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Ionic Thiocyanate (SCN^-) Production from 4-Hydroxybenzyl Glucosinolate Contained in *Sinapis alba* Seed Meal

VLADIMIR BOREK AND MATTHEW J. MORRA*

Soil & Land Resources Division, P.O. Box 442339, University of Idaho, Moscow, Idaho 83844-2339

Meal produced from *Sinapis alba* seed by crushing to remove oil contains a glucosinolate that when hydrolyzed produces phytotoxic allelochemicals; however, the responsible compounds and pathways for their production have not been elucidated. *S. alba* seed meal and partially purified extracts containing 4-hydroxybenzyl glucosinolate were included in experiments to identify and monitor enzymatically released products using GC−MS and HPLC−MS. The initial product, 4-hydroxybenzyl isothiocyanate, was unstable in aqueous media, showing a half-life of 321 min at pH 3.0, decreasing to 6 min at pH 6.5. More alkaline pH values decrease the stability of 4-hydroxybenzyl isothiocyanate by promoting the formation of a proposed quinone that hydrolyzes to SCN^-. Measurement of SCN^- showed stoichiometric release from *S. alba* meal at 48 h when buffered at pH values as low as 4.0, demonstrating that SCN^- production in soil is not only probable but likely responsible for observed phytoxicity of the meal.

KEYWORDS: Glucosinolates; *Sinapis alba*; allelochemicals; mustard; phytotoxic; sinalbin

INTRODUCTION

Glucosinolates are organic anions possessing a β-D-thioglucose moiety, a sulfonated oxime, and any one of a variety of aliphatic or aromatic R groups. At least 120 structurally different glucosinolates have been identified in 16 different families of angiosperms (*1*). Although glucosinolates themselves possess limited biological activity, enzymatic degradation by thioglucoside glucohydrolase (EC 3.2.3.1), or myrosinase, results in the formation of a number of compounds, including isothiocyanates, nitriles, SCN^-, oxazolidinethione, epthionitriles, and organic thiocyanates. The products formed from any one glucosinolate are controlled by both the chemistry of the glucosinolate itself as well as reaction conditions such as pH, Fe^{2+} concentration, and the presence of specifier proteins and coenzymes (*2*).

There is currently intense interest in using glucosinolate-containing plants as a source of pesticidal compounds to replace synthetic organic compounds for pest control, since insecticidal, nematicidal, fungicidal, and phytotoxic effects of the tissues have been documented (*3*, *4*). Although glucosinolates are found in all plant parts, they are most concentrated in the seed. Cold crushing the seed for oil removal produces a meal product that contains not only high concentrations of glucosinolates but preserves myrosinase activity such that water addition immediately results in the formation of biologically active hydrolysis products (*5*). Meals stored in the absence of water retain glucosinolates and myrosinase activity and thus can be used as a soil amendment of possible value in controlling soil-borne plant pests.

One such glucosinolate-containing meal that appears to have potential to act as an herbicide is that produced from *Sinapis alba* seed. Ascard and Jonasson (*6*) showed that *S. alba* meal effectively inhibited emergence of several annual weed species; however, the responsible allelochemicals were not identified. Sinalbin (4-hydroxybenzyl glucosinolate), the primary glucosinolate within *S. alba* meal, is expected to produce the corresponding isothiocyanate. Although it is generally assumed that complete decomposition to SCN^- occurs only at alkaline pH values (*7*), there have been reports that SCN^- is produced at pH values from 3 to 7 (*8*−*10*). The production of SCN^- from the primary glucosinolate in *S. alba* seed meal is noteworthy given the fact that this anion has been shown to be phytotoxic (*11*−*13*). Our objective was to elucidate the pathways of sinalbin hydrolysis and identify those allelochemicals of possible importance in controlling soil-borne plant pests. We focused on the production of SCN^- because of its possible role in controlling weed species.

MATERIALS AND METHODS

Seed Meal Preparation. All analyses and experiments were performed with meal remaining after seed from the *S. alba* cultivar IdaGold (*14*) was cold pressed to remove approximately 90% of the oil (*15*). The remaining oil was removed by performing three extractions with petroleum ether that involved shaking 500 g of the meal with 500 mL of petroleum ether and filtering through a Büchner funnel. The final filtration cake was washed with 250 mL of petroleum ether, allowed to air-dry, and homogenized in a blender.

