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Investigation of Foreign Amylase Adulteration in Honey Distributed in Japan by Rapid and Improved Native PAGE Activity Staining Method

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Abstract: Foreign amylase addition to honey in an effort to disguise diastase activity has become a widespread form of food fraud. However, since there is no report on the investigation in Japan, we investigated foreign amylases in 67 commercial honeys in Japan. First, the α-glucosidase and diastase activities of honeys were measured, which revealed that only α-glucosidase activity was significantly low in several samples. As both enzymes are secreted from honeybee glands, it is unlikely that only one enzyme was inactivated during processing. Therefore, we suspected the presence of foreign amylase. α-Amylase in honey were assigned using protein analysis software based on LC-QTOF-MS. As a result, α-amylases from *Aspergillus* and *Geobacillus* were detected in 13 and 6 out of 67 honeys, respectively. To detect foreign amylases easily, we developed a cost-effective method using native PAGE. Conventional native PAGE failed to separate the α-amylase derived from honeybee and *Geobacillus*. However, when native PAGE was performed using a gel containing 1 % maltodextrin, the α-amylase from honeybee did not migrated in the gel and the α-amylase could be separated from the other two α-amylases. The results from this method were consistent with those of LC-QTOF-MS method, suggesting that the novel native PAGE method can be used to detect foreign amylases.

Key words: foreign amylase, honey adulteration, food fraud, LC-QTOF-MS, native PAGE

INTRODUCTION

Honey is one of the oldest nutritious foods, as represented in the beekeeping relief in the ancient Egyptian tomb of Pabasa.[1)] Owing to its long history, honey has been the subject of ongoing research, which has revealed fructose and glucose to be the main components of honey and that honey contains several kinds of enzymes in addition to various nutritional components.[2)] Honey has several health benefits. It is suitable for recovering from fatigue and replenishing energy after exercise. Additionally, since it contains abundant oligosaccharides, phenols, and carbohydrates, it is expected to have an intestinal regulation effect.[3)] Honey contains a good balance of amino acids, minerals, and proteins, as well as a large amount of polyphenols, including quercetin, which shows strong antioxidant and antibacterial effects.[4)] α-Amylase in honey is one of the oldest studied honeybee enzymes. This enzyme is thought to be found in honey after being secreted from the secretory glands of honeybees. It has been isolated, purified, and studied for its various properties, including pH, temperature, and kinetics parameters.[5)6)7)] α-Amylase activity in honey is standardized as diastase activity, which is reported as diastase number (DN) and used as an indicator of honey freshness as the CODEX Standard.[8)] The quality threshold is usually set at 8 DN or higher, and 3 DN or higher for honeys such as citrus-derived with low natural enzyme content. Honeybee has three α-glucosidase isozymes; among these, α-glucosidase III is considered to be involved in honey production.[9)] Because this α-glucosidase is secreted from honeybee glands like α-amylase, it is likely to be present in honey.[10)] α-Glucosidase activity, like diastase activity, is sometimes measured for a honey quality control.[11)]

The history of honey is entangled with the history of the fight against food fraud. Good quality honey, such as Manuka honey from New Zealand, has long been traded at a high price.[12)] Therefore, methods of falsifying honey, such as diluting it with syrup, are rampant.[13)] Inexpensive sugars or industrial syrups, such as corn, high fructose corn, glucose, sucrose, and inverted syrups, are commonly used for adulteration. Since the 1900s, when invert sugar was made from starch, syrup has been a staple of honey adulteration. Therefore, the main method for identifying syrup in honey in the past was to detect starch-derived polysaccharides by thin layer chromatography (TLC).[14)] Subsequently, stable carbon isotope ratio analysis (SCIRA) was developed to identify compounds derived from C3 plants.[15)] Recently, nuclear magnetic resonance method, which is a non-targeted and exhaustive survey of the constituents of honey, has also been used.[16)] However, with the increasing diversity of syrup

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making methods, distinguishing syrup additions from honey using these methods has become difficult.

Diluting honey with syrups as described above decreases diastase activity, although diastase activity is also reduced in honey that has been improperly stored for long periods or overheated during the manufacturing process. α-Amylases from sources other than honeybees (foreign amylases) are sometimes added to disguise reduced diastase activity.[17)18)] One method for detecting foreign amylase is to compare the diastase activity determined using the Schade method with that using the Phadebas method.[19)] The Schade method is a classical diastase activity assay that uses soluble starch as a substrate.[20)] In contrast, the Phadebas method uses Phadebas® test tablets as the substrate.[21)] In the presence of foreign amylase in the honey, the two assays give different results because the substrate specificity is different from that of the honeybee-derived amylase. Recently, foreign amylase was reportedly discovered by a comprehensive analysis of honey proteins using nano liquid chromatography–tandem mass spectrometry (LC-MS/MS).[22)] α-Amylases from *Aspergillus niger* and *Bacillus amyloliquefaciens* were detected in some honeys sold in Europe.

