Factors Affecting the Dissolution and Degradation of Oriental Mustard-Derived Sinigrin and Allyl Isothiocyanate in Aqueous Media

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Sinigrin, the predominant glucosinolate in the oriental mustard *Brassica juncea*, is mainly degraded upon the enzymatic action of myrosinase under normal conditions to give allyl isothiocyanate (AITC) in an aqueous media. Because AITC is considered to be the principal nematicidal ingredient in *B. juncea*, its stability in aqueous media is an important issue in achieving efficient nematode control. Pure sinigrin and AITC were found to be relatively stable in buffered water in the pH range of 5.00−7.00 but less stable at pH 9.00. Both sinigrin and AITC were more stable in soil water (supernatant of a 1:1 water/air-dried soil mixture) than in buffered water at the same pH range of 5.00−9.00. Sinigrin dissolved from the mustard bran or ground seed into water very quickly and was degraded by codissolved myrosinase to AITC. The AITC that formed from the degradation of sinigrin was found to be more stable in the soil water than in the buffered water. Buffer capacity was considered to be one of the factors that contributed to the stabilization of AITC in the soil water, but other unknown factors from both bran or seed and soil may also have contributed to the stabilization.

Keywords: *Sinigrin; allyl isothiocyanate; HPLC; pH; soil water; buffer capacity; mustard; nematicidal*

INTRODUCTION

Sinigrin, the major glucosinolate present in the oriental mustard *Brassica juncea*, has been reported to be biologically active against many agricultural pests (Chew, 1988; Brown and Morra, 1995; Manici et al., 1997). Sinigrin is rapidly degraded by the enzymatic action of myrosinase to yield allyl isothiocyanate (AITC), allyl thiocyanate, and allyl cyanide (Chew, 1988; Duncan, 1991). However, under nearly neutral conditions, AITC is the predominant degradation product (Gil and MacLeod, 1980; Duncan, 1991), which is generally considered to be the actual cause of sinigrin's biological activity (Figure 1) (Chew, 1988; Brown and Morra, 1995; Manici et al., 1997).

In a search for alternative soil treatments to control soil-borne pests, a product based on oriental mustard bran, a byproduct in mustard milling factories, is currently under development at our Research Centre. Toxicological assays in vitro in our laboratory showed that the nematicidal activity of this mustard bran-based product was positively correlated to sinigrin/AITC content, and the bran had higher nematicidal activity than equivalent pure AITC in similar tests (data not shown). Our field study also showed that the product strongly suppressed the population density of the root lesion nematode, *Pratylenchus penetrans*, and significantly increased the yield of sweet corn production (data not shown).

Stability of the active ingredient in aqueous media is important in controlling nematodes. Pure AITC in aqueous media has been reported to decompose to allyl allyldithiocarbamate, which further degraded to diallyl tetra- and pentasulfide and other degradation products (Kawakishi and Namiki, 1969). In their study, the half-life of AITC in an acetate buffer (pH 5.2) at 37 °C was ∼5 days. Pure chemical standards or plant extracts have been used to study mechanisms of the enzymatic conversion of sinigrin to AITC (Gil and MacLeod, 1980; Uda et al., 1986; Duncan, 1991). However, the dissolution, stability, and degradation of in situ plant-derived sinigrin in aqueous media at different pH values have received little attention. A study of in situ plant-derived sinigrin and AITC in soil water would provide practical information on the chemistry of these natural products.

The effect of pH on the stability of sinigrin and AITC was of interest to us, not only because the enzymatic conversion of sinigrin to AITC was found to be pH-dependent but also because we found that further degradation of AITC was also affected by pH. Soil is a complex system in which many factors, including soil water, pH, and microbial activity, have direct impact on the fate of sinigrin and AITC (Brown et al., 1991). In their study, Brown et al. (1991) incorporated rapeseed meal (*Brassica napus* L.) into the soil and found that glucosinolates degraded rapidly, yielding isothiocyanates, which further degraded at least partially to ionic thiocyanates. It was also reported that degradation of sinigrin in soil gave mainly allyl nitrile and AITC, the ratio of these two degradation products being dependent on the soil pH and the presence of Fe^{2+} (Borek et al., 1994). However, when Borek et al. further studied the transformation of the glucosinolate-derived AITC and allyl nitrile in soil, they found that the half-lives of AITC in different soils were from 20 to 60 h and that the rate