Sinalbin Content of the Meal. The glucosinolate concentration of the defatted meal was determined using a method similar to that of the International Organization of Standardization (*16*). Defatted seed meal was weighed (200 mg) into 15-mL extraction tubes to which 500 mg

* Corresponding author. Tel: 208-885-6315. Fax: 208-885-7760. E-mail: mmorra@uidaho.edu.

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of 3-mm glass beads, 10 mL of 70% methanol/water solution, and 100 μL of internal standard [4-methoxybenzyl glucosinolate, obtained (*17*) from meadowfoam (*Limnanthes alba*) seed meal] were added. The detector response factor for 4-methoxybenzyl glucosinolate was determined by comparison with known concentrations of 2-propenyl glucosinolate having an assumed response factor of 1.0 (*16*). Extraction tubes were shaken for 2 h on a reciprocal shaker and centrifuged for 5 min at 1073*g* to precipitate the seed meal. The extract solution was transferred to columns containing 250 mg of DEAE anion exchanger and allowed to drain freely. The columns were washed twice with 1 mL of deionized water and finally with 1 mL of 0.1 M ammonium acetate buffer (pH 4.0). To the columns was then added 100 μL of a 1 mg/L sulfatase enzyme (Sigma-Aldrich, St. Louis, MO) solution and 100 μL of 0.1 M ammonium acetate buffer (pH 4.0). The columns were covered to prevent evaporation and allowed to stand with the enzyme for 12 h, after which time the samples were eluted into HPLC autosampler vials with two consecutive 750-μL volumes of deionized water.

A Waters 2695 HPLC separation module coupled with a Waters 996 photodiode array detector (PDA) and Thermabeam Mass Detector (TMD) was used for glucosinolate analysis. For quantitative purposes, all desulfoglucosinolates detected by PDA were measured at a wavelength of 229 nm. Separation was performed on a 250 × 2.00 mm, 5 μm, 125 Å Aqua C18 column (Phenomenex, Torrance, CA). The flow rate was 200 μL/min, with a methanol gradient starting at 0.5% and increasing to 50%. Glucosinolates were identified using a combination of expected retention behavior (time, sequence) and mass spectra.

4-Hydroxybenzyl Isothiocyanate Release from *S. alba* Seed Meal. Ten grams of the defatted meal was weighed into polypropylene centrifuge tubes to which was added 40 mL of deionized water. In one set of triplicate samples we added 10 mL of ethyl acetate as the extractant and 1 μL of decane (Sigma-Aldrich, St. Louis, MO) as the internal standard immediately after mixing the meal with deionized water. The mixtures were shaken and maintained at 22 ± 2 °C, and samples were removed periodically during a 96-h incubation period. In a second set of triplicate samples, the addition of 10 mL of ethyl acetate and 1 μL of decane was delayed until 30 min prior to each respective sampling time. At each sampling time the mixture was centrifuged for 10 min at 1677*g* and 250 μL of the supernatant was withdrawn for analysis. GC−MS analysis was performed using an HP 5890A gas chromatograph equipped with a 30 m × 0.32 mm i.d., 0.25 μm film HP-5MS capillary column (Agilent Technologies) coupled to an HP 5972 mass detector. Ethyl acetate extracts were manually injected into a split/splitless port (250 °C, 20-s split), and the temperature of the GC oven was programmed from 65 °C (isocratic 3 min) to 270 °C (isocratic 5 min) at a rate of 15 °C/min. The average linear flow rate of He at 250 °C was 35 cm/min. Data (total ion current) were corrected using decane as the internal standard and quantified using benzyl isothiocyanate as an external standard.

Extraction efficiencies for 2-propenyl, butyl, benzyl, and *tert*-octyl isothiocyanates were determined by combining 10 μL of each in duplicate 40-mL deionized water samples. The samples were treated in the same manner as described above, including both the immediate and delayed addition of ethyl acetate and decane. The amount of each analyte extracted using continuous or periodic extraction was determined using GC−MS as described for *S. alba* seed meal.

