitrile generation in *Brassica* plants. This suggestion appears to be at variance with our results, in particular, the ESP activity of the wild turnip seeds, which had the second highest ESP activity but the lowest $Fe^{2+}$ content. Seeds with significant ESP activity probably contain sufficient endogenous $Fe^{2+}$, and although actual levels were not reported (*22*), this suggests different $Fe^{2+}$ requirements between species, a topic discussed in greater detail later in the present study. Our study (**Table 1**) showed no relationship between total or $Fe^{2+}$ levels and ESP activity, although the activity paralleled the $Fe^{2+}$ concentrations as indicated by the $Fe^{2+}/Fe^{3+}$ ratios in all seedpowders except wild turnip.

The glucosinolate profiles of the crambe, cabbage, broccoli, and radish seedpowders agreed closely with previous studies (*1, 12, 14, 31, 33*). No glucosinolate profile could be located for this variety of wild turnip.

The lack of enhanced nitrile production in crambe and wild turnip seedpowders by Fe rules out nonenzymatic formation (*41*), and this is supported by the absence of nitriles in the unhydrolyzed controls, although enhanced formation of other hydrolysis products cannot be ruled out. This separation of *Brassica* seeds according to their response to $Fe^{2+}$ addition was also noted in an earlier study (*22*). These authors reported that seeds which appeared to have no activity showed major changes in hydrolysis products on addition of small amounts of $Fe^{2+}$. This led us to hypothesize that ESPs from different plant sources vary in their iron requirements. Supporting evidence was provided by a close scrutiny of the results of a study into *Arabidopsis* ESP (*15*). The *Arabidopsis* ecotype used to prepare recombinant ESP for their $Fe^{2+}$ addition studies was Tacoma (Tac), and upon hydrolysis this ecotype produced a profile similar to that exhibited by crambe (forming predominantly epithionitriles). The lack of response of *Arabidopsis* ESP to $Fe^{2+}$ addition was duplicated in the present study by the crambe ESP. However, confirmation that $Fe^{2+}$ is essential for ESP activity was provided by the strongly decreased levels of nitriles (simple and epithionitriles) produced on addition of the general chelator EDTA and the $Fe^{2+}$ specific

chelator BPDS. This dependency on $Fe^{2+}$ for ESP activity appears to be specific because no effect was found if $Fe^{3+}$ was added to any of the hydrolysis assays. The ineffectiveness of $Fe^{3+}$ to enhance nitrile formation has also been previously reported (*19*). That paper and our results were in direct contrast to studies on a protein related to ESP, thiocyanate-forming protein (TFP) (*21*), and of a recombinant *Arabidopsis* ESP (*17*) that found considerable enhancement of specifier protein activity following $Fe^{3+}$ addition. This discrepancy may be explained if any large increase in $Fe^{3+}$ produces a smaller but significant increase in $Fe^{2+}$ levels in the assay system. This is likely as both studies reported much less activity enhancement on $Fe^{3+}$ addition, and in the present study the $Fe^{3+}$ chelator, deferoxamine, caused a small but significant reduction in nitrile formation.

Ascorbic acid had no discernible effect on nitrile formation, but this was expected because ascorbic acid has several roles, one as a reducing agent and the other as a potent myrosinase activity enhancer, thereby increasing the formation of all hydrolysis products. It has been noted when ascorbate was added to crambe seedpowders considerable myrosinase activation occurred, but there was no increase in epithionitrile formation (*12*). The use of mercaptoethanol provided further evidence of differing $Fe^{2+}$ requirements for ESPs from different species. Mercaptoethanol addition to freshly prepared crambe and wild turnip seedpowders showed no effect on nitrile formation, whereas there was a dramatic increase in nitriles in broccoli and cabbage powders. It has been known for many years that when mercaptoethanol is added to aged crambe seedpowder, a restoration of nitrile formation occurs (*19*). Mercaptoethanol was also added to a model assay system that contained sinigrin and ascorbic acid together with an extract from Brussels sprouts (*42*). Without mercaptoethanol, allyl isothiocyanate was the only breakdown product of sinigrin. In its presence, epithionitriles were formed, and according to the authors this was due to activation of ESP. They suggested two possible mechanisms: (1) regeneration of the sulfhydryl groups or (2) reduction of the iron prosthetic group. No evidence was presented in support of either mechanism. To provide convincing evidence, a powerful $Fe^{3+}$-specific reducing agent, acidified $Sn^{2+}$ (*40*) was added to the hydrolysis assays. Crambe and wild turnip exhibited no increase in nitrile formation but broccoli and cabbage powders showed considerable enhancement in nitrile production. It would appear that this is the first time this reducing system has been added to glucosinolate hydrolysis assays, and it certainly provides convincing proof, due to the specificity of this reducing agent, that the conversion of $Fe^{3+}$ to $Fe^{2+}$ contributes significantly to variations in ESP activity.