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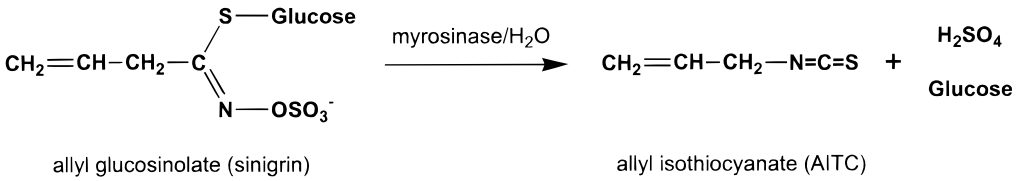

Figure 1. Enzymatic conversion of sinigrin to AITC by myrosinase.

of transformation did not significantly correlate to the soil pH (Borek et al., 1995).

The objectives of this study were to provide basic information on the kinetics of sinigrin and AITC in aqueous media, and the effect of pH on the stability and degradation of sinigrin and AITC in pure, buffered, and soil water. In our other study, we found that the biological activity of AITC was much higher when it was directly derived from mustard bran or seed than that of the same concentration of pure AITC (data not shown). Therefore, the other objective of this study was to elucidate the mechanism(s) underlying the observed enhanced activity of the plant-derived AITC. For this purpose, the stability of the released sinigrin from mustard bran and seed and its conversion to AITC were studied in relation to the stability of pure AITC in pure and buffered water, as well as in soil water under similar conditions.

MATERIALS AND METHODS

Extraction of Sinigrin from Mustard Bran and Seed. Sinigrin was extracted with four different methods to compare the efficiency of each extraction method.

(1) Soxhlet Extraction. One gram of finely ground seed or bran was weighed into coffee filter paper, wrapped, and put in a Soxhlet extractor. The extractor was equipped on a 150-mL round-bottom flask with 100 mL of 50% acetonitrile in water (v/v). The flask was heated with a heating mantel and kept boiling for 24 h at ~1 cycle per hour. The extract was then cooled to room temperature, filtered through an Ahlstrom No. 601-25 filter paper (Ahlstrom, Mt. Holly Spring, PA), rinsed with 50% acetonitrile, and made up to the mark in a 100-mL volumetric flask.

(2) Boiling Extraction. One gram of finely ground seed or bran was added to 100 mL of boiling 50% acetonitrile in a 200-mL round-bottom flask that was connected to a reflux condenser. After 30 min of boiling, the extract was cooled and filtered as described above.

(3) Extractions by Soaking in Hot or Cold Solvent. One gram of finely ground seed or bran was extracted by soaking in 100 mL of boiling (hot) or room temperature (cold) 50% acetonitrile for 30 min. The former (hot extraction) was not provided with additional heating.

(4) An additional extraction was also performed by adding 1.0 g of finely ground seed or bran into 100 mL of water at room temperature for 5 min. Extracts of methods 2−4 were cooled, filtered, and made up to 100 mL similarly as for method 1. All of the solutions obtained were then filtered through a 0.45 μm syringe filter (Gelman Sciences Inc., Ann Arbor, MI) before being analyzed by HPLC. HPLC grade water was used in all extractions and preparation of sample solutions.

Soil Water. A soil sample (loamy sand) was collected from Delhi, ON, Canada, and was air-dried and passed through a 2-mm sieve. Soil water was obtained by adding 1:1 water to the air-dried soil (w/w). After 24 h, the supernatant was filtered through an Ahlstrom No. 601-25 filter paper. The pH of the soil water was 6.90. Part of the filtrate (soil water) was also sterilized by boiling for 10−15 min and then cooled to room temperature. This water was used along with the unboiled water to prepare sinigrin or AITC solutions to examine the microbial effect on the degradation of AITC in the soil water.

Dissolution and Degradation of Sinigrin from Mustard Bran or Seed. Ten grams of bran or ground seed was soaked in 1000 mL of HPLC grade water or soil water. An aliquot of 0.5 mL was sampled initially at 5 min and then every 15 min for 400 min thereafter. Samples were syringe-filtered and immediately analyzed by HPLC. The pH of the solution was measured every 5−10 min for 140 min with a pH meter (combination glass electrode) (model HI 931400, Hanna Instruments, Italy).

