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α-starch and glycyrrhizin denature the acrid raphides of Pinellia tuber in traditional processing

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Abstract

Objective: Pinellia tuber, the dried tuber of *Pinellia ternata*, causes severe acrid irritation in the mouth and throat when swallowed in its raw form. Based on the theory of traditional Chinese medicine, this acridity is reduced by heating or processing using ginger, licorice, alum, or lime. Although these detoxification methods have been known since ancient times, only little is known about the mechanisms by which they detoxify Pinellia tubers. In the present study, we aimed to reveal the effectiveness of α-starch, and glycyrrhizin in licorice for the detoxification of Pinellia tubers.

Methods: Previously, we found that intact raphides of raw Pinellia tuber have a lipophilic character, and denaturation of the raphides reduced their lipophilicity and acridity. Accordingly, we developed a raphide denaturation assay (RDA) to quantify the degree of denaturation of raphides by measuring the absorbance of the petroleum ether (PE) layer comprising the raphides. The effect of α-starch or the decoction of licorice (the root and stolon of *Glycyrrhiza uralensis*) on raphide denaturation was then determined using this assay.

Results: The treatment of raphides with α-starch markedly enhanced heat denaturation of the raphides. Licorice decoction, glycyrrhizin, and glycyrrhetinic acid significantly denatured raphides in a calcium-dependent manner at high pH. Glycyrrhizin and glycyrrhetinic acid are also attached to the denatured raphides.

Conclusions: α-starch in Pinellia tubers contributes to detoxification by heating. In the traditional processing method for Pinellia tubers using licorice and lime, glycyrrhizin in licorice and calcium ions play important roles in denaturing raphides.

Keywords: α-Starch, Glycyrrhizin, *Pinellia ternata*, Processing, Raphide

Graphical abstract: http://links.lww.com/AHM/A42.

Introduction

Pinellia tuber, the dried tuber of *Pinellia ternata* Breitenbach, is a crude drug registered in the 18th Edition of the Japanese Pharmacopoeia[1–2] and the Chinese Pharmacopoeia 2020 Edition[3]. Raw Pinellia tuber contains homogentisic acid, 3,4-dihydroxybenzaldehyde, polysaccharides, calcium oxalate, etc[4], and is widely used in Japanese traditional Kampo medicine, such as Hangekobokuto[5]. The administration of insufficiently boiled unprocessed Pinellia tubers causes very strong acrid irritation in the mouth and throat[6]. Owing to this severe irritation, the Chinese Pharmacopoeia uses the term "toxic" to describe Pinellia tuber[3]. According to recent studies, the acridity of Pinellia tubers is caused by insoluble needle-like crystals stored in idioblasts, called raphides, which are composed of calcium oxalate and proteins[7–8]. The protein isolated from raphides has been demonstrated to cause inflammation in the eyes of rabbits alone, and destruction of the needle-points of the crystals is reported to reduce the acridity of the raphides[7,9]. Therefore, raphide acridity may be indispensible to both the needle-like structure of the crystal and its stored protein.

Pinellia tuber-induced acridity is known to be decreased by being processed with ginger, alum, licorice and lime, or boiling for a certain period of time[10]. According to the textbook of traditional Chinese medicine, Pinellia tubers should be used after the detoxification process[11]. The Chinese Pharmacopoeia lists three types of processed Pinellia tuber: "*Fabanxia*: prepared Pinellia tuber", which is the dried tuber of *Pinellia ternata* after being soaked in the decoction of *Glycyrrhiza* root and the solution of lime at a pH value higher than 12; "*Qingbanxia*: Pinellia tuber prepared with alum," which is the dried tuber of *Pinellia ternata* after being soaked in 8% alum solution; and "*Jiangbanxia*: Pinellia tuber prepared with ginger", which is the dried tuber of *Pinellia ternata* after boiling the decoction fresh rhizome slices of *Zingiber officinale* (ginger) with alum[3]. In

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Japan, raw Pinellia tubers are commonly used instead of processed tubers[1–2]; however, based on specific instructions, it should be decocted for a sufficient period of time to remove its acridity. These methods of detoxifying Pinellia tuber have been developed throughout the history of traditional medicine in eastern Asia[10]; however, the mechanisms of detoxification, except for alum, which was reported to destroy the needle crystal through solubilization of calcium oxalate by aluminum ions[12], have not been thoroughly revealed.

One of the difficulties associated with the study of Pinellia tuber detoxification is the objective and quantitative evaluation of its acridity in causing irritation. Recently, we reported that the acridity of raphides correlates with their lipophilicity, and the degree of their deactivation could be evaluated by measuring the turbidity of the petroleum ether (PE) layer in the water/PE partition of raphide samples using the raphide denaturation assay (RDA) [Supplementary Figure 1, http://links.lww.com/AHM/A40][13–14]. Using RDA, oxalic acid in ginger was found to specifically denature the raphides to reduce their acridity. Oxalic acid may be the active component in the processing of Pinellia tubers using ginger[14].

In this study, we assessed the features of detoxification by boiling and processing using *Glycyrrhiza* root extract and lime.

