



Lactic Acid Bacterial Conversion of trans-10-Hydroxy-2-Decenoic Acid to 10-Hydroxydecanoic Acid in Royal Jelly for Enhanced Immunostimulatory Function

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(Degree)

博士 (農学)

(Date of Degree)

2025-09-25

(Date of Publication)

2026-09-01

(Resource Type)

doctoral thesis

(Report Number)

甲第9335号

(URL)

<https://hdl.handle.net/20.500.14094/0100498375>

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Doctoral Dissertation

Lactic Acid Bacterial Conversion of *trans*-10-Hydroxy-2-Decenoic Acid to 10-Hydroxydecanoic Acid in Royal Jelly for Enhanced Immunostimulatory Function

乳酸菌によるローヤルゼリー中の *trans*-10-hydroxy-2-decenoic acid を
10-hydroxydecanoic acid へと変換することによる腸管免疫機能の強化

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令和 7 年 7 月

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Abstract

Royal jelly (RJ) contains 10-hydroxydecanoic acid (10HDAA), which is expected to have immune activation effects, such as promoting M-cell differentiation. To enhance the use of RJ as a functional immunostimulatory food ingredient, it is desirable to increase the 10HDAA content in RJ. Our aim was to isolate bacteria capable of converting 10-hydroxy-2-decenoic acid (10H2DA) contained in RJ to 10HDAA, thereby applying the isolate for producing fermented RJ with a high 10HDAA content. A strain of lactic acid bacteria, strain M1 was successfully isolated from the digestive tract of queen bees, capable of converting 10H2DA to 10HDAA. It was identified as *Lactobacillus panisapium*. By cultivation of strain M1 in a protease-treated RJ broth, the fermented RJ (fRJ) that contained five times the amount of 10HDAA compared to conventional RJ was produced. Preliminary evaluations of fRJ's immune-stimulating effects revealed several benefits. It promoted the differentiation of M-cells, which play a crucial role in intestinal immunity, activated the phagocytic ability of macrophages, and increased IgA secretion in individuals with reduced salivary immunoglobulin (Ig) A secretion. Safety tests confirmed that fRJ is safe for consumption. FRJ acts on M-cells, which tend to decrease with age, and promotes salivary IgA secretion.

The enzyme was partially purified from *L. panisapium* cell extracts based on its 10H2DA-reducing activity. LC-MS/MS and phylogenetic analysis identified the enzyme as a water-soluble fumarate reductase (~40% identity to reductases from *Shewanella frigidimarina* and *Klebsiella pneumoniae*). The reductase was heterologously expressed in *Escherichia coli*, and the recombinant enzyme displayed higher activity toward fumarate compared to 10H2DA in the presence of FAD and NADH. The enzyme exhibited optimal stability at 50°C and pH 6.0 (MES–NaOH buffer). Structural modeling revealed the absence of a heme-binding domain, which may facilitate access to larger substrates like 10H2DA. A glycine residue unique to the *L. panisapium* reductase likely facilitates larger substrate binding, such as 10H2DA; however, substituting it with threonine in the single mutant enzyme reduced activity toward 10H2DA while maintaining activity for fumarate. Freeze-dried whole cells of *E. coli* transformants also effectively converted 10H2DA to 10HDAA, offering a scalable alternative to the recombinant enzyme.

This study provides new insights into the structure-function relationship of the *L. panisapium* reductase and demonstrates its potential for biocatalytic applications in producing 10HDAA-enriched Royal Jelly, contributing to

the development of functional foods.

Abbreviations

10H2DA, *trans*-10-hydroxy-2-decenoic acid; 10HDAA, 10-hydroxydecanoic acid; Ig A, immunoglobulin A; M-cells, microfold cells; RJ, Royal Jelly; gp2, glycoprotein 2; pRJ, protease-treated Royal Jelly; MRS, De Man-Rogosa-Shape; TOS, *trans*-galactosylated oligosaccharide; D-MEM, Dulbecco's Modified Eagle's Medium; PDA, photodiode array detection; ELSD, evaporative light scattering detection; MALDI/TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry ;nRJ, raw royal jelly; fRJ, fermented royal jelly; MRJP1, major royal jelly protein 1; SCR, screening; FACS, fluorescence-activated cell sorting; PE, Phycoerythrin; MFI, mean fluorescence intensity; VAS, visual analog scale; LB, Luria-Bertani; IPTG, isopropyl- β -D-(-)-thiogalactopyranoside; MES, 2-morpholinoethanesulfonic acid, monohydrate; ANOVA, analysis of variance; OYE, old yellow enzyme

Chapter 1 INTRODUCTION

Royal Jelly (RJ) is a nutrient-rich secretion produced by honey bees (*Apis mellifera*) after digesting pollen, nectar, and honey and secreted from the hypopharyngeal and mandibular glands. It is a substance consisting mainly of water (50-60%), protein (18%), carbohydrates (15%), lipids (3-6%), mineral salts (1.5%), and vitamins (Viuda-Martos et al. 2008), with various biological activities already reported, including antioxidant, antitumor, anti-aging antioxidant, antitumor, neurostimulatory, anti-inflammatory, and anti-aging effects (Kunugi & Ali 2019; Pasupuleti et al. 2017). In addition, enzyme-treated RJ products have demonstrated additional biological activities. For example, protease-treated RJ (pRJ) and its ether extract have been shown to enhance antigen-specific immunoglobulin (Ig) A responses and increase expression of the universal M cell marker glycoprotein 2 (gp2), respectively (Kai et al. 2013).

Raw RJ contains two characteristic medium-chain fatty acids, *trans*-10-hydroxy-2-decenoic acid (10H2DA) and 10-hydroxydecanoic acid (10HDAA) (Yu et al. 2023). 10H2DA is the predominant fatty acid in raw Royal Jelly, present at approximately 1.2–4.5% (Yu et al. 2023). In contrast, 10HDAA is present in low concentrations of 0.3-1.2% (Yu et al. 2023). Although similar in structure, their biological activities differ, and previous studies have shown that 10H2DA contributes to the longevity and reproductive capacity of queens and plays an important role in larval

51 development (Kunugi & Ali 2019). It has also been associated with anti-inflammatory, immunomodulatory,
52 antioxidant, and antimicrobial effects (Yu, Yang et al. 2023; Liao, Alrosan et al. 2024). In addition, 10H2DA exhibits
53 estrogen-like and filaggrin-producing effects, contributing to skin health and hormone balance (Maeda et al. 2022;
54 Mishima et al. 2025). Furthermore, bioconversion of 10HDAA to 10H2DA by a one-step whole-cell catalytic reaction
55 has been established for industrialization (Fang et al., 2024).

56 In contrast, 10HDAA is known to inhibit the release of nitric oxide and IL-10 from mouse macrophages (Takahashi
57 et al. 2013; Chen et al. 2016; Isayama et al. 2020) and has the ability to stimulate the immune system by increasing
58 the number of M cells in the Peyer's patches of the gut and enhancing antigen-specific IgA expression, an effect not
59 seen with 10H2DA (Isayama et al. 2013). 10H2DA and 10HDAA are the major fatty acids found in raw RJ, but
60 10HDAA has a stronger ability to stimulate mucosal immunity.

61 However, the concentration of 10HDAA in raw RJ is inherently low, limiting its potential for immune enhancement.
62 To address this, we hypothesized that the effect of RJ on mucosal immunity could be amplified by converting
63 10H2DA to 10HDAA. Although there are no existing reports on bioconversion by whole-cell or enzymatic processes
64 for this purpose, bioconversion is a promising approach. Specifically, an enzyme from *Clostridium sporogenes* that
65 reduces the carbon-carbon double bond of similar substrates such as cinnamic acid (Mordaka et al. 2018).

66 The aim of this study is to isolate bacteria capable of converting 10H2DA to 10HDAA from the honeybee gut flora
67 and to produce 10HDAA-enriched RJ using this isolate. Furthermore, to evaluate the immunostimulatory potential
68 of bio-treated RJ and verify its ability to enhance mucosal immunity by promoting M-cell differentiation, activating
69 macrophages, and increasing IgA secretion, and to identify the reductase enzyme involved in the conversion and
70 characterize it through heterologous expression in *E. coli*.

71

Chapter 2 Isolation and Identification of 10H2DA-Converting Bacteria

2.1. MATERIALS AND METHODS

Chemicals and cultivation media

Pepsin and protease YBFII were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan) and Yamada Bee Company Inc. (Okayama, Japan), respectively. The raw royal jelly standardized to contain 1.6% 10H2DA and 0.4% 10HDAA was obtained from Yamada Bee Company, Inc. (Okayama, Japan). All other chemicals including 10H2DA and 10HDAA were of analytical grade. De Man-Rogosa-Shape (MRS) broth (Becton, Dickinson, and Corp., Franklin Lakes, NJ) and *trans*-galactosylated oligosaccharide (TOS) propionate (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) were used for the isolation, cultivation, and enrichment of *Lactobacillus* species and *Bifidobacteria*, respectively. Anaerobic jar and anerobic gas producing pouch, Anaeropack® Kenki (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) were used for anaerobic cultivation. Dulbecco's Modified Eagle's Medium (D-MEM) (FUJIFILM Wako Pure Chemical Corp.) and fetal bovine serum from HyClone Laboratories Inc. were used for cultivation of cells of the macrophage-like strain J774.1. Fluoresbrite® polychromatic red microspheres (Polysciences Inc., Warrington, PA, USA.) were used to assess macrophage phagocytic capacity.

Isolation of 10H2DA converting bacteria

Twelve *Lactobacillus* spp. have been isolated from the honey stomach, bee gut, or bee bread (Wang et al. 2018). Royal jelly is a honeybee secretion that is used in the nutrition of larvae and adult queens. 10H2DA is a special and unique compound among the components of royal jelly (Yu et al. 2023), and we hypothesized that there are bacteria metabolizing royal jelly components in the bodies of bees or their larvae, and that they are involved in the conversion of 10H2DA to 10HDAA. Therefore, 10H2DA-converting bacteria, particularly lactic acid bacteria and bifidobacteria among the bacterial complex of the gastrointestinal tract. Digestive tracts were collected from worker and queen bees and bee larvae obtained from Yamada Bee Farm. Subsequently, one gram of the gastrointestinal tract was aseptically mixed with 9 mL of sodium potassium phosphate buffer (pH 6.0) in 15 mL-test tube. The mixed solution was

designated as a digestive tract sample.

The digestive tract samples from Larval and from worker and queen bees were diluted 10 to 100,000 times by same buffer and spread on De Man-Rogosa-Shape (MRS) agar plates and *trans*-galactosylated oligosaccharide (TOS) propionate agar plates. Plates were incubated in a gas generator for anaerobic culture using Anaeropack® Kenki at 35 °C for 96 h. Colonies grown well on each plates were inoculated into MRS liquid medium and incubated anaerobically, described above. For initial separation and characterization, isolated samples were tested for Gram stain and catalase activity. Single colony isolation was carried out by using TOS agar plate (once) at 37 °C for 72 h and MRS agar plate (repeated three times) at 35 °C for 96 h.

