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Electric Pretreatment of Garbage for the Methane Production

TOKUDA Hiroharu*1§, HOMMA Hiroto*1, IRIE Mami*2, USHIKUBO Akikuni*2 and NAKANISHI Kotoyoshi*1

*1 Department of Brewing and Fermentation, Junior College of Tokyo University of Agriculture, 1−1−1, Sakuragaoka, Setagaya-ku, Tokyo 156−8502
*2 Department of International Agricultural Development, Tokyo University of Agriculture, 1−1−1, Sakuragaoka, Setagaya-ku, Tokyo 156−8502

In this study, we investigated the effects of the electric pretreatment of garbage on biogas (methane) production. Both the garbage atomization, and garbage solubilization were enhanced by pretreatment with 30 V of direct electric current. Moreover, the number of contaminants attached to the surface of garbage components was decreased by electric pretreatment. Furthermore, garbage pretreatment with direct electric current enhances biogas (methane) fermentation.

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Key words: pretreatment, direct current, garbage, biogas, hydrogen

In Japan, approximately 20 million tons of garbage is discharged annually1, and approximately half of this is household waste. Although approximately 10% of garbage is reused as fodder or manure, most garbage is incinerated and ends up in landfills. One problem with this method of garbage disposal is that the very high water content of garbage lowers combustion temperature, leading to the formation of dioxin. In recent years, the pollution of soil and groundwater as well as the generation of an offensive odor around land reclaimed from garbage landfills have become serious problems. On the other hand, garbage is considered as a recyclable and renewable resource since it contains a large variety and amount of biological compounds such as polysaccharides and proteins, as well as mineral salts. These components can be converted into useful materials by fermentation processes. Based on this information, much research on the possible use of garbage as a source material for biogas production has been carried out and some fermentation processes for methane production from garbage are already in use2. However, many problems remain in the utilization of garbage as raw material for fermentation. These problems arise because of the various shapes and quality of garbage, and the many contaminants that adhere to garbage, which inhibit fermentation3.

In this study, we investigated the effects of the electric pretreatment of garbage on the physical properties of and the microorganisms adhering to garbage. We further investigate the possibility that methane could be produced from electrically pretreated garbage.

Materials and Methods

1. Garbage

Artificial garbage prepared according to methods described in the literature4 was used in this study. Carrots, cabbage, bananas, apples, grapefruit, horse mackerel, steamed rice, tea dregs, and egg shells were cut into small cubes (about 2−3mm per side), and mixed in a ratio 9:9:5:5:5:5:2:1 by weight.

2. Microorganisms

The methane fermentation sludge used was obtained from Hitachi Plant Construction & Services Co., Ltd. Before use for methane fermentation from garbage, the sludge was filtered by screening with a 2-mm-size mesh and habituated according to the following method.

Sludge (3.6ℓ) was placed in a continuously stirred...
tank (4 ℓ) and the air in the tank was replaced with nitrogen gas. The tank was then maintained at 37°C for 52 days. During this incubation, 120 ml of sludge was substituted for the same amount of garbage with or without electrical pretreatment every 24 hours. This habituated sludge was used as a starter for methane production from garbage.

3. Electric pretreatment of garbage

The apparatus used for the pretreatment of garbage is illustrated in Fig. 1. It is composed of two baths partitioned with a semipermeable membrane. The volume of each bath is 1 ℓ, and platinum electrodes (3.5 × 12.0 cm) were installed in each bath. Five hundred milliliters of phosphate buffer (M/30 KH₂PO₄-M/30 Na₂HPO₄; pH 7.0) was dispensed into both baths and incubated at 30°C. Wet garbage (315 g) was placed on either the cathode or anode side, and direct electric current (DC 30 V · 0.3 A) was supplied from a power supply (Model 200/2.0; Japan Bio-Rad, Japan). Following 24-h treatment with the electric current, the semipermeable membrane was removed, and the garbage suspension was mixed with the solution in the opposite bath. This mixture then served as the electrically pretreated garbage preparation. The electric energy requirement (Pt) for this pretreatment was approximately 0.78 MJ (Pt = 30 V × 0.3 A × 86,400 s). As control, 315 g of garbage soaked for 24 h in the same buffer at 30°C was used.

4. Methane production in batch culture

The methane productivity of each garbage component was estimated in batch culture. Habituated sludge (144 ml) and 96 ml of a component of electrically pretreated garbage were placed in a 300-ml Erlenmeyer flask. The gas phase in the upper layer of the flask was replaced with nitrogen gas and anaerobic culture was carried out at 37°C. Evolved gas was collected in an inverted measuring cylinder over water and measured of its volume. As control, instead of a component of electrically pretreated garbage, a component of untreated garbage was used.

5. Continuous methane production

In order to measure continuous methane production from garbage, 3.6 ℓ of habituated sludge and 120 ml of electrically pretreated garbage were placed in a stirred reactor. Following the replacement of the gas phase in the upper layer of the reactor with nitrogen gas, anaerobic culture was carried out at 37°C. After the batch culture, continuous culture was started by feeding electrically pretreated garbage and drawing the fluid culture with pumps at a rate of 120 ml/day. As control, untreated garbage was fed instead of electrically pretreated garbage.

6. Analytical methods

The dry weight of the garbage was measured at room temperature after drying the garbage at 80°C for 24 h. The particle size of the garbage was measured using a testing sieve (Tokyo Screen Co., Japan). The apertures of the sieve mesh were 180, 300, 425, and 600 μm. The number of contaminants in the garbage was determined by plating aliquots of suspensions of the garbage on nutrient broth agar plates followed by a 48-h incubation at 30°C. The chemical oxygen demand (CODcr) of the filtrate of the garbage suspension was measured with a COD analyzer (HACH Co., USA, DR 2000). The total solid (TS) and volatile solid (VS) of the garbage were analyzed by conventional methods⁶. The composition of the generated biogas was determined by gas chromatography (Hitachi Co., Tokyo, G 3900) under the following conditions: column, SHINCARBON ST 50/80; carrier gas, helium; flow rate, 30 ml/min; column temperature, 40—200°C (10°C/min); detector, TCD.

Results and Discussion

1. Effect of electric pretreatment on garbage properties

We first tested the effect of electric current application on the pH of the solutions in the chambers of the apparatus to be used for garbage treatment. When 30 V of direct electric current was applied to the baths, the pHs of the solutions in the

![Fig. 1 Apparatus for the electric pretreatment of garbage](image)

Garbage was pretreated with 30V of direct electric current for 24h.
anode and cathode gradually reached approximately 2 and 12, respectively, after approximately 3 h; no change in pH was observed thereafter. It was possible to maintain the pHs of the solutions in both baths by applying direct electric current as low as 5 V. However, when the current application was terminated, the pHs of the solutions in both baths gradually returned to their initial values. Moreover, the pHs of the solutions could be immediately returned to their initial values by mixing the solutions from the two baths together. Based on these results, it appears that direct current application is a suitable method for garbage pretreatment with an acid or alkali solution. By using an adequate buffer solution whose pH has been optimized for methane-producing microorganisms, it is possible to start methane production immediately after garbage pretreatment. As this equipment can pretreat garbage without requiring wastewater recovery and neutralization, the negative environmental impact of this pretreatment method should be less than that of other chemical methods.

Next, the influence of electric pretreatment on garbage properties was examined. First, the weights of the garbage samples before and after the electric pretreatment were measured and the rate of solubilization of the garbage was calculated using the following equation.

$$\text{RS(\%)} = \left(\frac{\text{Wb} - \text{Wa}}{\text{Wb}}\right) \times 100$$

RS: rate of solubilization.
Wb: weight before pretreatment.
Wa: weight after pretreatment.

Fig. 2 shows the solubilization rate of the garbage. This figure reveals that the solubilization rate of the garbage was enhanced by electric pretreatment, particularly following pretreatment with DC 30 V (cathode), after which the solubilization rate reached about 60%.

Secondly, the particle size distributions of the garbage samples with and without electric pretreatment were measured (Fig. 3). In Fig. 3, “untreated” indicates the particle size distribution of the garbage immediately after preparation. Approximately 60% of the electrically pretreated garbage (DC 30 V (cathode)) had a particle size of 2 mm or less. In contrast, more than 80% of the garbage without pretreatment (i.e., soaked in buffer) had a particle size greater than 2.8 mm. These results indicate that electric pretreatment causes garbage atomization that is set off following the swelling of garbage particles.

Finally, the CODcr values for all components of the garbage suspensions before and after pretreatment were measured. This analysis showed that the electrical pretreatment markedly enhanced the CODcr values for horse mackerel, tea dregs, and bananas in the suspension (Fig. 4). These results show that some organic components are released from the garbage when it is electrically pretreated.
Fig. 4 Effect of electric pretreatment of garbage constituents on TS, VS and CODeC

Each value is the rate of change from the initial value. The rates of changes in TS ( ), VS ( ) and CODeC ( ), for individual garbage components, compared with initial values, are shown.

2. Effect of electric pretreatment on contaminants attached to the surface of garbage components

To generate useful materials from raw material sources using biochemical processes, a raw material is usually sterilized prior to its use. There are many reports on sterilization methods. However, most food wastes, such as garbage, are usually used as a raw material without sterilization, even though there are many kinds of contaminants attached to the surface of garbage. It is considered that most garbage contaminants are aerobic bacteria, and a large number of which die out during methane fermentation. Presently, it is suspected that the deterioration in garbage quality caused by contaminants constitutes an inhibitory factor for fermentation. However, little is known about the sterilization of contaminants attached to garbage or about the influence of the deterioration in garbage quality on fermentation productivity.

We have reported that the application of direct electric current has a bacteriostatic or bactericidal action against certain microorganisms. Therefore, it is expected that this electric method for the pretreatment of garbage will also be effective for not only garbage solubilization, but also the sterilization of contaminants in garbage. To test this hypothesis, the numbers of contaminants in the garbage before and after electrical pretreatment were determined (Table 1). The number of contaminants in the garbage was $2 \times 10^5$ (cells/g wet-garbage) even directly after its preparation. This value reached $3 \times 10^6$ (cells/g wet-garbage) and an offensive odor was generated after incubating for 24 h at room temperature. The garbage sample was then pretreated by the electric method and the change in the number of contaminants was determined. The number of contaminants began to decrease immediately after the application of direct electric current and continued to gradually decrease thereafter. No living cells were detected within 6 hours of electric pretreatment.

The combined data indicate that garbage pretreatment with direct electric current has beneficial effects on 1) solubilization, 2) the extraction of organic substances, and 3) the sterilization of contaminants. Further studies are needed to clarify the mechanism underlying these effects. It is considered that some amounts of heat and/or hydrogen gas are generated by electrochemical reactions during the electrical pretreatment of the garbage; however, since they are useful as an energy resource, it is also necessary to develop a method for their recovery, to reduce the operating cost of electric pretreatment.

Clearly, the method of garbage pretreatment presented here is very promising as a novel method that enables efficient methane fermentation and reduces the amount of fermentation residues.