Notably, inexpensive honey is on the market in Japan. Therefore, we conducted market research on 67 honeys available in the Japanese market, including imported honeys. First, the presence or absence of camouflage was evaluated by enzymatic activity, and then a quadrupole time-of-flight mass spectrometer (LC-QTOF-MS) was used to confirm the addition of foreign amylase. Furthermore, native PAGE activity staining method was examined as a simpler method. In Japan, the addition of foreign amylase to honey as an adulterant has not been prevalent; thus, no detection method for foreign amylases has been established. This study is the first to investigate foreign amylases in honey sold in Japan and use native PAGE as a detection method.

MATERIALS AND METHODS

Honey samples. We purchased 67 honey samples at supermarkets or online stores in Japan. The country of origin, honey source, and price of all purchased honey are presented in Table 1. Samples were stored at room temperature.

Sample preparation. After dissolving honey (4 g) in Dulbecco's phosphate-buffered saline (PBS: Nacalai Tesque, Inc., Kyoto, Japan), the volume was adjusted to 20 mL. The supernatant (10 mL) was placed in 10 kDa Amicon® Ultra 15 Centrifugal Filters (Merck KGaA, Darmstadt, Germany) after centrifugation for 10 min (15,000 × *g*, 10 ˚C). PBS (10 mL) was added to the concentrate obtained after centrifugation for 30 min (5,000 × *g*, 10 ˚C). PBS (10 mL) was added to the obtained concentrate and centrifuged for 20 min (5,000 × *g*, 10 ˚C). This operation was repeated six times to remove sugars. The volume of the obtained concentrate was made up to 2 mL with water and the solution was centrifuged for 10 min (15,000 × *g*, 10 ˚C). The supernatant obtained was used as the test solution.

Protein extraction and digestion. Trypsin/Lys-C Mix, Mass Spec Grade (Promega Co., Madison, WI, USA) digests of protein included in the test solution were analyzed using an LC-QTOF-MS (LC: Nexera X2 series, Shimadzu Co., Kyoto, Japan; QTOF-MS: 5600+, SCIEX, Framingham, MA, USA). First, the test solution (500 μL) was dried using a centrifugal evaporator and then dissolved in 100 μL of 50 mM Tris-HCl buffer (pH 8.0) containing 6 M urea. After denaturing the honey protein using a heat block at 95 ˚C for 30 min, 10 μL of 100 mM dithiothreitol (DTT) solution was added, followed by incubation at room temperature in the dark (30 min). Next, after adding 5 μL of 1 M iodoacetamide solution and incubation at room temperature in the dark (30 min), 500 μL of 50 mM Tris-HCl buffer (pH 8.0) was added and confirmed the resulting pH to be 8.0. To decompose honey proteins into peptide fragments, 2 μL of 0.2 μg/μL Trypsin/Lys-C Mix solution was added and incubated the solution at 37 ˚C for 16 h. Afterwards, 30 μL of 20 % trifluoroacetic acid was added to stop the enzymatic reaction. The solution was desalted using MonoSpinC18 (GL Sciences Inc., Tokyo, Japan) according to the manufacturer's instructions. The eluate was dried in a centrifugal evaporator, dissolved in 100 μL of 0.1 % formic acid, and analyzed with LC-QTOF-MS.

Enzyme activity. The α-glucosidase activity was measured as follows. Fifty μL of the honey extract was added to 50 μL of 100 mM acetate buffer (pH 5.3) containing 50 mM sucrose and incubated at 37 ˚C for 30 min. After stopping the reaction by adding 50 μL of 1 M Tris-HCl buffer (pH 8.0), the amount of liberated glucose was calculated using Glucose CII Test Wako (FUJIFILM Wako Chemicals Co., Osaka, Japan). One unit was defined as the amount of enzyme hydrolyzing 1 μmole of sucrose per min at pH 5.3 and 37 ˚C. The diastase activity was measured according to the method of The CODEX Standard for Honey.[8)]

LC-QTOF-MS. The analytes were separated using a reversed-phase C30 analytical column (2.0 mm I.D. × 150 mm; 3 μm particle diameter; Develosil RPAQUEOUS-AR, Nomura Chemical Co., Ltd., Aichi, Japan). The column temperature was maintained at 40 ˚C. Mobile phase A consisted of acetonitrile containing 0.1 % formic acid, and mobile phase B consisted of water containing 0.1 % formic acid. A linear gradient was set up starting with 2 % A to 50 % A over 82 min. After elution, in a washing step, A was increased to 90 % in 6 min and allowed to flow for 5 min. The flow rate was 0.15 mL/min, and the injection volume was 3 μL.

