



Inhibition of acetylcholinesterase and glutathione S-transferase of the pinewood nematode (*Bursaphelenchus xylophilus*) by aliphatic compounds

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ABSTRACT

To determine the nematicidal mode of action of aliphatic compounds against the pinewood nematode (*Bursaphelenchus xylophilus*), we evaluated the inhibition activity of 63 aliphatic compounds on *B. xylophilus* acetylcholinesterases (BxACEs) and glutathione S-transferase. In the primary inhibition assay using *B. xylophilus* crude proteins, more than 65% of BxACE inhibition activity was observed for C₆, C₉, C₁₀, and C₁₂ 2E-alkenals. Other compounds showed moderate or weak inhibition activity. The inhibition activity against 3 recombinant BxACEs was subsequently evaluated using active compounds in a primary inhibition assay. C₁₂ 2E-alkenal showed the strongest inhibition activity against BxACE-1, followed by C₉, C₆, and C₁₀ 2E-alkenals. The IC₅₀ values of C₁₂, C₆, C₁₀, and C₉ 2E-alkenal against BxACE-2 were 0.0059, 0.57, 0.86, and 0.99 mg/ml, respectively. C₁₂ 2E-alkenal showed the strongest inhibition activity against BxACE-3 followed by C₆ 2E-alkenal. In an inhibition activity test using glutathione S-transferase from the pinewood nematode, C₁₀, C₉, and C₆ 2E-alkenals and C₁₂ alkanolic acid showed >45% inhibition activity.

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1. Introduction

Pine wilt disease caused by the pinewood nematode *Bursaphelenchus xylophilus* is one of the most serious tree diseases in Asia and Europe, including South Korea, Japan, China, Taiwan, and Portugal [1–3]. This disease was first found in Gumsung Mt., Busan City in 1988 [4], and has become a serious threat to Korea's pine forests [5]. The total damaged area along the Korean peninsula includes about 5123 ha, and the number of infected trees covered about 7644 ha in 2011 [6].

Several different methods have been introduced to control this disease. Examples include felling and fumigation of disease-infected trees using metham-sodium, aerial spraying of synthetic pesticides, application of thiacloprid to control the insect vector *Monochamus alternatus*, felling and crushing of trees to prevent the survival of the *M. alternatus* larvae, and injection of nematicides (such as abamectin and emamectin benzoate) into trunks [3,7,8]. The total budget for the control of pine wilt disease was about US\$ 29.2 million in 2012 [9]. However, conventional pesticides or nematicides have many side effects, such as environmental pollution and toxicity to non-target organisms. Therefore, it is necessary to identify naturally occurring toxicants from plants that can be used for disease control, which in turn avoids the side effects of synthetic pesticides and nematicides.

Short chain aldehydes and corresponding alcohols are major constituents of volatile organic chemicals produced by plants when wounded and in response to insect attack [10,11]. Further, the antimicrobial and insecticidal activity of short chains aliphatic compounds has been reported [12]. Furthermore, Seo et al. [13] reported the nematicidal activity of aliphatic compounds against the pinewood nematode. However, studies on the primary modes of action of phytochemicals with nematicidal activity against pinewood nematode have not been conducted. To understand the primary modes of actions of phytochemicals, it is important to efficiently screen the effective nematicidal agents. In this study, we estimated the inhibition activity of short chain aliphatic compounds against acetylcholinesterase (ACE) and glutathione S-transferase (GST) of *B. xylophilus* to learn the nematicidal mode of action of aliphatic compounds.

2. Materials and methods

2.1. Pinewood nematode

B. xylophilus specimens from infected pine trees in the Donghae area of Gangwon province, Korea were separated by Baermann method [14], and confirmed by real-time species specific PCR [15]. The fungus *Botrytis cinerea* was cultured on potato dextrose agar (PDA) for rearing pinewood nematodes, which was separated by the Baermann method. Separated *B. xylophilus* organisms were washed with M9 buffer (Wormbook, <http://www.wormbook.org>) to remove any surface bacterial or fungal contaminants.

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2.2. Chemicals

Authentic compounds used for bioassays were commercially obtained or synthesized as shown in Table 1. A detailed description of the synthesis of test compounds is shown in our previous study [13].

2.3. Extraction of crude protein

Crude proteins from pinewood nematode were extracted using a Bullet Blender (Next Advance, Averill Park, NY). *B. xylophilus* (ca. 300 μ l) specimens were transferred to a 1.5-ml tube containing 500 μ l of protein extraction buffer (0.1 M Tris-HCl buffer, containing 20 mM NaCl and 0.5% Triton X-100; pH 7.8) and the metal beads (half of the total volume) and vigorously shaken for 1 min. To avoid protein degradation by protease, a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added to the extract. The extract was centrifuged at 17,000 \times g for 15 min at 4 °C, and crude protein was separated from the cell debris. The concentration of crude protein isolated from pinewood nematodes was estimated with Bradford reagent method by using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Bovine serum albumin (BSA) was serially diluted in 0.1 M Tris-HCl buffer (pH 7.8), containing 20 mM NaCl and 0.5% Triton X-100, and was used as the standard protein for the quantification.