Materials and methods

Materials

All crude drugs adhered to the quality control of the 18th Edition of the Japanese Pharmacopoeia[1]. The dried sliced form (approximately 2 mm wide) of Pinellia tuber (lot number, #009120002) and approximately 5 mm pieces of the cut dried root of *Glycyrrhiza uralensis* (Glycyrrhiza, lot number, #005819004) were purchased from Tochimototenkaido (Osaka, Japan). Soluble starch (β-form) was purchased from Hayashi Pure Chemicals (Osaka, Japan). The glycyrrhizin ammonium salt, glycyrrhetinic acid, glucuronic acid, and other salts were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of the low-starch Pinellia tuber suspension

Pinellia tubers (50 g) were milled using an electric mill (Y-308B, Yamamoto Denki, Fukushima, Japan) and filtered through a 150 μm mesh. The powder was mixed with H_2O (350 mL) and maintained at room temperature for 30 min. The mixture was then vortexed vigorously to obtain a thick suspension. The suspension was centrifuged at 18× *g* for 10 min to precipitate the bulk starch, and the supernatant containing the raphides was collected. H_2O (300 mL) was added to the precipitate and the suspension was vortexed vigorously. After centrifugation at 18 × *g* for 10 min, the second supernatant was separated and combined with the first supernatant. The combined supernatants were centrifuged at 1.0×10^3 × *g* for 10 min to precipitate the residue containing raphides. After removal of the supernatant, the residue was washed with H_2O (50 mL) and centrifuged twice. Of note, a remarkably small amount of raphides was found in the supernatant using a microscope. The final residue was re-suspended in H_2O (50 mL) to prepare low-starch Pinellia tuber suspension (LSPS) for subsequent use in the denaturation assay [Supplementary Figure 1, http://links.lww.com/AHM/A40]. The LSPS was prepared in conveniently sized packets and stored at –20°C until use.

PE extract of the raphides of Pinellia tuber

Powdered Pinellia tuber (30 g) was suspended in H_2O (120 mL) and allowed to stand at room temperature for 60 min. PE (40 mL) was then added and mixed vigorously. The suspension was kept at 4°C for 90 min, and three layers appeared. When shaken gently, the raphides in the middle layer moved into the upper PE layer in the same manner performed in the RDA. The cloudy upper layer was immediately collected to avoid contamination of the remaining middle layer. This collection procedure was repeated three times with fresh PE (40 mL), and all PE layers were combined and centrifuged at 1.0×10^3 × *g* for 5 min. The supernatant was removed, and the precipitated raphides were washed three times with fresh PE (20 mL), and then three times with H_2O (10 mL). The raphides were finally suspended in PE (7.0 mL) to create the PE extract (PEX) raphide suspension, and stored at –20°C in glass vials [Supplementary Figure 2, http://links.lww.com/AHM/A40]. An aliquot of the suspension was diluted with PE, and the OD_{660} was measured. Another aliquot of the suspension was dried and the weight of the raphides was precisely measured. The concentration of the PEX raphide suspension used in this study was 7.9 mg/mL.

Preparation of the α-starch solution

A 10% aqueous suspension of β-form (crystal) soluble starch was sealed in a plastic centrifuge tube (50 mL) and incubated at 100°C for 20 min. The suspension was mixed vigorously during the incubation period to prepare a homogeneous starch solution.

Assays of the denaturation activity against the raphides of Pinellia tuber

Either the PEX raphide suspension (180 μL) was added to an aqueous sample solution (4.0 mL) and then PE was thoroughly evaporated by a stream of air and mixed vigorously, or LSPS (0.50 mL) was added to the aqueous sample solutions (3.5 mL) and mixed. The reaction mixtures were pre-incubated for 30 min at 40°C or 100°C in sealed plastic centrifuge tubes. PE (3.0 mL) was then added at room temperature and vortexed vigorously (Supplementary Figure 3A, http://links.lww.com/AHM/A40). The mixtures were allowed to stand for 15 min until the upper, middle, and lower layers appeared (Supplementary Figure 3B, http://links.lww.com/AHM/A40). The tubes were then gently shaken for 10 s to raise the cloud of raphides from the middle layer to the upper layer. The upper PE layer containing the raphide cloud was collected, and the optical density at 660 nm (OD_{660}) was measured immediately (Supplementary Figure 3C, http://links.lww.com/AHM/A40). The procedure after the addition of PE was named RDA. When the raphides of Pinellia tubers are denatured by samples, their

lipophilicities decrease[15]; therefore, the OD_{660} of the PE layer decreases. This sample effect is referred to as raphide denaturing activity.

Preparation of the Glycyrrhiza decoction

Glycyrrhiza (3.0 g) was mixed with H_2O (36 mL) in a sealed plastic centrifuge tube (50 mL) and incubated in boiling water for 30 minutes. The suspension was then filtered through cotton gauze, and the filtrate was centrifuged at $1.0 \times 10^3 \times g$ for 10 min. The supernatant was collected and stored as a Glycyrrhiza decoction at –20°C until use. Part of the decoction was lyophilized to yield the extract, and the concentration of this decoction was 30 mg/mL. The concentration of glycyrrhizin in the extract was measured using the method described in the Japanese Pharmacopoeia 18th Edition[1]; this concentration was 9.6% (w/w). The extract (0.54 mg) was dissolved in methanol and used for fingerprint analysis, as shown in Supplementary Figure 4 (http://links.lww.com/AHM/A40).

Bindings of glycyrrhizin and glycyrrhetinic acid to the raphides

The PEX raphides suspension in PE (0.50 mL) was mixed with either the mixture of 5.0 mM glycyrrhizin aqueous solution (0.50 mL) and 3.5 mL of saturated aqueous solution of $Ca(OH)_2$ (A) or 8.0 mL of 0.94 mM glycyrrhetinic acid in saturated aqueous solution of $Ca(OH)_2$ (B). Thereafter, PE was evaporated using a stream of air. The reaction mixtures were mixed vigorously before pre-incubation.