To select strains capable of converting 10H2DA to 10HDAA (called as 10H2DA converting bacterium), isolates were anaerobically cultivated in MRS liquid medium containing 0.4% (w/v) 10H2DA at 35 °C for 96 h. After centrifugation of the culture, the cultural supernatants were analyzed by HPLC for the conversion. The concentrations of 10H2DA and 10HDAA were measured by Shimadzu prominence HPLC system (SHIMADZU Co., Kyoto, Japan) using a Sunniest C18 (4.6 mm i.d.× 250 mm; ChromaNik Technologies Inc., Osaka, Japan) at a flow rate of 1 mL/min at 40 °C. 10H2DA and 10HDAA were eluted with 0.1% TFA-acetonitrile (75:25, v/v) and were detected by photodiode array detection (PDA) and evaporative light scattering detection (ELSD), respectively. The retention times of 10H2DA and 10HDAA were 14.8 and 13.2 min, respectively (Fig. 1(a)). Finally, five strains could be selected as a 10H2DA converting bacterium.

Identification of strains capable of converting 10H2DA to 10HDAA

The protein mass spectra of the test bacteria obtained using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI/TOF MS) were compared with those of bacterial species data in the library at the Central Institute for Experimental Medicine and Life Science (Kanagawa, Japan). In addition, 16S rDNA sequencing, molecular phylogenetic analysis, genomic analysis, and average nucleotide identity testing were performed at Techno Suruga Laboratory Co., Ltd., (Shizuoka, Japan). Comparative analysis was performed for *Lactobacillus panisapium* and *Lactobacillus bombicola* which are closely related, described below.

122 The cellular fatty acid profile of *L. panisapium* comprised C_{18:1}ω9c, C_{16:0}, and *iso*-C_{19:0} as the major fatty acids
123 (Wang et al. 2018). The profile is one of properties is to classify the species and the closely related species, such as
124 *L. bombicola*, *Lactobacillus apis*, and *Lactobacillus helsingborgensis* (Wang et al. 2018). Preparation of cultivated
125 cells and their freeze dry cells and esterification of fatty acids were carried out according to the previously reported
126 procedures (Ikemoto et al. 1978). The cellular fatty acid methyl esters were dried, and the free fatty acids were
127 esterified with a hydrogen chloride–methanol reagent (5%–10%) for 3 h at 100 °C in a screw-cap tube. Methyl lauric
128 acid (C_{12:0}) was used as an internal standard. The esterified fatty acids were analyzed using a GC-MS-QP2010 Ultra
129 apparatus (Shimadzu, Kyoto, Japan) with a fused silica capillary column (InertCap 1MS; 0.25 mm × 30 m; GL
130 Science, Osaka, Japan). Helium was the carrier gas at a linear velocity of 35 cm/s. The column was held at 150 °C
131 for 2 min and then heated stepwise from 150 to 280 °C at 4 °C/min. The MS interphase and ion source temperatures
132 were 200 °C and 250 °C, respectively.

133 Melibiose, D-sorbitol, sucrose, maltose, raffinose, and rhamnose were added to tubes of pepton-yeast extract (PY)
134 medium as filter-sterilized solution, at a final concentration of 20.0 g/L. Growth was determined using the method as
135 described by Ma et al. (2017).

136

137 **Survival rates under simulated stomach and gastrointestinal conditions**

138 The isolated strains, after anaerobic cultivation in MRS medium at 35°C for 96 h, were washed with 0.5% (w/v)
139 NaCl solution. The bacterial cells were resuspended in the NaCl solution to a final concentration of 5%. Pepsin was
140 dissolved in this solution to achieve a concentration of 0.3% (w/v) and the pH was adjusted to 3.0 using 1N HCl.
141 This bacterial suspension was then mixed with 1 mL of pepsin solution and 0.5 mL of saline solution and preincubated
142 at 37°C. Subsequently, 100 µL of the bacterial suspension was combined with 500 µL of pepsin solution and 150 µL
143 of the NaCl solution, and the mixture was preincubated at 37°C for 1 h. A 2 µL sample of this mixture was spread on
144 MRS agar plates and incubated at 35°C for 96 h under anaerobic conditions.

145 To evaluate bile acid resistance, artificial bile acids were used. MRS medium was prepared with 0.3% Oxgall
146 (Bovine bile) by dissolving 60 mg of Oxgall in 20 mL of MRS medium. The bacteria grown in MRS medium were

seeded in 96-well microplates and centrifuged (3,000 ×g, 4°C, 10 min) to collect bacterial precipitates. These precipitates were then inoculated into MRS medium alone or MRS medium containing Oxgall, incubated under anaerobic conditions for 6 hours, and the absorbance (OD₆₆₀) of each well was measured

Bioconversion of 10H2DA to 10HDAA in royal jelly solution

The bioconversion of 10H2DA to 10HDAA in both untreated and protease-treated royal jelly solutions was investigated. Raw royal jelly (nRJ) was subjected to enzymatic degradation to produce protease-treated royal jelly (pRJ). For pRJ preparation, water was added to nRJ to achieve a 50% solution, which was then adjusted to pH 7.8 with NaOH. Protease YBFII was added to a final concentration of 0.5% (w/v), and the mixture was digested at 50°C for 2 h. Both nRJ and pRJ were diluted fivefold with purified water to prepare the fermentation mother liquor, which was adjusted to pH 6.5 using 2N NaOH and aerated with nitrogen gas to achieve a dissolved oxygen level of zero. Microbial culture medium constituted 10% of the total volume, and the mixture was incubated at 35°C for 120 h. During incubation, the pH was maintained at 6.5 with adjustments every 24 h, and nitrogen gas was bubbled through the solution for 30 min daily. Following fermentation, the royal jelly solution, now referred to as "fermented RJ," was sterilized by heating at 80°C for 20 min. The concentrations of 10H2DA and 10HDAA in the fermented solutions were quantified using HPLC.

Safety Analysis of Fermented Royal Jelly

In vitro, animal, and human safety studies were conducted on fermented RJ powder (lot: YHRP-M-201022-2), hereinafter referred to as fRJ. The fRJ was obtained by adding 25.8% excipient to the solid content of the fermented RJ solution, followed by lyophilization. In vitro and animal safety studies were conducted by the BioSafety Research Center Inc., (Shizuoka, Japan), and human overdosage studies were conducted by the Research Center for Immunological Analysis, Inc (Okayama, Japan).

In vitro reverse mutation studies were performed using bacteria and acute and repeated dose studies in animals. This study was approved by the Ethical Review Board of Yamada Bee Company, Inc. (approval no. 16000050).

2.2. RESULTS AND DISCUSSION

Isolation of lactic acid bacteria and bifidobacteria capable of converting 10H2DA to 10HDAA from honeybees

A total of four, seventy-three, and eighty gram-positive and catalase-negative strains were isolated from samples obtained from larvae, worker honeybees, and queen honeybees, respectively. Out of the 157 strains, five strains, no. QB 3-1-4, QB 3-1-9, QB 3-1-10, QB 3-2-4, and QB 9-1-6 demonstrated the ability to convert 10H2DA to 10HDAA. The conversion efficiency of no. QB 9-1-6 was approximately 100%, while the conversion efficiency of the other four strains were less than 50%. Consequently, these five strains were selected as candidate strains for further analysis.

Identification of 10H2DA converting strains

The five strains were tried to identify and classify, based on bacterial protein spectra using MALDI/TOF MS and 16S rRNA gene sequencing. Their mass spectral data did not match any of the tested bacterial protein spectra in the Bruker Daltonics MALDI Biotyper libraries (MBT Compass Library V12 and MBT Filamentous Fungi Library V5), making identification impossible. The 16S rRNA gene sequences of the five strains were 100% identical to each other. A homology search revealed that the strains had the highest identity (99.7%) with *L. panisapium* Bb2-3 (KX447147). Among the five isolates, strain QB9-1-6 was selected based on its 10H2DA conversion ability and was designated as strain M1. Phylogenetic analysis indicated that strain M1 belongs to a group consisting of *L. panisapium* and its closely related species, *L. bombicola*, *L. apis*, and *L. helsingborgensis* (Wang et al. 2018; Fig. 2). These related species can be classified according to their sugar assimilation ability and cellular fatty acid content (Wang et al. 2018). The cellular fatty acid profile of strain M1 comprised C_{18:1ω9c} (10%), C_{16:0} (20%), and *iso*-C_{19:0} (30%) as the major fatty acids (>10%). This profile differed from those of closely related species (Table 1). Sugar assimilation performance was found to be facultative towards D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, amygdalin, esculin, iron citrate, salicin, and D-cellobiose. Compared to previous findings on sugar assimilation in closely related species, strain M1 assimilated melibiose and sucrose but not maltose and D-sorbitol. These findings indicate that strain M1 belongs to the species *L. panisapium*. Additionally, strain M1 showed resistance to gastric and bile acids.

197

198 **Bioconversion of 10H2DA to 10HDAA in nRJ and pRJ broth**

199 When a diluted solution of nRJ was used as the fermentation broth, the growth of strain M1 was poor, and the
200 conversion reaction did not proceed effectively (Fig. 1). This finding could be attributed to the function of proteins
201 such as major royal jelly protein 1 (MRJP1) (Brudzynski and Sjaarda, 2015), which is an RJ protein with potential
202 bactericidal activity. Another reason may be that the proteins in nRJ are not in a form that can be used by the M1
203 strain, but are enzymatically degraded to pRJ, which is converted to a polypeptide that can be used. The amount of
204 10H2DA in the culture supernatant gradually decreased and was completely converted to 10HDAA by day 5 of
205 incubation. Culturing was performed under anaerobic conditions; the growth of M1 bacteria and sufficient conversion
206 of 10H2DA to 10HDAA did not occur in the presence of oxygen (Fig. 1). The protein, lipid, and total sugar content
207 of royal jelly are 12-15%, 3-6%, and 10-16%, respectively (Chen & Chen, 1995). Given that high conversion was
208 observed in protease-treated pRJ broth, it is likely that strain M1 cannot efficiently utilize untreated royal jelly
209 proteins as a carbon, nitrogen, and/or energy source.

210

211 **Safety analysis of fermented royal jelly as a food supplement**

212 **Reverse mutation test using bacteria**

213 The number of reverse mutant colonies in the fRJ-treated group was no more than twice that in the negative
214 control group for either the -S9- or +S9-treated strains. No growth-inhibitory effects were observed on the test
215 strains at either dose. The positive control induced a significant reversion of mutation in each test strain

216 **Acute toxicity test**

217 No animals from either sex died during the observation period. Abnormalities in general condition or weight
218 changes were not observed in any treatment group. In males, cysts and depressed foci in the kidneys were observed
219 in the control group, diverticula in the small intestine (ileum) in the 10-HDAA 51.9 mg/kg group, and transhepatic
220 nodules in the liver in the 103.8 mg/kg group. These findings were considered spontaneous changes commonly
221 observed. No abnormalities were observed in females. There were no deaths in either sex across the 26.0, 51.9, or

103.8 mg/kg dose groups of 10HDAA. No changes in general condition, body weight, or necropsy findings could be attributed to the administration of the test substance.

