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Original article

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

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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 10^8 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 C_{18} 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 ethanol–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, [^{15}N]-ammonia and deutero-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, $(M+NH_4)^+$] and 315 [$M+(NH_3)_2\ H]^+$ suggesting a molecular mass of 280 Da. Analyses with [^{15}N]-ammonia and deutero-ammonia confirmed the molecular weight and demonstrated the presence of

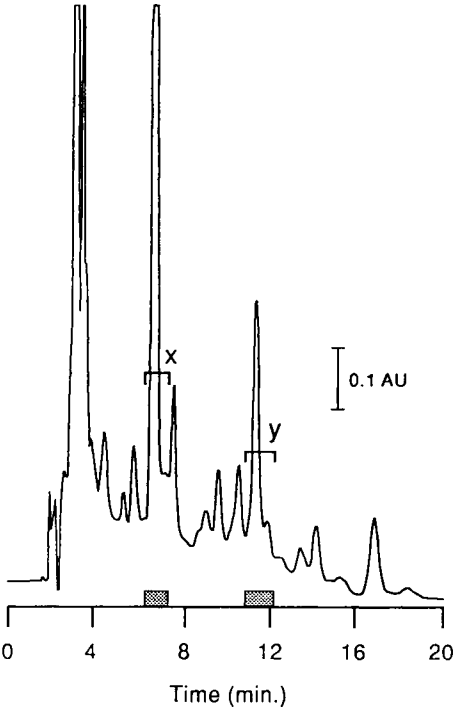

Fig 1. Reversed-phase high performance liquid chromatography trace (220 nm) of extract of *Ascosphaera apis*. Column: IBM octyl (150 x 4.6 mm; 5-μm particle size); solvent: 80% methanol/water containing 0.1% trifluoracetic acid, eluting at one ml/min. Column temp = 33°C. Hatched areas represent active antimicrobial fractions when bioassayed with *Bacillus larvae* and correspond to peaks **x** and **y**.

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-

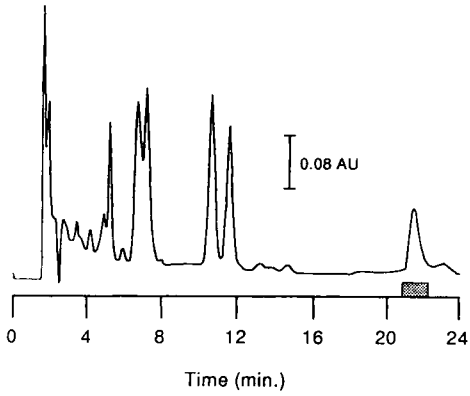

Fig 2. Reversed-phase high performance liquid chromatography trace (220 nm) of peak **y** (fig 1). Conditions as in figure 1, except the solvent was 75% aqueous methanol with 0.1% trofluoracetic acid. Hatched area (active compound) was subsequently analyzed by mass spectrometry.

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.

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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 était 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 (C_{18} suivi par du gel de silice) pour purifier les composés antimi-

crobiens. Toutes les fractions ont subi un test biologique, qui consistait à appliquer des quantités égales (25 µl de 5 ou 10 ml) du matériel à tester sur des disques de papier placés au centre de plaques de gélose à l'infusion de cœur-cerveau (additionnées de 0,1 mg/l d'hydrochlorure de thiamine et ajustées au pH 6,6), qui avaient été ensemencées avec une suspension de spores (environ 4 x 10^7 spores) de *B larvae*. Après incubation des plaques pendant 72 h à 34 °C, on pouvait observer des zones d'inhibition de croissance, là où les disques de papier contenaient l'antibiotique actif. Une purification plus poussée a été obtenue par analyse en chromatographie liquide haute pression en phase inverse (HPLC) sur une colonne C_8 éluée avec une solution aqueuse de méthanol à 80%, renfermant 0,1% d'acide trifluoracétique au débit d'un ml/min. Elle a été contrôlée par détection par absorption UV à 220 et 248 nm (fig 1). Celle-ci a montré 2 zones actives. La moins polaire des 2, «y», a été repurifiée avec une solution aqueuse de méthanol à 75% (fig 2). Le composé actif, dont le temps de rétention était de 21,44 min, a été examiné en spectrométrie de masse. En utilisant plusieurs gaz en ionisation chimique on a observé, par analysé en spectrométrie de masse, une masse moléculaire de 280 Da pour le composé. La méthylation du composé avec du diazométhane, suivie par l'analyse par chromatographie phase gazeuse-spectrométrie de masse, a montré que l'ester méthylique était le linoléate de méthyle; le composé non dérivé est donc l'acide 9,12 octadécadiénoïque (acide linoléique). Testé à l'aide de notre test biologique, l'acide linoléique était actif à la concentration de 2,5 µg par disque. L'identité du composé marqué «x» sur la figure 1 n'a pas pu être déterminée en raison de la faible quantité présente. La preuve apportée ici que l'acide linoléique est capable d'inhiber la bactérie qui provoque la loque américaine pourrait constituer 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 Myzelien 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% wäßriges Methanol gegen n-Hexan, und b) n-Butanol gegen Wasser, und schleßlich 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 107 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)

mit einer C8 –Säule, eluiert mit 80% wäßrigen Methanol mit einem Gehalt von 0,1% Trifluoressigsäure (1 ml/min) und überwacht in UV-Absorption bei 220 und 248 nm (Abb 1). Dies ergab zwei aktive Bereichte; der weniger polare von beiden, «y», wurde nochmals mit wäßrigem Methanol gereinigt (Abb 2). Die aktive Komponente, gewonnen bei einer Elutionszeit von 21,44 min, wurde mittels Massenspektrometrie untersucht. Bei Verwendung mehrerer Reagens-Gase ergab die «Direct exposure probe»-Massenspektrometrie eine Molekularmasse der Komponente von 280 Daltons. Eine Methylierung der Substanz mit Diazomethan gefolgt von Gaschromatographie-Massenspektrometrie zeigte, daß es sich bei dem Methylester um Methyllinoleat handelte und daß daher der gesucht Komponent 9,12-Oktadekadiensäure (= Linolsäure) war. Geprüft in unserem Biotest, erwies sich Linolsäure bei einer Konzentration von 2,5 mg per Scheibe als wirksam. Die in Abb 1 mit «x» bezeichnete Substanz konnte wegen der geringen Substanzmenge nicht bestimmt werden. Unser Nachweis, daß Linolsäure das Bakterium hemmen kann, welches die Amerikanische Faulbrut versursacht, könnte die Grundlage für eine sichere, wirksame und billige Kontrolle dieser Krankheit bilden.

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

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