The mass spectrometer was operated in positive ion mode. Ionization was performed by electrospray ionization, and the mass analysis conditions were set as follows: mode, information-dependent acquisition; desolvation temperature, 500 ˚C; ion spray voltage, 4,500 V; curtain gas pressure, 25 psi; nebulizer gas pressure, 50 psi; turbo gas pressure, 50 psi; mass range, *m/z* 50 to 1,000.

Database settings. The data files were processed by ProteinPilot version 5.0 software (SCIEX) using the Paragon algorithm.[23)] All searches were performed against the Uniprot/SwissProt database (taxonomy: all, sequence number: 568,744, download date: 20210714). Among the obtained proteins, those with an "unused" score of 2.0 or more and peptides (95 %) of 2 or more were counted as proteins with a reliability of 99 %.

Native PAGE activity staining method. Each of the test solution (10 μL) was mixed with an equal volume of Native Sample Buffer (Bio-Rad Laboratories Inc., Hercules, CA,

Table 1. The results of enzyme activities and detected foreign amylases in honey samples.

No.	Origin	Country	Price (yen/100g)	α-Glucosidase activity (mU/g)	Diastase activity (DN)	Detected foreign amylase	
						LC-QTOF-MS	Native PAGE
1	Blended	China	39.7	<5	8.7	*Geobacillus*	*Geobacillus*
2	Blended	China	45.2	<5	8.3	*Geobacillus*	*Geobacillus*
3	Blended	China	49.8	8	10.0	*Aspergillus*	*Aspergillus*
4	Blended	China	49.7	<5	15.6	*Aspergillus*	*Aspergillus*
5	Blended	Thailand	59.7	<5	13.3	*Aspergillus*	*Aspergillus*
6	Blended	Myanmar	69.1	201	11.2	N.D.	N.D.
7	Blended	China	49.8	8	10.2	*Geobacillus*	*Geobacillus*
8	Blended	China	59.8	17	12.5	*Aspergillus*	*Aspergillus*
9	Blended	China	59.7	6	13.2	*Aspergillus*	*Aspergillus*
10	Blended	China	36.8	<5	13.8	*Aspergillus*	*Aspergillus*
11	Blended	China	59.2	<5	10.2	*Geobacillus*	*Geobacillus*
12	Blended	China	49.8	25	10.6	*Aspergillus*	*Aspergillus*
13	Blended	China	55.4	<5	11.2	*Geobacillus*	*Geobacillus*
14	Blended	China	59.2	<5	7.3	*Geobacillus*	*Geobacillus*
15	Blended	China	49.8	49	9.1	*Aspergillus*	*Aspergillus*
16	Blended	China	72.3	82	7.7	*Aspergillus*	*Aspergillus*
17	Blended	Vietnam	147.2	30	3.8	N.D.	N.D.
18	Blended	Brazil	217.1	76	7.3	N.D.	N.D.
19	Blended	China	107.8	161	12.0	N.D.	N.D.
20	Blended	Mexico	154.0	38	5.6	N.D.	N.D.
21	Lotus	Japan	892.6	101	6.6	N.D.	N.D.
22	Lotus	Japan	370.3	1243	16.6	N.D.	N.D.
23	Blended	China	143.2	125	7.7	N.D.	N.D.
24	Blended	Hungary, Canada	224.5	224	7.6	N.D.	N.D.
25	Blended	Canada	263.3	310	8.7	N.D.	N.D.
26	Sunflower	Ukraine	185.3	52	5.3	N.D.	N.D.
27	Blended	China, Argentina	185.3	145	7.1	N.D.	N.D.
28	Lemon	Itary	383.1	34	6.6	N.D.	N.D.
29	Acacia	Itary	383.1	72	6.7	N.D.	N.D.
30	Acacia	Hungary	383.1	23	5.8	N.D.	N.D.
31	Multifloral	Japan	285.5	212	19.2	N.D.	N.D.
32	Cherry Blossoms	Japan	518.5	435	14.9	N.D.	N.D.
33	Mandarin orange	Japan	592.6	504	18.6	N.D.	N.D.
34	Lavender	France	1600.0	32	4.5	N.D.	N.D.
35	Rosemary	France	1600.0	167	7.1	N.D.	N.D.
36	Acacia	Japan	835.0	420	10.9	N.D.	N.D.
37	Multifloral	Japan	670.0	415	14.4	N.D.	N.D.
38	Acacia	China	182.7	99	10.7	N.D.	N.D.
39	Lotus	China	179.2	48	7.4	*Aspergillus*	*Aspergillus*
40	Blended	China	111.6	<5	<2.0	N.D.	N.D.
41	Lotus	China	238.7	142	6.4	N.D.	N.D.
42	Blended	Romania, Ukraine	199.2	68	5.4	N.D.	N.D.
43	Blended	Argentina, Canada, Hungary, Japan	275.2	72	7.0	N.D.	N.D.
44	Blended	China	119.2	37	6.5	*Aspergillus*	*Aspergillus*
45	Blended	Canada	199.2	27	4.6	N.D.	N.D.
46	Acacia	Japan	948.0	271	11.1	N.D.	N.D.
47	Multifloral	Japan	552.0	7	3.3	N.D.	N.D.
48	Multifloral	Japan	756.0	8	4.6	N.D.	N.D.
49	Blended	China	67.8	34	10.1	*Aspergillus*	*Aspergillus*
50	Blended	Argentina, New Zealand	164.5	78	11.7	N.D.	N.D.
51	Lotus	China	150.0	47	6.6	N.D.	N.D.
52	Acacia	China	162.0	41	6.4	N.D.	N.D.
53	Acacia	Switzerland	392.0	464	10.6	N.D.	N.D.
54	Acacia	Hungary	420.0	<5	6.4	N.D.	N.D.
55	Blended	Myanmar	119.8	26	4.4	N.D.	N.D.
56	Blended	China	78.9	5	<2.0	N.D.	N.D.
57	Acacia	Japan	816.7	480	11.6	N.D.	N.D.
58	Lotus	Japan	883.3	573	16.0	N.D.	N.D.
59	Multifloral	Japan	555.6	435	11.0	N.D.	N.D.
60	Acacia	Japan	549.7	582	15.4	N.D.	N.D.
61	Acacia	Japan	900.0	277	9.3	N.D.	N.D.
62	Lotus	Japan	954.5	413	8.9	N.D.	N.D.
63	Acacia	Japan	1035.7	396	6.1	N.D.	N.D.
64	Lotus	Japan	1035.7	531	8.5	N.D.	N.D.
65	Multifloral	Japan	638.6	68	3.1	N.D.	N.D.
66	Time	Greece	1000.0	206	7.2	N.D.	N.D.
67	Blended	China	89.8	25	7.0	*Aspergillus*	*Aspergillus*