LSPS (1.5 mL) was mixed with either the mixture of 5.0 mM glycyrrhizin aqueous solution (0.75 mL), 1.0% $Ca(OH)_2$ suspension (2.0 mL), and 1.75 mL of H_2O (C), or a mixture of 5.0 mM glycyrrhetinic acid in saturated aqueous solution of $Ca(OH)_2$ (1.25 mL), 1.0% $Ca(OH)_2$ suspension (2.0 mL), and 1.25 mL of H_2O (D). The reaction mixtures (A)–(D) were pre-incubated at 40°C for 30 minutes.

PE (3.0 mL) was added to each reaction mixture and mixed vigorously. The sample mixture was then centrifuged at $1.0 \times 10^2 \times g$ for 5 min to separate the upper, middle, and lower layers. The middle layer was carefully collected and washed thoroughly three times with a saturated aqueous solution of $Ca(OH)_2$. After washing, the residue was re-suspended in 50 µL of a saturated aqueous solution of $Ca(OH)_2$ and centrifuged at $4.3 \times 10^3 \times g$ for 5 min. Finally, the supernatants were collected and mixed with 50 µL of EtOH/2 M HCl (4:1) to serve as the negative control groups (A2–D2).

The residues were finally suspended in 100 µL of EtOH/2 M HCl (4:1), and then centrifuged at 4.3×10^3 $g \times$ for 5 min. The supernatants were collected as sample groups (A1–D1) for analysis by thin-layer normal phase chromatography (TLC). For the standards, each 5.0 mM aqueous solution of glycyrrhizin or glycyrrhetinic acid in saturated aqueous solution of $Ca(OH)_2$ was diluted with 1.5 times the amount of EtOH/2 M HCl (4:1). Thereafter, the supernatants (10 µL) were spotted onto TLC plates (Silica gel 60 F_{254}, Merck Millipore, Burlington, MA, USA) and eluted with *n*-BuOH/H_2O/acetic acid (7:2:1.4) for glycyrrhizin or with ethyl acetate/PE/acetic acid (4:11:1) for glycyrrhetinic acid. Images of the plates were captured under ultraviolet light at 254 nm.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's multiple test was used to compare the differences among all pairs among multiple groups or between the normal control group and other groups among multiple groups. Student's *t* test was used to compare two independent groups. All analyses were conducted using Mac Statistical Analysis Ver 3.0 (Esumi, Tokyo, Japan). Data are expressed as mean ± standard deviation (SD). $P < 0.05$ was considered to indicate significance.

Results

Raphide denaturation by boiling

The PEX raphides were pre-incubated for 30 min at 40°C or 100°C with or without starch. According to the RDA, after 30 min of boiling, single PEX raphides maintained 52% OD_{660} of the PE layer compared with the normal control, which was pre-incubated at 40°C. The addition of 2.5% of α-starch lowered the OD_{660} of the PE layer to 9% of the normal control value. The result of boiling with 2.5% glucose did not significantly differ from the pre-incubation of single PEX raphides at 100°C. The denaturing activity of α-starch was also observed after pre-incubation at 40°C. Neither β-starch nor glucose exhibited significant denaturing activity at 40°C (Figure 1).

Dose-dependence of α-starch on raphide denaturation by boiling

After the α-starch solution was diluted with H_2O and pre-incubated with PEX raphides for 30 min at 100°C, RDA was performed. α-Starch had a dose-dependent decrease in OD_{660} based on RDA, and its IC_{50} value was approximately 0.53% (Figure 2).

Raphide denaturing activity of glycyrrhiza decoction

The LSPS was pre-incubated with the Glycyrrhiza decoction for 30 min at 40°C. The RDA revealed that a 17% lower OD_{660} of the PE layer was observed relative to the normal control without Glycyrrhiza decoction with statistical significance ($P < 0.001$) (Figure 3A). When $Ca(OH)_2$, NaOH, or $CaCl_2$ was mixed with the reaction mixture of LSPS and Glycyrrhiza decoction, strong denaturing activity was observed when combined with $Ca(OH)_2$. This interaction between Glycyrrhiza decoction and $Ca(OH)_2$ was not observed in NaOH or $CaCl_2$. Single $Ca(OH)_2$ displayed significant raphide denaturing activity at approximately 50% of the normal control group value. In contrast, single NaOH or $CaCl_2$ exhibited a significant increase in OD_{660} in the RDA (Figure 3B).

Dose-dependence of the Glycyrrhiza extract and glycyrrhizin on the raphide denaturing activity

After the Glycyrrhiza decoction was diluted with H_2O and pre-incubated with LSPS and $Ca(OH)_2$ for 30 min at 40°C, RDA was conducted. Glycyrrhiza decoction exhibited a

