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Toxicity of Glucosinolates and Their Enzymatic Decomposition Products to *Caenorhabditis elegans*

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Abstract: An aquatic 24-hour lethality test using *Caenorhabditis elegans* was used to assess toxicity of glucosinolates and their enzymatic breakdown products. In the absence of the enzyme thioglucosidase (myrosinase), allyl glucosinolate (sinigrin) was found to be nontoxic at all concentrations tested, while a freeze-dried, dialyzed water extract of *Crambe abyssinica* containing 26% 2-hydroxyl 3-butenyl glucosinolate (*epi*-progoitrin) had a 50% lethal concentration (LC_{50}) of 18.5 g/liter. Addition of the enzyme increased the toxicity (LC_{50} value) of sinigrin to 0.5 g/liter, but the enzyme had no effect on the toxicity of the *C. abyssinica* extract. Allyl isothiocyanate and allyl cyanide, two possible breakdown products of sinigrin, had an LC_{50} value of 0.04 g/liter and approximately 3 g/liter, respectively. Liquid chromatographic studies showed that a portion of the sinigrin decomposed into allyl isothiocyanate. The results indicated that allyl isothiocyanate is nearly three orders of magnitude more toxic to *C. elegans* than the corresponding glucosinolate, suggesting isothiocyanate formation would improve nematode control from application of glucosinolates.

Key words: Caenorhabditis elegans, Crambe abyssinica, enzyme, *epi*-progoitrin, glucosinolate, myrosinase, physiology, sinigrin, thioglucosidase.

Glucosinolates are naturally occurring compounds found primarily in plants of the family Cruciferae, where they are thought to serve as repellents to potential pests (5,10). Enzymatic decomposition of glucosinolates may occur rapidly within the tissues of plants when the tissues are damaged, and it may occur by soil microorganisms when plant material decomposes. Recent work suggests that the products of this complex degradation, such as organic cyanides (i.e., nitriles) or isothiocyanates, are the actual causes of toxicity (2). Although several studies examining the nematicidal potential of glucosinolates on cruciferon plant extracts have been performed (9,14–19,21–23), these studies have not distinguished potential glucosinolate toxicity from the toxicity of the decomposition products or between decomposition products.

An objective of this work was to quantify the toxicity to the free-living nematode *Caenorhabditis elegans* of a commercially available glucosinolate, allyl glucosinolate (sinigrin), and of a glucosinolate-containing extract of *Crambe abyssinica.* A second objective was to quantify the toxicity of the enzymatic decomposition products of these glucosinolates in order to determine which, if any, of the decomposition products elicited the greatest toxicity.

Caenorhabditis elegans is an excellent test invertebrate for the rapid toxicological bioassessment of a variety of chemicals in aquatic medium (26,29), soils (6–8), and on agar plates (20,26–30). Its suitability for such work is enhanced by its ease of culture and maintenance in the lab, as well as the enormous body of knowledge that exists about its basic biology (31).

In this study, the *C. elegans* aquatic 24-hour lethality test developed by Williams and Dusenbery (29) was used to assess allyl glucosinolate (sinigrin) and *Crambe* extracts in both the presence and absence of the glucosinolate-hydrolyzing enzyme thioglucosidase (myrosinase). In addition, this lethality test was used to assess the toxicity of pure samples of allyl isothiocyanate and allyl cyanide (a nitrile).

Studies such as this may lead to greater

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use of standardized *C. elegans* toxicity test protocols as initial screens for assessing the potential of various compounds for pest control. Although *C. elegans* is a free-living species, it is known to be a potential pest to mushroom crops (13), and the evolution of rhabditid nematodes may have included other parasitic forms (24). *Caenorhabditis elegans* also has the advantage of being much easier to culture in the lab than most parasitic nematodes.

Materials and Methods

Cultures of wild type strain N2 *Caenorhabditis elegans* were maintained in the dauerlarva state at 20 C in M9 buffer (3). Two days before beginning a test, several hundred dauerlarvae were transferred to a petri plate containing K-agar (28) with a mature lawn of *Escherichia coli* strain OP50 (3) as a food source. After two days at 20 C, the nematodes had developed into synchronous adults that were then transferred with a sterile platinum wire to aquatic test samples (29).

Test solutions consisted of the following commercially available compounds dissolved in deionized water: sinigrin monohydrate (allyl glucosinolate; Sigma, St. Louis, MO), allyl cyanide (98% pure; Aldrich, Milwaukee, WI), and allyl isothiocyanate (94% pure; Janssen Chimica, Beerse, Belgium). *Crambe abyssinica* seed extract was prepared by blending seeds in a 5:1 ratio (100 C water:seed) (v/v) for 8 minutes followed by centrifugation at 500× g for 10 minutes. The supernatant was dialyzed (12,000 molecular weight cutoff) for 24 hours against four volumes of water at 4 C and then freeze-dried to produce a solid that was approximately 26.5% 2-hydroxyl 3-butenyl glucosinolate.

