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Immunochemical Authentication of Manuka Honey Using a Monoclonal Antibody Specific to a Glycoside of Methyl Syringate

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ABSTRACT: Leptosperin, a novel glycoside of methyl syringate, is exclusively present in manuka honey derived from the *Leptospermum* species *Leptospermum scoparium*. Quantification of leptosperin might thus be applicable for authentication of honey. The concentration of leptosperin has high linearity with antibacterial activity. We established a monoclonal antibody to leptosperin and characterized the antibody in detail by a competitive enzyme-linked immunosorbent assay (ELISA), comparing the results with those of the high-performance liquid chromatography (HPLC) method for validation. The antigen in manuka honey was confirmed as leptosperin by HPLC fractionation with quantitation by an ELISA. Leptosperin contents of 50 honey samples were analyzed by an established ELISA, which can handle 20 samples (duplicate) on one 96-well plate. Significant coincidence with the chemical quantitation was observed. Immunochemical quantitation of leptosperin would be an economical and facile method for the possible authentication of manuka honey, allowing many honey samples to be processed and analyzed by an ELISA simultaneously.

KEYWORDS: *manuka honey, chemical marker, authentication, leptosperin, monoclonal antibody*

INTRODUCTION

Honey has been used for medicinal purposes since ancient times. Among all the varieties of honey, manuka honey is known to exhibit strong nonperoxide-dependent antibacterial activity. Because of its possible medical uses, manuka honey is consumed with bread and tea and used to manufacture candy. In general, most honey is derived from the nectar of multiple sources, particularly flowers. This has given rise to varieties of honey known as even "monofloral" honey.

Manuka honey is consumed as one variety of premium honey. To maintain quality and protect consumers, authentication processes have been introduced. There are two main authentication processes. One is the "real" antibacterial activity against *Staphylococcus aureus*, and the other is the quantitation of methylglyoxal, which is the major antibacterial chemical in manuka honey. The former is further classified as nonperoxide- or peroxide-dependent antibacterial activity. These authentications provide information about the antibacterial activity directly or indirectly at the moment of testing, but unfortunately, the level of the main bactericide, methylglyoxal, is not constant.[1] The level of reactive methylglyoxal may decrease via reactions with other components. On the other hand, the precursor, dihydroxyacetone, in the honey may continually supply "fresh" methylglyoxal during incubation and/or storage.[1] This indicates that the activity/level of methylglyoxal may be very different when the consumer actually purchases the honey.

Leptosperin (leptosin), a glycoside of methyl syringate (methyl 3,5-dimethoxy-4-hydroxybenzoate), is a unique compound present in manuka honey.[2] This novel glycoside has a gentiobiose linked with the 4-hydroxy moiety of methyl syringate via a β-glycoside bond. The name, leptosperin, has been used instead of leptosin to avoid confusion with other leptosin(s), isolated from the marine fungus *Lestoshaeria* species.[3,4]

Previous studies have shown that leptosperin is a plausible authentication marker for manuka honey because the concentration of leptosperin in the honey has a good linearity with nonperoxide-dependent antibacterial activity.[2,5] The level of leptosperin in honey remains stable at temperatures between 37 and 50 °C for a month. These characteristics suggest that measurement or classification of leptosperin would be an appropriate indicator for authentication of manuka honey. In past studies, leptosperin has been detected and quantified by high-performance liquid chromatography−photodiode array (HPLC−PDA) or ultraviolet light (HPLC−UV) detection and HPLC-tandem mass spectrometry (LC−MS/MS). These chemical methods provide accurate data. In particular, mass spectrometry improves sensitivity and selectivity. On the other hand, a large number of samples cannot be examined simultaneously in contrast with an enzyme-linked immunosorbent assay (ELISA). Moreover, the cost of investment for