Stability of 4-Hydroxybenzyl Isothiocyanate in Buffered Media. Partially purified 4-hydroxybenzyl isothiocyanate was prepared by suspending 500 g of *S. alba* seed meal in 2 L of deionized water and extracting the mixture with 500 mL of ethyl acetate for 24 h. The ethyl acetate extract was separated by decanting the top organic layer after centrifugation, dried with 100 g of anhydrous sodium sulfate overnight, and concentrated under vacuum at laboratory temperature. The crude 4-hydroxybenzyl isothiocyanate extract was further purified by preparative column chromatography on silica gel (500 g). Elution was achieved in a stepwise fashion using six 100-mL aliquots of eluent composed of pentane and methylene chloride at ratios of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. Content of 4-hydroxybenzyl isothiocyanate within the fractions was verified by GC−MS using instrumentation and conditions as described previously. Fractions containing 4-hydroxybenzyl isothiocyanate were combined and concentrated under vacuum at laboratory temperature producing a yellowish, viscous fluid displaying only 4-hydroxybenzyl isothiocyanate and pentane/methylene chloride solvent peaks in the GC chromatogram. We were unable to further concentrate 4-hydroxybenzyl isothiocyanate using vacuum distillation because of its instability.

We tested the pH stability of 4-hydroxybenzyl isothiocyanate by incubating 25 μL of partially purified extract dissolved in 25 mL of eight different buffers with pH values ranging from 3.0 to 6.5. We used 0.1 M buffers prepared by mixing 0.2 M sodium citrate and citric acid solutions in precalculated ratios ranging from 4 mL sodium citrate and 46 mL citric acid to 41 mL sodium citrate and 9 mL citric acid in a total volume of 100 mL. Actual pH values of the buffers of 3.03, 3.52, 4.02, 4.49, 5.00, 5.46, 5.91, and 6.52 were verified using an Orion model 420A pH meter (Orion Research, Boston). At specific times during the incubation, a 1-mL sample was withdrawn from the buffered reaction solution with a syringe and injected into a Waters Integrity HPLC system (2695 separation module, 996 PDA, and TMD) equipped with a 150 × 2 mm i.d., 5 μm Aqua C-18 column (Phenomenex). The instrument was operated at a constant flow rate of 200 μL/min with a gradient from 5 to 35% of methanol during each 30-min run. Half-lives for 4 hydroxybenzyl isothiocyanate were estimated from straight lines obtained by plotting the natural logarithm of the normalized concentration versus time. This experiment was repeated twice with two different meal extracts acquired by the same procedures from the same seed material. Half-lives from only one of the experiments are reported, since the results for both experiments were similar.

Release of SCN^- from *S. alba* Seed Meal. Ten grams of defatted *S. alba* meal were weighed into a 250-mL polyethylene bottle to which was added 200 mL of deionized water or a citrate buffer solution (pH of 4.0, 5.0, 6.0, or 7.0) prepared as described previously. The samples were placed on a reciprocating shaker for 48 h, during which time 5.0-mL aliquots were removed periodically to determine the time course of SCN^- release. Each 5-mL aliquot was placed in a 50-mL centrifuge tube and 40.0 mL of a methanol:deionized water (2:1, v:v) solution containing 1% acetic acid was added. The tubes were shaken vigorously for 15 min and centrifuged for 5 min at 1073*g*, and 5 mL of the supernatant was filtered through a 25-mm, 0.2-μm GD/X membrane (Whatman) into a beaker. One milliliter of the filtered sample was then transferred to an HPLC autosampler vial to which was added 0.50 mL of a 0.01 M Fe^{3+} solution and 100 μL of a 0.1 M HCl solution. The vials were capped, shaken, and immediately analyzed using a Waters Integrity HPLC system equipped only with a 5-μm, 10 × 2 mm i.d. Aqua C-18 precolumn (Phenomenex). A 50-μL sample was injected and isocratically eluted using a 10% methanol solution pumped at a flow rate of 0.5 mL/min. Absolute concentrations of SCN^- in the unknown samples were determined following the same procedure as described above, except that 10.0 g of *S. alba* meal from which the glucosinolates had been removed with repeated methanol extraction was substituted for the unaltered meal. Amounts of a KSCN stock solution containing 10−100 μmol of SCN^- were added to the meal/buffer mixtures prior to the initial shaking, and a separate standard curve was prepared for each buffer pH.