Test solutions containing thioglucosidase (Sigma, enzyme activity of 200 units/g) were prepared as above, with the enzyme added to achieve a concentration of 1 μg/g of either sinigrin or *Crambe* seed extract. Tests were performed using freshly made solutions in microtiter wells (Falcon 3047) with 0.5 ml solution in each. Additional tests were performed with sinigrin (5, 10, and 20 g/liter) and enzyme (1 μg/g of sinigrin) at pH conditions and a ferrous ion concentration adjusted to promote either total allyl cyanide formation (pH 4.0, 10 mM ferrous ion) or allyl isothiocyanate formation (pH 10.0, no ferrous ion) (2,5, 25), followed by the nematode test. Four adult *C. elegans* were added to each well, and mortality was assessed after 24 hours (28,29). Each test was replicated at least 10 times, and LC_{50s} (concentration resulting in 50% mortality) were determined for each compound by probit analysis (11).

In order to determine the actual breakdown products of sinigrin with thioglucosidase obtained under the test conditions, an analysis was conducted (without nematodes) to determine the composition of the solution over the test period. The concentrations of sinigrin, *epi*-progoitrin (a glucosinolate) and allyl isothiocyanate in solution were quantified by liquid chromatography (1).

Results

In the absence of thioglucosidase, sinigrin was not toxic to *C. elegans* up to the greatest concentration tested (80 g/liter = 193 mM) (Fig. 1). In contrast, the addition of thioglucosidase resulted in an increased toxicity of at least two orders of magnitude

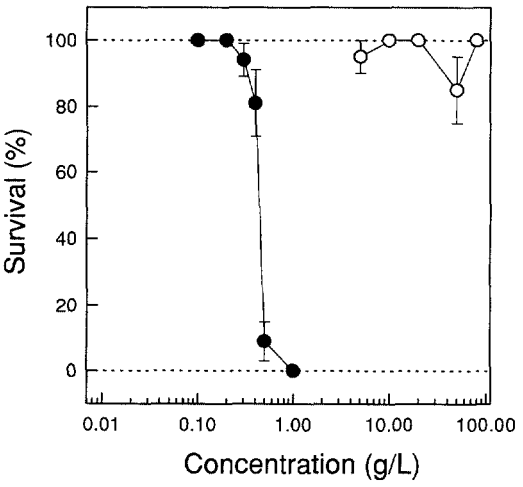

Fig. 1. Percentage of *C. elegans* survival after 24 hours of exposure to concentrations of sinigrin (○) and sinigrin with thioglucosidase (●).

(LC_{50} = 0.5 g/liter = 1.2 mM). A control with enzyme alone was found to be nontoxic.

Exposure to *Crambe* seed extract resulted in a mortality range between 5 and 50 g/liter (LC_{50} = 18.5 g/liter) (Fig. 2). In contrast to the results using pure sinigrin, the toxicity was not significantly changed by addition of thioglucosidase.

Under certain chemical conditions, thioglucosidase will convert glucosinolates into organic cyanides (i.e., nitriles) or isothiocyanates. Two possible products from the decomposition of sinigrin are allyl cyanide and allyl isothiocyanate. Figure 3 shows the concentration-response curves for these two compounds after 24 hours using the *C. elegans* toxicity test. Exposure to allyl isothiocyanate at concentrations between 0.01 and 0.1 g/liter resulted in nematode mortality (LC_{50} = 0.04 g/liter = 0.5 mM), while allyl cyanide was less toxic, having an LC_{50} of approximately 3 g/liter (45 mM). The sinigrin and thioglucosidase tests (at concentrations of 5, 10, and 20 g/liter of sinigrin), which were incubated under known pH conditions and controlled ferrous ion concentrations, were toxic where allyl isothiocyanate was expected to predominate (high pH, no ferrous ion). No mortality occurred in solutions where allyl cyanide was expected to predominate (low pH, 10 mM ferrous ion) (data not shown).

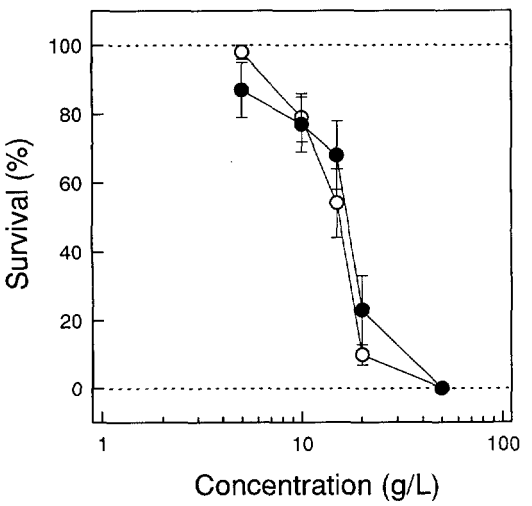

FIG. 2. Percentage of *C. elegans* survival after 24 hours of exposure to concentrations of *Crambe* extract (○) and *Crambe* extract with thioglucosidase (●).