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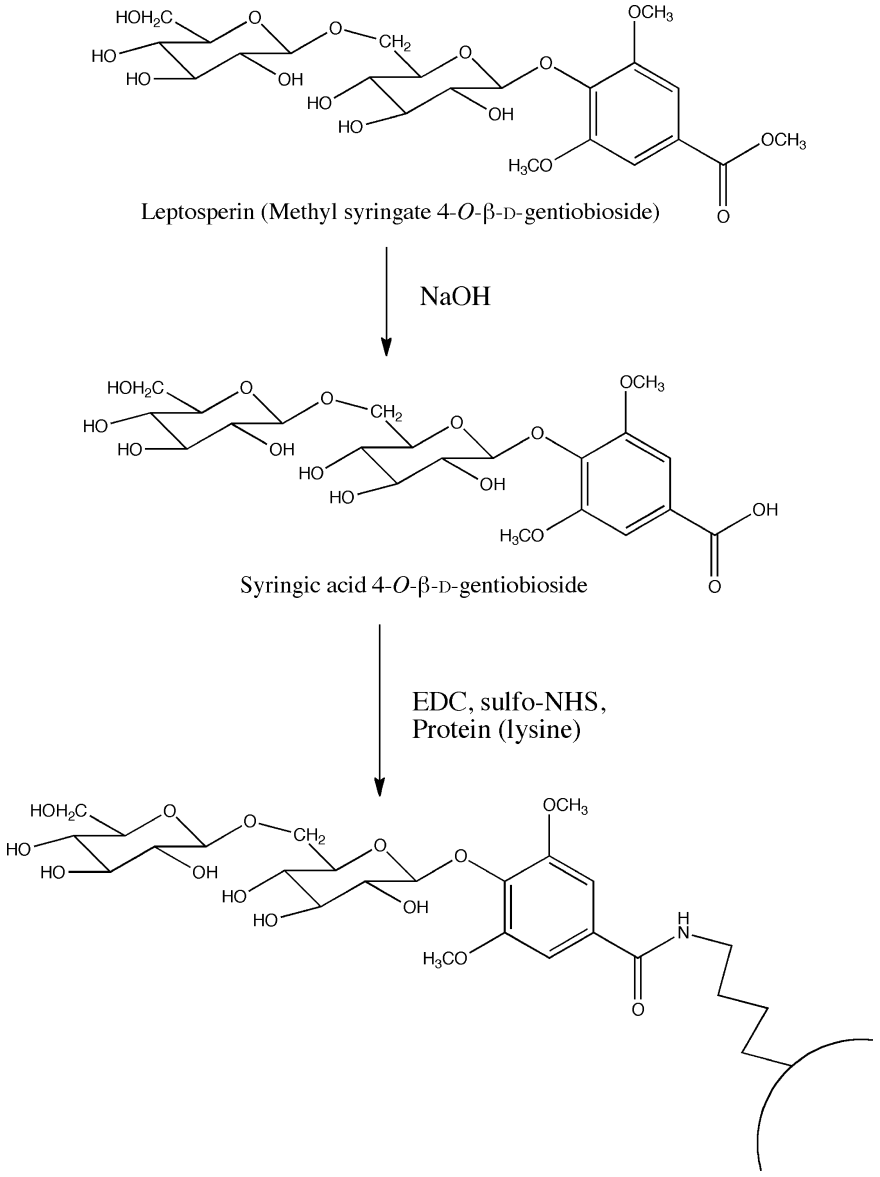

Figure 1. Scheme for the preparation of the immunogen to leptosperin. The methyl ester of leptosperin was hydrolyzed with a 1 M NaOH solution, and the successive syringic acid 4-*O*-*β*-D-gentiobioside was used as a hapten and conjugated with carrier protein, keyhole limpet hemocyanin (KLH), using carbodiimide through the carboxyl group of the hapten. Mice were immunized with the conjugated KLH, and monoclonal antibodies were then established.

HPLC or LC−MS/MS is much higher relative to that for an ELISA.

Recent studies have demonstrated the preparation and application of an antibody for the detection of phytochemicals or their derivatives.[6−10] Immunochemical approaches, in particular an ELISA, may allow the handling of multiple samples. The cost of an ELISA is much lower than that of HPLC or LC−MS/MS. For example, to quantify an antigen by an ELISA, only a microplate reader is needed.

In this paper, on the basis of the specific monoclonal antibody against leptosperin, we established an ELISA to quantify leptosperin levels in honey for the first time.

EXPERIMENTAL PROCEDURES

Materials and Methods. Methyl syringate was purchased from Alfa Aesar, Johnson Matthey Co. (Ward Hill, MA). Maltose, sucrose, and glucose were obtained from Nacarai Tesque Inc. (Kyoto, Japan). 2-Methoxybenzoic acid and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), 5-hydroxymethyl-2-furaldehyde (HMF), and gentiobiose were purchased from TCI Co., Ltd. (Tokyo, Japan). 4-Hydroxyphenylacetic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sulfo-*N*-hydroxysuccinimide (sulfo-NHS), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and keyhole limpet hemocyanin (KLH) were purchased from Pierce (Thermo Fisher Scientific K. K., Yokohama, Japan). Leptosperin was isolated from manuka honey as described previously.[2] Twenty certified honey samples were obtained from the Unique Manuka Factor Honey Association (UMFHA). Other kinds of honey samples were obtained from retail stores in Japan, China, Brazil, and New Zealand between 2011 and 2014.