3. Biogas production from electrically pretreated garbage

Biogas production from garbage with and without electrical pretreatment was examined in continuous culture in the absence of pH control. Fig. 5 shows the biogas production with time. No garbage was supplied to the tank when the pH of the fermentation liquid became 6.5 or less. The concentrations of methane and carbon dioxide in the biogas were $55 \sim 60\%$ and $40 \sim 45\%$, respectively. The biogas productivity per garbage with and
without electric pretreatment was changed daily and was not necessarily higher than the literature data. Up to 27 days of fermentation, higher biogas productivity was obtained for the untreated garbage. The total amount of biogas generated from the untreated garbage was somewhat higher than that generated from the pretreated garbage. However, biogas productivity decreased rapidly after that, and the generation of biogas stopped on the 39th day. On the other hand, biogas productivity from the pretreated garbage was stable for a while and continuous biogas production was successfully achieved for over 42 days. Consequently, the total amount of biogas generated from the electrically pretreated garbage was about 1.2 times that generated from the untreated garbage.

In order to clarify the factors that enhance the total amount of biogas, the fermentation efficiencies of all components of the garbage samples with and without electric pretreatment were examined. Fig. 6 shows the relative biogas productivities of the different garbage components. Each biogas productivity is expressed relative to the productivity of the untreated sample. Pretreatment with DC 30 V (cathode) improved the relative biogas productivity of cabbage, steamed rice, grapefruit, carrots, and horse mackerel. For cabbage and steamed rice, the biogas productivities of the pretreated samples were more than twice as high as those of the untreated samples. However, electric pretreatment was not as effective on the biogas productivity of bananas, apples, tea dregs, or egg shells (data not shown). Except in for bananas and tea dregs, the increases in both the solubilization rate and the amount of extracted organic substances caused by electric pretreatment were associated with the improvement in biogas productivity from individual garbage components. From these data, we draw the following conclusions.

1. The improvement in total biogas production from pretreated garbage mainly arises from an improvement in the biogas productivity of the 5 components shown in Fig. 6. 2. Some materials eluted from each garbage component by electric pretreatment may induce certain interactions inhibitory to biogas production in the prometaphase of fermentation. Further research is needed to clarify the details. 3. Garbage pretreated with direct electric current is suitable for a long series of biogas fermentation.

The improvement in total biogas (methane) productivity from electrically pretreated garbage seems to lead to an efficient and low-cost disposal of garbage in the future. This method of electric pretreatment of garbage is considered applicable to other agricultural wastes for the purpose of manufacturing raw materials for fermentation.

**Conclusions**

A method for the electric pretreatment of garbage was developed for the purpose of permitting methane generation from garbage. The method involves the treatment of garbage with 30 V of direct electric current for 24 h. Garbage
solubilization and the elution of organic materials from garbage were improved by this method. Furthermore, the contaminants attached to the garbage components were sterilized, and the amount of biogas (methane) generated from the electrically pretreated garbage was increased compared with that generated from the untreated garbage. This method of using a low electric current for garbage pretreatment to produce biogas (methane) may also be applicable to the pretreatment of other raw materials.

References

メタン発酵における原料生ごみの電解処理

徳田宏晴**・本間裕人**・入江満美**
牛久保明邦**・中西毅慶**

＊1 東京農業大学短期大学部醸造学科
（〒156-8502 東京都世田谷区桜丘1-1-1）

＊2 東京農業大学国際食料情報学部国際農業開発学科
（〒156-8502 東京都世田谷区桜丘1-1-1）

生ごみを原料としたバイオガス（メタン）発酵において、試料生ごみの電解前処理効果について検討した。生ごみ試料を電解処理（直流30 V・24 h）すると、試料の微粒子化が促進され、その可溶化率が向上するとともに、有機性成分の溶出が認められた。また、電解処理により、試料生ごみに付着している細菌数が減少した。さらに、原料生ごみを電解処理するとバイオガス（メタン）発酵の生産性が向上することも明らかとなった。

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Deodorizing Effects of Wild Grass Extracts against Methanethiol

URABE Kimiko*1§, HIGASHI Rie*1, INADA Taki*1, MINAMINO Kanako*1, NADAMOTO Tomonori*1 and ONO Kouki*2

*1 Department of Food Science and Nutrition, School of Human Cultures, The University of Shiga Prefecture, 2500, Hassaka-cho, Hikone 522–8533
*2 Department of Food and Nutrition, Gifu City Women’s College, 7–1, Hitoichibakita-machi, Gifu 501–0192

We investigated the deodorizing effects of hot-water extracts of samples of 26 species of wild grasses native to Hikone City, Shiga Prefecture against methanethiol (CH₃SH), and compared them with the deodorizing effects of methanol extracts of the samples. The deodorizing activities were determined from the amounts of CH₃SH remaining using headspace gas chromatography. Each of the 5.0 mg hot-water extracts and methanol extracts was added to CH₃SH and then examined. As a result, deodorizing effects stronger than those of green tea were observed in hot-water extracts of samples of 12 species and methanol extracts of samples of 11 species. Furthermore, deodorizing activities of 100% were found in eight species. Among them, the deodorant active substances of Glechoma hederacea [2], Artemisia capillaries [3], Porentilla fragarioides [4], Gnaphalium affine [5], Senactis annuus [6], and Oenothera biennis [8] were more effectively extracted by hot-water extraction than methanol extraction. These substantial deodorizing effects of hot-water extracts of wild grasses have not been reported to data. This experiment suggests that substances originating from plants are promising for possible deodorant applications.

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Key words: dedorizing effect, methanethiol, wild grass, hot-water extract, methanol extract

Recently, there has been much attention focused on finding new usages for natural substances that are environmentally safe and have positive physiological effects on human beings. Plants possess various, functional, substances, and they are considered to be a potential source of deodorants. Edible plants, herbs, and herb vegetables have been used for cooking and in preservation for some time now. They have also been used to improve odor problems in the past. For example, there have been many reports concerning their suppression of odor, in other words, deodorizing effects[4–6]. So, we aimed at studying the deodorizing effects of wild grasses, which are commonly around us, in our gardens and yards but are not usually used as food sources. We previously reported on the deodorant effects of methanol extracts of wild grasses (42 species) around us including so-called “weeds” against methanethiol (CH₃SH). As a result, methanol extracts from 14 species of wild grasses showed stronger deodorizing effects than sodium copper chlorophyllin (SCC), which is widely used as a deodorant[4]. To the best of our knowledge, none of these plants have been reported to have deodorizing properties. On the other hand, vegetables or wild edible plants are commonly boiled for cooking and commonly used as medicinal herbs. Therefore, the purpose of this present study is to investigate the deodorizing effects of hot-water extracts of wild grasses and to compare them with those of methanol extracts of wild grasses.

Materials and Methods

1. Wild grasses and extraction procedure

The wild grasses that grow naturally in Hikone City, Shiga prefecture were gathered between the months of April and October. As shown in Table 1, we examined 26 species from twelve families. The species of wild grass was identified on the authority of two pictorial books of the Japanese flora[16,17].
parts of the wild grass used were the aerial parts (the leaves, flowers, and stems), because the parts are generally used for food. The wild grass was washed in running water, and the water on the surface was wiped with a paper towel. The wild grass, cut into small pieces, was placed into an Erlenmeyer flask with about ten times its amount of distilled water, and left to boil for one hour. The mixture was filtered, and the filtrate was concentrated by an evaporator under reduced pressure at 40°C, then lyophilized, and used as a hot-water extract sample. The preparation of methanol extracts was carried out as follows:  

1. Shredded wild grass was soaked in an Erlenmeyer flask with about ten times the amount of methanol; and
2. was allowed to stand for seven days at room temperature;  
3. after filtration, the filtrate was evaporated under reduced pressure at 40°C and was used as a methanol extract sample.

2. Measurements of deodorant activities

The method of Tokita et al.13 was slightly modified and used in this study. 1 ml of CH·SH standard solution (1 mg/ml in benzene) was dissolved in 99 ml of ethanol and kept in a freezer (−20°C) until use. 2.9 ml of 0.1 M sodium phosphate buffer (pH 7.5) was placed in a 30 ml glass vial and mixed with the indicated amount of test sample. Immediately after the addition of 0.1 ml of CH·SH ethanol solution, the vial was sealed with rubber and aluminum caps and stirred for 5 s with a Vortex mixer. The mixture was incubated in a water bath at 37°C for 15 min and 2 ml of the headspace gas was then analyzed by gas chromatography (model GC-14 B, Shimadzu Corp., Japan) with a flame photometric detector. The gas chromatography conditions were the same as those described in our previous report. The amount of CH·SH was calculated from a standard curve drawn up in advance. Deodorizing activity for each sample was expressed as follows:

Deodorizing activity (%) = (C-S)/C × 100

C : The amount of CH·SH without the sample (control)
S: The amount of CH$_2$SH with the sample. All experiments were performed in triplicate. The values of coefficient of variation (%) were under 13%.

3. Chemicals

p-Coumaric acid was purchased from ICN Biochemicals Inc., rosmarinic acid, apigenin, and rutin from Chromadex Inc., caffeic acid from Sigma-Aldrich Chemicals Co., USA, myricetin, naringenin and (+) catechin from Funakoshi Co., Japan, and chlorogenic acid and hesperidin from Nacalai Tesque Inc., Japan. A standard solution of CH$_2$SH (1 mg/mL in benzene) and other chemicals were obtained from Wako Pure Chemical Inc., Japan.

Results and Discussion

1. Deodorizing activity of wild grass extracts

The extracts (5.0 mg) from 26 species of wild grasses were examined for deodorant activity against CH$_2$SH as shown in Table 2. For comparison, the values of methanol extracts were also shown in the table, though some data had reported in previous paper\(^3\). Green tea, well known as a deodorizer, was used as a reference. The deodorant activities of hot-water and methanol extracts from green tea were 78.6% and 72.4%, respectively. Twelve out of 26 hot-water extracts from species of wild grass had higher deodorizing activities than green tea. The following eight hot-water extracts in particular had a deodorizing activity of 100%: *Artemisia capillaries* \(^3\), *Artemisia feddai* \(^7\), Gnapalium affine \(^5\), Stenacis annuus \(^6\), *Glechoma hederacea* \(^2\), *Euphorbia maculata* \(^1\), *Oenothera biennis* \(^8\) and *Porentilla fragarioides* \(^4\).

In the case of methanol extracts, eleven species had higher deodorant activities than green tea, and a deodorizing activity of 100% was found in eight wild grasses, namely, *Artemisia capillaries* \(^3\), *Artemisia feddai* \(^7\), *Eclipta prostrata* \(^11\), Gnapalium affine \(^5\), *Stenacis annuus* \(^6\), *Glechoma hederacea*.

