Enzyme-assisted Extraction of Bioactive Phytochemicals from Japanese Peppermint (Mentha arvensis L. cv. ‘Hokuto’)

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Abstract: In this study, we provide a method for obtaining essential oil from Mentha arvensis L. in large quantities. Three types of polysaccharide-degrading enzymes were investigated, namely cellulase A “Amano” 3, cellulase T “Amano” 4, and hemicellulase “Amano” 90. The optimum extraction conditions were the combined use of 2 wt% cellulase T and 2 wt% hemicellulase 90, and 3 h of incubation. Enzyme-assisted extraction increased the amount of the essential oil from 2.2 mL to 3.0 mL, compared with the amount extracted without an enzyme.

Key words: Japanese peppermint, enzyme-assisted extraction, essential oil, l-menthol, cellulase

1 Introduction

The genus Mentha, comprising more than 25 species, grows naturally in all continents except Antarctica, and they are found mostly in tropical and subtropical regions¹. Mentha arvensis, M. piperita, M. longifolia and M. spicata are cultivated in many continents, such as Asia, Europe, America, and Australia, for the production of essential oils¹⁻⁵. The essential oils extracted from Mentha are widely used in food, flavors and fragrances, cosmetics, and pharmaceutical industries. Mint oil has various biological activities, such as antimicrobial, anti-acetylcholinesterase, antioxidant, and insecticidal activities⁶⁻¹⁰. Mint oils are also important in medicine for both internal and external applications. Regarding their internal applications, they are widely employed in the treatment of flatulence, nausea, and gastralgia¹¹. Regarding their external applications, they are used in treating rheumatism, neuralgia, congestive headache, and toothache. Consequently, the international demand for mint essential oils has been on the rise over the past few years.

Recently, many enzyme-assisted extraction techniques have been successfully applied for the extraction of essential oils. The use of enzymes for the extraction of essential oils from some plants, such as black pepper, cardamom, cumin, coriander, and bay, has been reported¹²⁻¹⁷. Sowbhagya et al. reported that the application of enzymes, such as cellulase, pectinase, and protease, to garlic resulted in a two-fold increase in the yield of volatile oils¹⁸. Hosni et al. also demonstrated that the application of enzymes to thyme increased the yield of essential oils¹⁹. Although the enzyme-assisted extraction procedure has been proven to increase the yield of essential oils, there are no reports on its application on Japanese peppermint. The present study investigated enzyme-assisted extraction to improve the extraction efficiency of essential oils from Japanese peppermint.

2 Experimental

2.1 Reagents

Lead (II) acetate trihydrate and gallic acid monohydrate were obtained from Kanto Chemical Co. Inc (Tokyo, Japan). Phenol and quercetin dihydrate were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Folin-Ciocalteu reagent was obtained from Kishida Chemical Co. Ltd (Osaka, Japan). Cellulase A “Amano” 3 from Aspergillus niger, cellulase T “Amano” 4 from

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Trichoderma viride, and hemicellulase “Amano” 90 from Aspergillus niger were purchased from Amano Enzyme (Nagoya, Japan). All other chemicals were commercially obtained.

2.2 Plant material and enzyme treatment

The leaves of Japanese peppermint (Mentha arvensis L. cv. ‘Hokuto’) were collected in September 2017 from an experimental field at Kitami Hakk Tsusho Co. Ltd. (43.54° N and 143.54° E). The collected leaves were shade-dried at room temperature. The dried leaves were ground by a rotor mill using a ZM 100 purchased from Nihonseiki Kaisha Ltd. (Tokyo, Japan), and the powder was stored at 5°C. The enzymatic treatment was performed according to the following procedure: a 3.0 g portion of ground leaves was mixed with 30 mL aqueous solutions of three enzymes at different concentrations, viz., 0.1, 1.0, 2.0, 5.0, and 10.0% (w/w), individually. The mixtures were incubated at 40°C with shaking at 120 strokes/min for 1, 3, 6, 12, and 24 h, individually.

2.3 Solvent extraction of volatile components

Three milliliters of n-hexane was added to the mixture containing 3.0 g of the dried leaves after enzymatic treatment, and the resultant mixture was shaken at 120 strokes/min for 10 min. After centrifugation at 5000 rpm for 10 min, the supernatant hexane phase including volatile components was recovered. The volatile components were extracted three times using 3 mL of n-hexane in each case. n-Tridecane was added to the n-hexane extracts as an internal standard for GC-FID and GC-MS analyses, and a final 10 mL solution was prepared by the addition of n-hexane.