Repeated dose test

None of the animals died during the experiments. Differences in body weight or body weight gain were not significantly different between the control and test groups in either sex during the treatment period. Urinalysis showed higher total urinary sodium excretion in males in the 51.9 mg/kg/day group of 10-HDAA and in females in the 26.0 and 51.9 mg/kg/day groups than those in the other groups. However, no change in blood sodium concentration was observed, which was presumably a result of excretion of the sodium contained in the test substance (approximately 7.5%) and was not considered to be a toxic effect. The number of positive ketone bodies and urine protein in males in the 13.0 mg/kg/day and higher dose groups increased dose-dependently. In females, the number of positive ketone bodies in the 26.0 and 51.9 mg/kg/day groups, and urine protein in the 51.9 mg/kg/day group increased dose-dependently. Despite these increases, toxic effects related to increased ketone and urine protein levels were not considered to be present, as blood biochemical tests showed no evidence of abnormal glucose metabolism, starvation, or renal damage, and histopathological tests showed no evidence of liver, pancreas, or kidney damage. No other abnormalities were found in the general condition, body weight, food consumption, hematology, blood biochemistry, ophthalmology, organ weight, necropsy, or histopathology, which could be attributed to administration of the test substance.

Human overdose test

Twenty patients consented to participate in the screening (SCR) and twelve of the enrolled participants consumed the study diet, among which, 10 participants completed the study and two participants withdrew from the study for personal reasons. One of the 10 participants (ID: 4) did not participate in V1 due to fever but was included in the final case count as he had participated in V0 and V2. The analyses included 3 males and 7 females with a mean age of 40.1 ± 4.5 years and a mean body mass index of 21.8 ± 1.2 for males and 20.5 ± 2.3 for females. Adverse events were graded by the investigators on the following four-point scale: 'relevant', 'probably relevant', 'probably not relevant', and 'not relevant'. The number (incidence) of adverse events was 6/12 (50.0%), of which 1/12 (8.3%)

247 was confirmed as adverse event (rashes) for which a causal relationship could not be excluded. The participant
248 developed a 'rash' during consumption of the test food (day 17) and took his anti-allergic medication, but the
249 symptoms did not subside and the rash spread all over body; hence, he consulted a dermatologist on day 24 of
250 consumption. The dermatologist suggested no connection to the test food but could not completely rule it out. The
251 participant was treated with a steroid/antihistamine combination, and the symptoms abated, but continued two
252 weeks post-study. The timing and duration of symptoms were not related to the duration of test food consumption,
253 suggesting a causal relationship was unlikely.

254 No serious adverse events or reactions leading to death were observed. Adverse events or reactions leading to
255 discontinuation of the study were not observed. No clinically relevant findings were observed during physical
256 examination, physiological examination, urinalysis, hematology, or blood biochemistry. In summary, no adverse
257 events were clearly causally related to the test product, and no adverse events of moderate or greater severity or
258 clinically concerning changes in laboratory values were observed. These findings suggest that it is safe for healthy
259 adults to consume excessive amounts (five times the dose) of fRJ-containing foods.

2.3. FIGURES AND TABLE

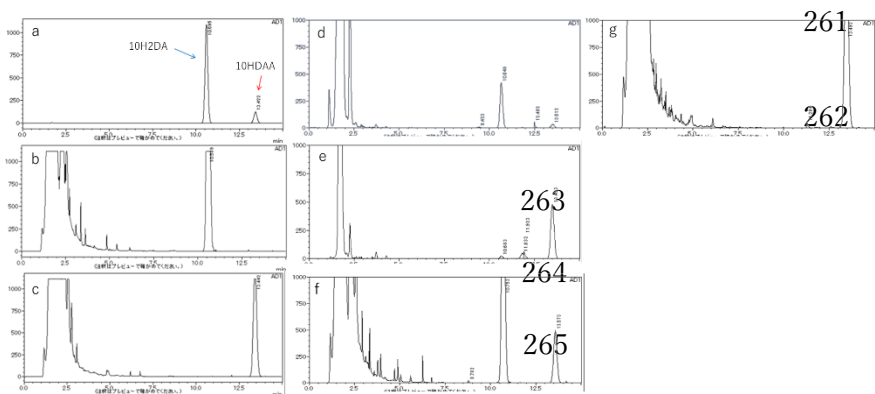


Figure 1. HPLC separation and determination of 10-hydroxy-2-decenoic acid (10H2DA) and 10-hydroxydecanoic acid (10HDAA)

HPLC separation and determination of 10-hydroxy-2-decenoic acid (10H2DA) and 10-hydroxydecanoic acid (10HDAA) in the cultural supernatants and raw royal jelly (RJ) and protease-treated RJ. The isolate, strain M1, was anaerobically cultivated (b-e), as described in the main text. In addition, bioconversions of 10H2DA to 10HDAA were compared under aerobic and anaerobic conditions (f and g). (a) Standards, 10H2DA (retention time [min], 10.5), and 10HDAA (13.5); (b) control (without cultivation); (c) cultural supernatant in strain M1; (d) cultural supernatant in raw RJ broth; and (e) cultural supernatant in enzyme-treated RJ broth. (f) Aerobic conditions and (g) anaerobic conditions.

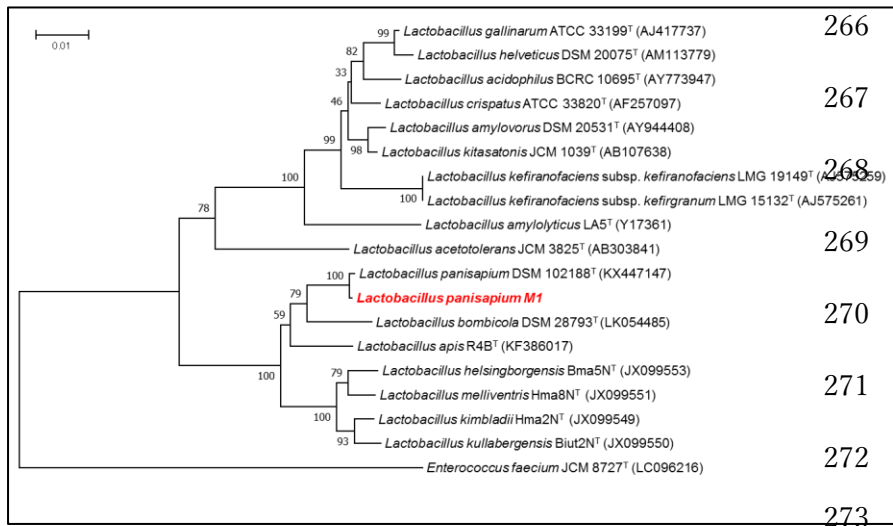


Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences

16S rRNA gene sequences showing the relationship between strain M1 and its phylogenetically close relatives. Bootstrap values based on 1,000 replications are listed as percentages at the branching points.

274 **Table 1. Comparison of cellular fatty acid content (%s) of strain M1 with its phylogenetically**
275 **closest relatives.**

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	2.53 ± 0.24	1.3	0.8	0.6	0.9
C _{15:0}	0.202 ± 0.11				
C _{16:0}	20.632 ± 1.07	16.2	15.2	13.0	18.6
C _{18:0}	5.044 ± 0.44	5.1	3.3	5.5	6.0
Unsaturated					
C _{18:1 n9}	52.318 ± 0.70	54.8	69.0	64.6	63.0
C _{18:1 n7}	7.65 ± 0.39	4.9	3.9	5.4	-
C _{19:1}	5.73 ± 0.31				
Branched-chain					
C _{19:0 iso}	5.26 ± 0.44	10.5	2.1	7.0	-

276 # Strains: 1, strain M1; 2, *L. panisapium* strain Bb 2-3^T; 3, *L. bombicola* DSM 28793^T; 4, *L. apis* LMG 26964^T;
277 5, *L. helsingborgensis* DSM 26265^T (Wang et al. 2018).

278

Chapter 3 Evaluation of Bioactivity in fRJ's Immune-stimulating Effects

3.1 MATERIALS AND METHODS

Evaluation of M cell differentiation-promoting activity

The ability of fRJ to promote M cell differentiation, as previously reported for 10HDAA, was evaluated. Using a method described by Isayama et al. (2020), monolayer-treated Caco-2 cells were co-cultured with pRJ or fRJ (1.7 mg/well), and the expression levels of glycoprotein 2 (GP2), a marker of mature M cells, were analyzed using fluorescence-activated cell sorting (FACS).

Measurement of macrophage phagocytosis

The phagocytic ability of fRJ was assessed using cells of the macrophage-like strain J774.1. The J774.1 cells were cultured in D-MEM containing 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA) and 1% penicillin-streptomycin-glutamine (Thermo Fisher Scientific K.K., Tokyo, Japan).

fRJ (lot: 20200512) was prepared in the culture medium at a concentration of 1 mg/mL. The solution was heated at 37 °C for 15 min and sterile filtered through a 0.20 µm filter.

Two milliliters of Fluoresbrite® polychromatic red microspheres (Polysciences Inc.) (5.68×10^9 beads/mL) were measured and centrifuged at $2000 \times g$ for 5 min, and the supernatant was separated. Two milliliters of culture medium was added to the precipitated beads to obtain a suspension of 4×10^8 beads/mL.

The J774.1 cells were harvested every 3 to 4 days at $1-2 \times 10^5$ cells/mL and used for testing. Cells were seeded at 2×10^5 cells/well in 48-well plates and incubated for approximately 16 hours at 37 °C in a 5% CO₂ incubator. After pre-culture, the medium was removed, and the prepared fRJ was added. fRJ was incubated in a 5% CO₂ incubator at 37 °C for 1 h. Phycoerythrin (PE)-labelled polystyrene bead suspension was added to reach 4×10^6 beads/well and incubated in a 5% CO₂ incubator at 37 °C for 2.5 h. After incubation, the cells were collected, and the phagocytosis rate and mean fluorescence intensity (MFI) were measured using a flow cytometer.

Means and standard deviations were calculated using basic statistics. Statistical analyses were performed using JMP

304 5.1 with a two-sided significance level of 5% for all tests. The calculated data are presented in tables and graphs as
305 mean $\pm$ standard deviation. The phagocytosis rate and MFI were compared between the control and fRJ groups using
306 Dunnett's test

307

308 **Effect on salivary IgA in clinical study**

309 The effect of orally administered fRJ on salivary IgA levels was tested in healthy adults. The study was approved
310 by the Ethical Review Board of Yamada Bee Company, Inc., on January 11 and July 27, 2021 (approval no. 2020027)
311 and was conducted in accordance with the principles of the Declaration of Helsinki. All participants provided written
312 informed consent and 12 participants with low IgA secretion rates were selected for the study. This study was an
313 open-label study and the test food was fRJ containing 20 mg 10HDAA per three tablets as a standard.

314 Participants administered three tablets of the test sample once a day for 3 weeks. The primary endpoint was the
315 rate of salivary IgA secretion (g/min), and the secondary endpoints were salivary IgA concentration (g/mL), visual
316 analog scale (VAS) questionnaire (sleep quality, fatigue, and stress levels), bowel frequency, and cold symptoms.
317 Tests were performed before ingestion and at 1, 3, 7, 14, 21, and 35 days after ingestion (2 weeks after the end of
318 ingestion), and each item was scored. Measurements are presented as mean $\pm$ standard deviation and comparisons
319 were made using a paired t-test.