<table>
<thead>
<tr>
<th>Wild grass</th>
<th>Hot-water extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyebane</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Indian pennywort</td>
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<td>100</td>
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<td>Kawarayomogi</td>
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<td>100*</td>
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<td>100</td>
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<td>100</td>
</tr>
<tr>
<td>Daisy-fleabane</td>
<td>100</td>
<td>100*</td>
</tr>
<tr>
<td>Himeyomogi</td>
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<td>100*</td>
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<tr>
<td>Evening primrose</td>
<td>100</td>
<td>51.9*</td>
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<td>Harujion</td>
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<td>Saw-thistle</td>
<td>84.9</td>
<td>29.9*</td>
</tr>
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<td>American false daisy</td>
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<td>100*</td>
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<td>Flax-leaf fleabane</td>
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<tr>
<td>Dokudami</td>
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<tr>
<td>Burweed</td>
<td>60.4</td>
<td>54.3*</td>
</tr>
<tr>
<td>Hananigana</td>
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<td>Common purslana</td>
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<td>Dayflower</td>
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<tr>
<td>Common sorrel</td>
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<td>63.2</td>
</tr>
</tbody>
</table>

Experimental procedures are described in the text. *Reported values (Urabe et al., 2003).*
Euphorbia maculata [1] and Porentilla fragarioides [4], Glechoma hederacea [2], Euphorbia maculata [1] and Porentilla fragarioides [4], which had deodorizing activities of 100% in the previous report5, had deodorizing activities of 100% in this experiment as well. This confirms that these wild grasses have high deodorizing activities regardless of growing time or place. As a result, almost half of the grasses tested had higher deodorizing activities than green tea in either hot-water or methanol extracts. However, correlation between the values of hot-water and methanol extracts, excluding wild grasses with a deodorizing activity of 100%, both in hot-water and methanol extracts, was substantially low (r = 0.22).

2. Comparison of deodorizing effects of wild grass extracts

In order to compare the deodorizing effects of 16 extracts from wild grasses, which had a deodorizing activity of 100% in Table 2, we measured the deodorizing activities of the samples, decreasing gradually step by step from 1.0 mg to 0.25 mg. The results are shown in Table 3. For six species of wild grasses, namely, Artemisia capillaries [3], Gnapalium affine [5], Stenacis annuus [6], Glechoma hederacea [2], Oenothera biennis [8] and Porentilla fragarioides [4], those extracted by hot water had approximately the same as or twice the deodorizing activity as those extracted by methanol. It was considered that hot-water extracted deodorizing activity substances are more effective than methanol. As none of these hot-water extracts have been reported to have deodorant properties, it is expected that they will be good sources of deodorizer in the future.

On the other hand, the methanol extract of Eclipta prostrata [11] had a high deodorizing activity of approximately 20 times that of the hot-water extract, 85.6% in 0.25 mg of methanol extract and 83.8% in 5.0 mg of hot-water extract. On the basis of this data, it was proposed that Eclipta prostrata [11] possibly contains an unstable substance, obtained through the process of heating, or a hydrophobic substance difficult to extract by water. Although it is not clear whether Eclipta prostrata [11] is used for food, it has been used as a drug6. It is possible to effectively obtain a substance with substantial deodorant activity from a methanol extract.

3. Deodorizing Activity of Polyphenols

The constituents of plants with deodorizing activities have been isolated are various, including polyphenols7, 8, 9, diterpenes10, biphenyl compounds11, etc. Among them, polyphenols are considered to contribute materially to the deodorizing effects of

<table>
<thead>
<tr>
<th>Wild grass</th>
<th>Extract</th>
<th>Amounts of addition (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Eyebane</td>
<td>Hot-water</td>
<td>86.2 (%)</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>100</td>
</tr>
<tr>
<td>Indian pennywort</td>
<td>Hot-water</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>58.2</td>
</tr>
<tr>
<td>Kawarayomogi</td>
<td>Hot-water</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>100</td>
</tr>
<tr>
<td>Potentil</td>
<td>Hot-water</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>37.0</td>
</tr>
<tr>
<td>Jersey-cutweed</td>
<td>Hot-water</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>30.8</td>
</tr>
<tr>
<td>Daisy-fleabane</td>
<td>Hot-water</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>43.5</td>
</tr>
<tr>
<td>Himeyomogi</td>
<td>Hot-water</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>–</td>
</tr>
<tr>
<td>Evening primrose</td>
<td>Hot-water</td>
<td>100</td>
</tr>
<tr>
<td>American false daisy</td>
<td>Methanol</td>
<td>100</td>
</tr>
</tbody>
</table>

– : Not tested
wild grasses. In this study, the amounts and species of polyphenols included, in some wild grasses with high deodorizing activities, have not yet been reported in detail. However, from pharmacologic and physiologic actions, some substances with high deodorizing activities, including polyphenol in wild grasses, have been reported\textsuperscript{10,11}. Tokita et al.\textsuperscript{10} reported the deodorant activities of some polyphenols, but their measurement results for deodorizing activity were different from ours in this study. So, we once again measured the deodorizing activities of some polyphenols. Table 4 shows our results. In Tokita’s report, the deodorizing activities of the polyphenols were as followed: catechol (95%), gallic acid (100%), protocatechuic acid (7%), caffeic acid (30%), apigenin (15%), kaempferol (0%), rutin (2%), myricetin (90%), and naringenin (0%). Some differences in deodorizing activity were observed but the same tendency was observed in the extent of deodorizing effects. Below, we examine the wild grasses that have substantial deodorizing activities for the possibility or even the slightest possibility of having polyphenol and then we will look into the deodorant applications of these wild grasses.

*Ooyomogi* (*Artemisia Montana*) and *yomogi* (*A. princeps*) of the asteraceae family contain dicafeeylquinic acids (DiCQA) in large amounts. *Artemisia capillaries* \textsuperscript{[3]}, a closely related plant, is confirmed to contain a substantial amount of DiCQA (7% in the extract)\textsuperscript{10} and *Artemisia feddeai* \textsuperscript{[7]}, also possibly contains DiCQA. Recently, the scavenging activities of 5-CQA and 3, 5-diCQA, which are CQA derivatives, have been reported\textsuperscript{13}. According to the report, 5-CQA of 2 μM (0.71 ng) and 3, 5-diCQA of 1 μM (0.52 mg) had very low activities of 4% and 0%, respectively. However, in our experiment, 1.0 ng of 5-CQA (2.8 μM) had a much greater activity of 52.7%, which is substantially different from the value reported by Negishi. Judging from the activity of 5-CQA in this report, we suppose that the deodorizing activity depends upon the amount of 5-CQA included in *Artemisia capillaries* \textsuperscript{[3]} or *Artemisia feddeai* \textsuperscript{[7]}.

*Nishikisou* (*Euphorbia humifusa*), which is a plant closely related to *Euphorbia maculata* \textsuperscript{[1]}, is confirmed to contain gallic acid\textsuperscript{10} and hydrolyzable tannins\textsuperscript{10}. On the basis of this fact, it can be assumed that *Euphorbia maculata* \textsuperscript{[1]} must also contain these two substances. Gallic acid has a deodorizing activity of 70.3% with the addition of 0.01 mg, which is a markedly high activity. Consequently, gallic acid is considered to greatly contribute to the deodorizing activity of *Euphorbia maculata* \textsuperscript{[1]}, varying in accordance with its abundance.

It is reported that the leaf of *Glechoma hederacea* \textsuperscript{[2]} contains 1.48% rosmarinic acid, which is a distinctive substance in the plants of the mint family\textsuperscript{10}. Furthermore, because it is reported to be stable against heating in the process of infusion, there presumably exists a considerable amount of rosmarinic acid in the hot-water extract that we

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Amount of addition (mg)</th>
<th>Deodorizing activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>protocatechuic acid</td>
<td>1.0</td>
<td>24.8</td>
</tr>
<tr>
<td>gallic acid</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>70.3</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>1.0</td>
<td>73.6</td>
</tr>
<tr>
<td>chlorogenic acid (5-CQA)</td>
<td>1.0</td>
<td>52.7</td>
</tr>
<tr>
<td>rosmarinic acid</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>48.1</td>
</tr>
<tr>
<td>apigenin</td>
<td>1.0</td>
<td>5.4</td>
</tr>
<tr>
<td>kaempferol</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>quercetin</td>
<td>1.0</td>
<td>58.1</td>
</tr>
<tr>
<td>quercetin-3-O-rutinoside (rutin)</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>myricetin</td>
<td>1.0</td>
<td>69.3</td>
</tr>
<tr>
<td>naringenin</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>catechol</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(+) catechin</td>
<td>1.0</td>
<td>20.9</td>
</tr>
</tbody>
</table>
used. We obtained 7.9 g of the hot-water extract from the fresh aerial parts of 100 g *Glechoma hederacea* [2] in this experiment. If rosmarinic acid (1.48 g) is contained in this extract (7.9 g), the ratio of rosmarinic acid to the extract is 18.7%. The deodorizing activity of rosmarinic acid was 48.1% with the addition of 0.1 mg in our result. Considering the content and the deodorizing activity of rosmarinic acid, rosmarinic acid is presumed to significantly enhance the deodorizing effect of *Glechoma hederacea* [2].

In this study, we proved that high deodorizing activity substances of some wild grasses were extracted by hot-water, rather than by methanol. In particular, hot-water extracts of *Gnaphalium affine* [5], *Glechoma hederacea* [2], *Artemisia capillaries* [3], *Stenacis annuus* [6] and *Oenothera biennis* [8] are known to be edible. Some wild grasses may contain polyphenol with high deodorizing activity. But a correlation between the deodorizing activities and polyphenol contents was not observed. Fractionation of potential extracts is essential for determining the phenolic or nonphenolic compounds responsible for the deodorizing activities. We are now analyzing the constituents contained in some wild grasses with high deodorizing activities.

