Isolation and identification of linoleic acid as an antimicrobial agent from the chalkbrood fungus, *Ascosphaera apis*

MF Feldlaufer 1, WR Lusby 1, DA Knox 2, H Shimanuki 2

1 Insect Neurobiology and Hormone Laboratory, Bldg 467 BARC-East, Beltsville, MD; 2 Bee Research Laboratory, Bldg 476 BARC-East, Beltsville MD 20705, USA

(Received 15 October 1992; accepted 4 December 1992)

**Summary** — Ethanol extracts of mixtures of mycelia and spores of *Ascosphaera apis*, the causative agent of chalkbrood disease in honey bee, was shown to contain an antimicrobial compound active against *Bacillus larvae*, the causative agent of American foulbrood disease. Purification by high performance liquid chromatography and analysis by mass spectrometry identified the active compound as 9,12-octadecadienoic acid (linoleic acid).

chalkbrood / American foulbrood / antimicrobial / linoleic acid / *Bacillus larvae*

**INTRODUCTION**

*Ascosphaera apis* is a heterothallic Ascomycete fungus that is the causative agent of chalkbrood disease in honey bee larvae. Dead larvae become overgrown with white mycelia and take on a characteristic chalk-like appearance (Gilliam and Vandenberg, 1990).

*A. apis* was first reported in the United States in solitary bees by Baker and Torchio (1968) and by the mid-1970s was distributed in honey bees across most of North America (Menapace and Wilson, 1976). During field studies in New Jersey, a decline in the number of honey bee colonies infected with the bacterium *Melissococcus pluton*, the causative agent of European foulbrood disease, was noticed to be coincident with an increase in chalkbrood disease (Herbert et al, 1987). We subsequently demonstrated that alcoholic extracts of *A. apis* inhibited the growth of laboratory-cultured *M. pluton*. In addition, the growth of *Bacillus larvae*, the bacterium that causes American foulbrood disease, was also inhibited when challenged with the fungal extract (Shimanuki et al, 1992). We now report the isolation and identification of 9,12-octadecadienoic acid (linoleic acid) as the predominant antimicrobial compound from *A. apis*. 
MATERIALS AND METHODS

Fungal material

A stock culture of a mated strain of *A. apis* (Maassen ex Claussen) Olive and Spiltoir, originally isolated from chalkbrood mummies received from Ohio in 1990, was maintained in the laboratory on potato dextrose agar (Difco) fortified with Bacto-yeast extract (4 mg/ml; Difco) at 30°C. Subcultures were made every 10–14 d. For this study, small blocks of agar from the stock culture containing both mycelia and spores were transferred to fortified potato dextrose broth plates (15 ml media per each 100 x 15 mm polystyrene Petri dish) and incubated at 30°C. Every 7–10 d mycelia and spores were removed from the broth with forceps, blotted on paper towels to remove excess broth, and stored refrigerated in ethanol (95%) until needed.

Bioassay

All extracts and fractions were tested for antimicrobial activity against *B. larvae*, the bacterial agent of American foulbrood disease. A stock spore suspension (≈ 2 x 10⁸ spores/ml) was prepared by mixing the dried remains of diseased honey bee larvae with sterile water in screw-top tubes. Before each use, the suspension was heat-shocked at 80°C for 10 min to kill any non-sporulating bacteria.

For the bioassay, 0.2 ml of the stock spores suspension was spread evenly over the surface of freshly prepared agar plates, which consisted of brain–heart infusion agar fortified with thiamine hydrochloride (0.1 mg/l; Difco) and adjusted to pH 6.6 with hydrochloric acid. Aliquots (usually 25 µl from 5 or 10 ml total volume) from all extracts and fractions were applied to paper assay discs (6.35 mm; No 740-E, Schleicher and Schuell) in a suitable solvent – either hexane, benzene or methanol. After drying, the discs were positioned in the middle of the agar plates and the plates were incubated at 34°C for 72 h. Zones of inhibition were clearly visible in active fractions, and the size of the zone was used to determine relative activity. Only the most active fractions were pursued.