Preparation of the Immunogen. Leptosperin was treated with a 1 M NaOH aqueous solution for 30 min at room temperature. After neutralization by the addition of Dowex 50W, the solution was centrifuged, and the modified leptosperin (syringic acid 4-*O*-*β*-D-gentiobioside, in the supernatant) was then purified by reversed-phase HPLC using a Combi-RP column (20 mm × 100 mm, Nomura Chemical Co. Ltd., Aichi, Japan) with a 0.1% acetic acid/CH_3CN

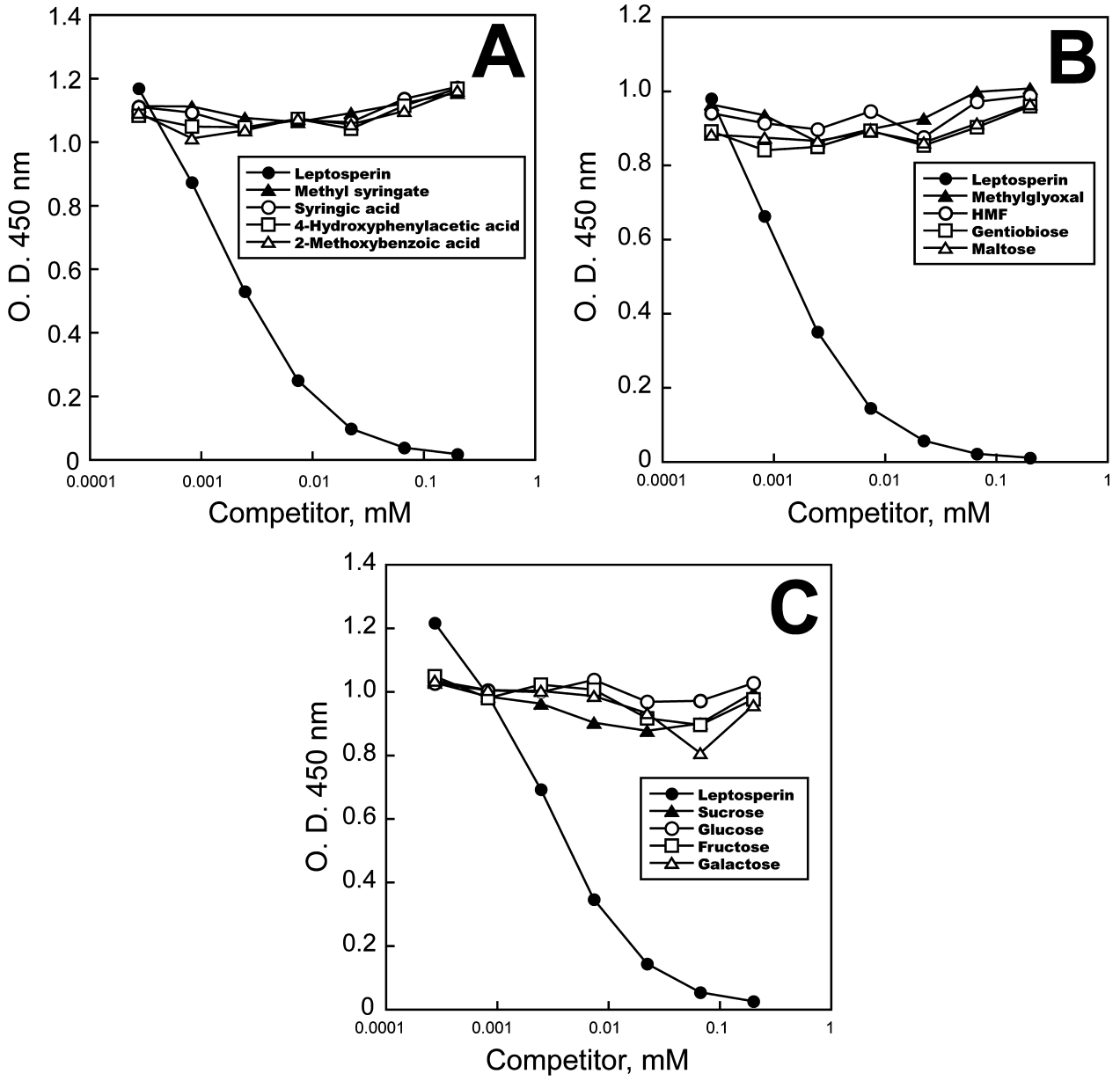

Figure 2. Characterization of monoclonal antibody 2H3 to leptosperin. The competitor and the antibody 2H3 were added to a well, which had been precoated with leptosperin-conjugated BSA. The antibody binding to the immobilized leptosperin-BSA was evaluated with a second antibody–peroxidase conjugate treatment. Each point represents the mean of duplicate determinations. (A) Cross-reactivity against phenolics: (●) leptosperin, (▲) methyl syringate, (○) syringic acid, (□) 4-hydroxyphenylacetic acid, and (△) 2-methoxybenzoic acid. (B) Cross-reactivity against aldehydes or disaccharides: (●) leptosperin, (▲) methylglyoxal, (○) HMF (5-hydroxymethyl-2-furaldehyde), (□) gentiobiose, and (△) maltose. (C) Cross-reactivity against saccharides: (●) leptosperin, (▲) sucrose, (○) glucose, (□) fructose, and (△) galactose.