References
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野草抽出物のメタンチオールに対する消臭効果

浦部貴美子*1・東 理恵*1・稲田多希*1

南野可奈子*1・濱本知恵*2・小野廉紀*2

＊1 滋賀県立大学人間文化学部生活栄養学科
（〒522-8533 滋賀県彦根市八坂町2500）
＊2 岐阜市立女子短期大学栄養学学科
（〒501-0192 岐阜県岐阜市一日市場北町7-1）

滋賀県彦根市内に自生している野草（26種類）の熱水抽出物について、悪臭物質のひとつであるメタンチオール（CH$_3$SH）に対する消臭効果を測定し、さらにメタンチオール抽出物の消臭率を比較検討した。消臭率は、ヘッドスペースガスを用いたガスクロマトグラフ法により残存するCH$_3$SH量から求めた。その結果、熱水抽出物およびメタンチオール抽出物5.0mg添加により、それぞれ12種と11種の試料に、緑茶（熱水抽出物78.6％、メタンチオール抽出物72.4％）より高い消臭効果が認められた。さらに、100％の消臭率を示した抽出物はそれぞれ8種類であった。このうち、カキドオシ（Glechoma hederacea）、カワラヨモギ（Artemisia capellaris）、キジムシロ（Porentilla fragarioides）、ハハコグサ（Gnaphalium affinis）、ヒメジョン（Stenactis annuus）、メマツヨイグサ（Oenothera biennis）に含まれる消臭活性成分は、メタンチオールよりも熱水によって効率よく抽出された。これら野草熱水抽出物の高い消臭性は、これまでのところ報告されていない。消臭効果の期待できる新しい植物由来の素材であることが示唆された。

（平成20年7月9日受理、平成21年3月9日受理）

Purification and Characterization of Polyphenol Oxidase from Japanese Butterbur (Petasites japonicus)

HAN Yunzhe*1, ZHAO Fushan*1, OGAWA Takeshi*2, OHTA Mayumi*2 and FUJITA Shuji*2§

* 1 Yanbian Institute of Agricultural Science, Longjing Jilin 133400, China
* 2 Laboratory of Food Science, Faculty of Agriculture, Saga University, 1, Honjoumachi, Saga-shi, Saga 840–8502, Japan

Polyphenol oxidase (o-diphenol: oxygen oxidoreductase, EC 1.10.3.1, PPO) in Japanese butterbur was purified about ~151-fold with a recovery rate of 11.3% by acetone fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration. The purified enzyme appeared as a single band on PAGE and SDS-PAGE. The molecular weight of the enzyme was estimated to be about 26,000 and 25,000 by gel filtration and SDS-PAGE, respectively. The purified enzyme quickly oxidized chlorogenic acid and (−)-epicatechin. The Km values of the enzyme were 0.14 mM for chlorogenic acid (pH 5.0, 30°C) and 0.7 mM for (−)-epicatechin (pH 8.0, 30°C). The optimum pHs were 5.0 and 8.0 for chlorogenic acid oxidizing (ChO) and (−)-epicatechin oxidizing (EpO) activities, respectively. In the pH range from 4.0 to 9.0, both ChO and EpO activities were stable at 4°C for 22 h. The optimum temperature of both activities was found at 30°C. Both activities were 50% inactivated after heat treatment at 60°C for 10 min. Both activities were strongly inhibited by L-ascorbic acid and l-cysteine at 5 mM.

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Key words: polyphenol oxidase, Japanese butterbur (Petasites japonicus), chlorogenic acid oxidase, (−)-epicatechin oxidase, purification and characterization

Enzymatic browning in raw fruits and vegetables is important in their preservation and processing, and is generally considered to be an undesirable reaction because it leads to unpleasant appearance and the concomitant development of off-flavors. Polyphenol oxidase (o-diphenol: oxygen oxidoreductase, EC 1.10.3.1, PPO) is found in most higher plants and is responsible for enzymatic browning. It catalyzes the oxidation of polyphenols in plants to o-quinones, which react with themselves or other phenolics to form a brown pigment. To prevent the browning that results in decreased marketability of the agricultural products, this enzyme has been characterized in many fruits and vegetables.17–11

Many researchers have demonstrated that PPO in plant tissues is localized in the chloroplast, particularly in the thylakoid membrane, or in the nongreen plastid-type component20,21. In the field of food chemistry, many researchers have also shown differences in the substrate specificity and some properties of PPO from various fruits and vegetables.

Japanese butterbur (Petasites japonicus) is one of the most common vegetables in Japan, and a brown scum caused by PPO always forms during its processing and/or cooking. However, details of the purification and properties of Japanese butterbur PPO have not hitherto been published. To determine the properties of PPO, pyrocatechol and 4-methycatechol are generally used as substrate12,13,14; however, chlorogenic acid (5-cafeoyl quinic acid) seems to be the normal substrate as it is widely distributed in plant tissues and is strongly oxidized by plant PPO.15,16,17. In Japanese butterbur, the presence of a few chlorogenic acid analogues has been reported, and the changes in absorption and difference spectra during the oxidation of Japanese butterbur extracts as well as

§ Corresponding author. E-mail: fujitas@cc.saga-u.ac.jp
of edible burdock extracts have indicated that the browning is caused by chlorogenic acid oxidation[38,20]. Therefore, the enzymatic browning of Japanese butterbur seems to be mainly due to the oxidation of chlorogenic acid analogues by endogeneous PPO. In our laboratory, it has been clarified that the purified PPOs of composite vegetables, such as garland chrysanthemum[30], leaf lettuce[30] and edible burdock[37], oxidize chlorogenic acid and (−)-epicatechin, however, the optimum pH of the enzyme differs depending on the type of substrate used. It is interesting that the same difference in optimum pH was observed in the purified PPO of Japanese butterbur. In this study, Japanese butterbur PPO was purified using chlorogenic acid as substrate, and some properties of the purified enzyme were determined.

Materials and Methods

1. Materials

Japanese butterbur was purchased at local markets in Saga Prefecture, Japan. DEAE-Cellulofine AL was purchased from Chisso, Tokyo, Japan. Butyl-Toyopearl 650 M, Phenyl-Toyopearl 650 M and Toyopearl HW 55-superfine (HW 55-S) were purchased from Tosco Co., Tokyo, Japan. The other reagents were purchased from Wako Pure Chemical Co., Osaka, Japan and Katayama Chemical Co., Osaka, Japan.

2. Assay of enzyme activity

(1) Chlorogenic acid oxidase (ChO) activity

The spectrophotometric method developed by Tono et al.[29] was employed to measure ChO activity. The mixture to be tested consisted of 0.5 ml of 0.4 mM aqueous solution of chlorogenic acid, 1 ml of 0.1 M citrate/0.2 M potassium phosphate buffer (McIlvaine buffer, pH 5.0) and 0.5 ml of the enzyme solution; it was incubated at 30°C for 5 min. After incubation, the reaction was stopped by adding 3 ml of 2% metaphosphoric acid solution. For the control, 0.5 ml of the enzyme solution was added to a mixture of 0.5 ml of 0.4 mM aqueous solution of chlorogenic acid, 1 ml of McIlvaine buffer (pH 5.0), and 3 ml of 2% metaphosphoric acid solution. The difference in absorbance at 325 nm (ΔA325) between the control and test solutions was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was expressed as ΔA325 of 0.1 per minute per milliliter of the enzyme solution (1 cm light path).

(2) PPO activity

PPO activity was measured by the colorimetric method[38]. The reaction mixture containing 0.5 ml of 10 mM aqueous solution of various polyphenols (see Table 2), 4 ml of McIlvaine buffer (pH 5.0), and 0.5 ml of enzyme solution. After 5 min of incubating the mixture at 30°C, the increase in absorbance at 420 nm (ΔA420) was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was defined as ΔA420 of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

3. Protein determination

Protein was determined by the method of Hartree[22] using bovine serum albumin (fraction V, Katayama Chemical Co., Osaka, Japan) as standard. In the chromatography, protein was determined by measuring the absorbance at 280 nm.

4. Enzyme purification

All steps of purification were carried out at 4°C. The edible part (leafstalk) of Japanese butterbur (3 kg) was homogenized in 1,500 ml of 0.1 M potassium phosphate / 0.1 M sodium phosphate buffer (0.1 M phosphate buffer, PB, pH 7.0) containing 2% NaCl, 1% l-ascorbic acid, and 1% polyvinyl polypyrrolidone. After filtering the homogenate through cotton cloth, the filtrate was centrifuged at 10,300 × g for 20 min, and acetone was added to the supernatant. The protein precipitate obtained from the 80% acetone fraction was collected by centrifugation at 10,300 × g for 20 min, dissolved in a small volume of 0.01 M PB (pH 7.0), and then dialyzed against the same buffer for 36 h with four or more changes of the dialysis medium. The dialyzed solution (crude enzyme) was applied to a DEAE-Cellulofine AL column (4 × 14.5 cm) equilibrated with 0.01 M PB (pH 7.0), and eluted with a linear gradient of sodium chloride (0 to 1.0 M NaCl in 0.01 M PB, pH 7.0). Enzyme activity was eluted with the buffer solution containing 0.5 to 0.8 M NaCl. The enzyme fractions were collected, brought to a 1 M ammonium sulfate concentration and applied to a Butyl-Toyopearl column (1.6 × 11 cm) equilibrated with 0.01 M PB (pH 7.0) containing 1 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1 to 0 M in 0.01 M PB, pH 7.0). The enzyme was eluted with the buffer solution containing 0.6 to 0 M ammonium sulfate. The enzyme active fractions were pooled and dialyzed against 0.01 M PB (pH 7.0) with four or more changes of the dialyzed medium. The dialyzed
enzyme solution was brought to a 1.5 M ammonium sulfate concentration and applied to a Phenyl-Toyopearl column (1.6 × 11 cm) equilibrated with 0.01 M PB (pH 7.0) containing 1.5 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1.5 to 0 M in 0.01 M PB, pH 7.0). The enzyme was eluted with the buffer solution containing 0.8 to 0.5 M ammonium sulfate. The enzyme active fractions were pooled and dialyzed against 0.01 M PB (pH 7.0) with four or more changes of the dialyzed medium. The dialyzed enzyme solution was applied to a Toyopearl HW 55-S (1.6 × 89 cm) column equilibrated with 0.1 M PB (pH 7.0) and eluted with the same buffer. The PPO active fractions were pooled and used for enzyme characterization.

5. Polyacrylamide gel electrophoresis (native PAGE)

The purified enzyme was electrophoresed by the method of Davis\(^\text{20}\), using 7.5% polyacrylamide gel at pH 9.0.

6. Molecular weight determination

The molecular weight of the purified enzyme was estimated by gel filtration and SDS-PAGE. Gel filtration was conducted using a Toyopearl HW 55-S column (1.6 × 89 cm), which was equilibrated and eluted with 0.1 M PB (pH 7.0), by the method of Andrews\(^\text{20}\), Chymotrypsinogen A (MW 25,000), egg albumin (MW 45,000), bovine serum albumin (MW 65,000), and γ-globulin (MW 125,000) were used as marker proteins at a flow rate of 20 ml/h. SDS-PAGE was carried out by the method of Laemmli\(^\text{20}\), using an SDS marker protein kit (Oriental Yeast Co., Tokyo, Japan) as standard.

7. Assay of enzyme properties

(1) Optimum pH

The effects of pH on ChO and EpO activities were determined at 30°C in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin buffers.

(2) pH stability

The enzyme was preincubated in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin buffers at 4°C for 22 h. Residual ChO and EpO activities were determined under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

(3) Optimum temperature

ChO and EpO activities were determined at various temperatures (20 to 60°C) in McIlvaine buffer (ChO: pH 5.0; EpO: pH 8.0).

(4) Thermal stability

The enzyme solution was heated at various temperatures between 30 to 80°C for 10 min and residual ChO and EpO activities were determined under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

(5) Effect of various compounds

ChO and EpO activities were measured in the presence (final concentration, 5 mM or 10 mM) and absence of various compounds under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

Results and Discussion

1. Enzyme purification

Enzyme was purified from homogenates of Japanese butterbur by acetone fractionation, and DEAE-Cellulofine AL, Butyl-Toyopearl 650 M, Phenyl-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Purification of PPO from Japanese butterbur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Volume (ml)</td>
</tr>
<tr>
<td>80% acetone sulfate</td>
<td>250</td>
</tr>
<tr>
<td>DEAE-Cellulofine AL</td>
<td>563</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650 M</td>
<td>563</td>
</tr>
<tr>
<td>Phenyl-Toyopearl 650 M</td>
<td>56</td>
</tr>
<tr>
<td>ToyopearlHW 55-S</td>
<td>700</td>
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</tbody>
</table>
Toyopearl 650 M and Toyopearl HW 55 - S chromatographies. Fig. 1 shows the typical elution pattern of the enzyme activity on the final Toyopearl HW 55 - S column. Enzyme activity showed one peak and the peak fractions from several columns were pooled as the purified PPO.