Extraction and initial purification

Mycelia and spores of *A. apis* were extracted in 95% ethanol (3 x 300 ml) and filtered through a coarse fritted glass filter. The combined filtrates were dried in vacuo and the residue partitioned between 70% aqueous methanol and *n*-hexane (countersaturated; 200 ml each phase). Each phase was back-extracted with the appropriate solvent and bioassayed. The majority of the activity resided in the methanolic phase, so after drying, the methanolic residue was partitioned between *n*-butanol and water (countersaturated; 200 ml each phase), back-extracting each phase. All activity was found to be in the butanol phase.

Column chromatography

The butanolic residue was eluted from a column (10 mm id) containing C₁₈ packing material (5 g; Waters; Milford, MA, USA) with 50-ml portions of varying concentrations of aqueous methanol (40–100% in 10% increments). The column was stripped with an additional 50 ml ethanol (95%). Activity was found in the 70, 80 and 90% fractions, so these were combined, dried, and eluted from a silica Sep-Pak (Waters) in an ethanolic–chloroform solvent system. Since all activity was found in the first 2 fractions (5 and 15%), these were combined, dried and eluted from another silica Sep-Pak using a less polar solvent system consisting of 5-ml fractions of varying concentrations of chloroform in benzene (5, 10, 20, 40, 60, 80 and 100%). The majority of antimicrobial activity was found in the 60% fraction, which was dried and further purified by liquid chromatography.

Instrumentation

Reversed-phase high performance liquid chromatography (HPLC) was performed on an IBM octyl column (150 mm x 4.6 mm; 5-µm particle size) eluted with aqueous methanol containing 0.1% trifluoracetic acid (TFA) at 1 ml/min (see figure legends for specific details). Effluent was monitored at 220 and 248 nm with a Waters 990 photodiode array detector, and either 1-ml frac-
tions or specific peaks were collected for bioassay.

Mass spectra of active fractions were obtained on a Finnigan 4500 mass spectrometer with samples being admitted via either a direct exposure probe or a coupled gas chromatograph fitted with a DB-1 fused silica column (30 m x 0.32 mm; 0.25 µm film; J and W Scientific; Folsom, CA, USA) and operated isothermally at 170 °C. Electron ionization (EI) spectra (70 eV) were collected at a source temperature of 150 °C. Source conditions for chemical ionization (CI) spectra were 0.6 Torr for ammonia, [¹⁵N]-ammonia and deuterio-ammonia, and 0.3 Torr for methane. Source temperature for all gases was 60 °C.

Chemicals

All solvents for extraction and purification were reagent grade, redistilled. Solvents for HPLC were from Burdick and Jackson (Baxter Scientific; Columbia, MD, USA). Authentic linoleic acid and its methyl ester (methyl linoleate) were obtained from Nu-Chek Prep (Elysian, MN, USA).

RESULTS

Separation of the fungal extract by reversed-phase HPLC yielded 2 areas that were active in the bioassay (fig 1). The less polar of the 2 areas, designated y and eluting = 12 min, was the most active even though it had less mass (as determined by UV peak area). Rechromatography of area y in a less polar solvent system (fig 2) revealed that it consisted of a number of peaks, only one of which was active (elution time = 21.44 min). Initial mass spectrometric examination of this compound by probe ammonia CI provided ions at m/z 298 [base peak, (M+NH₄)⁺] and 315 [M+(NH₃)₂H]⁺ suggesting a molecular mass of 280 Da. Analyses with [¹⁵N]-ammonia and deuterio-ammonia confirmed the molecular weight and demonstrated the presence of one exchangeable hydrogen. After methylation of the compound with diazomethane, analysis by gas chromatography–mass spectrometry provided a retention time and EI spectrum identical to that of authentic methyl linoleate, thereby confirming compound y as linoleic acid. Examination of authentic linoleic acid by reversed-phase HPLC revealed the retention time to be identical with that of our unknown. When tested in our bioassay against B larvae, authentic linoleic acid was shown to be ac-
tive at concentrations down to 2.5 μg per disc.

The more polar of the 2 active areas (fig 1) was designated x and upon rechromatography was shown to contain many peaks. While only 1 peak had antimicrobial activity, it was not present in sufficient quantity to permit identification by mass spectrometry.

