

Differing mechanisms of simple nitrile formation on glucosinolate degradation in *Lepidium sativum* and *Nasturtium officinale* seeds

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ABSTRACT

Glucosinolates are sulphur-containing glycosides found in brassicaceous plants that can be hydrolysed enzymatically by plant myrosinase or non-enzymatically to form primarily isothiocyanates and/or simple nitriles. From a human health perspective, isothiocyanates are quite important because they are major inducers of carcinogen-detoxifying enzymes. Two of the most potent inducers are benzyl isothiocyanate (BITC) present in garden cress (*Lepidium sativum*), and phenylethyl isothiocyanate (PEITC) present in watercress (*Nasturtium officinale*). Previous studies on these salad crops have indicated that significant amounts of simple nitriles are produced at the expense of the isothiocyanates. These studies also suggested that nitrile formation may occur by different pathways: (1) under the control of specifier protein in garden cress and (2) by an unspecified, non-enzymatic path in watercress. In an effort to understand more about the mechanisms involved in simple nitrile formation in these species, we analysed their seeds for specifier protein and myrosinase activities, endogenous iron content and glucosinolate degradation products after addition of different iron species, specific chelators and various heat treatments. We confirmed that simple nitrile formation was predominantly under specifier protein control (thiocyanate-forming protein) in garden cress seeds. Limited thermal degradation of the major glucosinolate, glucotropaeolin (benzyl glucosinolate), occurred when seed material was heated to >120 °C. In the watercress seeds, however, we show for the first time that gluconasturtiin (phenylethyl glucosinolate) undergoes a non-enzymatic, iron-dependent degradation to a simple nitrile. On heating the seeds to 120 °C or greater, thermal degradation of this heat-labile glucosinolate increased simple nitrile levels many fold.

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1. Introduction

Glucosinolates are sulphur-containing glycosides with aliphatic, aromatic, or indole side-chains found in brassicaceous plants. These plants include the economically important salad crops such as garden cress (*Lepidium sativum*) and watercress (*Nasturtium officinale*) as well as many brassica vegetables, e.g. broccoli (*Brassica oleracea* var. *italica*). Glucosinolate content in these brassicas is about 1% of dry weight in some tissues although the level is highly variable. The content can approach 10% in the seeds of some brassicas where glucosinolates may represent one half of the sulphur content (Fahey et al., 2001).

Upon tissue damage (e.g. in food preparation, chewing, or pest attack), the glucosinolates come into contact with the endogenous plant enzyme myrosinase, which hydrolyses the thioglucosidic

bond yielding aglycones. These unstable aglycones undergo a Lossen rearrangement to form isothiocyanates. Under the influence of specifier proteins that are present in many glucosinolate-containing plants including broccoli (epithiospecifier protein, ESP) and garden cress (thiocyanate-forming protein, TFP), alternative products such as simple nitriles, epithionitriles, and thiocyanates are formed at the expense of isothiocyanates (Matusheski et al., 2004; Burow et al., 2007; Williams et al., 2008; see also Fig. 4a and b). It was originally thought that simple nitrile formation was non-enzymatic and depended only on the ferrous ion level and low pH (Youngs and Perlin, 1967; Austin et al., 1968; Tookey and Wolff, 1970) or the thermolability of the parent glucosinolate (MacLeod et al., 1981). However, it is now accepted that these specifier proteins are responsible for nitrile formation by binding the sulphur of the aglycone, thereby preventing its participation in the Lossen rearrangement (Foo et al., 2000; Matusheski et al., 2006). In 2008, Bellostas et al. refocused attention on the formation of nitriles by non-enzymatic means. These authors reported that only small amounts of added Fe²⁺ were sufficient to degrade purified glucosinolates to simple nitriles. These results together

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with the earlier studies gave a strong indication that nitrile formation in some of these brassicas occurs by a non-enzymatic Fe^{2+} catalysed degradation of glucosinolates.

From a human health perspective, isothiocyanates are the most important of the products formed from glucosinolate degradation. There is convincing evidence that benzyl isothiocyanate (BITC), phenylethyl isothiocyanate (PEITC), and sulphoraphane (4-methylsulphinylbutyl isothiocyanate) are effective inhibitors of chemically induced tumours in one or more organ sites of rodents (Zhang and Talalay, 1994; Talalay and Zhang, 1996; Hect, 2000; Zhang, 2004). These isothiocyanates apparently induce carcinogen-detoxifying enzymes (Zhang et al., 1992; Zhang and Talalay, 1994; Talalay and Zhang, 1996). Examples of popular brassicas that are particularly rich in these isothiocyanates include garden cress (BITC) (Saarivirta, 1973; Cole, 1976; Daxenbichler et al., 1991; Burow et al., 2007) and watercress (PEITC) (Chung et al., 1992; Palaniswamy et al., 2003). By contrast, several investigations have shown that simple nitriles are ineffective as inducers of these detoxification enzymes (Matusheski and Jeffery, 2001; Basten et al., 2002; Mithen et al., 2003) and less effective as anti-proliferative agents (Nastuzzi et al., 2000). Thus, the potential health benefits of these cresses may be compromised by the formation of the inactive nitrile.

Garden cress seeds predominately contain benzyl glucosinolate, glucotropaeolin, which undergoes degradation to form three main products: phenylacetone nitrile (predominant product), benzyl thiocyanate, and benzyl isothiocyanate as shown in Figs. 1c and 4a (Gil and MacLeod, 1980a,b; Hasapis and MacLeod, 1982a,b; Burow et al., 2007). There is conflicting evidence as to whether watercress, which also predominantly contains an aromatic glucosinolate, gluconasturtiin (2-phenylethyl glucosinolate), forms simple nitriles. Several studies investigating seeds (Kaoulla et al., 1980) and mature plants (MacLeod and Islam, 1975; Wichtl and Bisset, 1994) found significant levels of phenylacetone nitrile while Cole (1976) and Daxenbichler et al. (1991) reported only isothiocyanate forma-

tion on hydrolysis. Reports of formation of simple nitriles in watercress are unexpected because seed powder or seedlings of watercress show no evidence of specifier protein (ESP/TFP) activity (Cole, 1976; Kaoulla et al., 1980).