A summary of the typical purification of the enzyme is given in Table 1. Finally, the enzyme was purified ~ 151-fold with a recovery rate of 11.3%, relative to that of the crude enzyme.

2. Characterization of Japanese butterbur PPO

The purified enzyme produced a single band on native PAGE and SDS-PAGE (Fig. 2). These results suggest that Japanese butterbur PPO was purified to a homogeneous state. The molecular weight of the enzyme was estimated to be about 26,000 and 25,000 by gel filtration and SDS-PAGE, respectively (Fig. 3). These results indicate that the purified PPO is a monomer protein. The molecular weight is smaller than those of the PPOs of composite vegetables such as head lettuce (MW 56,000) 17, garland chrysanthemum (MW 45,000) 19, leaf lettuce (MW 46,000) 20, and edible burdock (MW 40,000) 21, all of which mainly oxidize chlorogenic acid. The molecular weight is also smaller than those of the PPOs of apples (MW 65,000) 16, banana pulp (MW 41,000) 19, and Japanese pears (MW 56,000) 21, but is almost the same as that of another edible burdock PPO (MW 25,000) 22 which mainly oxidize pyrogallol and phloroglucinol.

As shown in Table 2, the purified Japanese butterbur PPO quickly oxidized not only chlorogenic acid but also (-)-epicatechin. The enzyme had a low activity toward other o-diphenols such as pyrocatechol, catechin and caffeic acid, but had no activity toward dopamine or DOPA. A similar substrate specificity was observed in the PPOs of composite vegetables such as head lettuce 17, garland chrysanthemum 19, leaf lettuce 20 and edible burdock 21, and of Japanese pears 21. The enzyme also had no activity toward trihydroxybenzenes such as pyrogallol, gallic acid, and phloroglucinol, or m-diphenols such as resorcinol. The Km values

![Fig. 2 SDS-PAGE (A) and native PAGE (B) of purified enzyme](image)

**Fig. 2** SDS-PAGE (A) and native PAGE (B) of purified enzyme


![Fig. 3 Estimation of molecular weight of the enzyme by gel filtration on Toyopearl HW 55-S (A) and SDS-PAGE (B)](image)

(A) M.W : molecular weight, V0 : void volume of the column, Ve : elution volume of the substance, 1 : r-globulin (160,000), 2 : bovine serum albumin (65,000), 3 : egg albumin (45,000), 4 : chymotrypsinogen A (25,000), E : purified enzyme.

(B) 1 : Cytochrome c hexamer (M.W : 74,400), 2 : Cytochrome c tetramer (49,000), 3 : Cytochrome c trimer (37,200), 4 : Cytochrome c dimmer (24,800), E : purified enzyme.
Table 2 Substrate specificities of enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>100</td>
</tr>
<tr>
<td>(−)−Epicatechin</td>
<td>94</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>42</td>
</tr>
<tr>
<td>Catechol</td>
<td>39</td>
</tr>
<tr>
<td>Catchin</td>
<td>37</td>
</tr>
<tr>
<td>Dopamin</td>
<td>9</td>
</tr>
<tr>
<td>nDopa</td>
<td>2</td>
</tr>
<tr>
<td>Resorcinolcinol</td>
<td>0</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>0</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0</td>
</tr>
</tbody>
</table>

(Michaelis constant) of the enzyme, measured using chlorogenic acid (pH 5.0, 30°C) and (−)-epicatechin (pH 8.0, 30°C) as substrates, were 0.14 and 0.7 mM, respectively (Fig. 4). These values were lower than those of leaf lettuce PPO<sup>80</sup> and edible burdock PPO<sup>81</sup>.

Several PPOs show different optimum pHs for different substrates: for example, the optimum pHs of garland chrysanthemum PPO<sup>80</sup> for chlorogenic acid and epicatechin were 4.0 and 8.0, respectively. The optimum pHs of sweet pepper PPO<sup>82</sup> for chlorogenic acid and pyrocatechol oxidation were 4.0 and 7.0, respectively. Fig. 5(A) shows the effects of pH on the ChO and EpO activities of the purified Japanese butterburPPO. The optimum pHs for ChO and EpO were 5.0 and 8.0, respectively. These optimum pH differences were also observed

![Fig. 4 Lineweaver-Burk plots of chlorogenic acid (A) and (−)-epicatechin (B) oxidation by the enzyme.](image)

V : Rate of the enzyme reaction, S : substrate concentration.

![Fig. 5 Effects of pH on the activity (A) and stability (B) of enzyme](image)

○ : ChO, chlorogenic acid oxidase activity; ● : EpO, (−)-epicatechin oxidase activity.

(A) PPO activity was measured at 30°C in McIlvaine (pHs 3.0 to 8.0) and Atkins & Pantin (pH 9) buffers.

(B) PPO was preincubated in McIlvaine (pHs 3.0 to 8.0) or Atkins & Pantin (pH 9) buffers at 4°C for 22 h. Residual ChO and EpO activities were measured under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).
in composite vegetables such as head lettuce\textsuperscript{5}, leaf lettuce\textsuperscript{10} and edible burdock\textsuperscript{11}, and eggplants\textsuperscript{10}. On the other hand, the purified Japanese pear PPO\textsuperscript{9} had an optimum pH of 4.2 for the two substrates. The acidic optimum pHs for chlorogenic acid have also been reported for PPOs of eggplants\textsuperscript{8}, apples\textsuperscript{8} and leaf lettuce\textsuperscript{12}, which were found in the pH range of 4.0 to 5.0, for chlorogenic acid oxidation. In comparison with this, the optimum pHs of the PPOs of banana pulp\textsuperscript{13}, banana peel\textsuperscript{13}, and guava\textsuperscript{14} were found to be near neutrality using (-)-epicatechin, pyrocatechol, 4-methylcatechol, and dopamine as substrates. Therefore, the ChOs in these plants were assumed to be acidic PPOs. Fig. 5 (B) shows the pH stabilities of the ChO and EpO activities of the purified PPO. Both activities were stable in the pH range of 4 to 9; ~70% of enzyme activity remained after incubation in solutions of various pHs from 3 to 9 for 22 h. The pH stability of the purified enzyme was different from that of garland chrysanthemum PPO\textsuperscript{14}, the purified enzyme being stable in the pH range from 5 to 11. The pH stability of the purified enzyme was also slightly different from that of edible burdock PPO\textsuperscript{13}, the enzyme being stable in the pH range from 5 to 7.

As shown in Fig. 6 (A), the purified enzyme showed a single optimum temperature with a maximum activity at 30°C, which is similar to the optimum temperatures of the PPOs of head lettuce\textsuperscript{11} and leaf lettuce\textsuperscript{10}, but is slightly different from that of edible burdock PPO\textsuperscript{13} (20°C). ChO and EpO had slightly different thermal stabilities (Fig. 6 (B)). After heat treatment at 60°C, 70% of the EpO activity, but only about 50% of the ChO activity remained. The thermal stabilities of both ChO and EpO activities were relatively approximated, as observed in head lettuce\textsuperscript{7}, garland chrysanthemum\textsuperscript{11} and leaf lettuce\textsuperscript{10}.

The effects of various compounds on the purified enzyme activity are listed in Table 3. The ChO and EpO activities were markedly inhibited by sodium diethylthiocarbamate, KCN, and NaF at 5 and 10 mM. The presence of metal ions (Zn\textsuperscript{2+}, Cu\textsuperscript{2+}, Ba\textsuperscript{2+}, and Mn\textsuperscript{2+}) at 10 mM reduced ChO activity by 70%. The complete inhibition of ChO and EpO activities was induced by L-ascorbic acid and L-cysteine at 5 and 10 mM, respectively. Similar effects of these compounds were found for the PPOs of Japanese pears\textsuperscript{9}, head lettuce\textsuperscript{9}, garland chrysanthemum\textsuperscript{11}, leaf lettuce\textsuperscript{11} and edible burdock\textsuperscript{11}. Sodium chloride also markedly inhibited the ChO and EpO activities.

Considering the above-mentioned different effects of pH, temperature and various compounds on the ChO and EpO activities of the purified enzymes of composite vegetables and Japanese pear PPO, it appears that these enzymes may have separate sites for the two activities.

**Conclusion**

The PPO of Japanese butterbur was purified approximately 151-fold with a recovery rate of 11.3% using chlorogenic acid as a substrate. The molecular weight of the purified PPO (MW
Table 3 Effects of various compounds on the enzyme activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>ChO&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 mM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sodium diethylthiocarbamate</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>KCN</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>81</td>
<td>62</td>
</tr>
<tr>
<td>ZnSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>83</td>
<td>26</td>
</tr>
<tr>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>67</td>
<td>19</td>
</tr>
<tr>
<td>BaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td>MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td>NaF</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>NaCl</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> ChO: chlorogenic acid oxidase. The enzyme activity was determined at pH 5 using Mcllvaine buffer.


25,000) is smaller than those of the PPOs of composite vegetables such as head lettuce (MW 56,000)<sup>17</sup>, garland chrysanthemum (MW 45,000)<sup>17</sup>, leaf lettuce (MW 46,000)<sup>17</sup>, and edible burdock (MW 40,000)<sup>17</sup>; however, the substrate specificity of the purified PPO, and the effects of pH, temperature, and various compounds on the purified enzyme activity were similar to those on the enzymes of the composite vegetables. These results suggest that the browning of Japanese butterbur is caused by the oxidation of chlorogenic acid and its analogues, and that L-ascorbic acid and L-cysteine are effective inhibitors of such enzymatic browning.