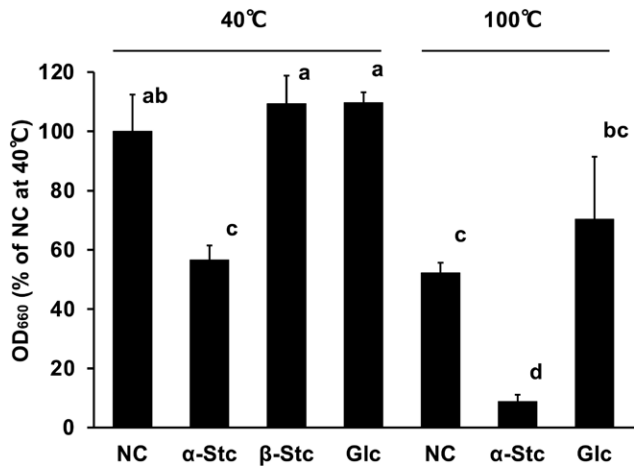

Figure 1. Denaturation of the raphides by boiling with or without starch. PEX raphide suspension (180 µL) was added to 4.0 mL of H_2O (NC) or 2.5% aqueous solution of α-Stc, which was prepared by diluting 10% α-starch solution as described in the Materials and methods section, 2.5% aqueous suspension of β-Stc, or 2.5% aqueous solution of Glc. Petroleum ether was thoroughly evaporated before pre-incubation at 40°C or 100°C for 30 min. The RDA was then performed. Data are expressed as mean ± SD (n = 3) based on the % of the NC group incubated at 40°C. Different letters above the columns indicate significant treatment differences based on Bonferroni's multiple test (P < 0.05). α-Stc: α-Starch; Glc: Glucose; NC: Normal control; PEX: Petroleum ether extraction; RDA: Raphide denaturation assay.

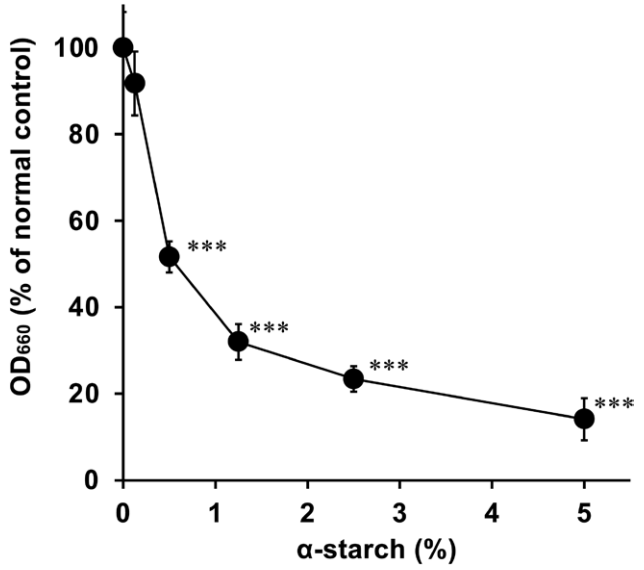

Figure 2. Dose-dependence activity of α-starch in the RDA. α-starch solution was diluted with H_2O, and the PEX suspension (180 µL) was added to each sample solution (4.0 mL). Petroleum ether was thoroughly evaporated before pre-incubation at 100°C for 30 min. Thereafter, the RDA was conducted. Data are expressed as mean ± SD (n = 3) based on the % of NC group incubated at 40°C. ***P < 0.001 *vs* normal control group based on the Dunnett's multiple comparison test. NC: Normal control; PEX: Petroleum ether extraction; RDA: Raphide denaturation assay.

significant decrease in OD_{660} in RDA at more than 1.9 mg extract/mL (Figure 4A). We proceeded to focus on glycyrrhizin as the active ingredient of the raphide denaturing factor in the Glycyrrhiza decoction, as glycyrrhizin is registered as the marker compound in both the Japanese and Chinese Pharmacopoeias[1,3]. Glycyrrhizin exhibited significant raphide denaturing activity in the presence of $Ca(OH)_2$ in a concentration-dependent manner, with an IC_{50} value of 0.33 mM (Figure 4B).

pH dependence of the raphide denaturing activity of glycyrrhizin

The pH dependence of the raphide denaturing activity of glycyrrhizin with calcium ions was investigated. LSPS was pre-incubated with $CaCl_2$ in the reaction mixtures at pH 8.0–12, with adjustments performed using borate-Na buffers. The raphide denaturing activity was observed to increase as the pH of the reaction mixture increased. Notably, the activity significantly decreased at pH 10 and then increased in accordance with the pH value of the reaction mixture (Figure 5). Glycyrrhizin alone, without $CaCl_2$, did not exhibit raphide denaturing activity at pH 12 (data not shown).

Raphide denaturing activity of glycyrrhizin and glycyrrhetinic acid

LSPS was pre-incubated with glycyrrhizin, glycyrrhetinic acid, or glucuronic acid in the presence of $Ca(OH)_2$, followed by RDA. Glycyrrhizin displayed significant raphide denaturing activity. Glycyrrhetic acid, the aglycone of glycyrrhizin, also exhibited statistically equal activity as glycyrrhizin, whereas glucuronic acid did not exhibit any activity (Figure 6).

Bindings of glycyrrhizin and glycyrrhetinic acid to the raphides

Raphides denatured by $Ca(OH)_2$ with glycyrrhizin or glycyrrhetinic acid were collected, washed, and dissolved in EtOH/2 M HCl (4:1). Glycyrrhizin and glycyrrhetinic acid were detected in the solution of the denatured raphides in HCl, whereas the negative control using the supernatant of $Ca(OH)_2$ suspension did not contain these compounds. The spot at the starting point on the plate was derived from impurities in the HCl reagent (Figure 7).

Calcium ion dependence of the raphide denaturing activity of glycyrrhizin

When LSPS was pre-incubated with glycyrrhizin and either $CaCl_2$ or $SrCl_2$ at pH 12, $SrCl_2$ did not exhibit any significant raphide denaturation activity (Figure 8).

Discussion

In our previous study using RDA, oxalic acid was identified as the main active constituent in the ginger extract for the detoxification of the Pinellia tuber in the processing of *Jiangbanxia*[14]. RDA was demonstrated to be highly reproducible, and its result aligned with the sensitivity of the gustatory tests to acrid irritation. In this study, using RDA, we investigated the features of the detoxification of Pinellia tuber by boiling and the traditional processing of *Fabanxia*.