The prospect that these two related species form simple nitriles from structurally similar glucosinolate substrates by different pathways presented an opportunity to learn more about the mechanisms involved in nitrile production. Knowledge of these nitrile forming mechanisms in cresses will provide valuable information, most notably on processing conditions and guidelines for human consumption. Raw consumption or mild heat treatment may maximise the formation of isothiocyanates and hence confer health benefits when eating these salad crops.

After confirming simple nitrile formation in both species, a comparison was made of: (1) the effect of heat treatments and (2) the addition of iron and chelators on nitrile formation in both cress seed powders. Together with data on the effects of heating on ESP and myrosinase activity and the levels of endogenous iron, our results provide evidence for simple nitrile formation occurring by different mechanisms in these species.

2. Results

2.1. Glucosinolates

Glucotropaeolin was confirmed to be the principal glucosinolate present in the garden cress seeds. With a level of $101.3 \mu\text{mol/g}$ FW, it comprised 95% of the total glucosinolates; gluconasturtiin (2-phenylethyl glucosinolate) contributed the remaining 5% (Fig. 1a). In the watercress seeds, the major glucosinolate was gluconasturtiin at $54.0 \mu\text{mol/g}$ FW, constituting 80% of the total glucosinolate pool. Glucoibarin (7-methylsulphonylheptyl glucosinolate), glucohirsutin (8-methylsulphonyloctyl glucosinolate), and glucotropaeolin were also present (Fig. 1b).

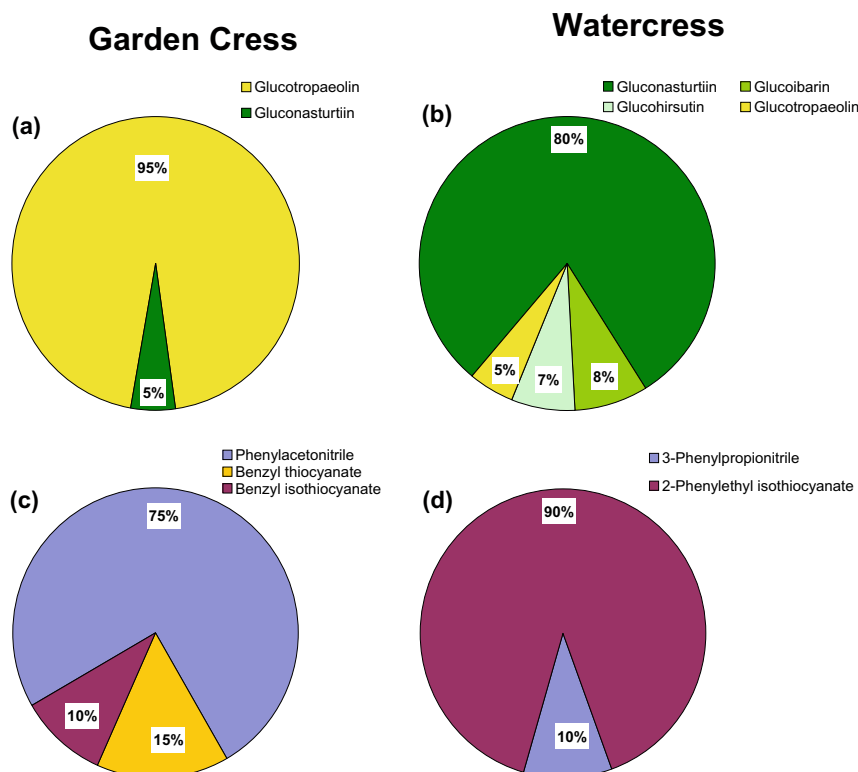


Fig. 1. Percentages of glucosinolate content and glucosinolate degradation products formed on 1 h autolysis in garden cress (a and c) and watercress (b and d) seeds. Data are the mean of replicates where $n = 3$.

2.2. ESP and myrosinase activities, total and ferrous iron content of cress seed powders

The original work on *Crambe abyssinica* ESP utilised the formation of epithionitrile versus simple nitrile during hydrolysis of epi-progoitrin (2(S)-2-hydroxy-3-butenyl glucosinolate) as a measure of ESP activity (Tookey, 1973). Activity in cress seeds and seed powders of other well-known brassicas was assessed in this study for comparison using purified myrosinase and epi-progoitrin as substrate. Garden cress, crambe and broccoli seed powders showed significant ESP activity but none was detected in watercress seed (Table 1).

Because we were interested in the myrosinase activity *in situ*, measuring the activity in the defatted seeds was preferred over the activity of the isolated enzyme, which is more commonly reported (Wilkinson et al., 1984; Ludikhuyze et al., 2000). The presence of known (e.g. ascorbic acid, MgCl₂, and iron) and possibly unknown components in seeds that are important for myrosinase activity gave a valuable advantage to this approach (Verkerk and Dekker, 2004). The myrosinase activity of the watercress seed powder was by far the lowest of the seed powders tested with its activity being significantly lower ($p = 0.014$; $R^2 = 0.811$) than that measured for garden cress seeds.

Endogenous iron concentrations in the seed powders were measured to determine if the levels were sufficiently high to affect simple nitrile formation (Table 1). Watercress had the highest total iron level at 189 mg/kg compared to 146 mg/kg in the garden cress. Interestingly, the garden cress seeds contained equal amounts of Fe²⁺ and Fe³⁺, whereas watercress seeds contained predominantly Fe³⁺ (78% of the total iron).

