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Novel assay of antibacterial components in manuka honey using lucigenin-chemiluminescence-HPLC

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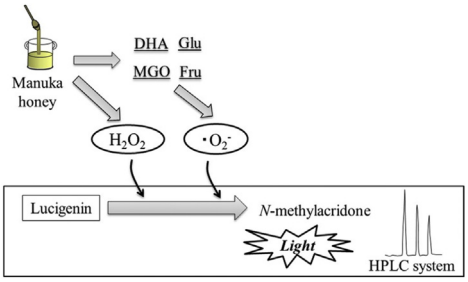
HIGHLIGHTS

- Antibacterial components in manuka honey by HPLC with lucigenin-CL.
- Five antibacterial compounds measured via generation of reactive oxygen species.
- Simple, sensitive and useful for quality control and analysis of antibacterial honey.

GRAPHICAL ABSTRACT



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ABSTRACT

Five components (hydrogen peroxide, methylglyoxal, dihydroxyacetone, fructose and glucose) of New Zealand manuka honey (*Leptospermum scoparium*) were analyzed using lucigenin chemiluminescence high-performance liquid chromatography (lucigenin-CL-HPLC). We focused on active oxygen species produced from the components in order to easily detect these five components contained in manuka honey. H_2O_2 and O_2^- generated from these components were identified by lucigenin-CL and electron spin resonance (ESR), and the bactericidal effect of ROS was confirmed using *E. coli*. The previously reported assays for Manuka honey components have low specificities and require complicated preprocessing methods. As our results, the detection and identification of these components were possible within 30 min in lucigenin-CL-HPLC system, without any special treatment. It is considered that lucigenin-CL-HPLC is useful for the quality control and the analysis of various honey.

1. Introduction

Manuka honey is the major medical grade honey currently approved for clinical application [1–3]. This substance is produced from the manuka bush (*Leptospermum scoparium*), a plant indigenous to New Zealand and Australia. The honey used as a source for medical-grade manuka honey is collected from its natural environment. To date, the factors identified as contributing to the antibacterial activity of honeys are the high sugar concentration, hydrogen peroxide (H_2O_2), methylglyoxal (MGO), dihydroxyacetone (DHA), antibacterial peptides, phenolic compounds, and low pH [4]. Manuka honey has been made all over the world for use in elucidating the bactericidal effect of honey [5–7]. However, the exact mechanism of manuka honey action that leads to bacterial cell death is unknown. The antibacterial activity of manuka honey is often expressed as an industry standard phenol-equivalent scale, the so-called unique manuka factor (UMF). This factor represents the concentration of a phenol solution yielding a similar zone of

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growth inhibition as the honey when tested in a radial diffusion assay with *Staphylococcus aureus* as the target microorganism [8]. Although the UMF designation suggests that the indicated level of activity is due to a manuka-specific compound, the UMF assay only measures the level of antibacterial activity; this scale is not informative regarding the identity of the components involved. The antibacterial activity of manuka honey has been demonstrated against multiple target species, including *Bacillus subtilis*, *Escherichia coli*, extended-spectrum beta-lactamase-producing organisms (ESBL), *S. aureus* (including methicillin resistant *S. aureus*, MRSA), *Enterococcus faecium* (including vancomycin-resistant enterococci, VRE), *Pseudomonas aeruginosa*, *Helicobacter pylori*, and others [9–11]. UMF has been reported to correlate with the levels of various manuka honey components such as MGO and leptosin [12–16]. The high levels of MGO in manuka honey are formed by conversion of DHA, which is present at exceptionally high concentrations in the nectar of *L. scoparium* flowers. This conversion occurs non-enzymatically at a slow rate during storage of the honey [4]. Based on a strong correlation between the MGO levels and the ability of honey to inhibit the growth of *S. aureus*, it has been suggested that MGO is fully responsible for the non-peroxide antibacterial activity of manuka honey. However, other work has demonstrated that the non-peroxide antibacterial activity of manuka honey is not solely attributable to MGO [3]. Thus, other non-MGO components appear to contribute to the antibacterial activity of manuka honey.