フキ（Petasites japonicus）のクロロゲン酸酸化酵素の精製と性質

韓 雲哲**・趙 福善*・小川剛史**
太田真由美**・藤田修二**

*1 延辺農業科学研究
**2 佐賀大学農学部

フキのポリフェノール酸化酵素をアセトン沈殿、イオン交換クロマトグラフィー、嫌気クロマトグラフィーおよびゲル濾過により精製した。これらの操作により、本酵素はアセトン分画後の粗酵素液の約115倍に精製され、回収率は11.3%であった。本酵素はPAGEおよびSDS-PAGE上に単一のバンドを示し、電気泳動的に均一であった。精製酵素の分子量はゲル濾過およびSDS-PAGEによりそれぞれ26,000および25,000と推定された。本酵素はクロロゲン酸および（−）-エピカテキンを強く酸化し、クロロゲン酸（pH 4.0, 30℃で測定）および（−）-エピカテキン（pH 8.0, 30℃で測定）の酸化反応時のミカエリス定数はそれぞれ、0.14 mMおよび0.7 mMであった。本酵素のクロロゲン酸酸化活性（ChO）および（−）-エピカテキン酸化活性（EpO）の最高pHはそれぞれ4.0および8.0であった。ChO, EpOの両活性ともpH 4〜9の範囲で、4℃, 22時間安定であった。両活性とも最適温度は30℃であり、60℃で10分間の加熱処理に対して安定であった。両活性とも5 mMのL-アスコルビン酸およびL-システインにより強く阻害された。

（平成21年1月8日受付，平成21年3月11日受理）
Effects of Postharvest Ethanol Vapor Treatment on Quality of Sudachi (Citrus sudachi hort. ex. Shirai) Fruit

NOMA Yuta*1, SUZUKI Yasuo*1*, TERAI Hirofumi*1 and YAMAUCHI Naoki*2

*1 Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657–8501
*2 Faculty of Agriculture, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753–8515

Sudachi (Citrus sudachi hort. ex. Shirai) fruit is a kind of acid citrus. It rapidly degreens at room temperature. Ethanol treatment retards ripening and senescence of harvested horticultural products. We investigated the effects of postharvest ethanol vapor treatment with ethanol pads on the quality of sudachi fruit to prolong the storage period. Mature green sudachi fruit were packed in a perforated polyethylene bag without (control) or with a 0.3, 0.6, 1, 3 or 6 g ethanol pad as the ethanol vapor treatment and stored at 20°C in darkness. The chlorophyll contents of fruit treated with a 1 g ethanol pad decreased more slowly than those of the control fruit, accompanying suppression of loss of fresh weight. There were no significant differences in the internal quality between the control and ethanol vapor-treated fruit, including total soluble solids (TSS), titrable acidity (TA), TSS / TA ratio, pH, L-ascorbic acid content, and L-dehydroascorbic acid content in the pulp juice. However, peel browning of fruit treated with 3 and 6 g ethanol pads occurred. From these results, it is demonstrated that ethanol vapor treatment of sudachi fruit could retard degreening and suggest the possibility that treatment could prolong the storage period without negative impacts on internal quality.

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Key words: chlorophyll degradation, degreening, ethanol vapor treatment, quality, sudachi

クロロフィル分解，脱緑，エタノール蒸気処理，品質，スダチ

Sudachi (Citrus sudachi hort. ex. Shirai) fruit is a kind of acid citrus similar to lime and yuzu fruit. Green sudachi fruit is preferred by consumers. It has a characteristic aroma, to which limonene, α-pinene, decanal, citronellall and others contribute. It is harvested at the green stage because favorable change in aroma occurs at that time. The characteristic aroma disappears as the fruit turns yellow. Sudachi fruit rapidly changes from green to yellow at room temperature. Therefore, the green color is an important index of fruit quality, and it is necessary to maintain the green of the peel during marketing and storage for consumer acceptance. A long-term storage method for green sudachi fruit has been reported to be a combination of low temperature, sealed packaging with 0.02 mm thick low-density polyethylene film, and curing. This combination keeps sudachi fruit green for up to 4 to 6 months without loss of the characteristic aroma.

Citrus is categorized as a non-climacteric fruit, which shows no change in its low CO₂ and ethylene production rates during ripening. However, ethylene has been reported to be necessary for the ripening of citrus fruit, accompanying degreening of the peel color, because exogenous ethylene induces the degreening of the peel and ethylene response inhibitors, including 2, 5-norbornadiene, silver nitrate, and 1-methylecyclopropene (1-MCP), inhibit degreening. Ethylene can be used for the degreening of citrus whose green color is undesirable, such as lemon, tangerine, and orange. On the other hand, in the case of citrus whose green color is desirable, such as lime, sudachi, and kabosu, ethylene must be removed or kept away from it.

Ethylene promotes the ripening and senescence of harvested horticultural products, which affect their quality. Ethanol treatment retards ethylene-induced ripening and senescence of products including tomato, avocado, muskmelon, broccoli, and cut carnation. In this research, we investigated the

§ Corresponding author, E-mail: ysuzuki@kobe-u.ac.jp
effects of postharvest ethanol vapor treatment with ethanol pads, which is a simple and cost-effective method, on the quality of sudachi fruit to ease postharvest handling and prolong the storage period, emphasizing the green color of the peel.

**Materials and Methods**

1. **Plant materials and ethanol vapor treatment with an ethanol pad**

Mature green sudachi fruit (*Citrus Sudachi* Hort. ex. Shirai) were purchased from the Japan Agricultural Co-operatives in Tokushima Prefecture. They were classified according to uniformity in peel color, shape, and size, and injured or yellowing fruit were removed. After measured fresh weight, four fruit were placed in a polyethylene bag (100 mm × 140 mm), thickness 0.04 mm with two holes (diameter 5 mm) on each side in the presence or absence (control) of a 0.3, 0.6, 1.3, or 6 g ethanol pad (Antimold Mid®, Freund Industrial Co., Ltd., Japan) as the ethanol vapor treatment. The bags were stored at 20°C in darkness until analyses. An ethanol pad allows ethanol vapor to diffuse gradually. A 0.3, 0.6, 1.3, or 6 g ethanol pad contains 0.18, 0.36, 0.6, 1.8, or 3.6 g of ethanol, respectively\(^{10}\). All of the experiments were conducted immediately after removing the fruit from the bags after 0, 1, 3, 4, 5, 7, and 9 days in storage (DIS). Because the degreening rate varies in sudachi fruit, two of four fruit from each bag that received an average score regarding color were selected for analysis. All experiments were conducted in triplicate; each two fruit from three different bags were used for each treatment in the experiments.

2. **Measurement of peel color**

Changes of peel color were measured with the chlorophyll content and peel browning. Chlorophyll was assayed by the method of Moran\(^{11}\). Fruit peel (1.0 g) was immersed in 20 mL N, N-dimethylformamide and stored at 5°C in darkness for 24 h. The chlorophyll content was determined by measuring the absorbance of the solvent at 647 and 664 nm. Peel browning was visually assessed according to the ratio of the browning area to the surface area of the fruit peel. The scale ranged from 0 to 10: 0 = 0% browning, 1 = 10% browning, 2 = 11–20% browning, 3 = 21–30% browning, 4 = 31–40% browning, 5 = 41–50% browning, 6 = 51–60% browning, 7 = 61–70% browning, 8 = 71–80% browning, 9 = 81–90% browning, and 10 = 91–100% browning. The higher scores indicate a change of color from green to brown.

3. **Chemical analyses**

Total soluble solids (TSS), titrable acidity (TA), juice pH, and ascorbic acid contents in the pulp juice of sudachi fruit were measured. Fruit juice extracted by hand-pressing using two layers of cheesecloth from peeled fruit was applied. TSS was measured with a portable Brix meter RA-250 (Kyoto Electronics Manufacturing Co., Ltd., Japan) and expressed as % Brix in the juice. TA was determined by titrating a mixture of 1.0 mL juice and 9.0 mL distilled water with 0.1 N NaOH and a phenolphthalein indicator. TA was calculated as the percentage of citric acid equivalents. Juice pH was measured with a twin pH meter B-212 (Horiba, Ltd., Japan). L-ascorbic acid (AsA) and L-dehydroascorbic acid (DHA) contents were assayed according to the method of Roé\(^{12}\).

4. **Statistical analysis**

Gram was used as units for statistical analysis of loss of fresh weight. In comparison of loss of the fresh weight (g) of control fruit and fruit treated with a 1 g ethanol pad, examination of significant differences was performed for both indices by analysis of variance first and then by Tukey’s multiple comparison test \((P<0.05)\), using a software package (Statcel-The Usefull Addin Forms on Excel-2nd ed., Japan).

**Results**

1. **Effects of ethanol vapor treatment on degreening of sudachi fruit**

Changes in the chlorophyll \(a\) and chlorophyll \(b\) content in the peel of the sudachi fruit treated with ethanol vapor at various concentrations are shown in Fig.1. The change in chlorophyll content in the peel corresponded to the visual change from green to yellow. The chlorophyll \(a\) content in control fruit decreased linearly from 0 to 7 DIS. The contents in fruit treated with 0.3 and 0.6 g ethanol pads also decreased in a manner similar to that of the control. The decrease in fruit treated with a 1 g ethanol pad was slower; the content in fruit treated with a 1 g ethanol pad was significantly more than that in control fruit at 4 and 5 DIS. Changes in the chlorophyll \(b\) content were almost the same as those in chlorophyll \(a\) content. These results indicate that ethanol vapor treatment of
sudachi fruit with a 1 g ethanol pad could suppress the degradation of chlorophyll in fruit peel. The chlorophyll \(a\) and \(b\) contents in fruit treated with 3 and 6 g ethanol pads and other quality described below were not measured after 3 DIS because peel browning was remarkably; that of fruit treated with 3 and 6 g ethanol pads started to occur at 3 DIS and 1 DIS, respectively (data not shown).

2. Effects of ethanol vapor treatment on loss of fresh weight of sudachi fruit

Fig. 2 shows changes in the loss of fresh weight, which is described as %, of the sudachi fruit treated with various ethanol pads. The loss of fresh weight (%) of fruit treated with a 1 g ethanol pad tended to be less than that of the control fruit. On the other hand, the loss of fruit treated with 0.3 and 0.6 g ethanol pads tended to decrease similarly to that in the control. Furthermore, the effect of ethanol vapor treatment on the weight loss (g) of fruit treated with a 1 g ethanol pad was statistically analyzed in order to better understand the effects. Gram instead of % was used as units for statistical analysis. There was significantly difference between the weight loss (g) of the control fruit and that of fruit treated with a 1 g ethanol pad at \(P < 0.05\) (data not shown).

3. Effects of ethanol vapor treatment on internal quality of sudachi fruit

Changes in the internal quality of sudachi fruit, including those in the total soluble solids (TSS), titratable acidity (TA), TSS / TA ratio (Fig. 3), pH (Fig. 4), L-ascorbic acid (AsA) content, and L-dehydroascorbic acid (DHA) content (Fig. 5), in the pulp juice of the fruit treated with various ethanol pads were measured. There were no significant differences in the internal quality between the control and ethanol vapor-treated fruit. In both the control and ethanol-treated fruit, the TSS, TA, TSS / TA ratio, and juice pH were stable throughout the storage period. The AsA contents in the control and ethanol-treated fruit remained constant throughout storage, while the DHA contents were slightly increased in the control and ethanol-treated fruit.
**Fig. 3** Effects of ethanol vapor treatment on total soluble solids (TSS) (A), titrable acidity (TA) (B), and TSS / TA ratio (C) in the pulp juice of sudachi fruit during storage at 20°C.

The sudachi fruit treated without (control) (●) an ethanol pad or with a 0.3 g (□), 0.6 g (△), or 1 g (○) ethanol pad were stored at 20°C. Measurements were conducted in triplicate. The results presented are the means ± S.D. Some error bars and symbols are hidden by symbols. No data on fruit treated with a 3 g or 6 g ethanol pad are shown in this figure because the peel of at least one sudachi fruit in each treatment turned brown at 3 and 1 DIS, respectively.