For RDA, we prepared two types of raphide samples, LSPS and PEX raphides. LSPS was not exposed to the low polar solvent, and was stored under intact conditions compared with PEX raphides. As starch was not completely removed from LSPS, the influence of starch

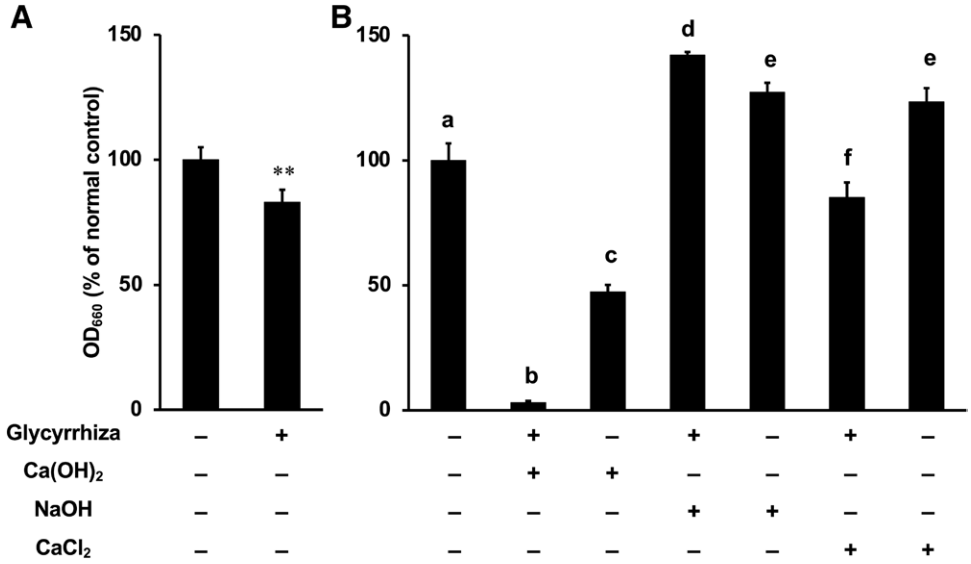

Figure 3. Raphide denaturing activity of the Glycyrrhiza decoction and its interaction with salts. (A) Glycyrrhiza decoction (54 mg/mL) or H_2O (each 2.0 mL) was mixed with LSPS (0.50 mL) and H_2O (1.5 mL), and pre-incubated at 40°C for 30 min. Thereafter, the RDA was conducted. Data are expressed as mean ± SD (n = 3) based on the % of the normal control group without Glycyrrhiza decoction and salts. $^{**}P < 0.001$ *vs* normal control group based on the Student's t test. (B) LSPS (0.50 mL) was mixed with the combinations of Glycyrrhiza decoction (54 mg/mL) (2.0 mL), 1.0% $Ca(OH)_2$ aqueous suspension (1.0 mL), 0.4 M NaOH aqueous solution (0.34 mL), or 0.4 M $CaCl_2$ aqueous solution (0.34 mL), and the volume of each reaction mixture was adjusted to 4.0 mL with H_2O. The reaction mixtures were pre-incubated at 40°C for 30 min, and then the RDA was conducted. Data are expressed as mean ± SD (n = 3) based on the % of normal control group without Glycyrrhiza decoction and salts. The different letters over the columns represent significant treatment differences based on Bonferroni's multiple test ($P < 0.05$). LSPS: Low-starch Pinellia tuber suspension; RDA: Raphide denaturation assay.