These results suggest that a simple analytical method with high sensitivity is required to detect the antibacterial components of manuka honey. Several analytical methods for the components of manuka honey and monofloral honeys have been reported to date [17–23]. However, techniques for detecting MGO face issues such as low specificities and complicated preprocessing methods. Simple analytical methods for the simultaneous detection of antibacterial components like MGO, DHA, fructose (Fru), glucose (Glu), and H_2O_2, have not been reported. We have focused on reactive oxygen species (ROS) produced from these five components to permit easy detection of these compounds in manuka honey. We report here that superoxide (O_2^-) are generated from four of these five components (with the exception of H_2O_2), and have used the resulting values to estimate the antibacterial content. We subsequently analyze the levels of all five components using a lucigenin chemiluminescence method (lucigenin-CL) for detection of ROS. Lucigenin-CL is used primarily for the measurement of ROS such as O_2^- [24]. Previously, we used lucigenin-CL for a high-sensitivity measurement of alkaline phosphatase as the substrate for reducing sugars and dihydroxyacetone phosphate (DHAP) [25]. The technique takes advantage of the reaction mechanism of lucigenin-CL, such that O_2^- activates a reducing substance, in turn leading to reduction of lucigenin, which then emits light. Lucigenin also permits the detection of hydrogen peroxide by a similar mechanism [26]. Thus, lucigenin-CL is expected to be of use in the analysis of the ROS-generating antibacterial components contained in manuka honey.

In this study, in order to obtain a convenient and highly sensitive analytical method for five components of manuka honey, we developed a novel analytical method using the lucigenin chemiluminescence high-performance liquid chromatography (lucigenin-CL-HPLC), which was based on a ROS detection system. In addition, we studied the antibacterial activity against *E. coli* of the MGO and DHA contained in samples of the same manuka honey. By correlating MGO and DHA antibacterial activity with detection of O_2^-, we verified the validity of lucigenin-CL-HPLC as a specific analytical method for detecting components with antibacterial activity.

2. Materials and methods

2.1. Reagents

Bis-*N*-methylacridinium nitrate (lucigenin), bis-(2,4,6-trichloro phenyl) oxalate (TCPO), and 2-bromo-4'-nitroacetophenone were obtained from Tokyo Chemical Industry Co., Ltd. MGO and microperoxidase were obtained from Sigma-Aldrich Co. LLC. DHA, hydrogen peroxide, Glu, Fru, acetonitrile, and superoxide dismutase (SOD) were obtained from Wako Pure Chemical Industries, Ltd. Catalase was obtained from Nacalai Tesque, Inc. All other reagents were of analytical grade.

2.2. Honey samples

Manuka honeys were kindly provided by Mr. Iwasaki et al., Medical Incubation System Co., and Tokyo, Japan. Manuka honeys in this study were used 100% New Zealand UMF 10+ (#1) and 100% New Zealand UMF 15+ (#2) that had been stored for less than a year at the time of testing, and UMF 10+ (#3) and UMF 15+ (#4) that had been stored for 3 years. Separate preparations of manuka honey UMF 10+ (#5), UMF 15+ (#6), MGO 100+ (#7) and MGO150+ (#8) were obtained from 100% Pule New Zealand Ltd and Manuka Health New Zealand Ltd. these honeys had been stored for 3 years at the time of testing. Following arrival in our laboratory, these manuka honeys were stored at room temperature. For each honey, a sample (0.1–0.5 g) of the substance was dissolved in analytical grade water (1 mL) and filtered through a hydrophilic membrane with pore size of 0.45-μm (Kurabo Industries Ltd, Osaka, Japan). Manuka honey #4 was used for all assays. The other manuka honeys were used for only the lucigenin-CL-HPLC.

2.3. Enzyme reactions

Each solution of catalase and SOD was formulated by dissolving the respective enzyme (100–10,000 units/assay) in analytical grade water. A honey sample was combined with an enzyme solution and the mixture was incubated for 30 min at 37 °C.

2.4. TCPO/ANS-CL assay for H_2O_2

The ANS solution (pH 9.0) was formulated as 0.02% 8-anilinonaphthalene-1-sulfonic acid (ANS), 0.1% bovine serum albumin (BSA), and 0.2 mol/L barbital. Sample solution (10 μL) was mixed with 100 μL of ANS solution and 100 μL of 0.05 mmol/L TCPO in ethyl acetate [27,28]. The combination was mixed and allowed to equilibrate for 10 s, and the intensity of luminescence was measured over a 10-s interval using a BLR-301 luminescence reader (Aloka; Tokyo, Japan).

2.5. Lucigenin-CL assay for O_2^- and reducible components

The lucigenin solution was formulated as 2.4×10^{-5} mol/L lucigenin, 0.16 mol/L KOH, and 0.02% Triton X-100. Sample solution (10 μL) was mixed with 100 μL of lucigenin solution [24,29]. The combination was mixed and allowed to equilibrate for 10 s, and the intensity of luminescence was measured over a 10-s interval using a BLR-301 luminescence reader (Aloka).

2.6. Detection of O_2^- and reducible components using electron spin resonance (ESR) spectrum

The lucigenin solution was used here as the spin trapping solution. An aliquot (200 μL) of spin trapping solution was combined with 5 μL of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and then