RESULTS AND DISCUSSION

Glucosinolates in *S. alba* Meal. As expected, sinalbin was the major glucosinolate in *S. alba* meal, constituting approximately 93% of total glucosinolate content (**Figure 1**). The measured concentration of sinalbin in defatted meal was 152 ± 5.2 μmol/g (mean value ± variance of five replicates). The meal also included (2*R*)-2-hydroxybut-3-enyl glucosinolate (3.6 μmol/g) and five unidentified glucosinolate peaks with a total estimated glucosinolate concentration of approximately 6.4 μmol/g. Concentrations of indolyl glucosinolates that could potentially produce SCN^- as a result of the hydrolytic instability of their respective isothiocyanates (*18*) represented a total of only about 1 μmol/g of defatted seed meal. The simplicity of the glucosinolate profile in *S. alba* meal thus facilitates our ability to determine a likely precursor for glucosinolate hy-

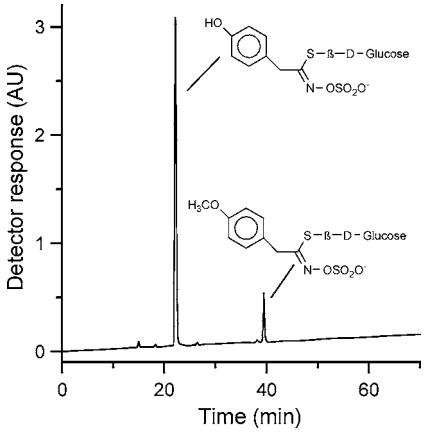

Figure 1. HPLC chromatogram showing the dominance of 4-hydroxybenzyl glucosinolate contained in *S.* alba seed meal extracts, along with a peak for the internal standard, 4-methoxybenzyl glucosinolate. Peaks represent respective desulfoglucosinolates of each structure shown.

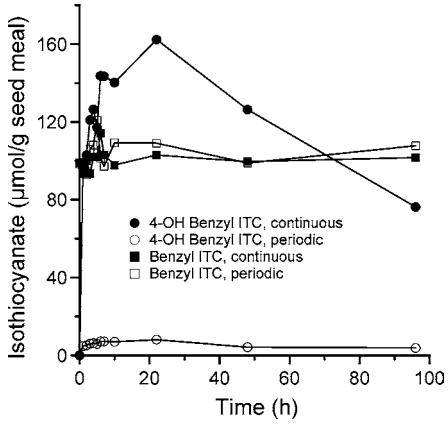

Figure 2. Continuous and periodic extraction into ethyl acetate of 4-hydroxybenzyl isothiocyanate resulting from hydrolysis of sinalbin contained in *S. alba* seed meal as compared to similar extractions of benzyl isothiocyanate from aqueous solution. 4-Hydroxybenzyl isothiocyanate incubations contained no seed meal but are expressed on a weight basis for comparison purposes only.

drolysis products that might be identified. Most important is the fact that low concentrations of indolyl glucosinolates eliminate the possibility that these compounds can serve as precursors of significant amounts SCN^- that might be measured in hydrolyzed extracts.

4-Hydroxybenzyl Isothiocyanate Release from *S. alba* Seed Meal. We observed a dramatic difference between the relatively high yield of 4-hydroxybenzyl isothiocyanate obtained by continuously extracting into ethyl acetate as compared to periodic measurements made by adding ethyl acetate 30 min prior to each respective sampling time (**Figure 2**). Maximum 4-hydroxybenzyl isothiocyanate extracted during the continuous procedure was 162 μmol/g seed meal at 24 h, whereas less than 10 μmol/g was extracted at any one time in the periodic analyses. In contrast, when continuous and periodic extractions were performed with benzyl isothiocyanate, comparable concentrations of the compound were measured in the ethyl acetate extracts irrespective of the procedure (**Figure 2**). 2-Propenyl, butyl, and *tert*-octyl isothiocyanates showed extraction yields similar to that of benzyl isothiocyanate, ranging from at least 98% for all isothiocyanates in the continuous extraction to a low of 83% for 2-propenyl isothiocyanate in the periodic extraction.