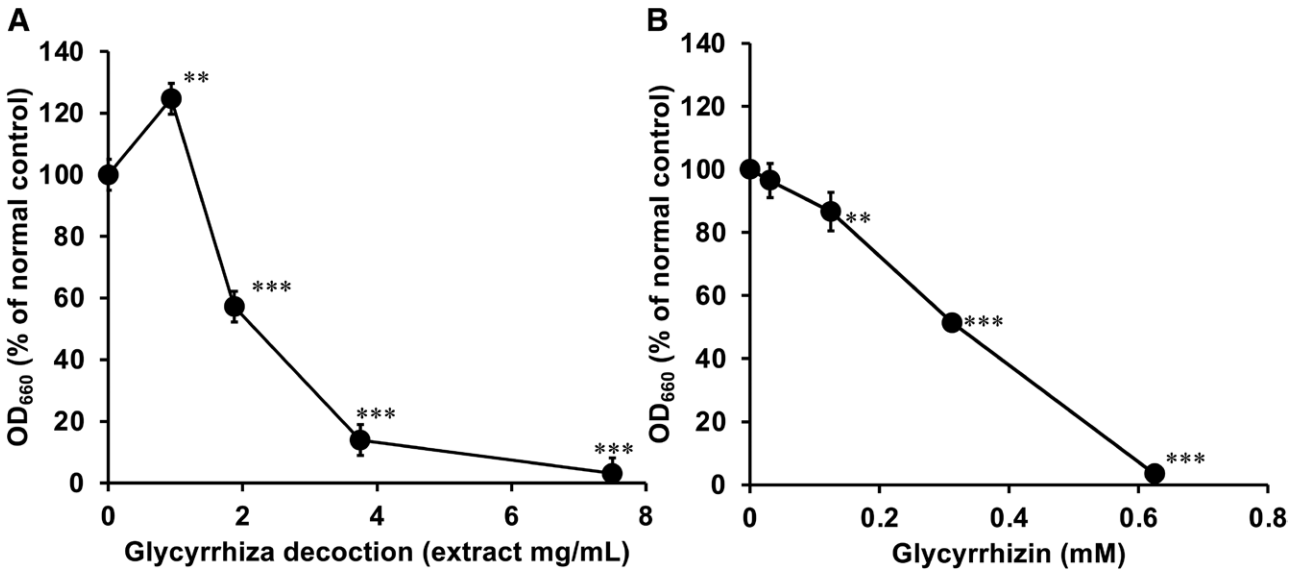

Figure 4. Dose-dependence activity of Glycyrrhiza extract (A) and glycyrrhizin (B) in the RDA. Diluted Glycyrrhiza decoction or glycyrrhizin was diluted with H_2O, and each sample solution (2.5 mL) was mixed with LSPS (0.50 mL) and 1.0% $Ca(OH)_2$ aqueous suspension (1.0 mL). The reaction mixtures were pre-incubated at 40°C for 30 min. Thereafter, the RDA was conducted. Data are expressed as mean ± SD (n = 3) based on the % of normal control group without any samples and $Ca(OH)_2$. $^{**}P < 0.01$ and $^{***}P < 0.001$, statistically significant differences compared with normal control group based on Dunnett's multiple comparison test. LSPS: Low-starch Pinellia tuber suspension; RDA: Raphide denaturation assay.

in the experiments performed at high temperature could not be negligible. On the other hand PEX raphides were more purified than LSPS and starch was not identified as an impurity in PEX raphides. However, the surface of PEX raphides easily became lipophilic owing to exposure to PE. We adopted these complementary raphide samples to increase the reliability of the results.

Detoxification was first examined by boiling. To observe the effect of heating alone, purified PEX raphides were employed for this experiment. When the purified raphides were boiled in water for 30 minutes, which is a common process for preparing decoctions in Japanese Kampo medicine[4], the raphides were found to be significantly denatured. However, approximately half of the raphides remained intact. On the other hand, when α-starch was added to the purified raphides and boiled in water, denaturation was significantly enhanced in a concentration-dependent manner.

It was reported that when powdered Pinellia tuber was boiled in water for 30 min and centrifuged, the precipitate retained strong acridity[15], which implied that the acrid ingredient in Pinellia tuber would originally be thermo-stable to a certain extent. On the other hand, the decoction of raw Pinellia tuber prepared using the common Japanese decocting procedure is known to have almost no acridity. It may be because the raphides stored in the intact idioblasts cannot contact and interact with the starch in the tuber during the decoction procedure and preserve the acridity until the end of boiling, while the raphides released from the destroyed idioblasts freely interact with the starch dissolved in the decoction and are denatured to lose their acridity. In addition, lightly roasted dried raw Pinellia tuber powder was found to preserve its acridity, whereas the acridity was removed when the powder was roasted after being mixed with 5% (w/w) of water (data not shown). Based on these results, we suggest that heat also plays a role in gelatinizing the starch in the Pinellia tuber in the presence of water during the boiling detoxification process, as well as directly affecting the raphides to denature. However, the detailed mechanism of the interaction between raphides and starch remains unclear. The α-starch solution displayed significant denaturing activity at 40°C, whereas neither insoluble β-starch nor glucose displayed significant denaturing activity, suggesting that the starch molecule specifically interacts with raphides to denature them.

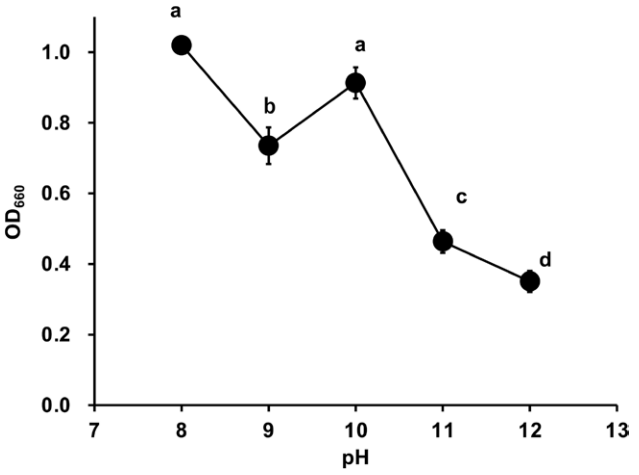

Figure 5. Effect of pH on the raphide denaturing activity of glycyrrhizin and calcium. The pH values of the aqueous solutions of boric acid (0.5 M) were adjusted to 8.0, 9.0, 10.0, 11.0, and 12.0, respectively, using 1 M NaOH. Each borate buffer (0.8 mL) was mixed with 0.4 M $CaCl_2$ aqueous solution (0.34 mL), 5 mM glycyrrhizin aqueous solution (0.50 mL), LSPS (0.50 mL), and H_2O (1.86 mL). The reaction mixtures were pre-incubated at 40°C for 30 min. Thereafter, the RDA was conducted. Data are expressed as mean ± SD (n = 3). Different letters above the columns represent significant treatment differences based on Bonferroni's multiple test ($P < 0.05$). LSPS: Low-starch Pinellia tuber suspension; RDA: Raphide denaturation assay.