mixture (85/15) at a flow rate of 5 mL/min. The modified leptosperin was then conjugated with KLH for an immunogen or BSA for a positive control antigen using EDC and sulfo-NHS. Briefly, 21 mg of modified leptosperin, 7.7 mg of EDC, and 8.7 mg of sulfo-NHS were dissolved in 700 μL of dimethylformamide, and then the mixture was incubated for 24 h at room temperature. Then, 350 μL of the reaction mixture reacted with KLH [8.5 mg/1.6 mL of 0.1 M phosphate buffer (pH 7.4)] for 4 h and then dialyzed against phosphate-buffered saline (PBS) for 3 days at 4 °C. The obtained leptosperin–KLH conjugate was stored at −80 °C until it was used. For the positive control antigen, BSA [13 mg/1.6 mL of 0.1 M phosphate buffer (pH 7.4)], instead of KLH, was mixed with 350 μL of reaction mixture and then dialyzed against PBS. The conjugation of leptosperin to BSA was confirmed by the loss of free amines by the reaction with 2,4,6-trinitrobenzenesulfonic acid (Thermo Scientific) according to the manufacturer's instructions and also an increase in absorbance at 262 nm, which was derived from absorption by the leptosperin moiety. The leptosperin–BSA antigen was used as the antigen for screening, cloning, and characterization by an ELISA.

Preparation of the Monoclonal Antibody. The monoclonal antibodies were prepared as described previously.[11] Three clones, 2H3, 2B5, and 1C10, which are all of the IgG1 class, were finally obtained. By simple characterization using culture media, the three antibodies showed strong reactivity against leptosperin and did not cross-react with gentiobiose, glucose, or methyl syringate. The 2H3 antibody from the clone was the most sensitive to leptosperin compared to the other two antibodies and then selected. The clone was cultivated, and the secreted antibody was purified from the cultured medium by a protein G column (HiTrap, Protein G HP, 1 mL, GE Healthcare) according to the manufacturer's instructions and then used for experiments.

Characterization of the Antibody by an ELISA. The leptosperin–BSA conjugate (5 μg/mL, 50 μL in PBS) was coated

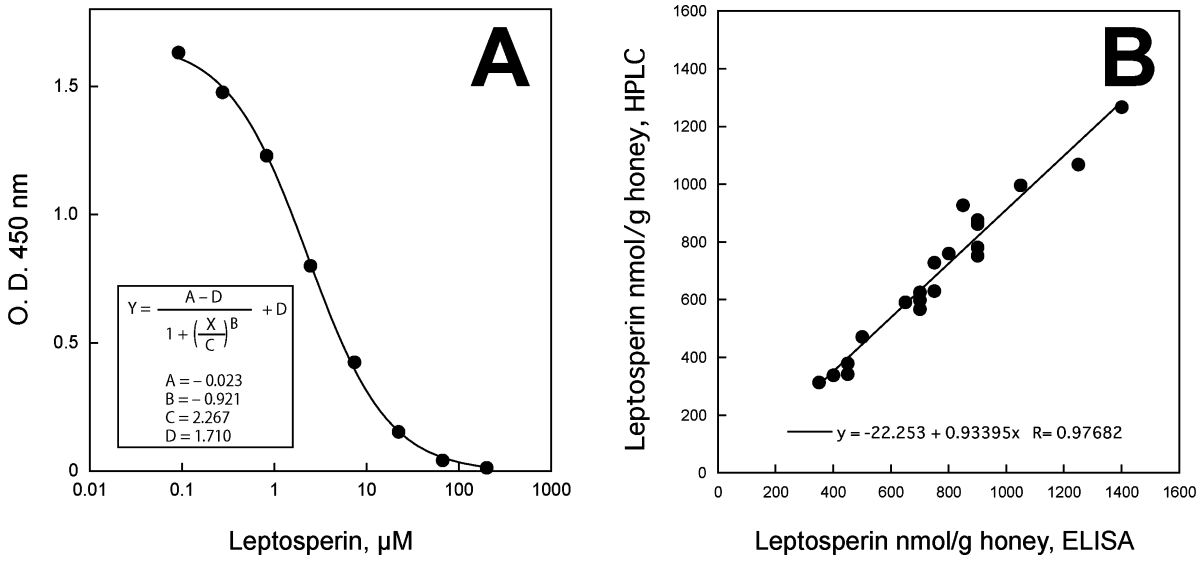

Figure 3. (A) Standard curve for an ELISA and (B) correlation between HPLC and ELISA. (A) A typical standard curve is shown with the equation (a four-parameter curve fitting). (B) The contents of leptosperin in the 20 certified manuka honey samples estimated by an ELISA significantly correlate with those determined by HPLC.[5] The ELISA for honey samples was performed four times, and the average was used.

