Isolation of *p*-hydroxycinnamaldehyde as an antibacterial substance from the saw fly, *Acantholyda parki* S.

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Abstract We purified an antibacterial substance from larvae of the saw fly, *Acantholyda parki* S., and identified its molecular structure as *p*-hydroxycinnamaldehyde. We then synthesized it by reduction of *p*-hydroxycinnamic acid. The antibacterial activity of the synthetic *p*-hydroxycinnamaldehyde was equal to that of the authentic substance. This molecule was found to have a broad antibacterial spectrum against not only Gram-negative, but also Gram-positive bacteria. Furthermore, it showed antifungal activity against *Candida albicans*. We suggest that this substance may play a role in the defense system of this insect. This is the first report of *p*-hydroxycinnamaldehyde of animal origin.

Key words: Insect immunity; *p*-Hydroxycinnamaldehyde; Antibacterial substance; Antifungal activity; Insect defense molecule; *Acantholyda parki* S.

1. Introduction

Dipteran and lepidopteran insects are known to synthesize a battery of defense proteins in response to bacterial challenge or body injury [1–5]. These include hemagglutinins and antimicrobial proteins showing broad spectra of activity against bacteria and fungi. They are encoded by independent genes that are selectively activated when insects become infected [6,7]. The *Rel* family of transcription factors, including molecules such as NF-κB, may participate in the activation of these defense protein genes [8,9].

The use of novel bioactive substances from natural sources for drug development is a promising field of current research. Insects constitute the largest group of extant organisms, and perhaps their individual number account for as much as 80% of all known fauna. Therefore, they are suggested to be a useful source of bioactive substances.

As mentioned above, several antimicrobial proteins have been purified from insects. However, since the molecular masses of these compounds usually exceed 3000 Da, it is difficult to use them for human therapy. It is necessary to focus on low-molecular-weight compounds for drug development, since these are generally more stable and effective, although few have been reported from insects.

Recently, Leem et al. [10] reported a novel antibacterial substance named 5-*S*-GAD derived from an extract of adults of the flesh fly, *Sarcophaga peregrina*. This is a small molecule with a molecular weight of 573, and has been shown to act as an inhibitor of protein tyrosine kinase [11,12].

Acantholyda parki S., a serious hymenopteran pest of *Pinus koraiensis* [13], has been used as a folk medicine in Korea. Although there has been no report about an antibacterial substance from this insect, several antibacterial peptides have been reported from another hymenopteran species, *Apis mellifera* (the honeybee) [14–16]. Therefore, we tried to isolate an antibacterial compound, possibly the counterpart of 5-*S*-GAD, from *A. parki* larvae. Although we were unable to detect any 5-*S*-GAD-like substance, we succeeded in purifying to homogeneity an antibacterial substance.

2. Materials and methods

2.1. Insect

A. parki larvae were collected from the Kyungki district of Korea in February 1997.

2.2. Immunization and preparation of crude extracts of A. parki larvae

One thousand saw fly larvae were anesthetized by keeping them at 4°C, and then individually pricked with a hypodermic needle that had been dipped in a suspension of *Escherichia coli* K12 594 (Str^r). The insects were kept at 27°C for 24 h, and then collected after being anesthetized. The anesthetized larvae were homogenized in 10 volumes of 0.1% (v/v) trifluoroacetic acid containing 10 μg/ml aprotinin using an ULTRA-TURRAX T25 homogenizer (IKA-Labortechnik, Germany). The resulting homogenates were ultracentrifuged for 30 min at 35 000×*g*, and the supernatants were filtered through Millipore AA filters (pore size 0.22 μm). The filtrate was used as a crude extract for purification of antibacterial substances.