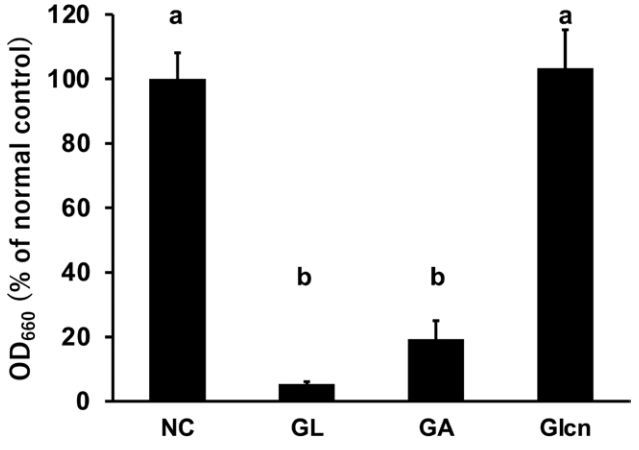

Figure 6. Raphide denaturing activity of GL, GA, and Glcn. Either 1 mM GL, 1 mM GA, or 2 mM Glcn aqueous solution (2.5 mL) was mixed with LSPS (0.50 mL) and 1.0% $Ca(OH)_2$ aqueous suspension (1.0 mL), and pre-incubated at 40°C for 30 min. Thereafter, RDA was conducted. Data are expressed as mean ± SD (n = 3) based on the % of NC group without any samples and $Ca(OH)_2$. The different letters over the columns represent significant treatment differences based on Bonferroni's multiple test ($P < 0.001$). GA: Glycyrrhetinic acid; GL: Glycyrrhizin; Glcn: Glucuronic acid; LSPS: Low-starch Pinellia tuber suspension; NC: Normal control; RDA: Raphide denaturation assay.

The irritative protein in raphides is reported to be a plant lectin[16]. Indeed, we isolated a protein from the purified raphides, which was identified as a c-type lectin encoded in the *P. ternata* gene; whose details will be provided elsewhere. The *P. ternata* lectin was reported to be denatured by being heated for 60 min to decrease water solubility by approximately half[17], which aligned with our result of raphide denaturation by heating without starch in this study. We suggest the denaturation of the raphides could be related to some changes in the lectin in the raphides. Lectin binds to sugar chains[18]. In contrast with starch, glucose did not significantly enhance the denaturation of raphides. Sugar chains of a certain length may be required to denature the raphides. Indeed, soluble dextrin exhibited a similar activity to starch (data not shown). *P. ternata* lectin may have a certain level of affinity for-with starch, which is enhanced by heating.

We succeedingly examined the raphide denaturing activity of Glycyrrhiza decoction and an aqueous solution of $Ca(OH)_2$, which are components of the traditional processing of *Fabanxia*. A single Glycyrrhiza decoction displayed significant but weak raphide denaturing activity; however, the activity was markedly enhanced by the addition of $Ca(OH)_2$. Neither the addition of NaOH nor $CaCl_2$ caused denature-enhancing activity (Figure 3), implying that a high pH value and calcium ions are required to enhance the raphide denaturing activity of Glycyrrhiza extract, which aligned with the process of *Fabanxia*. Glycyrrhiza extract exhibited significant and dose-dependent raphide denaturing activity at concentration greater than 1.9 mg/mL in the presence of $Ca(OH)_2$. At a low concentration (0.94 mg/mL), the Glycyrrhiza extract was found to increase OD_{660} in RDA (Figure 4A). However, the reason for this phenomenon remains unknown. LSPS contains raphides bound to idioblasts, which exist in the water layer as precipitates in RDA. The Glycyrrhiza extract may free raphides from binding to idioblasts and increase the concentration of free raphides.

Among the constituents of the Glycyrrhiza extract, we opted to first focus on glycyrrhizin. Glycyrrhizin showed significant raphide denaturing activity in the presence of $Ca(OH)_2$ in a concentration-dependent manner, with

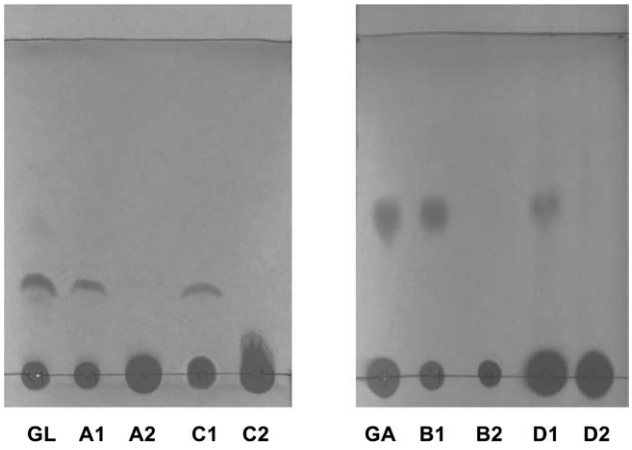

Figure 7. Binding of glycyrrhizin and glycyrrhetinic acid to the raphides. LSPS or PEX raphide suspension was reacted with glycyrrhizin or glycyrrhetinic acid in $Ca(OH)_2$ aqueous solution. The denatured raphides were extracted using EtOH/2 M HCl (4:1), and GL (left), or GA (right). The extract solution was analyzed using TLC as described in the Materials and methods section. A1, HCl/EtOH extract from denatured PEX raphides using GL; A2, negative control for A1; B1, HCl/EtOH extract from denatured PEX raphides using GA; B2, negative control for B1; C1, HCl/EtOH extract from denatured LSPS using GL; C2, negative control for C1; D1, HCl/EtOH extract from denatured LSPS using GA; D2, negative control for D1. GA: Glycyrrhetinic acid; GL: Glycyrrhizin; LSPS: Low-starch Pinellia tuber suspension; PEX: Petroleum ether extraction; TLC: Thin-layer chromatography.