Foreign amylase was detected by LC-QTOF-MS and native PAGE activity staining method.

USA). Subsequently, the solutions were loaded on the gel with 12 % polyacrylamide TGX FastCast Acrylamide Kit (Bio-Rad) containing 1 % maltodextrin (dextrose equivalent 4.0−7.0, Sigma-Aldrich, St. Louis, MO, USA). Tris/glycine buffer (FUJIFILM Wako Pure Chemical) was used as the running buffer. Electrophoresis was performed at a constant voltage of 200 volts for 60 min in ice water. After native PAGE, the α-amylases in honey were detected by performing activity staining. After electrophoretic migration, gels were incubated in 130 mM sodium acetate buffer (pH 5.3) containing 30 mM NaCl and 1 % soluble starch (1st Grade, FUJIFILM Wako Pure Chemical) at 40 ˚C for 30 min. Finally, gels were incubated with 7 mM iodine solution containing 0.24 M potassium iodide at room temperature for 30 min to determine activity of the α-amylases. Commercially available α-amylases derived from *Aspergillus oryzae* and *Bacillus licheniformis* (Sigma-Aldrich) were used as α-amylase markers.

In gel digestion. After native PAGE, the target bands were cut out using a scalpel and recovered. Five hundred μL of acetonitrile was added to dehydrate the gel. After removing the acetonitrile, the gel was dried in a centrifugal evaporator. Five hundred μL of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM DTT was added to the dried gel and incubated at 95 ˚C for 20 min. Alkylation was performed by replacing the solution with 500 μL of 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM iodoacetamide. After washing the gel with 500 μL of 50 mM Tris-HCl buffer (pH 8.0), about 500 μL of 50 % acetonitrile was added for dehydration. Next, 50 % acetonitrile was removed and the gel was dried in a centrifugal evaporator. After adding enough 50 mM Tris-HCl buffer (pH 8.0) to soak the gel, 2 μL of 20 μg/μL Trypsin/Lys-C Mix solution was added and incubated at 37 ˚C for 16 h. The liquid was discarded, 200 μL of 50 % acetonitrile containing 5 % trifluoroacetic acid was added, the mixture was shaken for 30 min, and the resulting solution was recovered. This operation was performed twice, and the recovered solution was dried in a vacuum centrifuge. After adding and dissolving 500 μL Tris-HCl buffer (pH 8.0), we added and mixed 30 μL of 20 % trifluoroacetic acid. The solution was desalted using MonoSpinC18 according to the manufacturer's instructions. The eluate was dried in a centrifugal evaporator, dissolved in 0.1 % formic acid, and analyzed with LC-QTOF-MS.