320 Additionally, the effect on human salivary IgA was also tested using a selection procedure that did not restrict
321 participants to those with a low IgA secretion rate. This study was conducted with the approval of the Ethical Review
322 Board of Yamada Bee Company, Inc. on July 27, 2021 (approval no. 2021007). This study was conducted in
323 accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent,
324 and 20 were selected for the study. This open-label study investigated the effect of fRJ on salivary IgA in healthy
325 adults. The test diet consisted of fRJ (13 capsules per day) containing at least 20 mg of 10HDAA. Twenty healthy
326 Japanese adults (men and women) aged 18 years or older consumed the fRJ-containing diet for 8 weeks, and the rate
327 of salivary IgA secretion (g/min) was assessed.

328

Statistical analysis

JMP 5.1 (SAS Institute Japan Ltd., Tokyo, Japan) was used for statistical analyses of clinical studies and GraphPad Prism version 7 (GraphPad Software, Boston, MA, USA.) for statistical analyses of in vitro studies. All tests were two-tailed and a significant level of 5% was taken as significant.

3.2. RESULTS AND DISCUSSION

fRJ is approximately more potent than pRJ in promoting M cell differentiation

The M cell differentiation-promoting effects of pRJ and fRJ were assessed using equal amounts of pRJ and fRJ. pRJ showed higher GP2 expression than that in DMSO. fRJ showed an additional higher expression population than that of pRJ (Fig. 3). This suggests that fRJ has a more potent effect in promoting M cell differentiation per unit weight, potentially reducing the required intake of RJ.

Functional assessment of fRJ (macrophages, IgA)

Based on the M cell differentiation-promoting effect and safety assessment tests of fRJ, a preliminary functional assessment was performed on macrophages and IgA, which are the immune cells that are downstream of M cells in the intestinal immune system.

Macrophage phagocytosis activity

Compared to the control, fRJ significantly increased the phagocytic rate and MFI at all tested volumes (Figs. 4 and 5). MFI was significantly higher in the fRJ-treated group than in the pRJ group at 0.25 mg/mL (Fig. 6).

Salivary IgA secretion rate

Twelve participants (4 men and 8 women) completed the study, with a mean age of 38.3 ± 15.2 years. After 1, 3, 7, 14, 21, and 35 days of ingestion, salivary IgA secretion rates were not significantly different from pre-ingestion levels (Fig. 7). However, participants with low salivary IgA secretion rates at pre-ingestion showed a significant increase on days 14 and 21 compared to pre-ingestion (Fig. 8). The number of bowel movements per day was averaged per week, and no significant differences were observed between the second and third weeks of ingestion

354 compared to the first week. Seven examinations and a cold symptom questionnaire were conducted, but the low
355 incidence of symptoms (three cases) made it unfeasible to evaluate these results relative to pre-ingestion. A
356 significant increase in salivary IgA secretion rate was also observed after 8 weeks of ingestion in studies where
357 participants were not limited to those with potentially low IgA secretion rates (Table 2).

358

359 These findings suggest that fRJ, a biotransformation of RJ, could be used as a food to strengthen the intestinal
360 immune system. In the future, we plan to verify the mechanism by which the effects demonstrated in this study
361 occur and the effect of fRJ on suppressing infection in humans.

362 **3.3. FIGURES AND TABLE**

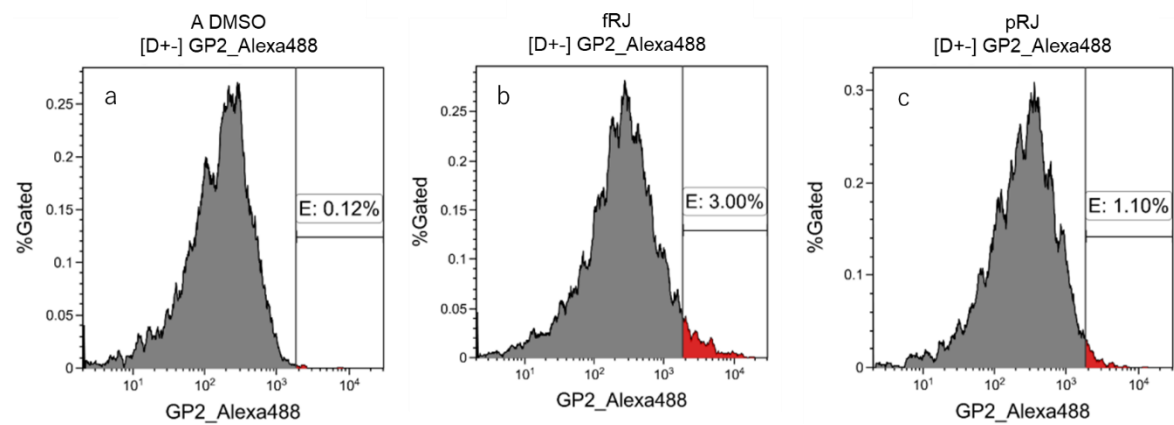


Figure 3. Assessment of M cell differentiation-promoting activity of RJ.

Representative flow cytometry results showing GP2 expression in Caco-2 cells treated with 0.85 mg/mL fRJ and pRJ. Comparison of the occupancy of GP2 high-expressing areas (red). Abbreviations: pRJ, raw royal jelly was subjected to protease degradation; fRJ, the protease-treated royal jelly was fermented by strain M1.

369

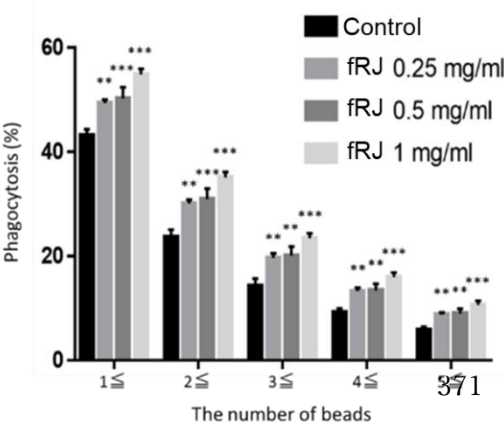


Figure 4. Phagocytosis rate by fRJ

Values are presented as mean ± SD. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 relative to the control using Dunnett's test. fRJ: fermented royal jelly. Abbreviation: fRJ, the protease-treated royal jelly was fermented by strain M1.

372

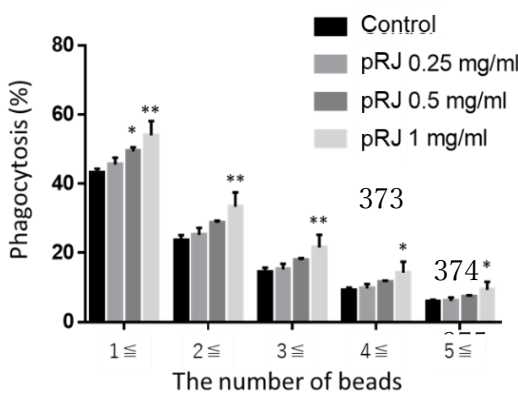


Figure 5. Phagocytosis rate by pRJ

Values are presented as mean ± SD. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 relative to the control using Dunnett's test. fRJ: fermented royal jelly. Abbreviation: pRJ, protease-treated royal jelly.

376

377

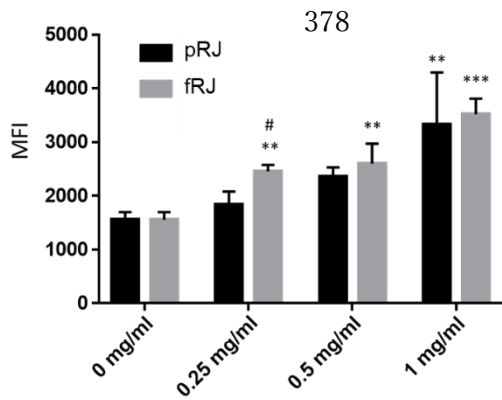


Figure 6. Average fluorescence intensities using fRJ and pRJ

Values are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to the control using Dunnett's test. Abbreviations: pRJ, raw royal jelly was subjected to protease degradation; fRJ, the protease-treated royal jelly was fermented by strain M1; MFI, mean fluorescence intensity.

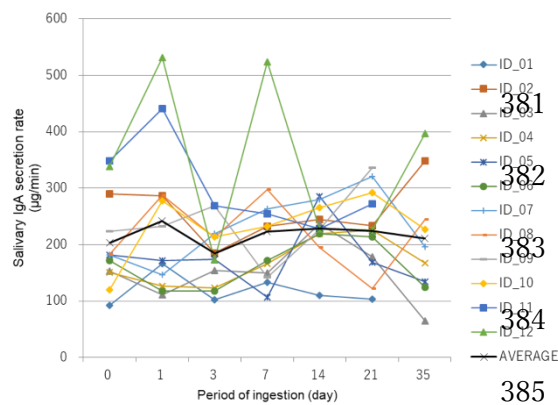


Figure 7. IgA secretion rate ($\mu\text{g}/\text{min}$) in 12 subjects

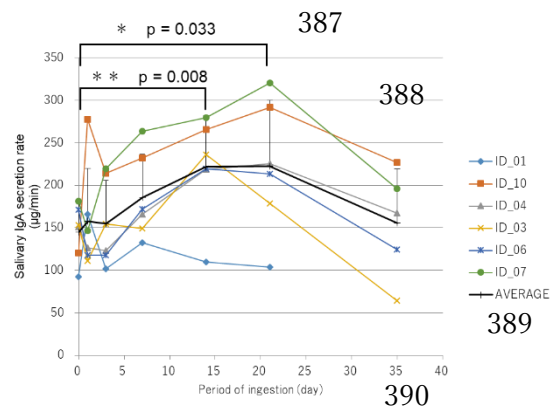


Figure 8. IgA secretion rate ($\mu\text{g}/\text{min}$) in 6 subjects

IgA secretion rate ($\mu\text{g}/\text{min}$) in six subjects with a low IgA secretion rate before starting treatment. * $p < 0.05$, ** $p < 0.01$ relative to the control

396 **Table 2. Salivary immunoglobulin (Ig) A levels and salivary secretion rate.**

397

Assessment items	Pre-ingestion	Period of ingestion			
		1 day	2 weeks	4 weeks	8 weeks
Salivary IgA secretion rate (µg/min)	201.140 ± 24.711	189.654 ± 20.743	193.952 ± 23.649	213.722 ± 29.211	237.591 ± 30.819*
Salivary IgA concentration (µg/mL)	584.544 ± 58.535	542.567 ± 41.040	516.148 ± 28.152	583.810 ± 41.482	644.681 ± 61.173
Salivary secretion rate (mL/min)	0.395 ± 0.057	0.368 ± 0.032	0.393 ± 0.046	0.374 ± 0.043	0.409 ± 0.051

400

401

402

403 Comparison with pre-ingestion; *p < 0.05 (paired t-test); mean ± SD.

404

Chapter 4 Heterologous Expression and Characterization of 10H2DA Reductase from *Lactobacillus panisapium* M1

4.1 MATERIALS AND METHODS

Organism and culture conditions

L. panisapium M1 (Itatani et al., 2024) was used as the source for a reductase for 10H2DA and its corresponding gene. Strain M1 was cultivated in De Man–Rogosa–Sharpe (MRS) broth (Becton, Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.4% (w/v) 10H2DA at 35°C for 96 h under anaerobic conditions, maintained using an AnaeroPack® Kenki (Mitsubishi Gas Chemical Co. Ltd., Japan) (Itatani et al., 2024). For recombinant plasmid construction and gene expression, *E. coli* strain HST08 (Takara Bio Inc., Kusatsu, Japan) was used as the host. The pCold I vector (Takara Bio Inc.) served as the cloning and expression vector for the reductase gene from strain M1. *E. coli* transformants were cultivated in Luria-Bertani (LB) broth (Lennox type, Nacalai Tesque, Kyoto, Japan), supplemented with ampicillin (50 µg/mL) as needed. Gene expression was induced with 0.1 mM isopropyl-β-D-(-)-thiogalactopyranoside (IPTG) when required.