Chemicals and Buffer Solutions. AITC was purchased from Aldrich (Milwaukee, WI) and sinigrin from Sigma (St. Louis, MO). HPLC grade water and acetonitrile (ACN) were from Caledon Laboratories Ltd., Georgetown, ON. All other chemicals were of reagent grade from commercial sources. Sinigrin and AITC were individually dissolved in buffer, pure water, or soil water so that the concentration was 1000 μg/mL. Buffers used in kinetics study were prepared as follows: Phosphate buffers of pH 5.00, 6.00, and 7.00 were prepared by adding 0.001 M $(NH_4)_2HPO_4$ solutions to 0.001 M KH_2PO_4 with simultaneous monitoring with a pH meter. The pH 9.00 buffer was prepared by adjusting the 0.001 M $(NH_4)_2HPO_4$ with 0.1 M NaOH. An additional phosphate buffer of pH 7.10 was prepared by mixing 0.005 M KH_2PO_4 and 0.005 M Na_2HPO_4 solutions. This buffer, along with 0.005 M $NaHCO_3$, was used as references for comparing the buffer capacity with the soil water. The buffer capacity (β) in this study is termed as the number of moles of HCl required to cause 1 L of the solution to change by 1 unit from its original pH. Titration and calculation of β followed Chiba's method (1979) with minor modifications.

Preparation of Sinigrin and AITC Samples for Chemical Degradation Studies. Sinigrin solutions (1000 μg/mL) were stored in glass volumetric flasks in a dark incubator at 25 ± 1 °C. Aliquots were taken at days 1, 5, 10, 20, 50, 80, and 120 and analyzed by HPLC in triplicate. AITC solutions were added and stored in HPLC autosampler vials (2 mL; Scientific Products and Equipment, Concord, ON) under the above conditions. All of the vials were fully filled and sealed with Teflon-lined caps (Scientific Products and Equipment) to avoid the loss of AITC due to volatilization. Three replicate samples were prepared for each solution, and the samples were analyzed by HPLC at days 1, 5, 10, 20, 50, 80, and 120.

HPLC Determination of Sinigrin and AITC. Sinigrin and AITC concentrations were analyzed by HPLC (HP 1100, Hewlett-Packard, Avondale, PA), using a Sphereclone ODS-2 column (Phenomenex, 5 μm, 15 cm × 4.6 mm) and a photodiode array detector. Data acquisition and analysis were performed using ChemStation software. A 3-cm precolumn packed with Spheresorb (Phenomenex) was connected prior to the analytical column. The mobile phase as follows was pumped at 1.0 mL/min: 1:99 = ACN/0.025 M NH_4OAc (v/v) was kept isocratic until 2:00 min and then linearly increased to 50:50 (v/v) at 2:30 min. This mobile phase was kept isocratic until 10:00 min and brought back to 1:99 at 12:00 min. There was a 2-min after-run between each sample injection. The detector was set at 228 nm (λ_{max}) for sinigrin and at 242 (λ_{max}) nm for AITC.

Data Analysis. In the kinetics study, concentrations (C) of sinigrin or AITC were transformed to ln(C) and fitted to the linear equations with time. The half-lives, $t_{1/2}$ (time at which sinigrin or AITC reached half of the original concentration), were then calculated according to the linear equations. The half-life values were used to compare the degradation rates of sinigrin or AITC at different pH and soil solutions.

Table 1. Sinigrin Content in Oriental Mustard Seed and Bran Determined after Extraction in Pure Water by Different Extraction Methods

	sinigrin (%, w/w)				
	Soxhlet extraction	boiling	hot soaking	cold soaking[a]	cold soaking[b]
seed	5.2	5.1	4.6	4.3	4.3
bran	1.9	2.2	1.7	2.1	1.8

[a] Cold soaking in 50% ACN. Numbers were corrected by adding the detected sinigrin (3.4%) and AITC concentration (0.9%). Analyzed by HPLC. [b] Cold soaking in water at room temperature for 5 min. Analyzed by capillary electrophoresis (method not published).

RESULTS AND DISCUSSION

Sinigrin contents in the bran and the whole seed of oriental mustard were between 1.7 and 2.2% and between 4.3 and 5.2%, respectively, depending on the method of extraction (Table 1). For the seed, the Soxhlet extraction method gave the highest sinigrin content of 5.2%, followed by boiling, hot soaking, and cold soaking in 50% ACN. We also found that at room temperature, pure water extracted the same amount of sinigrin in 5 min from the seed as did the 50:50 acetonitrile/water mixture at room temperature, that is, 4.3% (Table 1). The corresponding value was only slightly lower for the bran, that is, 1.8% compared to 2.1% (Table 1). Extraction with water was limited to 5 min to avoid the enzymatic degradation of sinigrin to AITC.