2.3. Formation of simple nitriles

Preliminary experiments had established that no significant pH changes occurred in seed powder hydrolysates. The pH of all the homogenates stayed within 0.1 units of the initial samples over the course of the 1 or 6 h hydrolyses (6 h results not shown).

On hydrolysis of the garden cress seed powders, the major substrate, glucotropaeolin gave rise to three products: phenylacetone nitrile, benzyl thiocyanate, and benzyl isothiocyanate with relative percentages of 75:15:10, respectively (Figs. 1c and 4a). When the hydrolysis time was increased to 6 h both the levels of phenylacetone nitrile and benzyl thiocyanate decreased but not significantly so. This was matched by an insignificant increase in benzyl isothiocyanate levels. Only hydrolysis products of the major glucosinolate, gluconasturtiin were detected in watercress seed powder samples. The predominant degradation product was PEITC at 92%. Substantial quantities, 10%, of the simple nitrile 3-phenylpropionitrile were also detected (Figs. 1d and 4b). There was a small but insignificant decrease in hydrolysis products when the hydrolysis time was increased to 6 h for the watercress seeds.

2.4. Role of Fe²⁺/Fe³⁺ in glucosinolate degradation

To determine the role of Fe²⁺/Fe³⁺ in changes to hydrolysis product levels, all product concentrations were normalised and the levels after hydrolysis with ultrapure water assigned the value of 1.0. Other hydrolysis product levels on Fe²⁺/Fe³⁺ addition were given as ratios of these control levels (see Fig. 2a and b).

The addition of Fe²⁺ to garden cress seed assays caused a significant increase ($p < 0.0001$; $R^2 = 0.989$) in phenylacetone nitrile formation at the expense of BITC production which significantly decreased ($p < 0.0001$; $R^2 = 0.984$). These effects contrasted with those found after Fe³⁺ addition which produced no significant changes ($p = 0.158$; $R^2 = 0.429$) (Fig. 2a).

The addition of Fe²⁺ to the watercress samples produced a dramatic and significant increase ($p < 0.0001$; $R^2 = 0.980$) in simple nitrile formation. An increase, slightly smaller in magnitude but still significant ($p = 0.0002$; $R^2 = 0.979$), was observed for nitrile formation on Fe³⁺ addition. There were no significant changes in PEITC formation either on Fe²⁺ ($p = 0.0742$; $R^2 = 0.590$) or Fe³⁺ ($p = 0.3253$; $R^2 = 0.238$) addition (Fig. 2b).

2.5. Iron chelators and glucosinolate degradation

To study the role of iron in the mechanisms involved in simple nitrile formation in greater detail, three chelators: (1) EDTA, a chelator of both Fe²⁺ and Fe³⁺ as well as numerous other cations, (2) BPDS (bathophenanthroline disulphonic acid), a chelator of Fe²⁺, and (3) deferoxamine, a chelator of Fe³⁺ were added to the hydrolysis product assays. Fig. 2a and b illustrates the changes in hydrolysis product levels on addition of these chelators to the samples.

The addition of 20 mM EDTA or 10 mM BPDS to the garden cress sample significantly reduced phenylacetone nitrile production ($p = 0.0001$; $R^2 = 0.980$) and significantly increased BITC production ($p = 0.0001$; $R^2 = 0.981$). However, deferoxamine had no effect ($p = 0.512$; $R^2 = 0.109$ for simple nitrile; $p = 0.424$; $R^2 = 0.249$ for isothiocyanate) on the hydrolysis products (Fig. 2a).

The addition of the chelators to watercress assays produced significant decreases in simple nitrile formation, with BPDS addition causing the largest reduction ($p = 0.0007$; $R^2 = 0.957$) although it did not change ($p = 0.1668$; $R^2 = 0.109$) PEITC formation (Fig. 2b).

2.6. Thermal effects on ESP and myrosinase activities and glucosinolate degradation products

As the seed powder of *C. abyssinica* was known to contain ESP, it was included in the experiments that monitored changes in ESP and myrosinase activities with increasing time-temperature treatments. The ESP and myrosinase activities of garden cress and *C. abyssinica* seed powders were almost identical in their response to the time-temperature heat treatments. The already

Table 1

Distribution of ESP activity,^a myrosinase activity,^b and total,^c ferrous,^c and ferric^d iron content in the seeds of five cruciferous species.

Species name	Common name	ESP activity ^a	Myrosinase activity ^b	Iron content ^{c,d}		
				Total ^c	Fe ²⁺	Fe ³⁺
<i>Lepidium sativum</i>	Garden cress	13.76 ± 0.94	176 ± 31	146 ± 9	71 ± 6	75 ± 9
<i>Crambe abyssinica</i>	Crambe	3.49 ± 0.47	167 ± 20	65 ± 3	48 ± 4	17 ± 5
<i>Brassica oleracea L. italica</i>	Broccoli cv. Saga	0.20 ± 0.06	174 ± 25	84 ± 11	43 ± 7	41 ± 11
<i>Nasturtium officinale</i>	Watercress	nd	29 ± 7	189 ± 6	41 ± 3	148 ± 5

nd = Not detected.

Data are the mean ± standard error of replicates, where $n = 3$.

^a Expressed as the ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed from purified epi-progoitrin.

^b Measured as µg sinigrin consumed/min/g (FW).

^c Expressed as mg/kg (FW).

^d Derived by subtraction of Fe²⁺ content from total iron content (mg/kg FW).