**Fig. 4** Effects of ethanol vapor treatment on pH in the pulp juice of sudachi fruit during storage at 20°C.

The sudachi fruit treated without (control) (●) an ethanol pad or with a 0.3 g (□), 0.6 g (△), or 1 g (○) ethanol pad were stored at 20°C. Measurements were conducted in triplicate. The results presented are the means ± S.D. Some error bars and symbols are hidden by symbols. No data on fruit treated with a 3 g or 6 g ethanol pad are shown in this figure because the peel of at least one sudachi fruit in each treatment turned brown at 3 and 1 DIS, respectively.

**Fig. 5** Effects of ethanol vapor treatment on L-ascorbic acid content (A) and L-dehydroascorbic acid (B) in the pulp juice of sudachi fruit during storage at 20°C.

The sudachi fruit treated without (control) (●) an ethanol pad or with a 0.3 g (□), 0.6 g (△), or 1 g (○) ethanol pad were stored at 20°C. Measurements were conducted in triplicate. The results presented are the means ± S.D. Some error bars and symbols are hidden by symbols. No data on fruit treated with a 3 g or 6 g ethanol pad are shown in this figure because the peel of at least one sudachi fruit in each treatment turned brown at 3 and 1 DIS, respectively.
Discussion

In the present study, it was found that the ethanol vapor treatment of sudachi fruit could retard degreening because the decrease in the chlorophyll contents of fruit treated with a 1 g ethanol pad was slower than that of the control fruit. While sudachi fruit is marketed and stored, degreening is the primary factor with the potential to reduce the commercial value of sudachi. Thus, this finding indicates that ethanol vapor treatment could be beneficial to prolong sudachi fruit quality. Degreening in citrus fruit is involved in ethylene through chlorophyll degradation by it([1]). Exogenous ethylene induces the degreening of the peel([3]). Also, it is suggested that endogenous ethylene is related with natural degreening of citrus because ethylene response inhibitor inhibits degreening, though ethylene production is extremely low([3]). Ethanol treatment of some horticultural crops suppresses ethylene-induced ripening and senescence, accompanying chlorophyll degradation. Ethanol vapor treatment retards the ripening of tomato fruit from mature green to red-ripe[4] and keeps florets of harvested broccoli green([5]). Furthermore, it has been suggested that this is due to the inhibition of ethylene synthesis and ethylene response by ethanol vapor treatment([6]). Thus, we assume that the retardation of the degreening of the sudachi fruit by ethanol vapor treatment, as demonstrated in this study, is due to the same mechanism. There are four known enzymes, chlorophyllase, Mg-dechelatase or Mg-dechelating substance, pheophorbide a oxygenase, and red chlorophyll catabolite reductase, in the catabolic pathway of chlorophyll during senescence and ripening. These enzymes are indispensable in the degradation of chlorophyll to a colorless blue-fluorescing intermediate([7]). In the present study, the retardation of chlorophyll degradation of sudachi fruit by ethanol treatment might be due to the inhibition of the activities and gene expression of the chlorophyll-degrading enzyme by ethanol treatment or by other unknown factors, related to ethanol that affect the metabolism of sudachi fruit. Further studies are needed to clarify these issues.

Loss of fresh weight after harvest is an important factor in degradation in the marketable quality of horticultural products. Ethylene and 1-MCP do not have any effect on loss of fresh weight of citrus fruit in storage([8-10]). However, ethanol treatment might be effective to suppress the weight loss during storage because the weight loss of fruit treated with a 1 g ethanol pad during storage was less than that of the control fruit (Fig. 3). 1-MCP treatment, even at an appropriate concentration, could induce some negative impact, including chilling injury (CI), decay, and off-flavor([11]). In contrast, ethanol treatment can decrease CI symptoms in cucumber([12]) and prevent the development of decay in grapes([13]) during storage. It is possible that ethanol vapor treatment may inhibit not only degreening but also postharvest disorders. Furthermore, ethanol vapor treatment of sudachi fruit at an appropriate concentration has no negative impact on the internal quality during storage because there were no significant differences of changes in TSS, TA, pH, and AsA during storage between the control fruit and fruit treated with a 1 g ethanol pad (Figs. 4, 5, and 6) and the flavor of the juice from ethanol-treated fruit was not different from one from control fruit by organoleptic test (data not shown). Ethanol vapor treatment of citrus fruit for storage could have several advantages in retaining quality when compared to even 1-MCP treatment.

Peel browning of sudachi fruit is the most common storage disorder. At the circulation level, its occurrence tends to increase when it is stored without curing or under extremely high CO2 conditions([14]). On the other hand, an appropriate 1-MCP treatment may prolong postharvest life, but treatment at higher concentration causes peel browning([15]). As indicated by our results, ethanol treatment also induced peel browning at higher concentrations than the optimum (Fig. 2). Ethanol treatment at a high concentration causes an increase in the permeability of cellular membranes([16]) and, furthermore, injures cells([16,17]). The peel browning observed in this study may be due to the membrane damages caused by the high concentrations of ethanol and not by the high CO2 conditions, because polyethylene bags for package were perforated. Further research on application of ethanol will need to be done in order not to cause peel browning.

Ethanol vapor treatment of horticultural products with an ethanol pad has advantages for storage in that it is simple and cost-effective. In this study, we demonstrated the possibility that ethanol vapor treatment of sudachi fruit could prolong the storage
period without a negative impact on the internal quality at ambient temperature. This finding is important and has widespread implications because it could also be available to other citrus, including lime, kabosu, and Nagato-yuzukichi, in which the green color is desirable to consumers. Further studies are needed for the commercial utilization of this treatment.

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Cheese-like Food Production from Various Nuts

HOMMA Hiroto*, TOKUDA Hiroharu* and NAKANISHI Kotoyoshi*

* Department of Brewing and Fermentation, Junior College of Tokyo University of Agriculture, 1−1−1, Sakuragaoka, Setagaya-ku, Tokyo 156−8502

Nuts are rich in vegetable proteins and lipids, and can be easily made into a milk-like emulsion such as coconut milk or hemp milk. In this study we attempted to produce a cheese-like food from various nuts instead of from cows’ milk. First, we investigated the curdling activity of various proteases on emulsions made individually from 13 species of nuts. The proteases bromelain, papain, subtilisin, thermolysin and a protease from *E. faecalis* could curdle the milk-like emulsion made from 12 species of nuts. The emulsions made from hemp nuts, coconuts, almonds, pine nuts and macadamia nuts curdled especially well. We then successfully produced cheese-like foods from 6 species of nuts (hemp nuts, coconuts, almonds, pine nuts, cashew nuts and macadamia nuts). Compared to cheese made from cows’ milk these nut-cheeses were softer, the curd yield from the emulsion was almost the same, the protein content was lower and the lipid content was higher. Hemp nut cheese was the most similar to cows’ milk-cheese in its composition and gave the highest yield of cheese among all of the tested nuts.

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**Key words**: cheese production, nuts, curd formation, protease

チーズ生産、ナッツ類、カード形成、プロテアーゼ

近年、中国やインドなどの人口大国でも経済的に豊かな中高級層の人口が急増し、食生活の欧米化が進んでいるためチーズやバターなど乳製品の消費が急速に拡大しているが、それに伴い原料である牛乳の増産が求められている。さらに、近年、消費者の価値観が高騰していることなどもあり、これ以上の増産は難しいと考えられている。そのため牛乳以外の原料を用いたチーズ類食品の開発などに興味がもたらされている。

このような背景から、牛乳に非常によく似た性質を有している豆乳が、牛乳の代替原料として注目を集めている。豆乳を利用したチーズ様食品の開発がこれまで盛んに試みられてきた。しかしながら、牛乳由来チーズの匹敵しうるような品質を有する豆乳チーズの製造は難しく、多くの研究すべき課題が残されている。筆者らは豆乳チーズの品質を改善すべく研究を行っており、これまでに新規な豆乳凝固活性を有する乳酸菌（*Enterococcus faecalis* TUA 2495 L）由来プロテアーゼを見出し、その酵素学的性質などを明らかにしてきた。現在も当該酵素を利用した豆乳チーズの品質改善について検討を進めているが、それと並行して豆乳以外の新規なチーズ原料の検索も試みてきた。

今回筆者らは、大豆と同様に植物性のタンパク質と脂質を豊富に含むナッツ類に注目した。ナッツ類の多くはココナッツやアーモンドなど独特の官能的に美味しい香りを有しており、加熱して圧縮することによりココナツミルクやヘンプミルク（麻の実より製造）などといった豆乳に類似したミルク様の乳液が簡便に得られることが知られている。さらにナッツ類は牛乳と異なり保存性も高く、輸送も容易である。しかしながら、これまでのところ、ナッツ類を原料とした酸・プロテアーゼ法によるチーズ製造についての報告はない。そこで本報告ではナッツ類のチーズ原料としての可能性を探ったところ、利用できる可能性が示されたので報告する。
実験方法

1. 使用原料

麻の実ナッツ（カナダ産），アーモンド（アメリカ産），カシューナッツ（インド産），クルミ（アメリカ産），ピーナッツ（中国産）は，新ナッツを使用した。白ゴマ（国産），黒ゴマ（国産），マカダミアナッツ（アメリカ産），松の実（中国産）は乾燥後を用いたものを使用した。大豆，カボチャの種（中国産），ビタミン（アメリカ産），ヘーゼルナッツ（アメリカ産）は乾燥後を用いたものを使用した。これらのナッツ類は，乾燥後に使用したものについては全て殻を剥ぐ後使用した。ココナッツはマレーシア産のココナッツミルク（缶詰）を使用した。

2. 使用酵素

プロテアーゼは，レンネット（SIGMA社製，牛子胃由来），微生物レンネット（SIGMA社製，Mucor miehei由来），プロアミン（MERCK社製，パラビオン（和光純薬製社），サボリシオン（SIGMA社製），サブチリシン（SIGMA社製）を使用した。これら市販酵素剤については，10mg/mlになるよう500mMのリン酸緩衝液（pH 6.0）に溶解して用いた。またEnterococcus faecalis TUA 2495 L株およびIAM 10065株の生産する凝乳活性を有するプロテアーゼを佐藤らの報告に基づき調製し，500mMのリン酸緩衝液（pH 6.0）で2倍希釈して使用した。

3. 乳液の調製および凝乳試験

ナッツ類50gに蒸留水100mlを加え，フードミル（Tescos社製）で破砕した。ただし白ゴマおよび黒ゴマについては蒸留水100mlでは流動性が低く破砕が困難だったため，蒸留水150mlを加えて破砕した。その後40℃で10分振搾しながらインキュベートし，塩布で包んでジュースプレス（Master Life社製）で圧搾して乳液を得た。この乳液5mlを試験管に入れて40℃で10分加熱し，酵素液1mlを加え，十分に振搾した。これを40℃にて静置し，乳液からのカード（固形物）の分離の有無を生したカードの凝固の度合いを経時的に観察した。