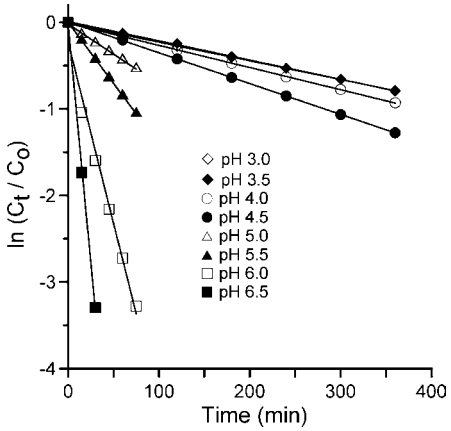

Figure 3. First-order plots for the disappearance of 4-hydroxybenzyl isothiocyanate incubated in buffered aqueous solutions with pH values ranging from 3.0 to 6.5. Plots for pH 3.0 and 3.5 are superimposed on each other in the graph.

Our results clearly indicate that 4-hydroxybenzyl isothiocyanate is unstable in aqueous media, and that isolation and purification require the use of nonreactive solvents. Similar attempts to isolate 4-hydroxybenzyl isothiocyanate have also been unsuccessful, leading Kjaer and Rubinstein (*19*) to trap the synthesized compound by continuously extracting into ether containing aniline, thereby converting the unstable isothiocyanate to a phenylthiourea derivative. The instability of 4-hydroxybenzyl isothiocyanate has been used quantitatively to measure sinalbin concentrations in seed meal of *S. alba* in a procedure that relies on sodium hydroxide addition for stoichiometric release of SCN^- (*7*). Although no data were provided, Gmelin and Virtanen (*8*) reported that 4-hydroxybenzyl isothiocyanate decomposes slowly in water at pH values as low as 7.0, producing SCN^- and what they presumed to be 4-hydroxybenzyl alcohol. Ensuing studies definitively identified 4-hydroxybenzyl alcohol as a major product of sinalbin hydrolysis (*9*, *10*, *20*) and showed that SCN^- production occurred at pH values as low as 3.0 (*10*). Our continued research was therefore directed at determining amounts of SCN^- produced at pH values relevant to what might be expected in agricultural soils.

Stability of 4-Hydroxybenzyl Isothiocyanate in Buffered Aqueous Solutions. A partially purified and concentrated seed meal extract containing 4-hydroxybenzyl isothiocyanate was dissolved in buffers ranging from pH 3.0 to 6.5. The half-life of 4-hydroxybenzyl isothiocyanate at pH 6.5 was the shortest at 6 min, increasing to 16, 49, 100, 195, 270, 312, and 321 min with decreasing pH values of 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, and 3.0, respectively (**Figure 3**). The hydrolytic instability of 4-hydroxybenzyl isothiocyanate, especially at higher pH values, explains its low extractability in unbuffered extracts of seed meal that had a pH of 5.3 and a sampling time of 48 h. Our data also confirm previous investigations indicating that alkaline pHs facilitate hydrolysis (*7*). However, we show that appreciable hydrolysis occurs at pH values as low as 3.0 and that in a soil environment buffered at pH values typically between 5 and 7, significant amounts of SCN^- production are expected in a relatively short time period.

The pH-dependency of the rate of the reaction as determined by 4-hydroxybenzyl isothiocyanate disappearance is plotted in **Figure 4**. The first-derivative plot of these data displays an inflection point near pH 4.5, most likely indicative of the pK_a value associated with the hydroxyl group of the compound. We propose that the rate of 4-hydroxybenzyl isothiocyanate hy-

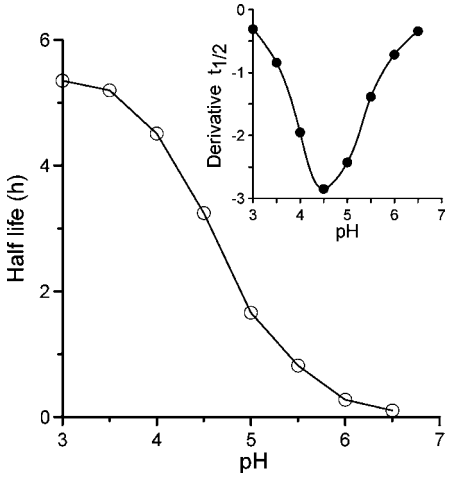

Figure 4. Half-lives for 4-hydroxybenzyl isothiocyanate plotted as a function of pH. Inset shows the first derivative of the half-life data displaying an inflection point at pH 4.5.