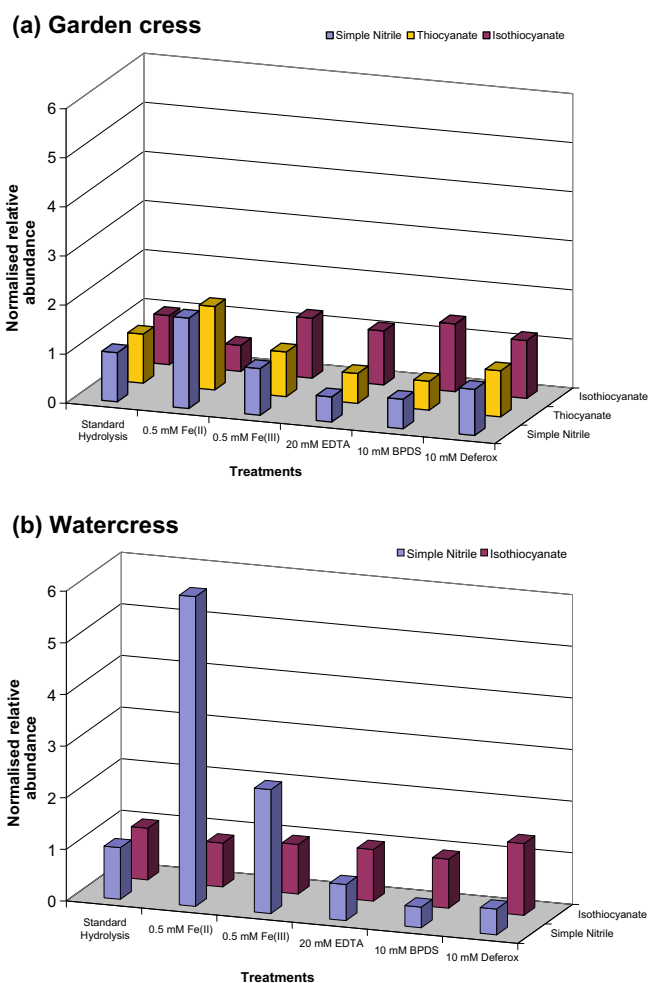


Fig. 2. Normalised percentage of total amount of glucosinolate degradation products formed after the addition of Fe^{2+} , Fe^{3+} , and chelators (EDTA, BPDS, and deferoxamine) to the autolysis mixtures of (a) garden cress and (b) watercress seeds. Data are the mean of replicates where $n = 3$.

low myrosinase activity of the watercress seed powders was readily removed by heating to 120 °C for only 90 min (Table 2).

Heating at 130 °C for 16 h significantly reduced the formation of all degradation products in garden cress seed ($p < 0.0001$; $R^2 = 0.997$ for simple nitrile; $p = 0.0002$; $R^2 = 0.978$ for isothiocyanate). When the boiled garden cress extract was incubated with purified mustard myrosinase the formation of the simple nitrile was significantly reduced ($p = 0.0001$; $R^2 = 0.983$) and production of the thiocyanate was nearly curtailed. The formation of BITC was enhanced three fold ($p < 0.0001$; $R^2 = 0.998$) (Fig. 3a).

Table 2

The effects of variable times and temperatures on ESP activity (expressed as ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed from purified epi-progoitrin) and myrosinase activity (measured as μg sinigrin consumed/min/g FW).

Seedmeal	Activity	Heat treatment (Temp °C/Time)					
		Control	120 °C 30 min	120 °C 90 min	100 °C 16 h	120 °C 16 h	130 °C 16 h
Garden Cress	ESP	13.76 ± 0.94	13.57 ± 1.35	10.46 ± 0.92	9.63 ± 0.85	nd	nd
	Myrosinase	176 ± 31	168 ± 25	177 ± 29	169 ± 29	30 ± 14	nd
Watercress	ESP	nd	nd	nd	nd	nd	nd
	Myrosinase	29 ± 7	10 ± 2	nd	nd	nd	nd
Crambe	ESP	3.49 ± 0.47	3.30 ± 0.52	2.88 ± 0.49	2.78 ± 0.31	1.20 ± 0.31	nd
	Myrosinase	167 ± 20	118 ± 18	105 ± 28	129 ± 23	29 ± 15	nd

nd = Not detected.

Data are the mean ± standard error of replicates, where $n = 3$.

Fig. 3b shows the results of varying hydrolysis times and heat treatments on watercress hydrolysis products and gluconasturtiin content. In seed powders heated at 130 °C for 16 h without addition of water no PEITC was formed. However, the unheated sample had levels of nitrile comparable with the control, while the heated sample without water produced large amounts of 3-phenylpropionitrile. Overall, the level of nitrile formation dramatically increased with temperature, i.e. 130 °C increased nitriles ten-fold when compared to the unheated control. Interestingly, heating for this time and temperature caused considerable degradation of the major glucosinolate in watercress, gluconasturtiin. There was approximately a 25% reduction in the glucosinolate by this thermal degradation mechanism (Figs. 3b and 4c).

3. Discussion

Gil and MacLeod (1980a) identified three glucosinolates in *L. sativum* seeds, namely allyl, 2-phenethyl (gluconasturtiin), and benzyl (glucotropaeolin) glucosinolates. The first two were found in only trace amounts with glucotropaeolin the major glucosinolate. This agrees with our study except we detected no allyl glucosinolate. The glucotropaeolin level (137.4 $\mu\text{mol/g}$) reported by O'Hare et al. (2005) agrees closely with the value determined for our garden cress seeds.