Cloning of the reductase gene from strain M1 and construction of the expression vector

Genomic DNAs were extracted and purified from *L. panisapium* M1, using NucleoSpin® Microbial DNA Kit (Takara Bio Inc.) according to manufacturer's instruction. The reductase gene from strain M1 was amplified by PCR using primers Frd_F (5'-CATATCGAAGGTAGGATGGCTAAATTTGTCTTTACACCAAGC-3') and Frd_R (5'-ACCGAGCTCCATATGCTATTTTGCTGAACGAACAAATTCAGC-3'). Simultaneously, the pCold I vector was linearized using primers pCold_1 (5'-CCTACCTTCGATATGATGATGATGATGATG-3') and pCold_2 (5'-CATATGGAGCTCGGTACCCTCG-3'). The amplified gene ligated with the linearized vector using the In-Fusion HD cloning kit (Takara Bio Inc.) to construct the recombinant expression plasmid (pColdI_Frd). The plasmid was introduced into *E. coli* strain HST08.

A site-directed mutation in the reductase from strain M1 was introduced to replace the glycine residue at position 269 with threonine (Thr269) based on structural comparisons between the reductase from strain M1 and the fumarate

431 reductase flavoprotein subunit from *Shewanella frigidimarina* (described below). To create this mutation, two
432 primers, Frd_F and M1Frd_G269T_anti (5'-AGTAATCAAGTAAACATGGTCTGTGTC-3') and
433 M1Frd_G269T_sense (5'-GTTTACTTGATTACTGAAGGACTTCGTGG-3'), were used along with Frd_F and
434 Frd_R to amplify the respective DNA fragments. These fragments were then assembled with the linearized
435 pCold I vector using the In-Fusion® HD Cloning Kit, resulting in the mutated expression plasmid
436 (pColdI_Frd_G269T). The sequence of the mutated construct was also verified, and the plasmid was introduced into
437 *E. coli* strain HST08.

438 *E. coli* transformants carrying either the wild-type or mutated reductase constructs were used to express
439 recombinant reductase enzymes for further characterization. For heterologous expression, transformants were
440 cultured in 1 L of LB broth at 37°C with shaking (140 rpm) until the optical density at 600 nm (OD₆₀₀) reached 0.4–
441 0.5. The culture temperature was then reduced to 15°C for 30 min, after which gene expression was induced by
442 adding 0.5 mM isopropyl-β-D(-)-thiogalactopyranoside (IPTG). The cultures were maintained at 15°C with shaking
443 (140 rpm) for an additional 24 h.

444

445 **Partial purification of reductase from *L. panisapium* M1 Wild-type reductase from *L. panisapium* M1**

446 All purification steps were carried out at 4°C, with centrifugation performed at 15,300×g for 10 min at the same
447 temperature. Cells (1 g) of strain M1, which had been harvested and frozen, were resuspended in 3 mL of 20 mM
448 Tris-HCl buffer (pH 7.5) (Buffer A) and 30 µL of 780 mM FAD solution. The cell suspension was ground with 3 g
449 of quartz sand in a pre-chilled mortar for 15 min. After centrifugation, the supernatant was collected and used as the
450 cell extract for subsequent purification steps. A typical summary of the wild-type reductase purification is presented
451 in Table 3 with detailed procedures by ion-exchange and hydrophobic chromatography steps included in its footnote.
452 The final purified preparation was analyzed by SDS-PAGE for protein identification (Fig. 9(a)).

453 **Recombinant reductase heterologously expressed by *E. coli***

454 Cells (0.12 g) of the *E. coli* transformants, harvested and frozen, were resuspended in 3.0 mL of Buffer A and 30
455 µL of 780 mM FAD solution. The cells were disrupted using an Ultrasonic Liquid Processor XL-2000 (Qsonica,

LLC., Newton, CT). After centrifugation, the supernatant (cell extract, 3.0 mL) was applied to a column (1.0×4.0 cm) of DEAE-Toyopearl (Tosoh Corp., Tokyo, Japan) equilibrate with buffer A. After washed step with 9 mL of buffer A, proteins were eluted with a linear gradient (0–0.75M) of NaCl in 66 mL of buffer A. Fractions of 1.5 mL were collected at a flow rate 1.0 mL min⁻¹. After addition of 50 µL of 780 mM FAD solution to each fraction, the reductase activity toward 10H2DA was measured. The high active fractions were pooled and dialyzed against 20 mM MES (2-morpholinoethanesulfonic acid, monohydrate)-NaOH buffer (pH 6.5) (buffer B). The dialyzed solution was applied to a GigaCap Q- 650 column (1.0×4.0 cm, Tosoh Corp.) equilibrate with buffer B. The liner gradient program and fractionation volume were followed the procedure outlined above. A typical summary of purification of the recombinant reductase is shown in Table 9(b). The purity of the SDS-denatured protein was verified by SDS-PAGE (Fig. 9(b)), as described below. The active fractions were pooled, and the protein concentration was measured using the Lowry method.

Enzyme assays

In this study, enzyme assay for a 10H2DA specific reductase in strain M1 was carried out, based on previously reported reductases from *Lactobacillus* spp. (Gao, Ren et al. 2012; Santamaría, Reverón et al. 2018) with modification. The reaction mixture, in a total volume of 1.0 mL, consisted of 90 µL of 50 mM potassium phosphate buffer (pH 6.0), 5 µL of 20 mM 10H2DA, 10 µL of 3 mM NADH, and 1 to 10 µL of enzyme solution. FAD was pre-added to the purified enzyme fraction, described above. The assay was performed in an anaerobic cuvette, which could be maintained in an anaerobic state by securely covering it with a round cuvette cap (BRAND GMBH + CO KG, Wertheim, Germany). The mixture was incubated at 40°C for 5 min, and then enzyme was added to initiate the reaction. Activity was monitored as a decrease in the absorbance of NADH at 340 nm. Activity was presented as the initial rate of NADH oxidation observed, using an extinction coefficient of $6.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for NADH at 340 nm. Activity units represent micromoles of NADH oxidized per minute.

Characterization of recombinant reductase

481 The enzyme activity toward 10H2DA was assayed using the recombinant reductase purified as described above,
482 unless otherwise stated.

483 **Effect of pH on enzyme activity and stability**

484 Enzyme assay was carried out at pH 5.0–8.0 in the following 100 mM buffers: potassium phosphate buffer (pH
485 5.0–8.0) and MES (2-morpholinoethanesulfonic acid, monohydrate)–NaOH (pH 6.0–7.5). The pH stability of the
486 recombinant reductase was determined by dialyzing the enzyme against the buffers, 50 mM sodium acetate buffer
487 (pH 4.5 and 5.0), 50 mM MES–NaOH buffer (pH 5.5–7.5), and 50 mM Tris–HCl buffer (pH 7.0–9.0) at 4°C for 15 h
488 and then measuring the residual enzyme activity at pH 6.0 and 40°C.

489 **Effect of temperature on enzyme activity and stability**

490 The temperature dependence of enzyme activity was examined over the range of 35–60°C. Thermal stability was
491 evaluated by preincubating the purified enzyme at various temperatures (30–60°C) for 30 min, followed by measuring
492 residual activity at pH 6.0 and 40°C.

493 **UV-visible spectrum of recombinant reductase**

494 The UV-visible absorption spectrum of the purified enzyme (2.4 mg/mL) dialyzed against buffer A was recorded
495 using buffer A as reference, with an Eppendorf® bio-spectrophotometer (Eppendorf AG, Hamburg, Germany).

496

497 **Detection of enzymatic reaction product by gas chromatography-mass spectrometry (GCMS)**

498 **Conversion of 10H2DA by the purified recombinant reductase**

499 The reaction mixture contained 126 mL of sodium potassium phosphate buffer (pH 6.0), 28 mL of purified enzyme
500 solution (1.2 mg/mL), 14 mL of 9 mM NADH, and 0.7 mL of 20 mM 10H2DA. After incubation at 40°C for 1.5 h.
501 The pH of the concentrated solution was adjusted to pH 3.0 with 3N HCl, and the solution was extracted with ethyl
502 acetate. The upper layer was collected and evaporated to dryness. The residue was dissolved in ethyl acetate, and the
503 reaction product was partially purified by TLC. TLC was performed on Merck silica gel 60F254 plates (Merck KGaA,
504 Darmstadt, Germany) and developed in the solvent hexane-ether acetate-acetic acid (15:5:1, v/v/v). The reaction
505 products were developed with 5% (w/v) sodium phosphomolybdate–ethanol. Here, the area on the TLC plate (Rf

value = 0.29) was scratched and the products were extracted with methanol. The collected products in the dried residue were incubated with *N,O*-bis(trimethylsilyl)trifluoroacetamide at 100 °C for 1.5 h. The trimethylsilylated products were analyzed by GC-MS as described below. The resulting trimethylsilylated products were analyzed by GC-MS using a GCMS-QP2010 Ultra system (Shimadzu, Kyoto, Japan). For GC-MS analysis, a fused silica capillary column (InertCap 1MS; 0.25 mm × 30 m; GL Science) was used with helium as the carrier gas at a linear velocity of 35 cm/s. The column temperature was programmed from 150°C (held for 2 minutes) to 200°C at a rate of 2°C/min, followed by a hold at 200°C for 50 minutes. Peaks corresponding to the trimethylsilylated derivatives of authentic 2HDA and 2-HDAA were observed at retention times of 14.8 and 13.2 min, respectively.

514

515 **Conversion of 10H2DA by freeze-dried *E. coli* cells including heterologous expressed reductase**

516 Since a system for regenerating NAD has not yet been established, the conversion of 10H2DA was performed
517 using whole cells. *E. coli* transformant was cultivated and the reductase was heterologously expressed. After
518 harvesting cells, they were incubated at -80 °C in eggplant flask. They were lyophilize using a freeze dryer
519 (EYELA FD-1000; Tokyo Rikakikai, Tokyo, Japan). The reaction mixture contained 210 mL of sodium potassium
520 phosphate buffer (pH 6.0), 200 mg of the freeze-dried cells, 0.4 mL of 780 mM FAD, 5.0 mL of 30 mM NADH, and
521 2.4 mL of 20 mM 10H2DA. It was incubated at 30°C for 6.0 h with mixing by magnetic hot stirrer at 100 rpm. After
522 centrifugation, the supernatant was collected. The reaction product was extracted and purified as described above.