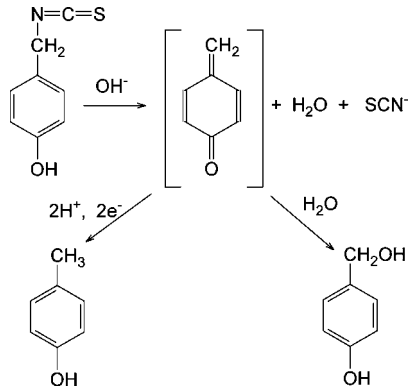

Figure 5. Proposed pathway for the transformation of 4-hydroxybenzyl isothiocyanate to products identified using chromatographic and spectroscopic techniques.

drolysis is controlled by pH, as it influences the formation of an unstable quinone intermediate (**Figure 5**). More alkaline pH values decrease the stability of 4-hydroxybenzyl isothiocyanate by promoting the formation of a quinone that hydrolyzes to form SCN^-. Support for the proposed pathway was obtained by identifying 4-methyl phenol and 4-hydroxybenzyl alcohol production when monitoring 4-hydroxybenzyl isothiocyanate disappearance in these buffered solutions (**Figure 6**).

We speculate from these data that any glucosinolate with the ability to create conjugated double bond structures (2-hydroxybenzyl glucosinolate) will behave in a similar fashion. In contrast, benzyl glucosinolates substituted in the meta position (3-OH benzyl glucosinolate) or those with functional groups that prevent electron delocalization (4-methoxybenzyl glucosinolate) will degrade to more stable isothiocyanates. The stability of the isothiocyanate has important implications for the use of seed meal products in pest control given the differences in biological activities of the glucosinolate hydrolysis products (*3*).

Ionic Thiocyanate Release from *S. alba* Seed Meal. *S. alba* seed meal was incubated with deionized water and buffer solutions ranging from pH 4.0 to 7.0 to quantify SCN^- production resulting from 4-hydroxybenzyl glucosinolate hydrolysis in the presence of a full component of meal constituents. Our results showed that SCN^- production occurred most slowly at pH 4.0, but that final concentrations determined at 48 h varied from a low at pH 6.0 of 143 μmol/g and a high in deionized water of 166 μmol/g of seed meal (**Figure 7**). The amount of

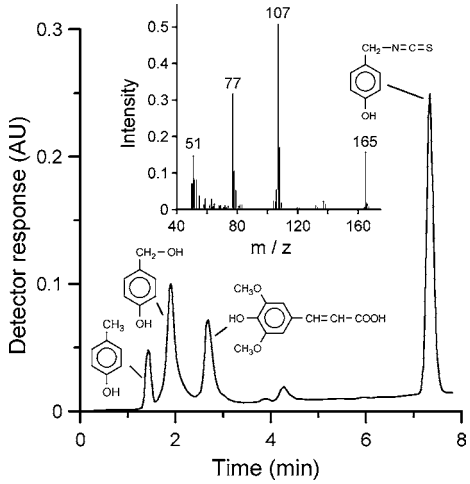

Figure 6. Products identified in the HPLC–MS chromatogram when 4-hydroxybenzyl isothiocyanate extracts obtained from *S. alba* seed meal were incubated at pH 4.5. The inset shows the mass spectrum for 4-hydroxybenzyl isothiocyanate. Sinapic acid, shown at a retention time of approximately 2.9 min, is produced from sinapine in a reaction unrelated to glucosinolate hydrolysis.