Recently several studies (*27–29*) have identified a group of AtNSPs in *A. thaliana*, which, in conjunction with myrosinase, were able to generate simple nitriles, but not epithionitriles. Addition of $Fe^{2+}$ increased the proportion of simple nitrile formed in assays, which utilized a range of glucosinolate substrates (*28*). $Fe^{2+}$ addition also increased proportions formed in the control assays, which did not contain AtNSP. No epithionitrile was detected in any of the reactions. $Fe^{3+}$ addition had no effect on simple nitrile formation in the control or NSP assays. Interestingly, the authors noted on addition of EDTA no influence on AtNSP activity when 4-methylsulfinylbutyl and 2-propenyl glucosinolates were used. In contrast, there was a slight but significant reduction in nitrile formation when 4-methylthiobutyl and benzyl glucosinolates were substrates. Even so, the authors still proposed that simple nitrile formation by AtNSP was not dependent on the presence of $Fe^{2+}$ or $Fe^{3+}$ ion. Another study (*29*) investigated the iron dependence of nitrile-specifier activity of another member of AtNSP (AtNSP2) and suggested

that $Fe^{2+}$ addition promoted AtNSP2 activity for both 2-propenyl and benzyl glucosinolate substrates, even though simple nitriles were formed in the absence of AtNSP2. High ESP activity and similar changes for both nitriles on Fe and chelator addition suggest limited, if any, contribution by AtNSP to simple nitrile formation in crambe and wild turnip. However, the limited ability of the broccoli and cabbage seedpowders to form epithionitriles in the ESP assays and the standard hydrolyses suggests a possibility of NSP activity in these plants. This may be particularly so for the cabbage powders as they possess substantial amounts of the substrate required for epithionitrile formation, sinigrin. The large and significant increases in simple nitrile and epithionitrile formation on $Fe^{2+}$ addition as shown in this study and the chelator-induced decreases indicated a high level of iron dependency in nitrile formation of these two seedpowders. This high $Fe^{2+}$ dependency together with confirmation of substantial ESP expression (*14*) in both broccoli and cabbage indicated that simple nitrile formation in these seedpowders is under ESP control. However, limitations of this experiment should be noted, particularly as the reported data concerning the NSP response to $Fe^{2+}$ addition was restricted to two of four possible *A. thaliana* NSP proteins. These limitations were augmented by the fact that to date these NSP proteins have been identified in only *Arabidopsis*.

Experiments by this laboratory (*18*) have shown that ESP activity in three broccoli cultivars increased up to day 2 after germination before decreasing again. This contrasted with activity changes in crambe seeds and seedlings, with seeds having the highest activity followed by a steady decrease with seedling age. The present work confirmed these findings as well as providing evidence that these changes were linked to a significant increase in $Fe^{2+}$ concentrations as indicated by $Fe^{2+}/Fe^{3+}$ ratio. The consistent increase in total iron levels shown by all of the seedlings tested was due to absorption of tap water on germination. Tap water was used for germination to duplicate growing conditions prevalent in the commercial sprout industry (*10*, *11*). It was originally thought that the increasing formation of ascorbic acid may produce the necessary reducing environment as suggested by earlier studies (*19*, *43*). However, the increasing levels of ascorbic acid in all tested samples during plant development, most notably crambe and wild turnip, eliminated this simple explanation.

An early study noted that the ability to form nitriles was partially lost in crambe seedpowder made from aged seed (*19*). Several years later, it was reported that there was a decreased ability to form epithionitriles in turnip seeds, aged for 1 year (*25*). Similar changes were noted by the same author in a later study of three ESP-containing species in which the author suggested differences between freshly harvested seed and stored commercial seed were due to a loss of ESP activity (*26*). In the present study all ESP-containing samples lost activity with storage. A concomitant loss in $Fe^{2+}$ content with storage, but no perceptible loss in total iron levels, confirmed that activity loss was due to the oxidation of $Fe^{2+}$ to $Fe^{3+}$.

The reduction in ESP activity in broccoli seedlings germinated from aged or stored seeds indicates a potential to grow seedlings and plants with decreased ESP. The use of aged or stored broccoli seeds to produce edible sprouts with considerably lower ESP activity has the potential to dramatically improve the health benefits of these sprouts, particularly if the poor germination rate can be overcome.

The work described here supports the view that $Fe^{2+}$ levels are essential in governing natural ESP activity. We have demonstrated that ESPs of several *Brassica* species have differing iron requirements for activity. In some cases the conversion of $Fe^{3+}$ to $Fe^{2+}$

drives ESP activity changes during early plant development. Oxidation of $Fe^{2+}$ to $Fe^{3+}$ accounts for the loss of ESP activity during seed aging or storage, and this has also been seen by previous investigators.

Our search for the role of iron in enzymatic nitrile formation has uncovered two distinctive responses. Several other investigators (*14, 22*) have noted similar alignments of ESP-containing species. One explanation could be that ESP exists in two forms, one that produces predominantly simple nitriles on glucosinolate hydrolysis and another mainly epithionitriles. The observation in our study that the cabbage seedpowder produced predominantly simple nitriles on hydrolysis even though it contains substantial amounts of terminal alkenyl glucosinolate substrate adds weight to this possibility. Another explanation could be that ESP has inactive and active forms as suggested recently (*44*). Other researchers hinted at another possibility based on whether ESP comprises two proteins (broccoli and cabbage) or one protein (crambe) (*14*). Whether this duplicity in ESP activity does occur naturally or is an artifact of the small number of ESP-containing plants tested so far remains to be elucidated.

As we gain more information about health-promoting phytochemicals such as isothiocyanates, it becomes increasingly important that we understand the mechanisms that control their formation and factors that promote the production of any alternative compounds so that breeding technology and agronomic conditions, food-processing parameters, or guidelines for human consumption may be developed to provide crops that offer greater health benefits.

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