RESULTS AND DISCUSSION

Estimation of foreign amylases by LC-QTOF-MS.

First, 67 honeys were measured for two types of enzymatic activity, and some honeys with diastase activity exceeding the CODEX standard were found with almost no detectable α-glucosidase activity (Table 1). These two digestive enzymes are secreted from the same secretory gland, and it is unlikely that only α-glucosidase activity would be inactivated under normal conditions, even considering their different temperature stabilities. Therefore, it was suspected that foreign amylase was added to those honeys to mask the inactivation of amylase. Peptide fragments obtained by tryptic digestion of honey were measured by LC-QTOF-MS, and the obtained data were analyzed by protein analysis software, ProteinPilot. As the results, *Aspergillus*- and *Geobacillus*-derived α-amylases were detected in 13 and 6 of the 67 honey samples, respectively (Tables 1; S1 and S2; see J. Appl. Glycosci. Web site). Although the database referenced in this study only enabled the identification of the genus, α-amylase derived from *Aspergillus* has been reported previously,[19] but this is the first study reporting the presence of α-amylase derived from *Geobacillus* in honey. *Geobacillus* is a highly thermostable bacterium found in hot springs, and α-amylase purified from it is also known to have extremely high thermostability.[24][25] One method of processing honey is collecting, heating, and concentrating immature honey, which is disguised as pure honey.[26] *Geobacillus*-derived α-amylase may be a suitable enzyme for compensating diastase activity after heat treatment because it is more thermostable than honeybee-derived α-amylase. All honey samples in which foreign amylase were detected showed high diastase activity (DN = 6.0 or higher), whereas the α-glucosidase activity was undetected or low (Table 1). Because α-glucosidase was not detectable, it is presumed that these honeys were heated or diluted by adding syrup.[18] Moreover, the prices associated with these honey samples were less than 200 yen per 100 g, which is low for honey in the Japanese market (100 yen = USA$0.72 as of 05/19/2023). Samples, 40 and 56, showed significantly low enzyme activities, but foreign amylase was not detected. This result was confirmed with inferior honey that had been heated or diluted with syrup. In addition, we investigated the presence of *Aspergillus*-derived proteins other than the detected α-amylase in honey samples using ProteinPilot against the Uniprot/SwissProt (taxonomy: *Aspergillus*, sequence number: 5,230, download date: 20220802), but no other *Aspergillus*-derived proteins were found. This result suggested that *Aspergillus* was not naturally present in honey as a mold but that only *Aspergillus*-derived α-amylase was present. If *Aspergillus* were present as a mold, other enzymes, such as proteases and lipases, would also be secreted. Thus, we verified that it was added as a foreign amylase. Uniprot did not have sufficient data on *Geobacillus* to conduct a similar survey. However, since there has been no report of *Geobacillus* existing in honey as an indigenous bacterium, it was considered that *Geobacillus*-derived amylase was also added as a foreign amylase.

Detection of foreign amylases by native PAGE activity staining method.

As foreign amylase was detected by LC-QTOF-MS, activity staining after native PAGE was performed as a confirmatory test. Separation of α-amylase in native PAGE was confirmed using honey samples 22, 2, and 3, in which foreign amylase was not detected, *Geobacillus*-derived amylase was detected, and *Aspergillus*-derived amylase was detected, respectively. In samples 2 and 22, α-amylases were detected at the same position (Fig. 1A). LC-QTOF-MS results showed different α-amylases in these samples. Therefore, 1 % maltodextrin was added to prepare the gel for electrophoresis, and native PAGE was performed. Subsequently, the α-amylase bands of samples 22 and 2 were detected at different positions, and the *Geobacillus*-derived α-amylase band was detected only in sample 2 (Fig. 1B). Assuming that the honeybee-derived α-amylase did not run in the gel and was present at the position (0), the part was cut out, digested in the gel, and

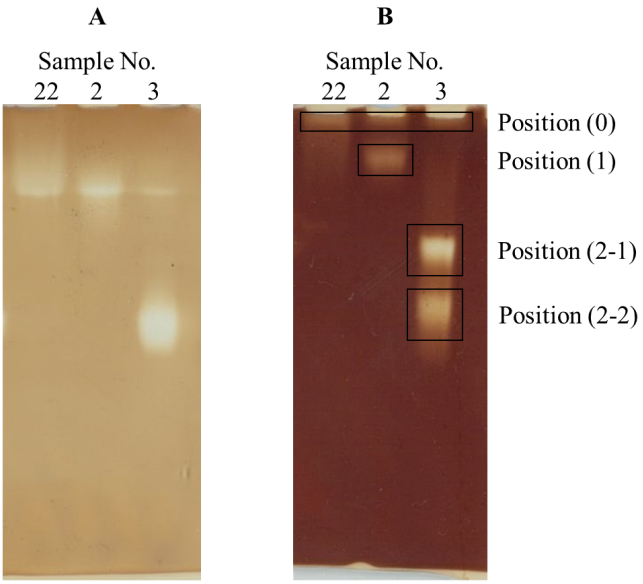

Fig. 1. Results of native PAGE using gels without (A) and with maltodextrin (B).