523

524 **Detection of reaction product, 10HDAA by GC-MS**

525 The collected products in the dried residue were incubated with *N,O*-bis(trimethylsilyl)trifluoroacetamide at 100
526 °C for 1.5 h. The trimethylsilylated products were analyzed by GC-MS as described below. The resulting
527 trimethylsilylated products were analyzed by GC-MS using a GCMS-QP2010 Ultra system (Shimadzu, Kyoto,
528 Japan). For GC-MS analysis, a fused silica capillary column (InertCap 1MS; 0.25 mm × 30 m; GL Science) was used
529 with helium as the carrier gas at a linear velocity of 35 cm/s. The column temperature was programmed from 150°C
530 (held for 2 min) to 200°C at a rate of 2°C/min, followed by a hold at 200°C for 50 min. Peaks corresponding

531 to the trimethylsilylated derivatives of authentic 10H2DA and 10HDAA were observed at retention times of 14.8 and
532 13.2 min, respectively.

533

534 **In silico analyses**

535 Sequence analysis was carried out using GENETYX-WIN software (ver. 14, Genetyx Co., Tokyo, Japan). Multiple
536 sequence alignment was done using CLUSTAL W version 2.1 (<http://clustalw.ddbj.nig.ac.jp/>). Automated
537 comparative modeling of the three-dimensional protein structure of the reductase from strain M1 was performed
538 using SWISS-MODEL (<http://swissmodel.expasy.org>) and visualized with the PyMOL molecular graphic system
539 (version 2.5.1). The model was based on the structure of fumarate reductase flavoprotein subunit from *Shewanella*
540 *frigidimarina* (PDB code: 1Y0P), with a sequence identity of 40% (182/448 amino acids) and a GMQE score of 0.82.

541

542 **Statistical analysis**

543 All experiments were performed in duplicate and replicated at least three times. All data were calculated as the
544 mean $\pm$ standard deviation. Analysis of variance (ANOVA) was selected to test value differences ($p < 0.05$). For
545 comparison of different groups, the data were analyzed using one-way ANOVA and Tukey's multiple comparison.
546 All calculations were conducted using GraphPad Prism software (ver. 6.02 for Windows, La Jolla, CA, USA).

547

548 **4.2. RESULTS AND DISCUSSION**

549 **Isolation and sequence analysis of 10-hydroxy-2-decenoic acid reductase from *L. panisapium* M1**

550 Strain M1 demonstrated 100% conversion efficiency of 10H2DA to 10HDAA when anaerobically cultivated in
551 MRS liquid medium containing 0.4% (w/v) 10H2DA at 35°C for 96 h (Itatani et al. 2024). The enzyme responsible
552 for converting 10H2DA to 10HDAA was identified based on its reducing activity, coupled with NADH oxidation in
553 the presence of FAD. Although several protein bands were observed in the SDS-PAGE analysis of the partially
554 purified active fraction (Fig. 9(a)), further attempts were made to identify the specific protein.

555 LC-MS-MS Mascot analysis indicated that the 53 kDa-protein (Fig. 9(a)) belongs to the FMN/FAD-binding

556 oxidoreductase family from *Lactobacillus* spp. Additionally, other detected 51 kDa- and 39 kDa-proteins (Fig. 9(a))
 557 included translation elongation factors (WP_103751762 and WP_103751563). Matched peptides from strain M1
 558 reductase corresponded to a hypothetical oxidoreductase (WP_103751687) from *L. panisapium*, with a protein
 559 coverage of 68.5%. A 1.4 kb fragment was amplified from the genomic DNA of strain M1 using the primers Frd_F
 560 and Frd_R. The fragment carried a 1,398 bp open reading frame encoding a polypeptide composed of 466 amino acid
 561 residues (accession no. LC853583) with a calculated molecular mass of 50.0 kDa, which is consistent with the wild-
 562 type enzyme on SDS-PAGE (Fig. 9(a)). A protein–protein BLAST search against the UniProtKB/SwissProt database
 563 indicated that the reductase from strain M1 probably belongs to the fumarate reductase family. Phylogenetic analysis
 564 revealed that the reductase is closely related to previously reported fumarate oxidoreductases, including those from
 565 *Lactobacillus bulgaricus* 2038 (ADY84870, 66% [307/465 a.a.]), *Shewanella frigidimarina* NCIMB 400 (ABI73517,
 566 40% [182/448 a.a.]), and *Klebsiella pneumoniae* NTUH-K2044 (BAH63234, 45% [204/448 a.a.]) (Fig. 10(a)). In *L.*
 567 *bulgaricus*, the reductase plays a role in converting fumarate to succinate, a key metabolic step during yogurt
 568 fermentation in association with *Streptococcus thermophilus* (Yamamoto et al. 2024). However, this reductase has
 569 yet to be purified and characterized. Living organisms utilize various strategies to reduce fumarate, most commonly
 570 through a membrane-bound quinol:fumarate oxidoreductase composed of three to four subunits (Iverson 2013).
 571 Another type of fumarate reductase consists of water-soluble periplasmic enzymes, such as flavocytochromes *c*
 572 (Bertsova et al. 2020). The reductases from *S. frigidimarina* and *K. pneumoniae* is classified as unknown donor: a
 573 water-soluble fumarate oxidoreductase, based on subunit composition and domain architecture in bacteria and yeast
 574 fumarate reductases (Rothery et al. 2003; Pankhurst et al. 2006; Bertsova et al. 2020).

575

576 **Structure analysis of 10-hydroxy-2-decenoic acid reductase from *L. panisapium* M1**

577 Multiple alignment of the reductase from strain M1 and the bacterial fumarate reductases from *L. bulgaricus*
 578 (ADY84870), *K. pneumoniae* (BAH63234), and *S. frigidimarina* (ABI73517) revealed that key amino acid residues
 579 involved in fumarate reduction with FAD were almost conserved in the reductase from strain M1 with the reductases
 580 from *K. pneumoniae* and *S. frigidimarina* (Fig. 10(b)) (Wardrope et al. 2006). Conserved residues in the reductase

581 from strain M1 included two histidine residues (His255 and His397), three arginine residues (Arg273, Arg294, and
582 Arg437), and one glutamic acid residue (Glu270), all of which are critical for fumarate reduction and succinate
583 formation with FAD (Fig. 10(b)) (Bertsova et al. 2022). In motif II, one amino acid residue differed among the
584 reductases. In *K. pneumoniae*, Ser731 is involved in binding the C4 carboxylate of fumarate, while in *S. frigidimarina*,
585 Thr377 serves a similar role (Fig. 10(b)) (Serebryakova et al. 2022). Both residues have hydroxyl groups that interact
586 with the substrate's carboxylic group, stabilizing the substrate-enzyme complex (Bogachev et al. 2012). However, in
587 the reductase from strain M1, this residue is replaced with Gly269, which is also present in *L. bulgaricus* strain 2038
588 (Fig. 10(b)). Glycine, being the smallest amino acid, lacks a bulky side chain and provides greater flexibility,
589 potentially reducing steric hindrance in the active site and facilitating the binding of larger substrates such as 10H2DA.

590 Structural modeling revealed that the predicted structure of the reductase from strain M1 closely resembles that of
591 the fumarate reductase from *S. frigidimarina* NCIMB 400 (PDB code 1Y0P), with 40% sequence identity and an
592 RMSD value of 0.634 (386 atoms) (Rothery et al. 2003). Both enzymes share a mixed α/β architecture with
593 similar α -helices and β -sheets. However, the reductase from strain M1 lacks the tetraheme-binding domain (Met1 to
594 His136) found in the *S. frigidimarina* reductase (Fig. 10(c)). The absence of this domain may allow larger molecules
595 like 10H2DA to more easily access the enzyme's active site, despite the enzyme's higher catalytic efficiency for
596 smaller molecules like fumarate.

597 Bertsova et al. (2022) have reviewed the structure aspects of soluble cytoplasmic NADH: fumarate
598 oxidoreductase including fumarate reductase from *K. pneumoniae*, which consist of three domains: old yellow
599 enzyme (OYE)-like-, FMN bind-, and FAD binding_2- domains (Bertsova et al. 2020; Bertsova et al. 2022). The
600 FAD-binding_2 domain contains a fumarate-binding site and a noncovalently bound FAD molecule. The catalytic
601 mechanism of fumarate reduction has been extensively studied in the soluble fumarate reductase (flavocytochrome
602 *c*, Fcc3) of the bacterial genus *Shewanella* (Leys et al. 1999; Reid et al. 2000).

603 X-ray structural studies of the *S. frigidimarina* reductase have identified three groups of functionally important
604 amino acid residues: those binding the C1 carboxylate (Arg414, His516, and Arg556; motif I) and the C4 carboxylate
605 (His377 and Thr389; motif II), as well as those forming the proton conductance channel (Glu390, Arg393, and

606 Arg414; motif III) (Fig. 10(b) and 10(d) (Bertsova et al. 2022). These residues are conserved in *K. pneumoniae*
607 fumarate reductase as well (Fig. 10(b) and Fig. 10(d)). In motif II, the Thr377 residue, which is critical for binding
608 the C4 carboxylate of fumarate, is replaced by Gly269 in the reductase from strain M1 (Fig. 10(b) and 10(d) (Bertsova
609 et al. 2022). Gly269 likely reduces steric hindrance and enhances substrate accessibility, potentially explaining the
610 reductase's ability to accommodate larger substrates like 10H2DA. To investigate the role of Gly269 in substrate
611 specificity, a mutant reductase (G269T) was constructed to restore a threonine residue at this position. The mutation's
612 effect on enzyme activity for 10H2DA is described in the following section

613

614 **Characterization of the purified recombinant reductase**

615 **Physicochemical properties**

616 The heterologous expression of the recombinant reductase with a histidine tag at N-terminal position was designed,
617 but the recombinant enzyme was not adsorbed to a nickel resin for affinity chromatography. Thus, the enzyme was
618 purified by ion-exchange chromatography, resulting in a 9.4-fold purification with an overall yield of 270% (Table
619 3(b)). SDS-PAGE revealed that cell extracts of the *E. coli* transformant contained a His-tag fusion polypeptide (Fig.
620 9(b)). After dialysis of the purified recombinant reductase, the spectrum of the reductase did not show the absorption
621 bands around 375 and 450 nm, which are characteristic of flavoproteins in which a flavin cofactor is covalently bound
622 to protein, as seen in previously reported reductases from *Vibrio harveyi* and *Shewanella oneidensis* (Fig. 11)
623 (Bertsova et al. 2022; Delavari et al. 2024).