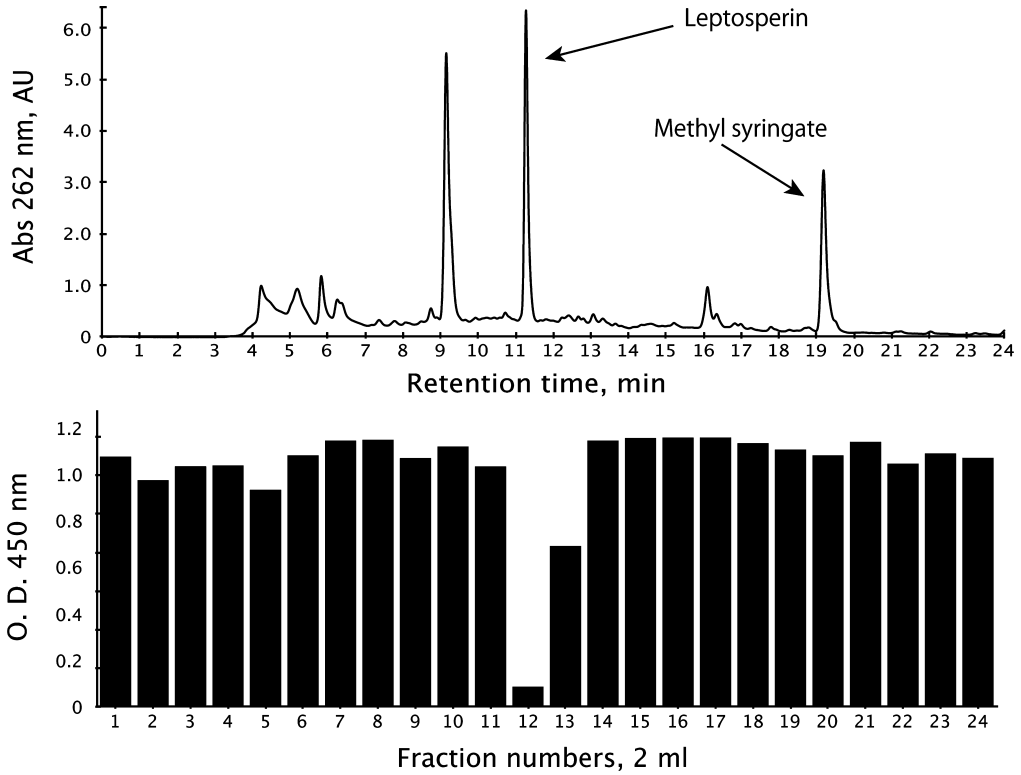

Figure 4. Fractionation and identification of antigen in manuka honey. An aqueous solution of manuka honey was injected into a semipreparative HPLC instrument, and the eluate was fractionated every minute (each 2 mL). The top panel shows the separation chromatogram with monitoring at an absorbance of 262 nm. The elution positions of leptosperin and methyl syringate are shown with arrows. The bottom panel indicates the immunoreactivity of fractionated samples estimated by a competitive ELISA.

on microtiter plate wells and kept overnight at 4 °C. The wells were washed with PBS containing 0.05% Tween 20 (PBST) and then blocked with 200 μL of 1% Block Ace (Dainihon Sumitomo Seiyaku, Osaka, Japan) for 1 h at 37 °C. Competitor (0–0.1 mM) or authentic leptosperin was mixed with the 2H3 antibody (0.02 μg/mL) in a well and further incubated for 2 h at 37 °C. The plate was washed and treated with 1/5000 diluted anti-mouse immunoglobulins labeled with peroxidase (DAKO) in PBST for 1 h at 37 °C. Color development was performed using 100 μL of 3,3′,5,5′-tetramethylbenzidine reagent (Popular, Nacalai Tesque) and terminated by the addition of 100 μL of 1 M aqueous phosphoric acid.