Position (0), α-amylase from honey bee; Position (1), α-amylase from *Geobacillus*; Position (2), α-amylase from *Aspergillus*. Sample No. 22: representative of honey sample in which no foreign amylase was detected. Sample No. 2: representative honey sample in which foreign amylases from *Geobacillus* were detected. Sample No. 3: representative honey sample in which foreign amylases from *Aspergillus* were detected.

measured by LC-QTOF-MS using ProteinPilot against the Uniprot/TrEMBL (protein name: amylase, sequence number: 310,568, download date: 20211214). Subsequently, honeybee-derived α-amylase was detected, suggesting that it was not migrated in the gel containing maltodextrin. We considered that native PAGE using this maltodextrin-containing gel was effective for detecting foreign amylase and tested all honey samples (Fig. 2, and Table 1). The results were consistent with those obtained by LC-QTOF-MS. No band was detected for honey samples in which only honeybee-derived α-amylase was detected, and for honey samples in which the *Geobacillus*-derived α-amylase was detected by LC-QTOF-MS, a band was detected at position (1), as shown in Fig. 1B. For honey samples in which *Aspergillus*-derived α-amylase was detected by LC-QTOF-MS, a band was detected at position (2−1) and (2−2), as shown in Fig. 1B. Based on these results, the α-amylase at position (1) was found to be derived from *Geobacillus,* and the α-amylase at positions (2−1) and (2−2) were found to be derived from *Aspergillus*. In addition, the presence of two *Aspergillus*-derived bands suggested the presence of isozymes (Fig. 1). Some α-amylases are known to adsorb to starch because they have a starch-binding domain at the C-terminus that binds to raw starch.[27)28)29)] Although there has been no report of this starch-binding domain existing in honeybee-derived α-amylase, it is thought that it bound very strongly to maltodextrin in the gel and did not move in the native PAGE analysis. These results suggest that the native PAGE activity staining method using maltodextrin-containing gel is effective for quick and easy detection of foreign amylases in honey samples.