ESP and myrosinase activities as well as endogenous iron levels were evaluated to determine mechanisms and factors involved in simple nitrile formation in the two cress species. Recently Burow et al. (2007) reported that a thiocyanate-forming protein (TFP) (which belongs to the same family as ESP) catalysed thiocyanate and simple nitrile formation in different organs of garden cress. Consistent with this and other previous studies (Cole, 1976; MacLeod and Rossiter, 1985) garden cress seeds tested in the present study had high ESP activity (Table 1) indicating that TFP may have a significant role in phenylacetone nitrile production (Fig. 4a). High rates of myrosinase activity (Table 1) further support the view that garden cress hydrolysis utilises an enzymatic mechanism for simple nitrile production. Recent studies (Wentzell and Kliebenstein, 2008; Burow et al., 2009; Kissen and Bones, 2008) have identified in the model plant *Arabidopsis thaliana* a group of nitrile specifier proteins (NSP) with sequences similarity to *L. sativum* TFP, that generate simple nitriles in conjunction with myrosinase. In a detailed characterisation of one member of this group Kissen and Bones (2008) revealed that NSP redirected the hydrolysis profile of a range of glucosinolates, including 2-propenyl glucosinolate to exclusively simple nitriles. The high ESP activity which indicated large amounts of epithionitrile formed as compared to the very small amount of simple nitrile in our study, confirmed by similar results in Burow et al. (2007) suggests only minimal contribution to simple nitrile formation by these nitrile specifier proteins in garden cress seeds.

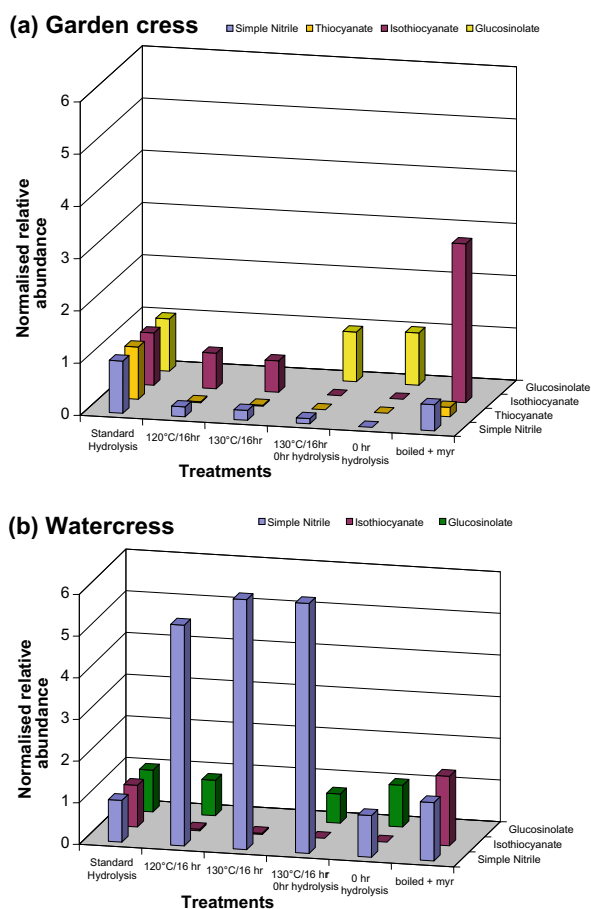


Fig. 3. Normalised percentage of total amount of glucosinolate degradation products formed on autolysis of: (a) garden cress and (b) watercress seeds following various heat treatments. (0 h hydrolysis refers to analysis of degradation products without addition of water). Data are the mean of replicates where $n = 3$.

It is well known that ESP and TFP require iron for their activity (Tookey, 1973; Hasapis and MacLeod, 1982a,b; Burow et al., 2006; Matusheski et al., 2006). The high total iron concentration present in garden cress seeds (Table 1) may not only be sufficient for TFP activity, but may also drive a limited iron-dependent non-enzymatic nitrile formation. The suggestion that specifier proteins require sufficient endogenous Fe^{2+} for natural activity was noted by MacLeod and Rossiter (1985). They reported that seeds of *Brassica napus* cv. Panter and *B. campestris* had no original ESP activity, but on the addition of Fe^{2+} activity was observed.

To determine the iron requirements for any possible simple nitrile formation mechanisms, solutions of Fe^{2+} and Fe^{3+} and selected chelators were added to the hydrolysis assays of garden cress and watercress (Fig. 2a and b). In agreement with earlier measurements by Hasapis and MacLeod (1982b) the addition of Fe^{2+} enhanced glucotropaeolin hydrolysis in garden cress seeds to phenylacetone and benzyl thiocyanate while reducing BITC production (Fig. 2a). Further confirmation that Fe^{2+} is essential for TFP activity was provided by the strongly reduced levels of phenylacetone and benzyl thiocyanate observed on addition of the general chelator EDTA and the Fe^{2+} specific chelator BPDS. This dependency on Fe^{2+} for specifier protein activity has been the subject of intense interest over the years, with studies verifying the link with ESP (Tookey, 1973; Burow et al., 2006; Matusheski et al., 2006) and TFP (Hasapis and MacLeod, 1982b; Burow et al., 2007). However, no effect was found if Fe^{3+} or its chelator, deferoxamine was added to the garden cress hydrolysis assay. The ineffec-

tiveness of Fe^{3+} to enhance nitrile formation was also reported by Tookey and Wolff in 1970. Their report and our results were in direct contrast to studies on TFP (Hasapis and MacLeod, 1982b) and ESP (Burow et al., 2006) which both found considerable enhancement of specifier protein activity following Fe^{3+} addition. This discrepancy may indicate that ESP and TFP (even from different cultivars of the same species) vary in their sensitivity to Fe^{2+} and Fe^{3+} or that the TFP in our study had optimum Fe^{3+} levels. The latter may be more likely as the Fe^{3+} level of the seeds in our study was high (Table 1) whereas Hasapis and MacLeod (1982b) reported that the seeds in their study were so low in iron as to have no significant effect on nitrile formation.