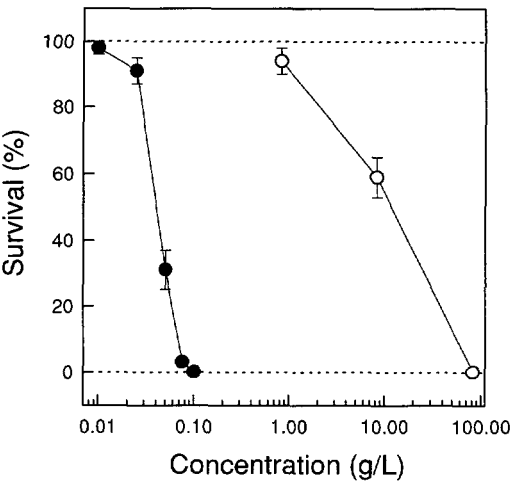

FIG. 3. Percentage of *C. elegans* survival after 24 hours of exposure to concentrations of allyl isothiocyanate (●) and allyl cyanide (○).

The concentration of isothiocyanate in the sinigrin with thioglucosidase solution was found to be 12 to 31% of the glucosinolate concentration after 24 hours. However, allyl isothiocyanate (both pure and produced by enzymatic decomposition) was found to be chemically unstable in the unbuffered aqueous solution, and disappeared from solution within 1 week at 4 C.

The pH of the solutions was typically in the range of 5.5 to 6.0. An exception was the solution with sinigrin and thioglucosidase, which after 24 hours had a pH of 4.0, probably due to sulfate production by the reaction mixture (12). Fluctuations within this range of pH have been found to have no effect on mortality in the *C. elegans* toxicity test (Donkin and Williams, unpubl. obs.).

DISCUSSION

Several authors have speculated that pesticidal effects from glucosinolates are largely due to their enzymatic decomposition products rather than the glucosinolates themselves (2,4,10,17,19). These decomposition products consist mostly of allyl isothiocyanate and allyl cyanide (i.e.,

nitrile), with their relative production depending on pH, ferrous ion, and glucosinolate side-chain structure (2,25). Borek et al. (2) found that acidic pH and 10 mM ferrous ion favor allyl cyanide production from enzymatic hydrolysis of sinigrin, while allyl isothiocyanate is the principal product at higher pH. Ferrous ion was found to inhibit the formation of both products at pH 4 and 6 (2), although Chew (5) reports that ferrous ion may preferentially promote nitrile formation.

Our results confirm that the enzymatic decomposition products of sinigrin rather than sinigrin itself, are toxic. The low-molecular-weight extract from *Crambe abyssinica* seeds, which contains many compounds other than glucosinolate, was toxic only at fairly high concentrations, and its toxicity was unaffected by incubation with thioglucosidase. It is possible that the principal glucosinolate in the *Crambe* extract, *epi*-progoitrin, was hydrolyzed into oxazdidinethione rather than isothiocyanate (5). Therefore, some unknown chemical in the extract not affected by the addition of thioglucosidase would appear to be the toxic agent in the *Crambe* extract.

Based on the results of other studies (2, 5,25), the conditions for enzymatic reaction of sinigrin in this study (pH >3.5, no ferrous ion) suggest that allyl isothiocyanate, rather than allyl cyanide, should be the primary decomposition product. Tests with pure allyl cyanide and allyl isothiocyanate support this prediction. If sinigrin were to have reacted completely in the presence of enzyme to form a stoichiometric amount of a product, then the concentration-response curve for the sinigrin and enzyme solution would resemble that of either pure allyl cyanide or allyl isothiocyanate, whichever was the primary product. Since pure allyl cyanide is less toxic than sinigrin with enzyme by 1.5 orders of magnitude, the primary reaction product cannot be allyl cyanide. Any deviation due to incomplete sinigrin reaction would be less toxic than the pure product. Pure allyl isothiocyanate is an order of magnitude more toxic than sinigrin with enzyme, and this result would be expected if, as the chromatographic evidence shows, sinigrin did not completely decompose to form this product. Since the enzymatic conversion was found to be 12–31% isothiocyanate, the toxic agent in the sinigrin-enzyme solutions appears to be allyl isothiocyanate. This finding is further supported by the testing of sinigrin with enzyme at pH conditions and a controlled ferrous ion concentration that found *C. elegans* mortality occurred only in those solutions where allyl isothiocyanate would be expected to predominate.

Caenorhabditis elegans aquatic toxicity tests support results of other researchers who found glucosinolates to be relatively non-toxic to nematodes except when reacted with thioglucosidase (14–17,19). However, a breakdown product of some glucosinolates—isothiocyanate—is relatively toxic to nematodes and might be useful as an effective nematicide.

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