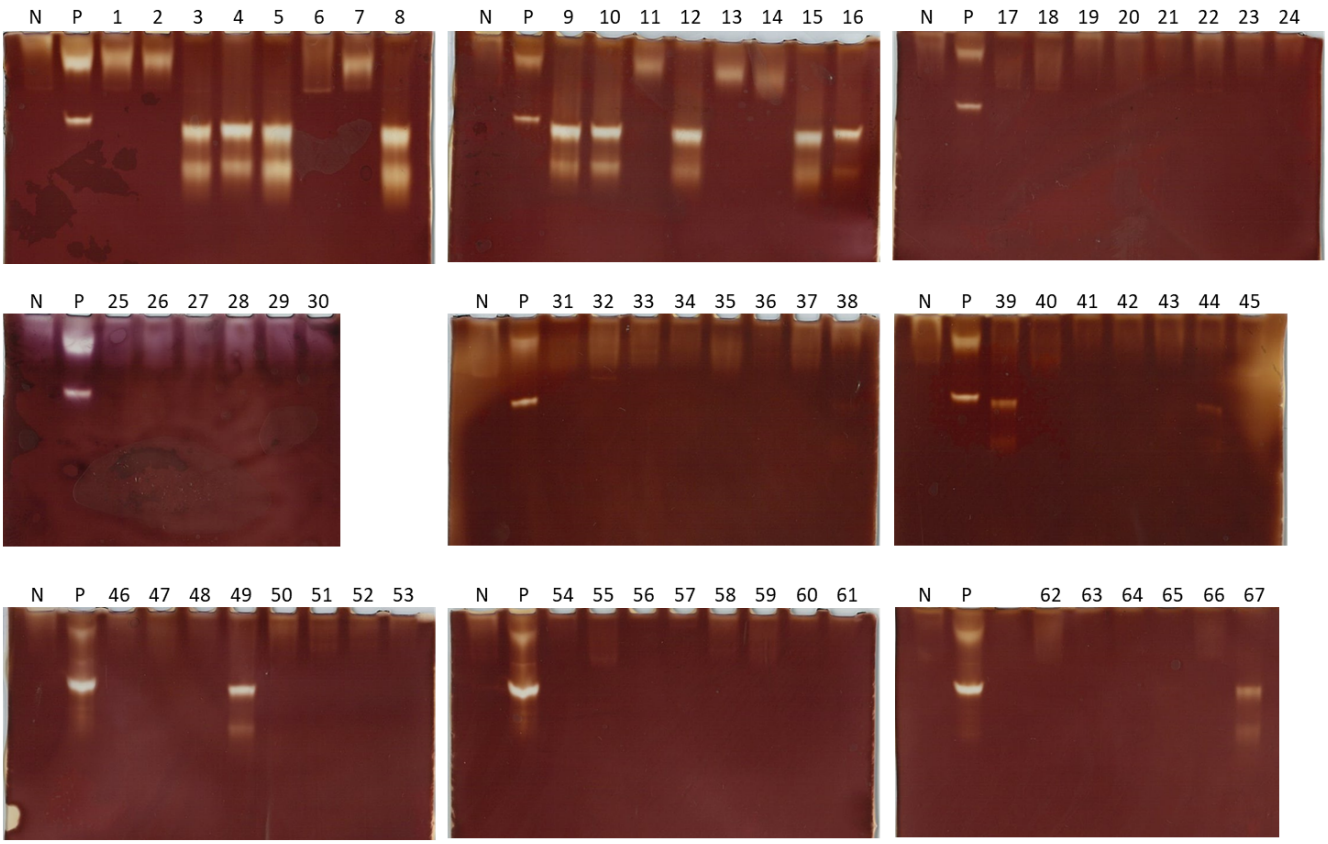

Fig. 2. Results of native PAGE activity staining using 1 % maltodextrin-containing gels.

Honey extracts were applied to native PAGE using 1 % maltodextrin-containing gels (12 % acrylamide) and foreign amylase was detected by active staining. N, negative control; P, positive control (upper band; α-amylase from *Bacillus licheniformis*, lower band; α-amylase from *Aspergillus oryzae*). No. corresponds to the number in Table 1.

CONCLUSIONS

From the results of this survey, it was found that foreign amylase was added to inexpensive honey distributed in Japan. Similar to the addition of syrup, the addition of foreign amylases camouflages reduced diastase activity due to processes such as heating. In Japan, only the TLC and SCIRA methods are used to identify adulterants in honey, which cannot detect the addition of foreign amylases to honey. Initially, we also used LC-QTOF-MS to detect foreign amylases; however, LC-QTOF-MS equipment are expensive and not versatile. Therefore, we report a novel verification method for the detection of these α-amylases in honey sold in Japan. We propose the native PAGE activity staining method using maltodextrin-containing gels as a facile and effective approach for detecting foreign amylases in honey. In our study, two types of foreign amylases were detected, but other α-amylases may be added to honey in the future. Therefore, it should be investigated whether a wide range of biogenic α-amylases can be separated from honeybee-derived α-amylases by native PAGE activity staining method.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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REFERENCES