When mustard bran was soaked in pure water at room temperature, sinigrin was very rapidly (2−5 min) dissolved and reached its highest level of 145 μg/mL at 2 min (Figure 2a). The dissolved sinigrin was rapidly degraded to AITC, presumably upon contact with the simultaneously released myrosinase enzyme. The concentration of formed AITC reached its highest level (44 μg/mL) at 54 min and slowly decreased afterward (Figure 2a). A concentration of 44 μg/mL indicates 0.44% AITC in bran (w/w), which is equivalent to 1.8% sinigrin in bran (w/w). This is 100% of the total sinigrin content (1.8%) found in mustard bran when extracted with pure water at room temperature for 5 min (Table 1). The highest AITC concentration was observed at 54 min (44 μg/mL), and the concentration decreased to half of its highest concentration at 400 min (Figure 2a). In soil water (pH 6.90), sinigrin dissolved in a similarly rapid fashion. However, the converted AITC was more stable in the soil water (Figure 2b), with AITC concentration holding constant at 49 μg/mL after 120 min (Figure 2b). The concentration of AITC at 400 min remained the same as it was when it peaked at 120 min (Figure 2b). This concentration implied 0.49% AITC in the mustard bran (w/w), which is equivalent to 2.0% sinigrin in the bran (w/w), which again is a quantitative conversion of the total sinigrin content (Table 1).

The mechanisms involved in the stabilization of AITC in soil water are not known. In the kinetics study, when pure sinigrin and AITC were in different buffer (pH 5.00−9.00) and soil water solutions, AITC was also found to be more stable in the latter (Figure 3; Table 2). Half-lives of AITC were 31, 34, 31, and 26 days in pH 5.00, 6.00, 7.00, and 9.00, respectively (Table 2). In sterilized soil water (Figure 3b, soil water/sterile), its half-life ($t_{1/2}$) was substantially longer at 43 days but not significantly different from that in the nonsterilized soil water ($t_{1/2}$ = 40 days), suggesting that microorganisms, if present, do not contribute significantly to the

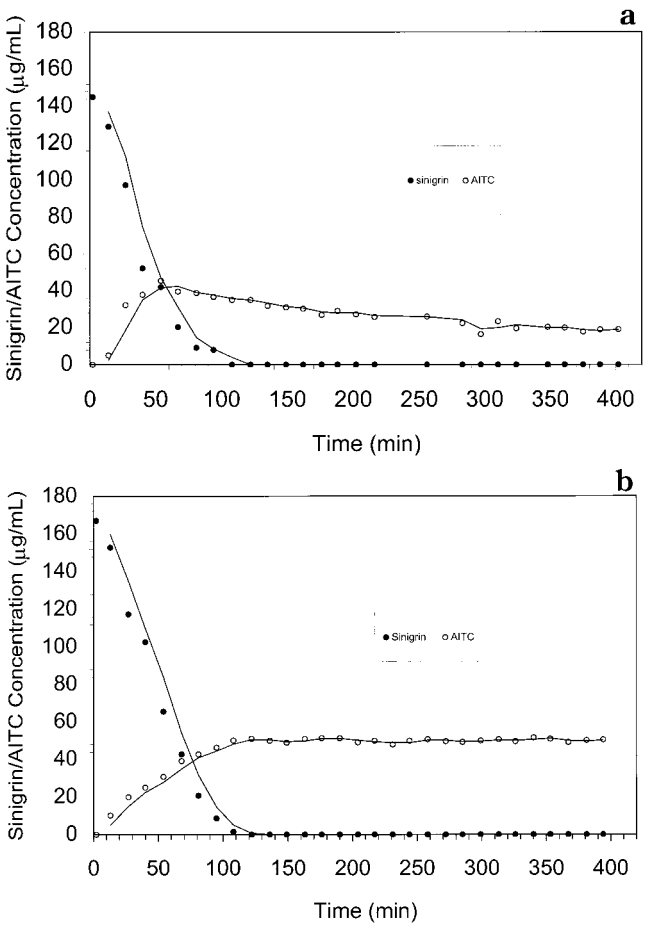

Figure 2. Dissolution of sinigrin from the oriental mustard bran and its degradation to AITC in pure water (a) and soil water (b).

degradation of this compound (Figure 3b; Table 2). The half-lives of pure sinigrin in water were >120 days except for the pH 9.00 buffer solution (Figure 3a). Sinigrin was found to degrade primarily to AITC even under such high pH. Concentrations of the formed AITC in the sinigrin/pH 9.00 buffer were 47, 72, and 46 μg/mL at days 50, 80, and 120, respectively.