2.3. Purification of the antibacterial substance

The crude extract of saw fly larvae was loaded onto an ODS-AM 12S50 cartridge (YMC, Kyoto, Japan). After washing the cartridge with 0.05% trifluoroacetic acid, the absorbed material was eluted with 10% (v/v) acetonitrile containing 0.05% trifluoroacetic acid. Each fraction was concentrated under vacuum to remove the acetonitrile and trifluoroacetic acid, and the residue was dissolved in autoclaved distilled water for measurement of the antibacterial activity. Fractions showing antibacterial activity were pooled and subjected to reverse-phase HPLC using a C18 column (YMC-Pack ODS-A, S-5, 120 Å, 250×4.6 mm; YMC) equilibrated with 0.1% trifluoroacetic acid. After washing the column with 0.1% trifluoroacetic acid, the absorbed material was eluted using a linear gradient of 0–20% acetonitrile in 0.1% trifluoroacetic acid. Fractions showing antibacterial activity were pooled and subjected to normal-phase HPLC using a TSK gel Carbon-500 column (150×4.6 mm; Tosoh, Tokyo, Japan), and the adsorbed material was eluted using a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid. At this stage, one peak containing antibacterial activity was eluted from the column. In order to test the purity of fractions containing high antibacterial activity, they were pooled and again subjected to reverse-phase HPLC using the same type of C18 column. The adsorbed material was eluted using a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid. The antibacterial substance was eluted as a single symmetrical peak without impurity, as detected by UV absorbance at 220 nm. The amount of antibacterial substance was determined by the method of Lowry et al. [17], with 4-hydroxycinnamic acid as the standard. All of the HPLC procedures were carried out using a HP 1050 HPLC system (Hewlett-Packard, California, USA).

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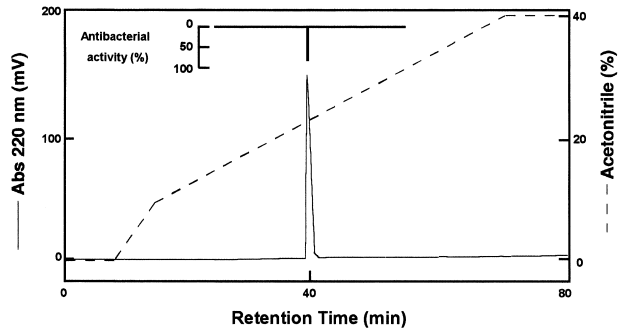

Fig. 1. Reverse-phase HPLC profile of antibacterial substance. The UV absorption at 220 nm was monitored (solid line). Fractions were concentrated and assayed for antibacterial activity (inset). Chromatographic conditions were: column, YMC-Pack ODS-A, S-5, 120 Å, C18 250×4.6 mm; solution A, 0.1% trifluoroacetic acid; solution B, 0.1% trifluoroacetic acid in 40% acetonitrile; linear gradient 25–100% solution B in solution A (dotted line); flow rate, 1 ml/min.

2.4. Assay of antimicrobial activity

Antibacterial activity was measured in liquid medium using *E. coli* K12 594 (Str^r) as the indicator bacterium. *E. coli* was grown in antibiotic medium (Difco, Michigan, USA). At the exponential phase of growth, cells were collected and suspended in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl and 0.2% (w/v) bovine serum albumin at a density of 2.5×10^8 cells/ml, and the suspension was then diluted 300-fold with antibiotic medium. The sample solution in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl and 0.05% (w/v) bovine serum albumin (10 μl) was mixed with 100 μl of the bacterial suspension in each well of a 96-well microtiter plate, and incubated at 37°C for 5 h. The absorbance at 650 nm was measured to assess bacterial growth using Emax Microplate Reader Precision (Molecular Devices Co., California, USA). As a positive control, cecropin B (Sigma, Missouri, USA) was used.

For the determination of the antimicrobial specificity, we used the various microbial strains. All of the bacterial strains were grown in antibiotic medium (Difco). *Candida albicans* KCTC 1940 was grown in Sabouraud medium (Difco).

2.5. Identification of p-hydroxycinnamaldehyde

The UV spectrum was analyzed using a Spectronic 3000 ARRAY spectrophotometer (Milton Roi, New York, USA). The IR spectrum was recorded using a DIP-140 spectrophotometer (Jasco, Tokyo, Japan). High-resolution mass spectra were measured using a JMS-SX 102A mass spectrometer (JEOL, Tokyo, Japan) in the electron impact (+) mode, using perfluorokerosene as the internal standard. All NMR spectra were recorded using a DMX 600 spectrometer (Bruker, Germany) equipped with a pulse-field gradient system.