DISCUSSION

The antimicrobial activity of fatty acids and their derivatives, mainly soaps, has previously been demonstrated and reviewed (see Kabara, 1978; and references therein). While linoleic acid is an important membrane constituent of most ascomycete fungi (Weete, 1974, 1980), our isolation of linoleic acid from A apis as an antimicrobial agent is an interesting example of how one bee pathogen – the fungus that causes chalkbrood disease – produces a ubiquitous compound that inhibits the growth of a competing bacterial pathogen. A related fatty acid, termed laetisaric acid (8-hydroxylinoleic acid), has been isolated from a basidiomycete fungus that has been shown to be fungicidal toward several plant pathogenic fungi (Bowers et al, 1986). Currently in the United States, only oxytetracycline is approved by the Food and Drug Administration (FDA) for the control of both foulbrood diseases of honey bee (Shimanuki, 1990). The activity of linoleic acid against foulbrood bacteria may form the basis of a new, effective and inexpensive control.

ACKNOWLEDGMENTS

The technical expertise of KR Wilzer Jr and DJ Harrison is greatly appreciated. Discussions with MJ Thompson were extremely helpful.

Résumé — Isolement et identification de l'acide linoléique comme agent antibactérien à partir du champignon du couvain plâtré (Ascosphaera apis). On a montré que des extraits à l'éthanol de spores et de myceliums d'Ascosphaera apis, champignon responsable du couvain plâtré chez les larves d'abeilles (Apis mellifera L), renfermaient un agent antimicrobien qui inhibait la bactérie Bacillus larvae, agent de la loque américaine. Le champignon a d'abord été multiplié au laboratoire sur un bouillon de dextrose de pomme de terre (additionné d'un extrait de levure à 4 mg/ml) à 30 °C. Puis on a procédé à une série de fractionnements à l'aide de solvants (a) mélange d'une solution aqueuse de méthanol à 70% et de n-hexane et b) mélange de n-butanol et d'eau) et de chromatographies sur colonne (C18 suivi par du gel de silice) pour purifier les composés antimi-
La base d'une méthode de lutte sans danger, efficace et bon marché.

couvain plâtré / loque américaine / antimicrobien / acide linoléique / Bacillus larvae

Zusammenfassung — Isolierung und Bestimmung der Linolsäure als eine antimikrobielle Substanz von dem Kalkbrutpilz, Ascosphaera apis. Es konnte nachgewiesen werden, daß Extrakte aus Sporen und Mycelien von Ascosphaera apis, dem Pilz, der die Kalkbrutkrankheit der Honigbiene verursacht, eine antimikrobielle Substanz enthält, welche den Bacillus larvae, den Erreger der Amerikanischen Faulbrut, hemmt. Der Kalkbrutpilz wurde zunächst bei 30 °C im Laboratorium in einer Kartoffel-Dextrosebrühe (ergänzt mit Bäckerhefe-Extract zu 4 mg/ml) vermehrt. Dann führten wir eine Serie von Fraktionierungen durch Lösungsmittel durch, und zwar a) 70% wäbiriges Methanol gegen n-Hexan, und b) n-Butanol gegen Wasser, und schließlich Säulenchromatographien (C_{18} gefolgt von Silika-Gel), um die antimikrobielle(n) Komponente(n) zu reinigen. Alle Fraktionierungen und Säulenfraktionen wurden in einem Biotest geprüft, indem gleichgroße Teile (25 μl von 5 oder 10 ml) des Testmaterials auf Papierscheiben ausgebracht und in das Zentrum von Infusions-Agarplatten (mit Zusatz von 0,1 mg/l Thiaminhydrochlorid, eingestellt auf pH 6,6) gelegt wurden. Der Agar war mit einer Sporensuspension (etwa 4 x 10^7 Sporen) von B larvae geimpft.

Nach Inkubation der Platten für 72 h bei 34 °C waren Wachstumshemmzonen auf den Agarplatten zu beobachten, wenn deren Papierscheiben das aktive Antibiotikum enthielten. Die weitere Reinigung erfolgte durch Reversed-phase-Hochleistungs-Flüssigkeitschromatographie (HPLC)

Kalkbrut / Amerikanische Faulbrut / Antimikrobielle Wirkung / Linolsäure / Bacillus larvae

REFERENCES

Baker GM, Torchio PF (1968) New records of Ascosphaera apis from North America. Mycologia 60, 189-190


Kabara JJ (1978) Fatty acids and derivatives as antimicrobial agents – a review. In: The Pharmacological Effect of Lipids (Kabara JJ, ed) Am Oil Chem Soc, Champaign, IL