Quantitation of Leptosperin in Honey Samples. For an ELISA, manuka honey was dissolved in water at a concentration of 0.02 g/mL of water. Nonmanuka honey was dissolved at a concentration of 0.1 g/mL. The samples were centrifuged, and supernatants were then used. Authentic leptosperin (0–0.1 mM) and the honey samples (duplicate) were analyzed by an indirect competitive ELISA as described above. The concentration of

leptosperin was calculated by MPM-III software (version 6.0, Bio-Rad) with a four-parameter curve fitting. For analysis of 20 manuka honeys, an ELISA was performed four times and the averages were used. Commercial honeys were analyzed twice, and the averages were used. For chemical measurement of leptosperin, HPLC−PDA was used as described previously.[5]

Fractionation of Honey by Semipreparative HPLC. Manuka honey (UMF10+) was dissolved in water (1 g/mL of aqueous 10% CH_3CN) and then centrifuged. The supernatant (100 μL) of the sample was injected into a semipreparative HPLC instrument. The separation was achieved using a Develosil ODS-HG-5 column (8 mm × 250 mm, Nomura Chemical Co. Ltd.) and a 0.1% formic acid (A)/CH_3CN (B) gradient system at a flow rate of 2 mL/min. The program was as follows: 90% A at 0 min, 70% A at 25 min, 90% A at 30 min, and 90% A at 55 min. The eluate was fractionated every minute (each 2 mL) in a test tube and dried with a centrifugal evaporator. The samples were dissolved in PBS, and 50 μL of the sample (duplicate) was then used for a competitive ELISA as described above.

Statistics. The relationship between two groups was calculated by IBM SPSS Statistics version 22 with Spearman's correlation.

RESULTS

Preparation of the Immunogen and Monoclonal Antibody. The methyl ester (-$COOCH_3$) of leptosperin was hydrolyzed by alkali, and successive demethylated leptosperin was conjugated via carboxylic acid with a carrier protein, KLH, using a conventional carbodiimide method (Figure 1). To prepare a positive antigen, conjugation of demethylated leptosperin to BSA was simultaneously performed. The conjugation was confirmed by a 20% loss of the free amino moiety on the BSA, which was estimated by reactivity against trinitrobenzenesulfonic acid. The increase in absorption at 262 nm also indicated the adduction (data not shown). The immunogen was injected into mice, and hybridomas between spleen cells and myeloma cells were prepared by a typical polyethylene glycol method. Three clones (2H3, 2B5, and 1C10), which secreted the respective antibody to the immunogen, were obtained, and the 2H3 clone was then selected on the basis of simple characterization by an ELISA.

Specificity of the Antibody to Leptosperin. The detailed characterization of the 2H3 antibody was performed by a competitive ELISA. As shown in panels A and B of Figure 2, the antibody recognized leptosperin but not its component, methyl syringate or gentiobiose. Though manuka honey contains some characteristic compounds, HMF, 4-hydroxyphenylacetic acid, 2-methoxybenzoic acid, and methylglyoxal,[12−14] they did not cross-react with the 2H3 antibody. The antibody did not react with other saccharides such as glucose, fructose, sucrose, and galactose, which are the major components of honey (Figure 2C). These results suggest that the established antibody specifically reacts with leptosperin.

Quantification of Leptosperin in 20 Certified Manuka Honey Samples. Using the antibody, we established an ELISA method for the detection and quantitation of leptosperin. The range of quantitation was approximately 0.3−200 μM (Figure 3A). Twenty certified manuka honey samples were analyzed by an ELISA and then compared with previous data by HPLC.[5] As shown in Figure 3B, a significant correlation between the results of ELISA and HPLC was observed ($r = 0.97$; $p < 0.001$). An ELISA tended to produce a value higher than that produced by HPLC. The fitting equation (Figure 3B, inset; $y = -22 + 0.93x$) indicated that approximately 22 nmol of leptosperin per gram of honey should be calculated in manuka honey by an ELISA; even actual leptosperin was under the HPLC detection limit. This result suggested that some contaminants present in honey interfered with the assay. The amounts of leptosperin in the honey correlated with their nonperoxide-dependent antibacterial activities or the content of methylglyoxal.[2,5,12] The amount of leptosperin measured by an ELISA also correlated with this (data not shown). These results indicate that immunochemical quantification of leptosperin may be applicable for authentication of manuka honey.