2.6. Synthesis of p-hydroxycinnamaldehyde

The method used for synthesis of *p*-hydroxycinnamaldehyde was that reported by Duran et al. [18]. *p*-Hydroxycinnamic acid (2.79 mg) was added to dimethylchloromethyleneammonium chloride (4.27 mg) in 15 ml of anhydrous tetrahydrofuran and stirred at room temperature for 30 min. Then, 33.33 ml of 1 M $LiAl(OtBu)_3H$ was added to the reaction mixture with CuI (3.17 g) at −78°C. For quenching of the reduction, 4 ml of 6 N HCl was added to the reaction mixture, and then detection was done using TLC with authentic *p*-hydroxycinnamaldehyde as an indicator. The resulting reaction mixture was separated by reverse-phase HPLC using C18 column (YMC-Pack ODS-A, S-5, 120 Å, 250×4.6 mm; YMC). All chemicals were purchased from Sigma.

Table 1
Summary of ^{13}C and ^1H NMR spectral data for the antibacterial substance in D_2O

Position	δC	δH
Olefinic CH-2	126.1	6.69 (1H, dd, J=15.7 Hz)
Aromatic CH-3,5	117.6	6.96 (2H, d, J=8.6 Hz)
Aromatic CH-2,6	132.8	7.66 (2H, d, J=8.7 Hz)
Olefinic CH-1	158.2	7.74 (1H, d, J=15.6 Hz)
Aromatic C-4	161.1	–
-CHO	199.5	9.51 (1H, d, J=8.2 Hz)

The proton signal multiplicity and coupling constant (J=Hz) is given in parentheses.

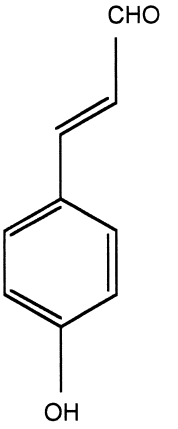

Fig. 2. Structure of *p*-hydroxycinnamaldehyde.

3. Results

3.1. Purification of an antibacterial substance from A. parki larvae

We tried to isolate the *A. parki* counterpart of 5-*S*-GAD, a low-molecular-weight antibacterial substance from *Sarcophaga*, from saw fly larvae using a method based on that described by Leem et al. [10]. One major and two minor fractions of antibacterial activity were eluted with 10% acetonitrile in 0.05% trifluoroacetic acid during of the ODS-AM 12S50 cartridge column chromatography. We focused on the major fraction, and tried to purify this substance further. Although we were unable to detect any *A. parki* counterpart of 5-*S*-GAD, we succeeded in purifying one substance with a yield of 20%, showing antibacterial activity, to homogeneity by repeated HPLC procedures (Fig. 1).

3.2. Molecular characterization of the antibacterial substance

The maximal absorption on the UV spectrum was observed at 233 and 325 nm. The IR absorption at 3120, 1645, 1600–1500, and 970 cm^{-1} was attributed to phenolic OH, conjugated carbonyl, the olefinic double bond and aromatic nucleus, and the *trans* form of the olefinic double bond, respectively. The molecular formula of this substance was determined to be $C_9H_8O_2$ on the basis of the electron impact (EI) high-resolution mass spectra (found: *m/z* 148.0521; calculated: *m/z* 148.0524), in combination with the NMR spectrum. By assignment of the ^1H NMR and ^{13}C NMR spectra, we determined the structure of this antibacterial substance to be 3-(4-hydroxyphenyl)-2-propenal (Fig. 2). All the NMR spectral data, summarized in Table 1, were completely identical to those reported previously. This substance is also called *p*-hydroxycinnamaldehyde, which has been purified from rhizomes of the plant *Alpinia galanga* [19], and also *Sarcophyta sanguinea* [20]. This is the first report of *p*-hydroxycinnamaldehyde isolated from an animal source.

Table 2
Antimicrobial activity of *p*-hydroxycinnamaldehyde

Microbe	IC_{50} (mM)
Aeromonas hydrophila KCTC 2358	0.15
Bacillus subtilis KCTC 3069	0.08
Enterococcus faecalis KCTC 1913	>0.43
Micrococcus luteus KCTC 1056	0.04
Mycobacterium smegmatis KCTC 2002	0.20
Staphylococcus aureus KCTC 1928	0.17
Enterobacter aerogenes KCTC 2190	>0.43
Citrobacter freundii KCTC 2006	0.19
Escherichia coli K12 594	0.05
Escherichia coli O157 H7 993	0.04
Klebsiella pneumoniae KCTC 2208	>0.43
Proteus vulgaris KCTC 2512	0.11
Salmonella enteritidis ACTC 13076	0.04
Shigella flexneri KCTC 2008	0.21
Candida albicans KCTC 1940	0.06