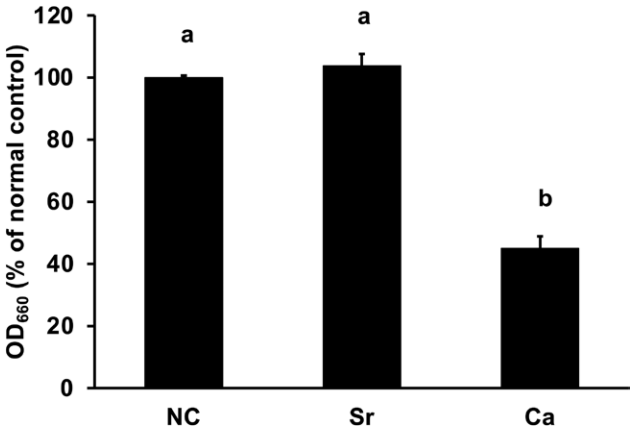

Figure 8. Calcium dependence of glycyrrhizin on raphide denaturation activity. Either 0.4 M $SrCl_2$ aqueous solution or 0.4 M $CaCl_2$ aqueous solution (0.34 mL) was mixed with 0.8 mL of 0.5 M borate-Na buffer (pH 12), 5 mM glycyrrhizin aqueous solution (0.50 mL), LSPS (0.50 mL), and H_2O (1.86 mL). The reaction mixtures were pre-incubated at 40°C for 30 min. Finally, the RDA was performed. Data are expressed as mean ± SD (n = 3) based on the % of the NC group without glycyrrhizin, $SrCl_2$, or $CaCl_2$. Different letters above the columns represent significant treatment differences based on Bonferroni's multiple test (P < 0.001). LSPS: Low-starch Pinellia tuber suspension; NC: Normal control; RDA: Raphide denaturation assay.

an IC_{50} value of 0.33 mM (Figure 4B). Glycyrrhizin also displayed the strongest denaturing activity at pH 12 (Figure 5), which was in accordance with the instructions for processing *Fabanxia* to maintain a pH greater than 12 in the reaction mixture.

Glycyrrhiza extract (1.9 mg/mL) exhibited raphide denaturing activity with approximately 57% of the control in RDA. As the concentration of glycyrrhizin in the Glycyrrhiza extract was 9.6%, the concentration of glycyrrhizin in the reaction mixture of the Glycyrrhiza extract (1.9 mg/mL) was estimated to be approximately 0.2 mM. From the concentration-dependent profile, 0.2 mM glycyrrhizin may exhibit approximately 71% of the control in RDA. Therefore, glycyrrhizin was estimated to have an approximately 80% contribution to the denaturing activity of the Glycyrrhiza extract. In addition, the estimated contribution of glycyrrhizin in 3.8 mg/mL of the Glycyrrhiza extract was 36%, using the formula applied above (Figure 4). Therefore, glycyrrhizin must be the main constituent in Glycyrrhiza to denature the raphides; however, besides glycyrrhizin, other active ingredients in the Glycyrrhiza extract can denature the raphides.

Glycyrrhizin is a glycoside that consists of glycyrrhetinic acid and glucuronic acid in its chemical structure. Glycyrrhetinic acid, the aglycone, displayed raphide denaturing activity to an equal degree as glycyrrhizin, whereas glucuronic acid displayed no activity (Figure 6). Glycyrrhetinic acid may serve as the site of the denaturing action of glycyrrhizin in the chemical structure. To further investigate the denaturing mechanism, we collected raphides denatured by glycyrrhizin or glycyrrhetinic acid with $Ca(OH)_2$ from the middle layer of RDA, and then extracted them under acidic conditions. TLC analysis revealed the detection of glycyrrhizin or glycyrrhetinic acid in the denatured raphides, implying that denaturation was induced by the binding of glycyrrhizin or glycyrrhetinic acid to the raphides (Figure 7). The pH profile of the denaturing activity of glycyrrhizin also displayed a small peak at pH 9 (Figure 5). This phenomenon could be related to the pH-dependent conformational changes of the lectin to interact with glycyrrhizin, and will be further assessed in future investigations. The interaction between raphides and glycyrrhizin was found to depend on calcium ions, specifically dicationic metallic ions (Figure 8). C-type lectin binds mannose in a calcium-dependent manner[18]. The calcium dependency of the interaction between raphides and glycyrrhizin may be related to the nature of lectin.

In summary, during the processing of *Fabanxia*, glycyrrhizin binds to the raphides *via* the interaction between lectin and glycyrrhetinic acid motif in the basic condition of pH 12 to denature the raphides and detoxify the Pinellia tuber. The detailed mechanisms by which the interaction with starch or the binding of glycyrrhizin denature raphides to lose their lipophilicity remain unclear. Investigations on the mechanisms of raphide denaturation could reveal how raphides cause acrid irritation. Further investigations are anticipated.

Conflict of interest statement

Toshiaki Makino is the editorial board member of this journal. Toshiaki Makino received grant support from Tsumura & Co., Kracie Pharmaceuticals, JPS Pharmaceuticals, Taisho Holdings, and Kobayashi Pharmaceuticals.

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Author contributions

Tsukasa Fueki, Takao Namiki, Koichiro Tanaka, and Toshiaki Makino designed this study. Tsukasa Fueki, Itsuki Nose, and Yan Liu performed the experiments. Tsukasa Fueki wrote the draft article, and Toshiaki Makino finalized the article. All authors have read and approved the final manuscript. All data were generated in-house and no paper mill was used. All authors agree to be accountable for all aspects of the work and ensure its integrity and accuracy.

Ethical approval of studies and informed consent

Not applicable.

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