Identification of the Antigen as Leptosperin in Manuka Honey. To confirm that the antigen in the honey was leptosperin, honey was fractionated by HPLC and the reactivities of the fractions with the antibody were then analyzed by a competitive ELISA. As shown in Figure 4, the antibody significantly reacted with fraction 12. Fraction 12 corresponded to a retention time of 11−12 min, which coincided with the elution time of leptosperin (11.5 min). This indicated that the antibody specifically reacts with leptosperin

Table 1. Quantitation of Leptosperin in Certified Manuka Honeys by HPLC and ELISA

				nmol/g of honey	
sample, information	certified	purchased	origin	HPLC[a]	ELISA
UMF[b] (Manuka Honey)					
1	UMF 15+	China	New Zealand	860	1023
2	UMF 15+	China	New Zealand	530	494
3	UMF 15+	China	New Zealand	758	752
4, similar label with samples 26 and 27	UMF 15+	Japan	New Zealand	835	930
5	UMF 15+	Japan	New Zealand	414	436
6	UMF 15+	Japan	New Zealand	579	508
7	UMF 12+	Japan	New Zealand	487	482
8, beech trees, blended, Southern Alps	UMF 5+	New Zealand	New Zealand	852	897
Active + (Manuka Honey or Jelly Bush Honey)					
9	Active 15+	China	New Zealand	396	381
10, certified chloramphenicol-free	Active 15+	China	New Zealand	262	288
11, stick type	Active 13+	Japan	New Zealand	483	446
12	Active 12+	New Zealand	New Zealand	294	326
13	Active 10+	New Zealand	New Zealand	147	179
14, 100% raw unpasteurized	Active 5+	New Zealand	New Zealand	1044	1099
15, jelly bush honey	Active 5+	Japan	Australia	58	83
MGO[c] (Manuka Honey)					
16	MGO 250+	Japan	New Zealand	690	673
17	MGO 250+	China	New Zealand	386	434

[a]Data (samples 1−17) determined by HPLC were acquired previously.[5] [b]UMF means unique manuka factor, which is used as an index of antibacterial activity. [c]MGO is used for an index of the level of methylglyoxal in honey.

Table 2. Quantitation of Leptosperin in Noncertified and Other Honeys by HPLC and ELISA

sample, information	certified	purchased	origin	nmol/g of honey HPLC[b,c]	nmol/g of honey ELISA[a]
Noncertified (or Self-Certified) Manuka Honey (or Jelly Bush Honey)					
18	–	Japan	New Zealand	113	135
19, "self-rigid inspection passed"	–	Japan	New Zealand	372	412
20, bottled in Japan, liquid type	–	Japan	New Zealand	438	364
21	–	Japan	New Zealand	767	806
22, with kiwi fruit (2%)	–	Japan	New Zealand	74	129
23, South Island	–	Japan	New Zealand	592	666
24, "high grade manuka honey, 92.5%"	–	Japan	New Zealand	720	665
25, with 0.25% propolis, 5+ certificated by a laboratory	–	Japan	New Zealand	258	223
26, same jar and label as sample 27	–	Japan	New Zealand	ND[d]	ND[d] (16)
27, same jar and label as sample 26	–	New Zealand	New Zealand	164	157
28, squeeze type, jelly bush honey	–	Japan	Australia	35	ND[d] (31)
Other Honeys (Non-manuka Honey)					
29, scottish heather honey	–	Japan	U.K.	ND[d]	ND[d] (41)
30	–	Brazil	Brazil	ND[d]	ND[d] (31)
31, buckwheat	–	Japan	Japan	ND[d]	ND[d] (19)
32, multifloral honey, Awaji Island	–	Japan	Japan	ND[d]	ND[d] (30)
33, multifloral honey	–	Japan	Japan	ND[d]	ND[d] (11)
34, kiawe tree	–	Japan	United States	ND[d]	ND[d] (27)
Other Honeys (Non-manuka Honey)					
35, acacia	–	Japan	Hungary	ND[d]	ND[d] (20)
36, asphodel	–	Japan	Italy	ND[d]	ND[d] (17)
37, agrumi (citrus)	–	Japan	Italy	ND[d]	ND[d] (16)
38, lavender (*Lavandula stoechas*)	–	Japan	Italy	ND[d]	ND[d] (15)
39, millefiori (Tuscan honey)	–	Japan	Italy	ND[d]	ND[d] (19)
40, eucalyptus	–	Japan	Italy	ND[d]	ND[d] (17)
41, cardoon (*Galactites tomentosa*)	–	Japan	Italy	ND[d]	ND[d] (21)
42, borragine (*Borago officinalis*)	–	Japan	Italy	ND[d]	ND[d] (28)
43, orange	–	Japan	Italy	ND[d]	ND[d] (20)
44, eucalyptus	–	Japan	Spain	ND[d]	ND[d] (18)
45, lemon	–	Japan	Spain	ND[d]	ND[d] (19)
46, lavender (*Lavandula stoechas*)	–	Japan	France	ND[d]	ND[d] (26)
47, sunflower (*Helianthus annuus*)	–	Japan	France	ND[d]	ND[d] (27)
48, oak (honeydew honey)	–	Japan	Spain	ND[d]	ND[d] (33)
49, fir (Abies) (honeydew honey)	–	Japan	France	ND[d]	ND[d] (31)
50, leather wood (*Eucryphia lucida*)		Japan	Australia	ND[d]	ND[d] (20)

[a]The calculated data are given in parentheses. [b]Data (for samples 18–34) determined by HPLC were acquired previously.[5] [c]Data (for samples 35–50) determined by HPLC were acquired previously.[2] [d]Not detected.

in manuka honey. In addition, syringic acid 4-*O*-β-D-gentiobioside (demethylated leptosperin) could not be detected.