624

625 **Identification of the reaction product from 10H2DA**

626 The GC retention times of trimethylsilylated authentic 10H2DA and 10HDAA were 14.7 min (stable molecular ion
627 peak, $M^+ - CH_3 = 315$; relative intensity, 69%) and 13.2 min ($M^+ - CH_3 = 317$; 100%), respectively (Fig. 12). Here, it was
628 impossible to clearly purify 10H2DA and its enzymatic reaction product by TLC. Thus, they were trimethylsilyl-
629 derivatized and analyzed as a mixture by GC-MS. The peak of the predicted enzymatic reaction product, 10HDAA
630 was certainly detected in the total ion-chromatogram at 13.2 min. The molecular ion and stable molecular ion peaks

631 appeared in the mass spectrum of the trimethylsilylated product appeared at m/z 332 (M^+ , 0.9%) and 317 (M^+-CH_3 ,
632 100%) (Fig. 12).

633

634 **Catalytic properties and stability in recombinant reductase**

635 The reductase could not utilize NADPH as the reductant, instead of NADH in the presence of FAD. The
636 recombinant reductase was stable in the pH range of 5.5 to 6.0. It showed high activity in the pH range of 6.0 to 7.0
637 (Fig. 13(a)). At pH 5–6 ranges, the molecules of 10H2DA are fully or partially deprotonated (Angelov and Hristov
638 2018), it may be easy to do the formation of an enzyme substrate complex via Arg437 residue of the reductase (Fig.
639 10(d)) and/or the reduction of the double bond ($C=C$) of 10H2DA. The activity of fumarate reductase from *K.*
640 *pneumoniae* demonstrates a sharp maximum at pH values 6.0–6.5 (Bertsova et al. 2020). Additionally, in wild-type
641 soluble fumarate reductase from *S. frigidimarina*, k_{cat} value at pH 6.0 is higher than neutral and weak alkali conditions,
642 while the affinity for fumarate is the highest at pH 9.0 (Pankhurst et al. 2006). The NADH:acrylate oxidoreductase
643 activity of acrylate reductase from *V. harveyi* was pH-dependent, with a maximum between pH 6.5 and 7.0
644 (Bertsova et al. 2022). To our knowledge, there is no information on thermo-stability and effect of temperature of
645 recombinant reductase and other reductases related to it. The recombinant reductase was stable up to 55°C and it
646 showed high activity at 45 to 50 (Fig. 13(b)).

647 The activity of the recombinant reductase toward fumarate was approximately two-fold higher than that toward
648 10H2DA (Table 1), suggesting substrate specificity for smaller molecules such as fumarate. This substrate preference
649 may be attributed to conserved amino acid residues in the substrate-binding motif, which are optimized for fumarate
650 binding in reductases from *S. frigidimarina* and *K. pneumoniae* (Bertsova et al. 2022). Interestingly, the recombinant
651 wild-type enzyme and the G269T mutant exhibited comparable activities toward fumarate (Table 1), but the activity
652 of the mutant enzyme toward 10H2DA was significantly decreased. These findings suggest that the Gly269 residue
653 in the strain M1 reductase plays a critical role in accommodating larger substrates like 10H2DA. However, neither
654 the wild-type nor mutant enzyme showed activity toward coumarate (Table 1), indicating potential structural or
655 mechanistic constraints. Under aerobic condition, fumarate reductase from *K. pneumoniae* reduce O_2 at a rate

656 compatible with that of the fumarate reduction with consumption of NADH (Bertsova et al. 2020). In this study, the
657 activity for 10H2DA was measured under anaerobic conditions as much as possible, but it was not possible to obtain
658 measurements at a wide range of the substrate concentrations that would allow accurate calculation of enzymatic
659 kinetic parameters such as K_m , V_{max} , and k_{cat} values.

660 In most fumarate reductases or succinate dehydrogenases, electrons required to reduce FAD (to the FADH⁻ form)
661 are transferred via an attached iron-sulfur protein or a chain of heme groups, such as those found in the reductase
662 from *S. frigidimarina* (Curtolo and Arantes 2023). The absence of these electron transfer components in the strain
663 M1 reductase suggests a simplified electron transfer mechanism. However, the exact sequence of hydride and proton
664 transfers to fumarate remains unclear and requires further investigation.

665

666 **Bioconversion of valuable compounds by fumarate reductase**

667 Resting cells containing the heterologously expressed reductase successfully transformed 10H2DA into 10HDAA,
668 achieving a molar yield of 10.9% (Fig. 12(b)). The bioconversion efficiency of the resting cells surpassed that of the
669 purified recombinant reductase, demonstrating the advantage of whole-cell systems for enhancing 10HDAA
670 production in Royal Jelly. This improved efficiency can be attributed to cofactor recycling mechanisms inherent to
671 whole cells, which eliminate the need for external cofactor supplementation or NAD-dependent glucose
672 dehydrogenase systems (Kumar et al. 2024). Whole cell systems offer significant advantages for industrial
673 applications (Romano et al. 2014; Filippucci et al. 2020), offering scalability and cost-efficiency. Furthermore, the
674 use of whole cells simplifies the bioconversion process and promotes sustainability, making it an ideal approach for
675 producing functional food ingredients like 10HDAA-enriched Royal Jelly. The next step is to improve production
676 efficiency further by examining various reaction conditions for the industrial use of this whole-cell system.

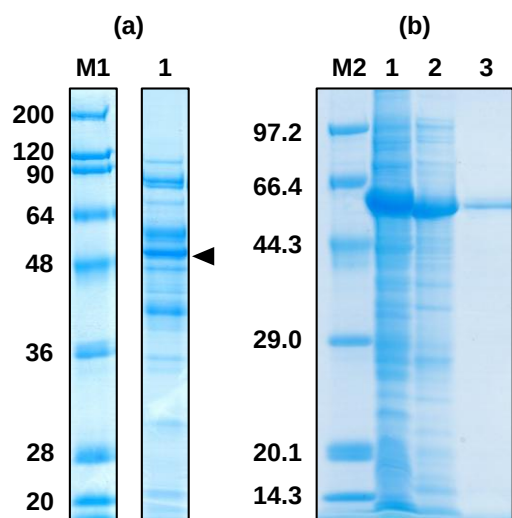
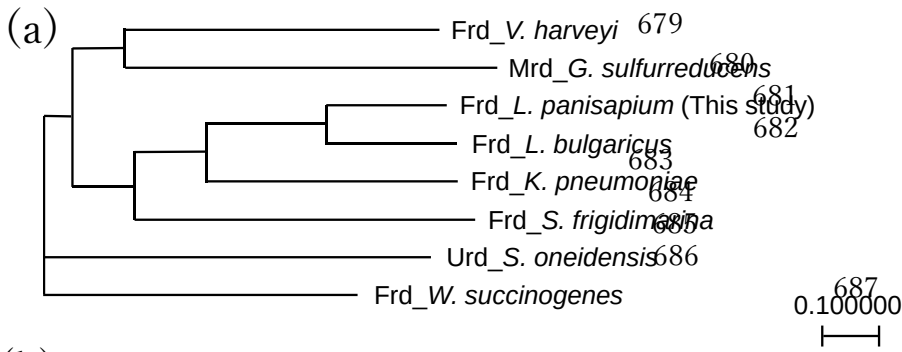


Figure 9. SDS-PAGE of partially purified reductase fraction (a) and purified recombinant reductase (b)

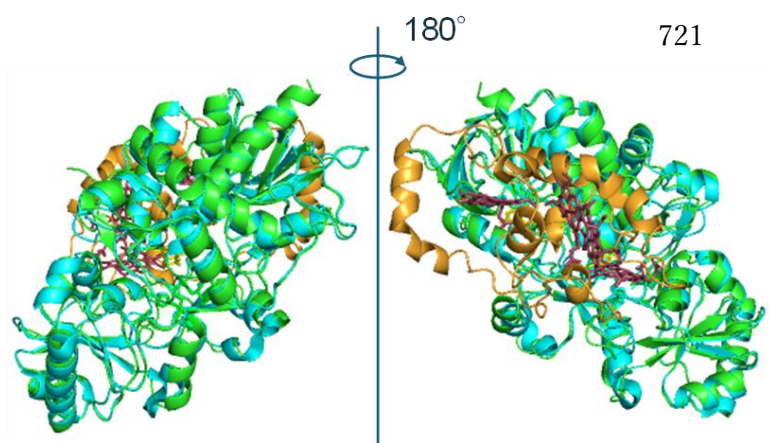
(a) Lane: M1, broad range protein marker (Takara Bio Inc., Kusatsu, Japan); 1, partially purified preparation by butyl Toyopearl chromatography. (b) Lane: M2, low range protein marker (Takara Bio Inc.); 1, cell extracts from *E. coli* transformant; 2, reductase active fraction by DEAE-Toyopearl chromatography; 3, final preparations of recombinant reductase purified by GigaCap Q-650.



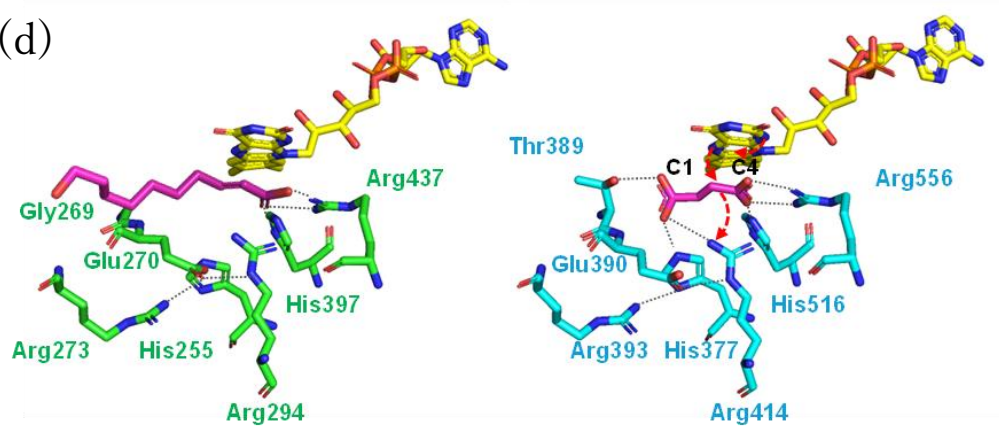
(b)