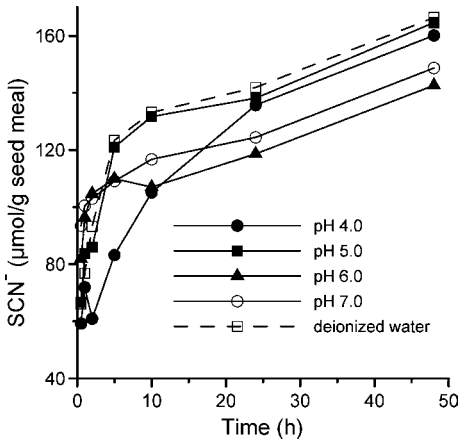

Figure 7. Ionic thiocyanate produced from *S. alba* seed meal incubated in deionized water and aqueous solutions buffered at pH values ranging from 4.0 to 7.0.

SCN^- expected on the basis of 4-hydroxybenzyl glucosinolate concentration in the meal and the assumption of its complete stoichiometric conversion to SCN^- is approximately 152 μmol/g of seed meal, thus indicating near complete conversion in 48 h at all pH values.

Results obtained with seed meal incubations confirm conclusions reached using 4-OH benzyl glucosinolate extracts, clearly indicating that 4-hydroxybenzyl isothiocyanate is rapidly hydrolyzed to SCN^- at pH values expected in most soils. In contrast, data from previous investigations conducted with purified sinalbin and myrosinase indicate that decreased pH values promote the formation of 4-hydroxybenzyl cyanide at the expense of 4-hydroxybenzyl isothiocyanate (*10*, *20*), thereby decreasing subsequent formation of SCN^- by approximately 50% at pH 3.0 as compared to pH 7.0 (*10*). Our results indicate that the presence of additional meal components moderate the influence of pH on the production of 4-hydroxybenzyl cyanide, thus preserving SCN^- formation. Application of *S. alba* seed meal to soil with the addition of sufficient water to promote glucosinolate hydrolysis is expected to produce an amount of SCN^- stoichiometrically equivalent to the amount of 4-hydroxybenzyl glucosinolate within the meal.

SCN^- production in soils amended with *S. alba* seed meal has significant consequences with respect to phytotoxicity and the use of meal as a bioherbicide. The herbicidal activity of SCN^- is well-known, and commercial formulations containing NH_4SCN have been marketed (*3*, *11*, *21*). Amendment rates necessary for weed control have been determined by a number of investigators for NH_4^+, K^+, and Na^+ salts with complete removal of all vegetative cover reportedly occurring for a period of 4 months when SCN^- is applied at rates of 270−680 kg/ha (*21*). Harvey (*22*) showed that higher rates of 1366 kg of SCN^-/ha were necessary for complete plant kill for 4 months, but a large percentage of the weeds were removed with only 137 kg of SCN^-/ha. Bissey and Butler (*23*) tested application rates that might alter wheat germination, finding that 342 kg of SCN^-/ha caused inhibition but that the effect was no longer observed at 69 d postapplication. Solutions of SCN^- sprayed directly on vegetative growth showed that cotton defoliation was possible using only 8.6 kg of SCN^-/ha (*21*).

Amounts of SCN^- contributed from *S. alba* seed meal used here, assuming complete stoichiometric conversion, would amount to 8.8, 17.7, and 35.3 kg of SCN^-/ha for amendment rates of 1000, 2000, and 4000 kg of meal/ha, respectively. Although glucosinolate concentrations in the *S. alba* meal used were not reported, Ascard and Jonasson (*6*) observed weed control effects with application rates of 1000−2000 kg/ha. We likewise have observed phytoxicity toward weed and crop species when meal was amended to greenhouse or field soils at rates from 1000 to 4000 kg of meal/ha (unpublished data), thereby providing the impetus for the work reported here. SCN^- rates provided in *S. alba* meal, although not as high as those used previously in phytotoxicity studies with soluble salts, provide SCN^- in amounts of potential value in weed control.

In addition to weed control benefits afforded by SCN^- produced as a result of glucosinolate hydrolysis, the meals contain between 5 and 6% N that when mineralized represents an important nutrient source to crop plants. Organic agriculture may thus benefit from the use of *S. alba* meal as a soil amendment both through weed control and as a nutrient source. Potential environmental effects appear minimal given that biological degradation of SCN^- has been observed in soils (*24*) and *S. alba* is typically grown as a condiment mustard for human consumption.

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