3.3. Synthesis of p-hydroxycinnamaldehyde

As it was difficult to purify *p*-hydroxycinnamaldehyde in quantity from saw fly larvae, we tried to synthesize it chemically. This was done by treating *p*-hydroxycinnamic acid with dimethylchloromethyleneammonium chloride and reduction of the resulting *p*-hydroxycinnamic dimethylchloromethyleneammonium salt with $LiAl(OtBu)_3H$ in the presence of CuI without protection of the hydroxyl group. The resulting reaction products were separated by reverse-phase HPLC, and the major product was identified as *p*-hydroxycinnamaldehyde with a yield of 64%. The synthetic *p*-hydroxycinnam aldehyde and the authentic substance coeluted as a single peak when a mixture of the two was subjected to HPLC. We also confirmed the structure of synthetic *p*-hydroxycinnamaldehyde on the basis of the ^{1}H-NMR and EI-MS spectra. We compared the antibacterial activities of synthetic and authentic *p*-hydroxycinnam aldehyde using *E. coli* as a target bacterium, and found that they were the same (Fig. 3).

3.4. Antimicrobial specificity of p-hydroxycinnamaldehyde

We found that this molecule was effective against both Gram-negative and Gram-positive bacteria, with similar IC_{50} values (0.04–0.08 mM) for *E. coli* K12 594, *Salmonella enteritidis*, *E. coli* O157 H7 933, *Bacillus subtilis* KCTC 3069, and *Micrococcus luteus* KCTC 1056 under the assay conditions we employed. Furthermore, the substance also exhibited antifungal activity against *Candida albicans* KCTC 1940 with an IC_{50} value of 0.06 mM. This is probably the first report of antimicrobial activities of *p*-hydroxycinnamaldehyde. The antimicrobial activity of *p*-hydroxycinnamaldehyde is summarized in Table 2.

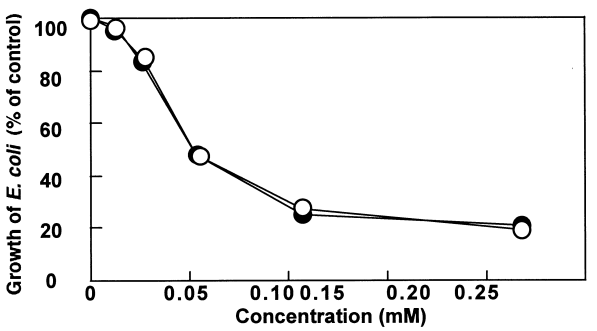

Fig. 3. Comparison of antibacterial activity between synthetic (○) and authentic *p*-hydroxycinnamaldehyde (●).

4. Discussion

p-Hydroxycinnamaldehyde has not been found previously in animals, including insects. However, ethyl *trans*-cinnamate has been found as a pheromone in the hairpencils of a number of lepidopteran species [21]. It is now known that insects are dependent on their various fruit and nut host species for the immediate precursors of the hairpencil herbal-scented compounds. However, we were unable to detect *p*-hydroxycinnamaldehyde in the leaf of *P. koraiensis*, which constitutes the diet of the saw fly, nor were we able to find any in the food content of their intestine. These insects generally winter below ground without feeding from the end of September. *p*-Hydroxycinnamaldehyde may be synthesized as a metabolite of some precursor from the leaf of *P. koraiensis*. Otherwise, it is possible that *p*-hydroxycinnamaldehyde may be synthesized from L-phenylalanine in this insect, as is the case in plants [22,23]. In plants, *p*-hydroxycinnamaldehyde is a precursor of lignin, which is an integral cell wall constituent and effective barrier against plant pathogens [24].

We suggest that this low-molecular-weight substance may play a role in the defense system of insects. Leem et al. have reported that 5-*S*-GAD would be rapidly synthesized upon infection with bacteria, and would suppress bacterial growth as a primary response, while the genes for various antibacterial proteins would subsequently be activated in response to H_2O_2 produced by 5-*S*-GAD [10].

p-Hydroxycinnamaldehyde was detected in four times larger amounts in immunized than in naive saw flies, suggesting that it is an inducible defense molecule produced in response to bacterial infection, integument injury, or both. Therefore we suggest that this compound may be related to the insect defense system, although many more experiments are needed to clarify this issue.

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