4. スターターの調製

スターターは，Lactococcus lactis subsp. lactis 154株，Lactococcus lactis subsp. cremoris 439株，Lactobacillus paracasei 1532株の3株の乳酸菌をそれぞれ個別にスキムミルク培地を用いて30℃で2日間培養し調製した。

5. チーズ製造

ナッツ類700〜750gに適量の蒸留水を加え，上記と同様の方法で1,000〜1,500mlの乳液を調製した。得られた乳液を40℃で30分加熱し，ここに酵素液を乳液に対して0.5%（v/v）添加した。酵素はココナッツについてはサブチリシンを，ほかのナッツについてはプロアミンを使用した。ここに乳酸菌スターター3種類を，使用した乳液に対して1%（v/v）ずつ計3%（v/v）添加した。その後，40℃で120分間保持し凝固させた後，適度に固まらせながら30分かけてゆっくりと培養を55℃に昇温させた（カッティングおよびクッキング処理）。ここにオートクレープ減圧（121℃10分）した20%（w/v）食塩水150mlを添加し，30分は静置した。

十分にホエーとカードが分離したところをモールド（直径11cm，高さ11.5cm，底部にまき糸を設置し，内側を上から下に流し込み，ホエーを排出させた。約1時間後，カードの上側にさらにしわを重石を乗せ，その重量を徐々に増加（1〜2時間の間隔で0.5kgずつ，計1.5kg）させた。カードがある程度しっかりしてからモールドの上部に入れ替えてさらにしわを取り替える操作を行い，重石を1.25kg増加して（計2.75kg）室温で約2週間静置した。その後再びモールドをしめ，さらに重石を2.5kg加える（計5.25kg）ホエーを排出させた。ホエーの排出が停止したら，モールドから出し得られた固形物をナッツチーズとした。

6. 一般分析

水分含量は，80℃で試料を乾燥させて恒量を求め，乾燥前後の重量差から水分含量を算出した。脂質含量はソックスレー法により求めた。全タンパク質量はケルダール法により定量した。このとき窒素とタンパク変換係数は，アーモンドでは5.18，その他のナッツでは5.30とした。

灰分は灰化法により求めた。すなわち，試料を電気炉を用いて550℃で2時間灰化させ恒量を求め，灰化後の重量から算出した。炭化物は差し引き法により，水分，脂質，タンパク質，灰分の含有量を全成分から差し引いて求めた。pHはpH電極（HORIBA社製pH METER M-12）により測定した。凝固度は凝固度に応じて求めた。すなわち，精製した試料10gに煮沸した蒸留水40mlを加えて振搾し試料溶液を調製し，これを0.1 N水酸化ナトリウム溶液で滴定することにより求めた。このとき，pH 7.0までの滴定量を滴定度1とし，pH 7.0〜8.3までの滴定量を滴定度IIとした。

実験結果および考察

1. 凝乳試験

13種類のナッツから乳液を調製して，そこに凝乳活性を有するプロテアーゼを添加し，カード（固形物）の形成の有無と生したカードの凝固の度合いを観察した。その結果，プロアミン，パラビオン，サボリシオン，サブチリシン，TUA 2495 LあるいはIAM 10065株由来プロテアーゼを添加した場合において，乳液の多くがカードとホエーに分離し，カードが明確に凝固することが観察された（Table 1）。さらに，ココナッツ，アーモンド，松の実あるいはマカダミアナッツを原料とした乳液において良好な凝固が認められ，反応液を水平に傾けても分離したカードの形状がよく崩れないほどしっかりと凝固しているのも観察された。一方，レネットおよび微生物レンネットを用いた場合には乳液の状態はコントロールとあまり差はなく，カードの形成は認められ
Table 1  Curd formation test of various curdling enzymes on the emulsions made from various nuts

<table>
<thead>
<tr>
<th>Protease</th>
<th>Time (h)</th>
<th>White sesame</th>
<th>Black sesame</th>
<th>Peanut</th>
<th>Walnut</th>
<th>Hazelnut</th>
<th>Hemp nut</th>
<th>Coconut</th>
<th>Almond</th>
<th>Pine nut</th>
<th>Pump seed</th>
<th>Cashew nut</th>
<th>Macadamia nut</th>
<th>Pistachio nut</th>
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<td>Rennet (From Mucor miehei)</td>
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Activity was evaluated based on curd conditions as follows: -, curd was not formed; ±, the emulsion separated into whey and curd and the curd was of low viscosity; +, the emulsion separated into whey and curd, and the curd was of high viscosity; ++, the emulsion separated into whey and curd and the curd kept its shape for more than 3 seconds when the test tube was canted horizontally; and ++++, the emulsion separated into whey and curd and the curd kept its shape for more than 10 seconds when the test tube was canted horizontally.

なかった。レンネットと微生物レンネットは極めて基質特異性の高いプロテアーゼであり、ナッツ類から得られる乳液中のタンパク質の構造はミルクゲーゾンとは異なるため、あまり特異性の高いプロテアーゼ等の酵素のほうが高い凝乳活性を示したものと推察されたう。また、松の実、カボチャの種、カシューナッツ、マカダミアナッツ、ビスタチオではプロテアーゼを添加しないコントロールの条件下において、降雨と通過することに影響が生じることが観察された。しかし、この場合生じた沈殿は主にコロイド粒子が沈殿したものと思われ、プロテアーゼ存在下において形成されるカーデは異なり全く凝固していなかった。アーモンドはコントロールでも乳液がホエーとカーデに分離し、カーデが凝固することが観察された。アーモンド自体に凝乳性プロテアーゼが含まれている可能性が推察された。以上より、ナッツ類を用いて調製した乳液においても酵素との組み合わせた場合には、カーデの形成が可能であることが示された。

2. チーズ製造試験

プロテアーゼと反応させることによりカーデを得られることができ示されたナッツ類の中から、麻の実、ココナッツ、アーモンド、松の実、カシューナッツ、マカダミアナッツの6種類のナッツを選択し、実際にチーズ製造を試みた。その結果、試験したすべてのナッツにおいて実際にチーズ様食品（以下「ナッツチーズ」とする）を製造することができた。また、もれたアーモンドを原料として
同様に製造を試みたが、ほとんどのカードを得ることができず、ナッツチーズは製造できなかった。製造に使用したナッツの量および得ることのできたナッツチーズの量をTable 2に示す。得られたナッツチーズの量は麻の実の乳液を用いた場合に最も多く、使用した乳液の1/3程度の重量のチーズが得られた。得られたナッツチーズの量が最も少なかったのはアーモンドを用いた場合であり、得られたチーズの重量は使用した乳液の1/10程度であった。他ナッツではおおむね使用した乳液のおよそ1/6程度のチーズが得られた。乳牛などからとられる一般的なチーズではカード収量は原料乳の12～20％程度であるのでナッツチーズの収量は乳牛チーズとあまり変わらないと思われる。

製造したナッツチーズは一般的な牛乳チーズよりも軟質の物が多く、最も軟質だったアーモンドチーズはクリーム状であった（Fig.1-A）。製造したチーズの中ではココナッツチーズが最も硬く形状がしっかりしていたが、パター程度の硬さであり一般的なチーズ分類に照らし合わせると軟質チーズに分類されると思われた（Fig.1-B）。そのため食感はほとんどのナッツチーズにおいてクリームチーズの極めて柔らかというものであった。また、油分が多いと感じられる物が多く、香りについては原料ナッツの香りと乳酸発酵によるヨーグルト様の香気を有している物が多く、特にココナッツチーズ、アーモンドチーズ、麻の実チーズは原料ナッツの香りが強く官能的に好ましかった。色については、ココナッツチーズ、麻の実チーズおよびカシューナッツチーズが白色で、松の実チーズとマカダミアナッツチーズは淡黄色、アーモンドチーズは淡褐色であった。

製造したナッツチーズの一般成分、pH、および滴定酸度をTable 2に示した。ナッツチーズのタンパク質含量は、麻の実チーズのみ17.2％であったがそれ以外はいずれも2～3％ほどであった。一般の乳牛から製造されるチーズのタンパク質含量は、クリームチーズで8.2％、カマンベールチーズで19.1％、チェダーチーズで25.7％という分析値が報告されており、ナッツチーズのタンパク質含量はかなり少ないという[8]。脂防含量はナッツチーズでは麻の実、カシューナッツを除いて60％前後であったが、牛乳チーズではクリームチーズで33.0％、カマンベールで24.7％、チェダーチーズで33.8％および30％前後であることが知られており、ナッツチーズは牛乳チーズの2倍近い脂質を含んでいることが示された[9]。ナッツチーズの中では麻の実チーズが水分40.8％、タンパク質17.2％、脂防35.8％と比較的乳牛を原料とする軟質チーズに近い値を示した。pHについてはナッツチーズにより4.41から5.47までの値を示した。牛乳から製造されるチーズではpHは5～6の間にいることが多く、ナッツチーズのpHはそれと同等からやや低い範囲にあるということが示された[10]。滴定酸度の結果をあわせて考えると、滴定酸度IIが低い物が多いことから、緩衝能が低く乳酸発酵によるpHの変動を受けやすいものと推察される。

今回製造したナッツチーズの中では、ココナッツチーズが硬度や香りの点で優れていた。また、原料あたりのチーズ

Table 2  Materials used for cheese production. The weight of the nuts used, the volume of water added, the emulsion volume and the wet weight of the cheese-like food that was produced are indicated.

<table>
<thead>
<tr>
<th>Material</th>
<th>Nuts (g)</th>
<th>Hemp nuts</th>
<th>Coconuts</th>
<th>Almonds</th>
<th>Pine nuts</th>
<th>Cashew nuts</th>
<th>Macadamia nuts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added water (mL)</td>
<td>1,400</td>
<td>0</td>
<td>1,500</td>
<td>1,500</td>
<td>1,500</td>
<td>1,400</td>
<td></td>
</tr>
<tr>
<td>Emulsion volume (mL)</td>
<td>1,500</td>
<td>1,200</td>
<td>1,050</td>
<td>1,500</td>
<td>1,500</td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td>Produced cheese (g)</td>
<td>564</td>
<td>220</td>
<td>92.4</td>
<td>225</td>
<td>249</td>
<td>265</td>
<td></td>
</tr>
</tbody>
</table>

*Canned coconut milk was used.

Fig.1 Appearance of nut-cheeses
A: Almond-cheese, B: Coconut-cheese
Table 3 Composition, pH and acid degree of nut-cheeses

<table>
<thead>
<tr>
<th>Content</th>
<th>Nut-cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemp nut</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>40.8</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17.2</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>35.8</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>3.8</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.5</td>
</tr>
<tr>
<td>pH</td>
<td>5.30</td>
</tr>
<tr>
<td>Acid degree I*</td>
<td>2.36</td>
</tr>
<tr>
<td>Acid degree II*</td>
<td>2.91</td>
</tr>
</tbody>
</table>

*Acid degree I indicates the volume of a 0.1 N NaOH solution required to neutralize 10 g of nut-cheese to pH 7.0.

*Acid degree II indicates the volume of a 0.1 N NaOH solution required to alkalize 10 g of nut-cheese from pH 7.0 to 8.3.

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