Frd_LP	196:AKAVLLASGGFGSSKELIKKYRPDLVDFKTTNPGATGDGLKLAQAQLEQMDFIQVH	255
Frd_Lb	196:AKAVILATGGFGASKEIVKKYRPDLGKYTTNAGATGDGLILGEEAGAELVQMDFIQVH	255
Frd_KP	660:TKSIVVATGGFSANSAMVVKYRPDLGKFTTNHKGATGSGIALLERIGAGTVDMGEIQIH	719
Frd_SF	318:ADAVILATGGFAKNNERVAKLDPSTKGFISTNPGAVGDGLDVAENAGGALKDMQYIQA	377
	 *.*** . . *.**.* . . **. **.***. . . . * ** *	
Frd_LP	256:PTAQTDDTHVYLIGELRGEGAILVNKAGKRFVNMNTRKIVSNSINDLHENGAYLIFDQ	315
Frd_Lb	256:PTAQTDEKTFDIGEAVRGEGAILVNKAGQRFINELDTRKVVSAAITGLNEDGAYLIFDQ	315
Frd_KP	720:PTVEQQTSS--YLISEIRGGGAILVNQGNRFFNEMETRDKVSAAIIALPEHFAYIVFDE	777
Frd_SF	378:PTLSVKGG--VMVTEAVRGNGAILVNREGKRFVNEITTRDKASAAILAQTGKSAYLIFDD	435
	** . . * ** * ** * ** * ** *.** . . **.***	
Frd_LP	316:GIRDNFTAVEFYDHIGLVSHGDTLSEIAEANGINGQNLNTVKTWNEAVASGNDQEFNRT	375
Frd_Lb	316:GVRDHSVKAVEFYDHIGLVHGATLEELAGKIGVDAAGLEATVASWNEAVKGQDDQAFGR	375
Frd_KP	778:HVRANKAADEYIAKGFVTSASSPRELAEKLGMDYHAFLATLECYNGAVEKQHDEQFGR	837
Frd_SF	436:SVRKSLSKIDKYIGLVAPTADSLVKLGKMEGIDGKALTETVARYNSLVSSGKDTDFERP	495
	. * . * * * . . * . * . * * * *	
Frd_LP	376:TGMDRAIDRGPFFAIHINPAIHHTMGGIHTIPKTEVLDTNGNVISGLYAAGEVSGGLHGN	435
Frd_Lb	376:TGMDRDISVAPYFAIHHPAIIHHTMGGHLINPETQVLGKDDKVIIEGLYAAGEVSGGLHGN	435
Frd_KP	838:TALRAPINEGPFHAIRIAPGVHHTMGGVTINTDGEVLNVAQQPIRGAYAAGEVVGGIHHG	897
Frd_SF	496:N-LPRALNEGNYAIEVTPGVHHTMGGVMIDTKAEVMNAKKQVIPGLYGAGEVTGGVHGA	554
	 ** . * * **** * .*. . * *.**** ** **	
Frd_LP	436:NRIGGNSIAETVIFGRQAGMQMAEFVRS	465
Frd_Lb	436:NRIGGNSVAETVVFGQAGIQASKFVRGEN	465
Frd_KP	898:NRIGGNAVADIIIFGTLAGHQAARARG--	925
Frd_SF	555:NRIGGNAISDIIITFGRLAGEEAAKYSKKN-	583
	.* . **.* **	

(c)



(d)



Frd_*L. panisapium* with 10H2DA

Frd_*S. frigidimarina* with fumarate

Figure 10. Phylogenetic analysis and multiple alignment of the reductase from *L. panisapium* M1 and bacterial fumarate reductases.

(a) Phylogenetic analysis of bacterial reductase. Abbreviations: Mrd_ *G. sulfurreducens* (source, methacrylate reductase from *Geobacter sulfurreducens* AM-1; accession no., AJY71931; identity with the reductase from *L. panisapium* M1, 30% [140/458 a.a.]), Urd_ *S. oneidensis* (Urocanate reductase from *Shewanella oneidensis* MR-1, Q8CVD0, 35% [160/454 a.a.]), Frd_ *W. succinogenes* (fumarate reductase from *Wolinella succinogenes* DSMZ 1740, CAE09288, 29% [140/481 a.a.]), Frd_ *S. frigidimarina* (fumarate reductase from *Shewanella frigidimarina* NCIMB 400, ABI73517, 40% [182/448 a.a.]), Frd_ *K. pneumoniae* (fumarate reductase from *Klebsiella pneumoniae* NTUH-K2044, BAH63234, 45% [204/448 a.a.]), Frd_ *V. harveyi* (fumarate reductase from *Vibrio harveyi* ATCC 33843, AIV07243, 33% [129/389 a.a.]), Frd_ *L. bulgaricus* (fumarate reductase from *L. delbrueckii* subsp. *bulgaricus* 2038, 66% [307/465 a.a.]), and Frd_ *L. panisapium* (10-hydroxy-2-decenoic acid reductase from *L. panisapium* M1, LC853583). (b) Multiple alignment of the reductases, Frd_Lp from *L. panisapium* M1, Frd_Lb from *L. bulgaricus* 2038, Frd_Kp from *K. pneumoniae* NTUH-K2044, and Frd_Sf from *S. frigidimarina* NCIMB 400. Conserved amino acid residues involved in fumarate reduction and consequently succinate formation with flavin were shown in red. (c) Superposition of the homology model of the reductase from *L. panisapium* M1 (green) with the structure of previously reported reductase from *S. frigidimarina* (cyan). The c-type tetraheme FAD-containing region of *S. frigidimarina* reductase and tetraheme were shown in light brown and radish brown. (d) Comparison of active sites. Active site of the reductase from *L. panisapium* M1 with 10-hydroxy-2-decenoic acid (Frd_Lp with 10H2DA) and fumarate reductase from *S. frigidimarina* with fumarate (Frd_Sf with fumarate). Each substrate and FAD were shown in magenta and yellow, respectively. Hydrogen bounds and hydride transfer are shown in dotted black line and red arrow, respectively.

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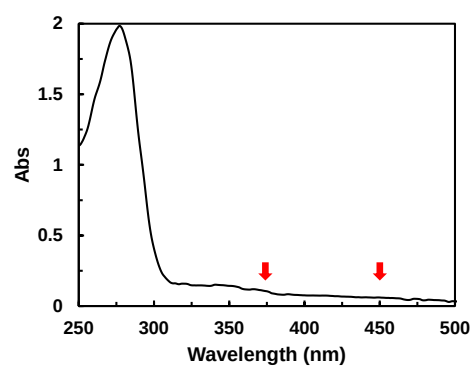


Figure 11. UV-Visible absorption spectrum of the purified recombinant reductase from *L. panisapium* M1

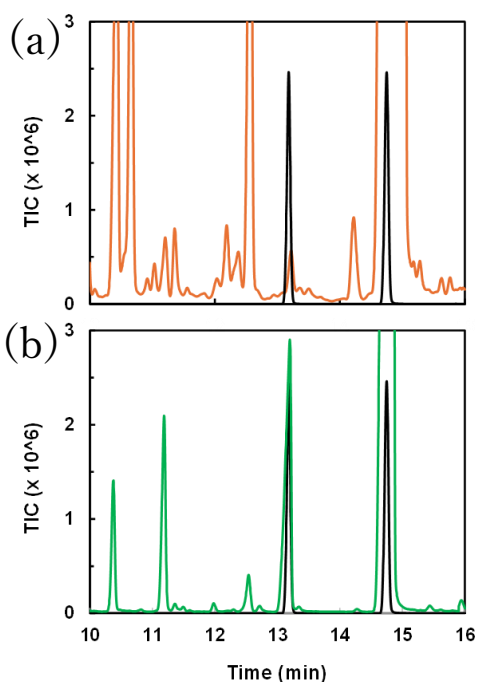


Figure 12. Total ion chromatogram (TIC) of trimethylsilylated authentic compounds, its enzymatic reaction sample, and its resting cell reaction sample

The trimethylsilylated authentic compounds (solid black line), its enzymatic reaction sample (solid orange line in (a)), and its resting cell reaction sample (solid green line in (b)). Peaks corresponding to the trimethylsilylated derivatives of authentic 10-hydroxy-2-decenoic acid and 10-hydroxydecanoic acid were observed at retention times of 14.8 and 13.2 min, respectively.

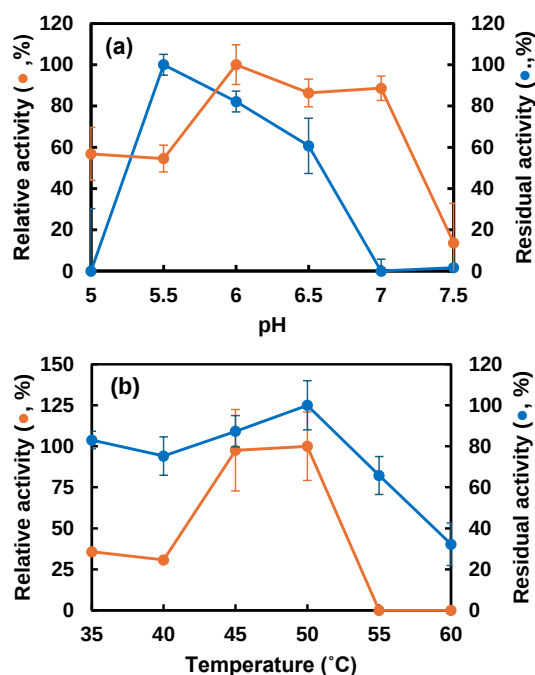


Figure 13. Effect of pH and temperature on the reductase activity

(a) pH stability and effect of pH on the reductase activity and (b) thermos-stability and effect of temperature on the reductase activity

Table 3. Summary of purification of wild type reductase and its recombinant enzyme

(a) Wild type reductase				
	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extracts	0.012	21.8	0.0005	100
DEAE-Toyopearl	0.001	4.9	0.0002	8
Butyl-Toyopearl	0.003	0.3	0.0098	23

(b) Recombinant reductase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extracts	0.078	31.1	0.0025	100
DEAE-Toyopearl	0.015	9.0	0.0017	19.2
GigaCap-Q650	0.210	8.9	0.0235	270

751

752

753 5. CONCLUSIONS

754 The low level of 10HDAA in RJ was a problem, but by using the M1 strain derived from the queen bee, it was
755 possible to produce fRJ with a fivefold increase over conventional RJ. The fRJ was more potent than pRJ in promoting
756 M cell differentiation. Preliminary tests also showed that fRJ has the ability to activate macrophage phagocytosis and
757 increase the rate of IgA secretion.

758 And in this study, a reductase from *L. panisapium* M1, responsible for the bioconversion of trans-10-hydroxy-2-
759 decenoic acid (10H2DA) to 10-hydroxydecanoic acid (10HDAA), was identified and characterized, along with its
760 recombinant and mutant forms. The reductase from strain M1 represents a distinct type of fumarate reductase,
761 differing from those of *K. pneumoniae* and *S. frigidimarina* in terms of sequence and structural features. Further
762 characterization and structural analysis of the enzyme can pave the way for functional modifications to enhance its
763 activity toward 10H2DA. Additionally, optimizing whole-cell bioconversion systems using *E. coli* transformants
764 offers a scalable approach for the production of 10HDAA-enriched Royal Jelly, supporting its application as a
765 functional food ingredient.

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872 **7. ACNOWLEDGMENT**

873

874 I would like to thank the many people who gave me guidance and encouragement in the preparation of this thesis.

875 My supervisor, Professor Takenaka of the Graduate School of Agricultural Science, Department of Applied
876 Biological Chemistry, Kobe University, provided me with appropriate guidance throughout. I would like to express
877 my deepest gratitude to him.

878 I am grateful to Professor Uno and Associate Professor Kimura of the same department for their appropriate advice
879 as sub-reviewers in the preparation of this paper. I would like to express my gratitude to them.

880 Finally, I would like to express my sincere gratitude to the members of the Laboratory of Environmental
881 Microbiology, especially Ms. Nao Takeda, for her invaluable advice and cooperation in the execution of this research.

882 I would like to express our sincere gratitude to them.
883