Application of the Determination of Leptosperin in Honey and Vodka by an ELISA. Leptosperin has been found almost exclusively in manuka honey and jelly bush honey.[2,5,12,15] As shown in Table 1, leptosperin was detected in almost all manuka honey samples by an ELISA. The data for certified and noncertified manuka honey are fairly linear with respect to data determined previously by HPLC ($p < 0.001$).[2,5] There were no strong signals from non-manuka honey (Table 2), even if the honey was rich in methyl syringate (sample 36, from Sardinia, Italy).[16] After taking each datum into consideration, we decided that 70 nmol of leptosperin/g of honey was the detection limit for this ELISA. For reference, calculated data are given in parentheses in Table 2. In addition, leptosperin was also found (approximately 4 μmol/mL) in vodka to which manuka honey had been added. The concentration in vodka was almost identical to that measured by HPLC in our previous report.[5]

DISCUSSION

The leptosperin levels in manuka honey correlated with the nonperoxide-dependent antibacterial activity of honey, which is used for authentication of manuka honey as UMF.[5] Because leptosperin is found exclusively in manuka honey,[2,12] the quantification of leptosperin could be an authentication marker for manuka honey. To detect the leptosperin, chemical methods are available, and these methods provide accurate measures of leptosperin levels.[5] Some products contain trace amounts of manuka honey, making it difficult to detect leptosperin using conventional HPLC–PDA (UV). However, mass spectrometry could resolve the problem. In contrast, immunochemical methods such as an ELISA can analyze many samples simultaneously; therefore, the cost of the required equipment, such as a conventional microplate reader, is lower than those required for chemical methods like HPLC–PDA (UV) or LC–MS/MS. Needless to say, a specific antibody is required for the construction of an ELISA.

There are several reports on antibodies to phytochemicals or their metabolites. Kawai et al. prepared antibodies to quercetin glucuronide and proved the localization of the metabolite in atherosclerotic plaques or the brain immunohistochemically.[7−9] Recently, a monoclonal antibody to liquiritin, a glucoside of liquiritigenin, has been developed to assist in the quality control of licorice products.[10] Antibodies developed specifically for a phytochemical may be useful for analyzing the metabolism of compounds and for the quality control of products. In this study, we aimed to obtain the antibody specific to leptosperin.

In preliminary experiments, we tried to prepare the antibody using an immunogen, which was prepared by conjugation of the carrier protein with leptosperin by a periodate oxidation method.[17] The obtained antibody recognized leptosperin; however, it also recognized its aglycone, methyl syringate (data not shown). In the study presented here, the methyl ester of methyl syringate in the leptosperin molecule was treated with a weak alkaline solution to expose the carboxyl moiety (-COOH); i.e., the methyl syringate moiety was transformed into a syringic acid moiety. Using the generated terminal COOH group, the leptosperin molecule was conjugated with a lysine residue of a carrier protein, KLH. The novel antibody that was obtained did not recognize gentiobiose, syringic acid, or methyl syringate (Figure 2). This result suggests that the antibody is highly specific for leptosperin.

We have found that the antigen in manuka honey completely coincides with the fraction containing leptosperin (Figure 4). Figure 3B and Tables 1 and 2 show good linearity between the data determined by HPLC and ELISA, indicating that the reliability of leptosperin analyses by an ELISA is sufficient for practical purposes.

The antibacterial activity is a key property of manuka honey, and this activity can be changed during storage, transport to a store, or its display at the store. A recent study showed that quantitation of leptosperin could identify falsified manuka honey or low-quality manuka honey[5] sold in the market. These findings suggest that the quantitation of leptosperin along with the measurement of antibacterial activity should be applicable for authentication of manuka honey. Immunochemical methods, such as an ELISA, using the antibody specific to leptosperin provide a simple method for novel immunochemical authentication of manuka honey.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; PDA, photodiode array; UV, ultraviolet light; MS/MS, tandem mass spectrometry; HMF, 5-(hydroxymethyl)-2-furaldehyde; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; KLH, keyhole limpet hemocyanin; UMFHA, unique manuka factor honey association; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20

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