In addition to the pH effect on the stability of AITC, the buffer capacity of soil water might have been another factor that contributed to the stabilization of AITC. The pH of soil water (6.90) was nearly the same as that of pure water (HPLC grade). When bran was soaked in pure water (Figure 4), the pH value of the suspension decreased markedly from 6.55 to 5.60 at 10 min, and it kept decreasing until ∼30 min to pH 5.41. The pH then increased slightly and stabilized at 5.75 (Figure 4). The pH of the soil water also decreased when mustard bran was added, but not as significantly as did the pure water. It decreased sharply from 7.00 to 6.45 at 10 min and then slowly decreased and stabilized at 6.10 after 40 min (Figure 4). Overall, the pH of the pure water decreased much more than that of the soil water, indicating a buffering effect by the soil water. A similar trend of decreased pH values was observed for the ground seed. When ground seed was added separately to pure or soil water, the pH values of both types of water drastically decreased until ∼30 min (Figure 4). The pH of the pure water continued to decrease and stabilized at 5.25 after 50 min, whereas the pH of the soil water decreased to 5.55 and increased slightly and

Table 2. Half-Lives of Sinigrin and AITC in Aqueous Media of Various pH Values

	sinigrin				AITC			
aqueous medium	slope	intercept	R^2	$t_{1/2}$, days	slope	intercept	R^2	$t_{1/2}$, days
pH 5.00	–[c]	–	–	>120	−0.0189	6.7955	0.972	31
pH 6.00	–	–	–	>120	−0.0165	6.7797	0.9558	34
pH 7.00	–	–	–	>120	−0.0175	6.7485	0.9405	31
pH 9.00	−0.0113	6.9445	0.9680	65	−0.0175	6.6654	0.9376	26
SW[a]	–	–	–	>120	−0.0165	6.8790	0.9826	40
SSW[b]	–	–	–	>120	−0.0146	6.8481	0.9823	43

[a] Nonsterilized soil water (pH 6.90). [b] Sterilized soil water (pH 6.90). [c] Not applicable.

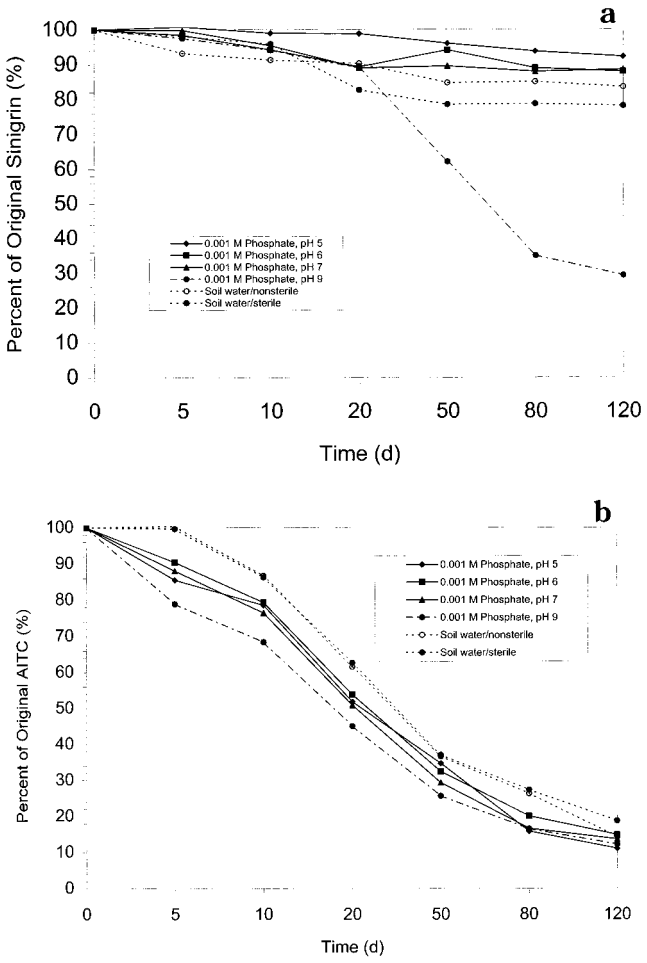

Figure 3. Degradation of sinigrin (a) and AITC (b) in different buffer solutions, sterilized soil water and nonsterilized water.