2.4. Inhibition assay against *B. xylophilus* crude protein extract

Aliphatic compounds were diluted in acetone to 100 mg/ml concentration. The protein solution (79 μ l), containing 30 μ g protein in 0.1 M Tris-HCl buffer (pH 7.8) mixed with 20 mM NaCl and 0.5% Triton X-100, was combined with 1 μ l of the chemical (final concentration of 1 mg/ml) and pre-incubated at room temperature for 10 min. The control reaction contained a solution of the protein and 1 μ l acetone without any chemical. The acetone concentration of all reactions was 1%. Then, 10 μ l of 10 mM acetylthiocholine iodide (ASChI, Sigma-Aldrich) as substrate and 10 μ l of

4 mM 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB, Sigma-Aldrich) as the colorimetric assay reagent dissolved in 0.1 M Tris-HCl (pH 7.8) containing 20 mM NaCl and 0.5% Triton X-100, were added to a pre-incubated blend of proteins and chemicals (with the final concentration of ASChI and DTNB being 1 mM and 0.4 mM). The residual activity of ACE, along with V_{max} (max velocity), was measured by following the reaction at 412 nm at 30 s intervals for 20 min at room temperature, by using the VersaMax microplate reader (Molecular Devices). The inhibition rate was calculated as a percentage with respect to the control by the following formula:

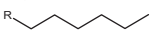
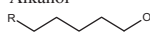
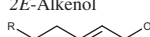
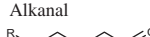
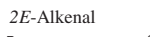

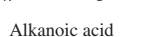
$$\% \text{ Inhibition} = 100 - (\text{Enzyme Activity of Treatment} / \text{Enzyme Activity of Control} \times 100)$$

All experiments were performed three times to determine the primary inhibition rate of ACE, which was then converted to the arcsine square root value for analysis of variance. The mean values of treatments were compared and analyzed using Scheffe's test [16].

2.5. Recombinant BxACEs expression

About 0.2 mg of pinewood nematodes was soaked in 200 μ l TRI reagent (MRC, Cincinnati, OH), and vigorously ground for 1 min using a Bullet Blender (Next Advance) and 0.5 mm metal beads. Total RNA layer was separated from genomic DNA or the protein layer by the BCP reagent (MRC, Cincinnati, OH), and precipitated using isopropanol (Sigma-Aldrich). The total RNA finally obtained was purified by rinsing with 70% ethanol. This purified total RNA was treated using DNaseI (Takara, Shiga, Japan) to remove all remaining genomic DNA, which had been used for the synthesis of single-stranded cDNA with the SuperScript first-strand cDNA synthesis system (Invitrogen, Carlsbad, CA). Three recombinant BxACEs (BxACE-1, -2, and -3) were expressed using the bEasyBac baculovirus expression system [17]. Each open reading frame (ORF) was amplified from the cDNA by PCR-amplification. All 3 ORFs included the signal peptide sequence and the His-tag sequence, but excluded the 3 cleavage site region to increase the expression efficiency. The amplified ORFs were cloned into the

Table 1
Aliphatic compounds tested in this study.^a

	Carbon length (Manufactory, purity)								
	C6	C7	C8	C9	C10	C11	C12	C13	C14
Hydrocarbon 	Merck 96%	Aldrich 99%	Aldrich 98%	Aldrich 97%	Wako 99%	Wako 99%	Wako 99%	Aldrich 99%	Wako 99%
Alkanol 	Wako 97%	TCI 98%	TCI 98%	Aldrich 98%	Aldrich 99%	TCI 98%	Aldrich 98%	Aldrich 97%	Aldrich 97%
2E-Alkenol 	Wako 95%	Synthetic 99%	Synthetic 99%	Synthetic 99%	Aldrich 97%	Synthetic 98%	Synthetic 97%	Synthetic 99%	Synthetic 95%
Alkanal 	TCI 98%	Wako 95%	Aldrich 99%	Aldrich 95%	Aldrich 99%	Aldrich 97%	Aldrich 92%	Aldrich 90%	Synthetic 92%
2E-Alkenal 	Aldrich 98%	Aldrich 94%	Aldrich 94%	Aldrich 97%	Synthetic 98%	Synthetic 98%	Synthetic 97%	Synthetic 99%	Synthetic 95%
Alkyl acetate 	TCI 98%	TCI 98%	Aldrich 99%	Synthetic 99%	Synthetic 99%	Synthetic 99%	Aldrich 97%	TCI 98%	Synthetic 99%
Alkanoic acid 	TCI 98%	TCI 96%	TCI 98%	TCI >90%	TCI 98%	TCI 98%	Aldrich 98%	TCI 98%	Aldrich 99%

^a Aliphatic compounds test in this study was well describe by Seo et al. [13].

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