1) A.Y. Naggar, G. Codling, J.P. Giesy, and A. Safer: Beekeeping and the need for pollination from an agricultural perspective in Egypt. *Bee World*, **95**, 107−112 (2018).
2) S. Bogdanov, T. Jurendic, R. Sieber, and P. Gallmann: Honey for nutrition and health: A review. *J. Am. Coll. Nutr.*, **27**, 677−689 (2008).
3) L. Jiang, M. Xie, G. Chen, J. Qiao, H. Zhang, and X. Zeng: Phenolics and carbohydrates in buckwheat honey regulate the human intestinal microbiota. *Evid. Based Complementary Altern. Med.*, **2020**, 6432942 (2020).
4) M.S. Hossen, M.Y. Ali, M.H.A. Jahurul, M.M. Abdel-Daim, S.H. Gan, and M.I. Khalil: Beneficial roles of honey polyphenols against some human degenerative diseases: A review. *Pharmacol. Rep.,* **69**, 1194−1205 (2017).
5) S. Babacan and A.G. Rand: Purification of amylase from honey. *J. Food Sci.*, **70**, c413−c418 (2005).
6) S. Babacan and A.G. Rand: Characterization of honey amylase. *J. Food Sci.,* **72**, C050−C055 (2007).
7) M.T. Rinaudo, C. Ponzetto, C. Vidano, and F. Marletto: The origin of honey amylase. *Comp. Biochem. Physiol. B Comp. Biochem.*, **46**, 253−256 (1973).
8) CODEX Alimentarius Commission: Adopting the draft revised standard for honey. *Alinorm,* **1** (25), 22−24 (2001).
9) M. Nishimoto, M. Kubota, M. Tsuji, H. Mori, A. Kimura., H. Matsui, and S. Chiba: Purification and substrate specificity of honeybee, *Apis mellifera* L., α-glucosidase III. *Biosci. Biotechnol. Biochem.*, **65**, 1610−1616 (2001).
10) M. Kubota, M. Tsuji, M. Nishimoto, J. Wongchawalit, M. Okuyama, H. Mori, H. Matsui, R. Surarit, J. Svasti, A. Kimura, and S. Chiba: Localization of α-glucosidases I, II, and III in organs of European honeybees, *Apis mellifera* L., and the origin of α-glucosidase in honey. *Biosci. Biotechnol. Biochem.*, **68**, 2346−2352 (2004).
11) B. Lichtenberg-Kraag: Evidence for correlation between invertase activity and sucrose content during the ripening process of honey. *J. Apic. Res.*, **53**, 364−373 (2014).
12) K.M. Rogers, K. Somerton, P. Rogers, and J. Cox: Eliminating false positive C4 sugar tests on New Zealand Manuka honey. *Rapid Commun. Mass Spectrom.*, **24**, 2370−2374 (2010).
13) R. Fakhlaei, J. Selamat, A. Khatib, A.F.A Razis, R. Sukor, S. Ahmad, and A.A. Babadi: The toxic impact of honey adulteration: A review. *Foods*, **9**, 1538 (2020).
14) J.W. White Jr.: Detection of honey adulteration by carbohydrate analysis. *J. AOAC Int.*, **63**, 11−18 (1980).
15) S. Soares, J.S. Amaral, M.B.P.P. Oliveira, and I. Mafra: A comprehensive review on the main honey authentication issues: Production and origin. *Compr. Rev. Food Sci. Food Saf.*, **16**, 1072−1100 (2017).
16) Rd.O.R. Ribeiro, E.T. Mársico, Cd.S. Carneiro, M.L.G. Monteiro, C.C. Júnior, and E.F.Od. Jesus: Detection of honey adulteration of high fructose corn syrup by Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR). *J. Food Eng.*, **135**, 39−43 (2014).
17) T. Damto: A review on effect of adulteration on honey properties. *SSRN* (2019).
18) A. Naila, W.N. Julika, S. Flint, A.Z. Sulaiman, A. Mohamed, and A. Ajit: Analysis of adulterants in international honey available in the Maldives. *ACS Food Sci. Technol.*, **1**, 1373−1380 (2021).
19) M. Voldřich, A. Rajchl, H. Čížková, and P. Cuhra: Detection of foreign enzyme addition into the adulterated honey. *Czech J. Food Sci.*, **27**, S280−S282 (2009).
20) J.W. White Jr.: Diastase in honey: The Schade method. *J. AOAC Int.*, **47**, 486−488 (1964).
21) L.P. Oddo and P. Pulcini: A scientific note on the Phadebas method for honeys with low enzyme content. *Apidologie*, **30**, 347−348 (1999).
22) T. Erban, E. Shcherbachenko, P. Talacko, and K. Harant: A single honey proteome dataset for identifying adulteration by foreign amylases and mining various protein markers natural to honey. *J. Proteomics*, **239**, 104157 (2021).
23) I.V. Shilov, S.L. Seymour, A.A. Patel, A. Loboda, W.H. Tang, S.P. Keating, and D.A. Schaeffer: The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol. Cell Proteomics*, **6**, 1638−1655 (2007).
24) S.A. Fincan and B. Enez: Production, purification, and characterization of thermostable α-amylase from thermophilic *Geobacillus stearothermophilus*. *Starch*, **66**, 182−189 (2014).
25) J.L. Uma Maheswar Rao and T. Satyanarayana: Purification and characterization of a hyperthermostable and high maltogenic α-amylase of an extreme thermophile *Geobacillus thermoleovorans*. *Appl. Biochem. Biotechnol.*, **142**, 179−193 (2007).

26) Y. Yuan, Z. Deng, B. Zhang, G. Li, J. Zhang, R. Liu, and H. Li: Quality evaluation and geographical classification of immature rape and acacia honeys in China. *J. Sci. Food Agric.*, **101**, 5446−5456 (2021).
27) K. Ohdan, T. Kuriki, H. Takata, H. Kaneko, and S. Okada: Introduction of raw starch-binding domains into *Bacillus subtilis* α-amylase by fusion with the starch-binding domain of *Bacillus* Cyclomaltodextrin glucanotransferase. *Appl. Environ. Microbiol.,* **66**, 3058−3064 (2020).
28) B.K. Tibbot, D.W.S. Wong, and G.H. Robertson: Studies on the C-terminal region of barley α-amylase 1 with emphasis on raw starch-binding. *Biologia Bratislava*, **57**, 229−238 (2002).
29) W. Yu, W. Zou, S. Dhital, P. Wu, M.J. Gidley, G.P. Fox, and R.G. Gilbert: The adsorption of α-amylase on barley proteins affects the *in vitro* digestion of starch in barley flour. *Food Chem.*, **241**, 493−501(2018).