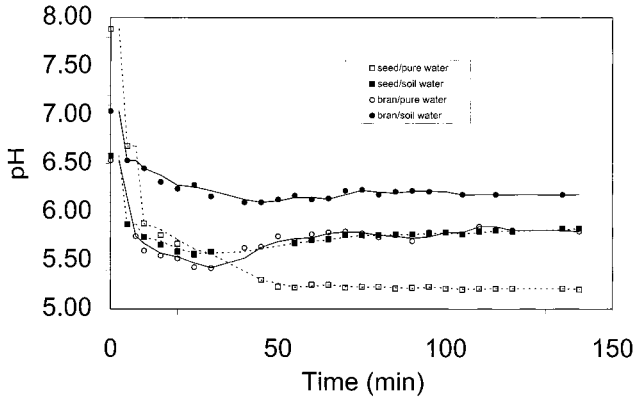

Figure 4. pH changes of pure and soil water when oriental mustard bran or seed was released.

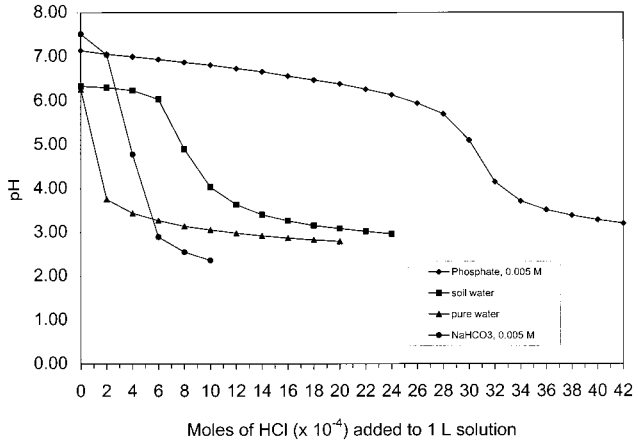

Figure 5. Buffer capacity of soil water as demonstrated by pH changes in relation to moles of HCl added.

stabilized at 5.75 after 55 min (Figure 4). Ground seed caused a larger pH decrease than the bran, due very likely to the higher sinigrin content (Table 1), which produced more sulfate during its enzymatic degradation (Figure 1). The change of pH caused by mustard bran or ground seed was different from what was observed in the pure sinigrin and myrosinase reaction reported by Gil and MacLeod (1980). In their study the pH value of a sinigrin/myrosinase solution (sinigrin concentration was 1×10^{-3} M) dropped to 3.30 after 60 min of reaction. They also found that at a pH <5.00, decomposition of sinigrin by myrosinase favored nitrile over isothiocyanate, but at pH >5.00, >90% of the product was AITC (Gil and MacLeod, 1980). We did not observe any pH <5.20 with either bran or seed in either pure water or soil water (Figure 4). Factors contributing to this difference are most likely from the plant material (bran or seed) other than sinigrin or other glucosinolates, because the total amount of sinigrin from the seeds in the present study was equal to what was used by Gil and MacLeod (1980).

The higher pH found in a soil water of either mustard bran or seed compared with that found in pure water indicated that the buffer capacity of soil water might be a factor that kept the pH of the solution steady at a higher level. As shown in Figure 5, the buffer capacity β for the soil water was 8×10^{-4}, whereas for the pure water it was only 1.8×10^{-4}. The buffer capacity of the pH 7.10 phosphate buffer was 26×10^{-4} (Figure 5). Higher buffer capacity, by definition, implies smaller changes in pH by the addition of an acid or base. In the case of mustard bran- or seed-derived sinigrin, higher pH values maintained by soil water might have caused the stabilization of AITC. However, other unknown factors from the plant materials (mustard bran or seed) and the soil might also have affected the stability of bran- or seed-derived AITC in the soil water.

Many studies have focused on the process of enzymatic degradation of sinigrin to AITC. Degradation of

pure AITC has also been studied, and degradation pathways have been proposed. AITC generated from the biomass of bran or seed may behave differently from the pure AITC because of coexisting plant components that may affect the chemistry and biological activity of AITC. Unidentified substance(s) in soil water may also contribute to the stability of sinigrin and AITC. Our observation of the enhanced nematicidal activity of the bran relative to that of pure AITC (on an equal AITC concentration basis) indicated such differences (data not shown). Also, when bran or ground seed is applied to the soil, it absorbs water, causing enzyme (myrosinase) activation and the subsequent release of AITC to the soil water. AITC in soil water, as our results suggested, was more stable than in pure water (Figures 2 and 3). This stabilization of AITC may also have contributed to the high activity of mustard bran or seed in the field (data not shown).

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