Experiments showed that heating at $>120^{\circ}C$ for 16 h was sufficient before the TFP activity in garden cress seeds was reduced to non-detectable levels. Myrosinase activities followed a similar trend in these seeds although some residual myrosinase activity remained in seeds heated to $120^{\circ}C$ for 16 h (Table 2). Various heat treatments on the seeds produced results that paralleled those reported by Matusheski et al. (2004) in broccoli sprouts and florets. These authors reported that heating sprouts to $70^{\circ}C$ for 20 min nearly abolished ESP activity while sufficient myrosinase activity was maintained to maximise isothiocyanate production. The results in our study were less clear cut, but heating to $120^{\circ}C$ for 90 min or 16 h certainly reduced TFP activity substantially (Table 2). At these time and temperature regimes, the myrosinase activity was also significantly reduced and thermal degradation of the glucotropaeolin to phenylacetone nitrile became an important factor (Fig. 3a). This higher temperature resistance of crucifer seeds to enzyme de-activation was foreseen by Matusheski and co-authors (2004) and several earlier studies (VanEtten et al., 1966; Hasapis and MacLeod, 1982a). Assuming the mechanism of simple nitrile formation in garden cress does not change with plant development and, as Matusheski et al. (2004) suggest, the heat resistance of specifier protein activity reduces with ontogeny, mild heat treatments of garden cress plants may optimise glucotropaeolin conversion to BITC at the expense of phenylacetone nitrile.

Although heating at $120^{\circ}C$ for 16 h reduced TFP activity to non-detectable levels (Table 2), garden cress seeds still had detectable levels of phenylacetone nitrile (Fig. 3a). This persistence in simple nitrile formation in heated garden cress seeds ($125^{\circ}C$ for 7.5 h) was also noted by Hasapis and MacLeod (1982a). This small amount of phenylacetone nitrile was probably formed by the thermal degradation of glucotropaeolin as outlined by MacLeod et al. (1981). Proof of this degradation was given when the unhydrolysed heated seeds showed small amounts of phenylacetone nitrile as compared to the absence of nitrile found on analysis of the unhydrolysed, unheated seeds (Fig. 3a). Boiling followed by the addition of non-TFP containing myrosinase (Fig. 3a) confirmed the integral role of TFP in simple nitrile formation with a substantial decrease in phenylacetone nitrile and subsequent large increase in BITC levels.

The large increase in 3-phenylpropionitrile in the watercress seeds at temperatures at or greater than $120^{\circ}C$ indicated a far more extensive thermal degradation (Figs. 3a, b and 4c) than that shown by the garden cress seeds. This is supported by MacLeod et al. (1981) who reported that gluconasturtiin was the most heat-labile glucosinolate forming considerable amounts of 3-phenylpropionitrile on heating to $125^{\circ}C$ for only 15 min. Closer examination of glucosinolate loss on heating at $130^{\circ}C$ (Fig. 3b), assuming it stoichiometrically degrades to the nitrile, showed an increase in 3-phenylpropionitrile nearly equal to that presented in Fig. 3b. This suggests that the large increase in nitrile formation at elevated temperatures was mainly due to thermal degradation of the major glucosinolate substrate, gluconasturtiin (Fig. 4c).

In 1980, Kaoulla and co-authors noted that the ESP assay performed on watercress seeds failed to detect any activity. Their results are consistent with our data where no ESP activity was

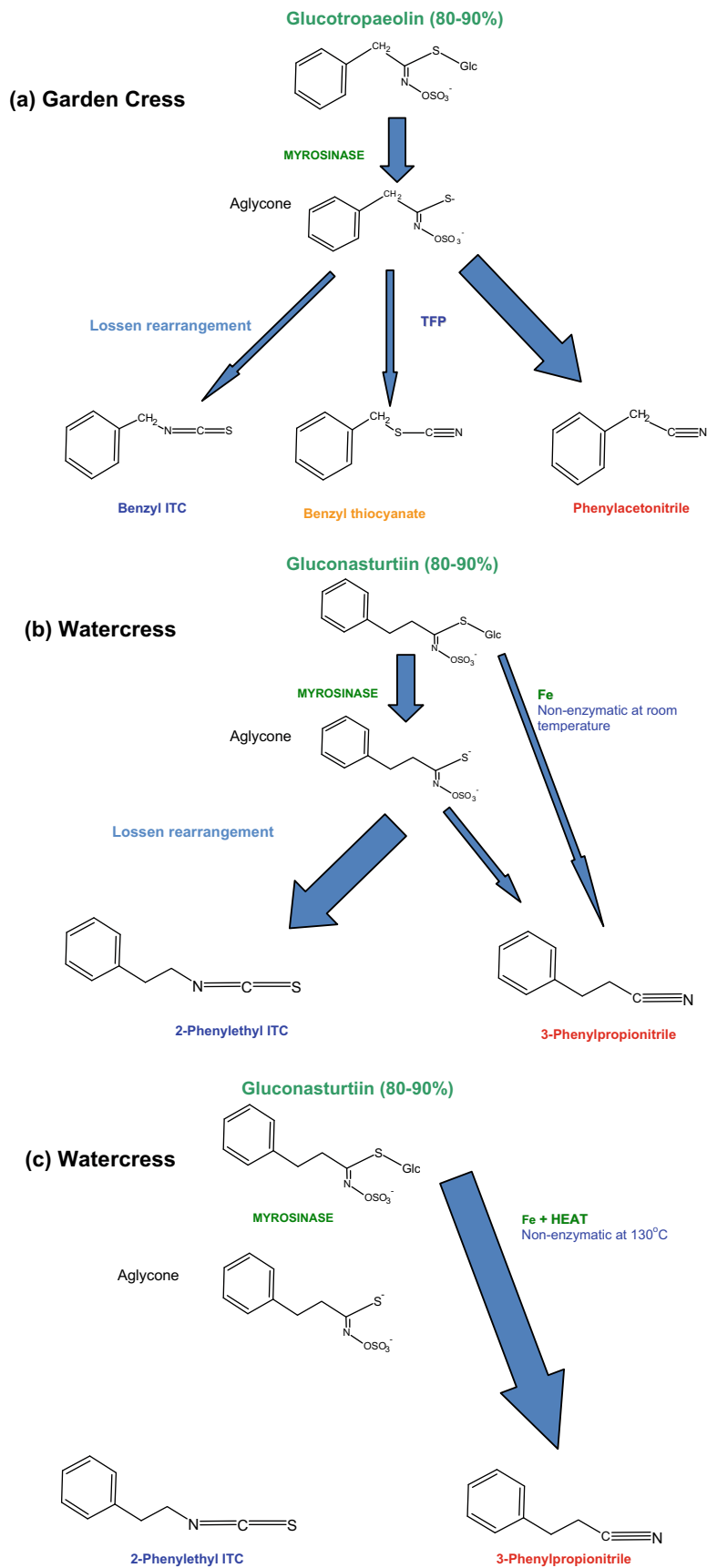


Fig. 4. Model of glucosinolate degradation of: (a) glucotropaeolin in garden cress seeds at room temperature, (b) gluconasturtiin in watercress seeds at room temperature, and (c) after water cress seeds had been heated to 130 °C overnight. Widths of arrows are proportional to the amounts of degradation product formed.

detectable in watercress seed (Table 1). This also agreed with the absence of epithionitrile production for the 8 week old seedlings as noted by Cole (1976). The very low myrosinase activity in watercress seeds found in our study (Table 1) is consistent with the earlier study of Wilkinson et al. (1984) who reported that watercress had the lowest activity of purified extracts from twelve mature vegetables.

Analysis of the watercress seeds showed the presence of significant amounts of the simple nitrile, 3-phenylpropionitrile (Figs. 1d and 4b), similar to the report by Kaoulla et al. (1980). Hofmann in 1874 isolated 3-phenylpropionitrile as a major component from mature watercress using steam distillation, confirmed later by MacLeod and Islam (1975) on plants of a similar age. Our results and these previous studies were in contrast to those reported by Cole (1976) and Daxenbichler et al. (1991) where glucosinolate degradation of watercress seeds and seedlings generated only isothiocyanates. In the latter investigations, the hydrolysis conditions favoured isothiocyanate production. Here we confirm that significant levels of 3-phenylpropionitrile were present in watercress seeds although other experiments in our study indicated that the nitrile was present in the seeds before hydrolysis was initiated (Fig. 3b). Significant levels of 3-phenylpropionitrile in the unhydrolysed watercress seeds, very low myrosinase activity together with high levels of endogenous iron led us to hypothesise that nitrile formation in this species may be non-enzymatic and dependent on iron as suggested in the earlier studies of Youngs and Perlin (1967), Austin et al. (1968) and Tookey and Wolff (1970).

The effect of the addition of either iron species on 3-phenylpropionitrile formation in watercress seeds was profound (Fig. 2b) with levels increasing many fold with no change in PEITC production. The results of chelator addition confirmed this Fe dependency. The lack of change in isothiocyanate formation indicated that simple nitrile production was not at the expense of PEITC but proceeded independently of any aglycone production. This aglycone independent formation, together with very low myrosinase activity would seem to preclude any significant contribution to simple nitrile formation by the recently identified group of nitrile specifier proteins (Wentzell and Kliebenstein, 2008; Burow et al., 2009; Kissen and Bones, 2008). There is some precedence for this non-enzymatic, iron-dependent simple nitrile production on glucosinolate degradation. In a series of experiments involving refluxing 2-propenyl glucosinolate with equimolar amounts of Fe^{2+} Youngs and Perlin (1967) reported the formation of allyl cyanide. They explained this by a glucosinolate-Fe intermediate which provided a route for concerted electron transfer to form nitriles. Later, Austin et al. (1968) showed glucosinolates with a hydroxyl group in the side chain degraded to simple nitriles and thionamides on addition of high Fe^{2+} concentrations. Recently, the formation of these thionamides was confirmed by Bellostas et al. (2008) utilising micellar electrokinetic capillary chromatography to study the non-enzymatic Fe^{2+} catalysed transformation of progoitrin. Together with results from other purified glucosinolates the authors reported that nitriles are the major products formed non-enzymatically at mild conditions of temperature and pH in the presence of as little as 0.25 M excess Fe^{2+} . An additional peak in the chromatogram the authors suggested was due to Fe^{2+} -glucosinolate complex with one hydrated acidic Fe^{2+} and either one or two glucosinolates.

The results obtained suggest that simple nitrile formation in watercress seeds may involve a completely different mechanism to that shown in the garden cress seeds. At room temperature, a non-enzymatic, iron-dependent chemical reaction provides the main simple nitrile formation pathway in watercress seeds. Strong evidence for this mechanism are: (1) high endogenous iron content, (2) low myrosinase activity, (3) a dramatic increase in nitrile levels on both Fe^{2+} and Fe^{3+} addition with no subsequent PEITC decrease, (4) an equally dramatic decrease on chelator addition also

with no PEITC increase, and (5) the presence of substantial 3-phenylpropionitrile in the unhydrolysed seeds. At temperatures of 120 °C or above the major nitrile producing pathway was the thermal degradation of the heat-labile gluconasturtiin. Assuming these nitrile forming pathways do not change with plant development, (an assumption based on persistent nitrile formation even in mature plants as noted by several earlier studies and a consistent low myrosinase activity at all stages of plant development), heating or cooking of watercress plants may reduce human health benefits as simple nitrile production may increase many fold. This very low myrosinase activity as well as its lability would also complicate the development of processing methods that optimise PEITC formation in watercress. More research needs to be conducted into these and other brassicas for the validity of these assumptions to be assessed.

As we gain more information about the health promoting phytochemicals such as BITC and PEITC, it becomes increasingly important that we understand the mechanisms that control their formation or the production of any alternative compounds so that food processing parameters or guidelines for human consumption may be developed to provide the public with salad crops that offer greater health benefits.

4. Experimental

4.1. Materials

Garden cress and watercress seeds were purchased from Eden Seeds and Royston Petrie Seeds Pty Ltd., respectively. Crambe seeds used for testing and preparation of epi-progoitrin were a gift from the Centre for Legumes in Mediterranean Agriculture, The University of Western Australia. The broccoli seeds (c.v. Saga) were a gift from OptiGrow Pty. Ltd. Organic solvents (HPLC grade) and most general purpose reagents were purchased from Biolab (Australia) Pty Ltd. Myrosinase purified from *Sinapis alba* and other chemical reagents, including phenyl/benzyl isothiocyanates, BPDS (bathophenanthroline disulphonic acid), deferoxamine and sinigrin were purchased from Sigma Aldrich (Australia). CHEB [(2S)-1-cyano-2-hydroxy-3,4-epithiobutane], CHB [(S)-1-cyano-2-hydroxy-3-butane] and epi-progoitrin for ESP activity measurements were purified from crambe seeds using previously described extraction and purification methods (Matusheski et al., 2001; Niedoborski et al., 2001; Rochfort et al., 2006).

4.2. Preparation of seed powders

The method used was that reported by MacLeod and Rossiter (1985). 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 stored in air-tight containers in a desiccator.

4.3. Analysis of glucosinolates

To 1 g of seed powder, 10 ml of boiling water was added and boiled for 5 min. The resultant slurry was sonicated for 5 min and the extract was filtered through Whatman No. 4 filter paper, and volumetrically adjusted to 20 ml with water (Rochfort et al., 2006). The samples were then stored at -20°C until HPLC analysis. The levels of glucosinolates were determined by HPLC as previously described by West et al. (2002). Quantification of glucotropaeolin and gluconasturtiin was based on commercially available high purity sinigrin and converted to glucotropaeolin or gluconasturtiin based on relative peak areas obtained with equimolar concentrations of sinigrin (O'Hare et al., 2008). For glucosinolate

identification by mass spectrometer the HPLC system was coupled to a Micromass ZMD quadrupole mass analyser.

4.4. Measurement of ESP activity

ESP activity was determined by incubating the glucosinolate epi-progoitrin with excess purified myrosinase enzyme and Fe^{2+} in the presence of the extract under study as previously described by Matusheski et al. (2004). ESP activity was defined as the ratio of epithionitrile (CHEB) to simple nitrile (CHB) formed. The GC was calibrated using standard curves of 1–100 mg/ml phenyl isothiocyanate (internal standard), CHB and CHEB in methylene chloride.

4.5. Measurement of myrosinase activity

Myrosinase activity present in the seed powders was measured by the hydrolysis of a known amount of sinigrin added to the extracts as previously described by Verkerk and Dekker (2004). The amount of convertible sinigrin was calculated after 20 min of exposure to the extracts. Myrosinase activity was expressed as μg sinigrin consumed/min/g (FW).

4.6. Analysis of glucosinolate hydrolysis products

To triplicate samples (0.25 g) of the seed powders was added 0.75 ml of ultrapure water (or solutions of iron or specific chelators). In the case of $\text{Fe}^{2+}/\text{Fe}^{3+}$ additions, iron solutions of 0.5 mM were added, consistent with the concentrations added by Burow et al. (2006) as well as giving a 2 M excess of iron in the assay system, consistent with Bellostas et al. (2008). The mixture was shaken at room temperature for 1 h (or 6 h) to allow hydrolysis to occur after which the appropriate internal standard (40 μl) was added. A 1 h incubation was used in this study to be consistent with the previous studies of Cole (1976, 1980) and MacLeod and Rossiter (1985). The hydrolysis products were extracted with 1 ml methylene chloride. After centrifugation, the extract was dried (anhydrous sodium sulphate) and stored ready for analysis.

Samples were analysed for hydrolysed products by GC–FID (flame ionisation detection) using the method of Matusheski et al. (2004), but modified by utilising 5 μl of methylene chloride extract injected with split mode (split ratio 1:30) into a Varian 3900 GC system. The flow path consisted of a 4 mm ID single gooseneck liner with nitrogen as the carrier gas. The GC was calibrated using standard curves of 1–100 mg/ml phenyl isothiocyanate (internal standard for garden cress) and benzyl isothiocyanate (internal standard for watercress). For product identification by MS, the column was coupled to an MSD 5975 mass spectrometer (Agilent Technologies, USA) with helium as the carrier gas. Products were identified using mass spectra and retention times of authentic standards and published MS spectra (Spencer and Daxenbichler, 1980).

4.7. Heat treatments

Samples of seed powder (1.5 g) were weighed into aluminium moisture dishes and placed in an oven at 120 °C for 30, 90 min and 16 h or at 130 °C for 16 h. On removal, the dishes were placed in a desiccator, allowed to cool and then weighed. The heat treated garden cress samples were analysed for hydrolysis products as described in 4.6 and for ESP and myrosinase activities. The watercress seed powders were treated similarly except for the sample heated at 130 °C for 16 h. This was divided into four sub-samples and the following treatments and measurements were performed: (1) analysis of hydrolysis products, (2) ESP and myrosinase activities, (3) glucosinolate content, and (4) analysis of hydrolysis products

(with and without the addition of ultrapure water and no (0 h) hydrolysis period).

Triplicate seed powder samples (0.25 g) were combined with 0.75 ml of ultrapure water and boiled for 15 s. The mixture was cooled, 100 μl of purified myrosinase solution (0.5 U/ml) was added plus 0.5 ml of water and allowed to hydrolyse for 1 h at room temperature. The hydrolysis products were determined as in Section 4.6.

4.8. Measurement of total and ferrous iron content

Total iron content of seed powder samples was determined in triplicate by inductively coupled plasma optical emission spectrophotometer (ICPOES) analysis following a digestion with a pre-mixed combination of nitric and perchloric acids containing vanadium as a catalyst.

Ferrous iron was determined by the method of Pierson and Clark (1984) modified by utilising aqueous solutions of the chelating agent, BPDS.

4.9. 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).

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