2.4 Determination of the total sugar in soaking solution

The total sugar content (TSC) was determined by a spectrophotometric assay using the phenol-sulfuric acid method. A calibration curve of glucose (ranging from 20 to 200 mg/L) was prepared, and the results, determined by the regression equation of the calibration curve \( y = 0.0011 x + 0.0287, R^2 = 0.9988 \), were expressed as mg glucose equivalents per gram of dried leaves (mg GE/g DW). The deproteinization of the soaking solution for the removal of enzymes was carried out using lead (II) acetate. A soaking solution (1 mL), 3 mL of saturated lead (II) acetate aqueous solution, and 21 mL of distilled water were mixed, and the mixture was maintained at room temperature for 20 min. After filtration through celite, 0.5 g of sodium carbonate was added to the filtrate for the removal of excess lead acetate. The solution was filtered through celite once more, and the new filtrate was used as the deproteinized solution. A 5% (w/v) aqueous phenol solution (2 mL) was added to 1 mL of the deproteinized solution in a test tube and stirred properly. Concentrated sulfuric acid (5 mL) was added to the mixture and immediately stirred for 10 s vigorously. The reaction mixture was left standing for 10 min and cooled in a water bath for 20 min. The absorbance of the reaction mixture at 490 nm was measured using a Hitachi U-2810 spectrophotometer. A control sample was also prepared because commercial enzymatic preparations generally use saccharides as excipients. All the samples were analyzed three times, and the mean value was calculated.

2.5 Determination of the total polyphenol and total flavonoid contents in the soaking solution

The total polyphenolic content (TPC) in the soaking solution was determined with the Folin-Ciocalteu reagent using the method of Othman et al. with some modifications. A calibration curve of gallic acid (ranging from 0 to 20 mg/L) was prepared, and the results, determined by the regression equation of the calibration curve \( y = 0.00227 x + 0.011, R^2 = 0.9983 \), were expressed as mg gallic acid equivalents per gram of dried leaves (mg GAE/g DW). Further, 2 mL of the deproteinized solution was added to 5 mL of a 10% (v/v) Folin-Ciocalteu reagent. The obtained solutions were mixed and incubated at room temperature for 8 min, after which 4 mL of 7.5% (w/v) sodium carbonate \((\text{Na}_2\text{CO}_3)\) solution was added. After 60 min, the absorbance was measured at 765 nm. All the samples were analyzed three times and the mean value was calculated.

The total flavonoid content (TFC) in the soaking solution was determined by a spectrophotometric assay according to the method of Djeridane et al. with some modifications. A calibration curve of quercetin (ranging from 0 to 20 mg/L) was prepared, and the results, determined by the regression equation of the calibration curve \( y = 0.0061 x + 0.006, R^2 = 0.9976 \), were expressed as mg quercetin equivalents per gram of dried leaves (mg QE/g DW). This method is based on the formation of a flavonoid-aluminum complex that has a maximum absorbance at 430 nm. Additionally, 2 mL of the deproteinized solution was mixed with 2 mL of 100 mM tris-HCl buffer (pH 7.4) and 0.3 mL of a sodium nitrite \((\text{NaNO}_2)\) solution (0.72 mol/L), and 0.3 mL of aluminum chloride \((\text{AlCl}_3)\) solution (0.75 mol/L) was added after 10 min of incubation at room temperature in the dark. Subsequently, the mixture was allowed to stand for 10 min, after which 2 mL of 1 M sodium hydroxide \((\text{NaOH})\) was added. After incubation at room temperature in the dark for 15 min, the absorbance of the reaction mixture was measured at 430 nm. All the samples were analyzed three times and the mean value was calculated.

2.6 Extraction and analysis of essential oils

For the enzymatic treatment, 50 g of the ground powder of the dried leaves was mixed with 500 mL of distilled water containing 1 g of cellulase T and 1 g of hemicellulase 90. The mixture was incubated for 3 h at 40°C, and thereafter, the whole mixture was subjected to hydrodistillation.
Enzyme-assisted Extraction of Japanese Peppermint Oil

J. Oleo Sci.

for 1 h. The hydrodistillation apparatus was purchased from Tokyo Seisakusho Co. Ltd (Tokyo, Japan). Subsequently, the extracted oil was collected after cooling to room temperature. The quantity of the essential oil was measured. This extraction was performed three times.

The identification and quantification of the essential oils and volatile components were carried out through GC-FID and GC-MS. The GC apparatus was a GC-2014 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID) and an InertCap Pure-Wax column (60 m × 0.25 mm i.d. × 0.25 μm film thickness; GL Sciences, Tokyo, Japan). Helium of chromatographic grade was used as the carrier gas at a flow rate of 1.0 mL/min. The inlet was operated in the split mode with a split ratio of 20:1. The temperature program used was as follows: initially 50°C, increasing at a rate of 5°C/min to 180°C, and maintained at this temperature for 10 min. The injector and detector temperatures were set at 250°C. The relative and absolute concentrations of l-menthol were calculated using an internal standard method based on the GC-FID peak areas, while those of the other volatile components were calculated based on the GC-FID peak areas using the FID response factor. The GC-MS analyses were carried out using a QP-2010 ultra gas chromatography-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a fused silica capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness). The temperature was initially 50°C, after which it was linearly increased to 180°C at a rate of 5°C/min. Helium gas at a flow rate of 1.0 mL/min was used as the carrier gas. The injector port and the ion source were maintained at 250°C. The mass spectrum was scanned from m/z 30-1000 amu. The peak identification was carried out using the NIST14 GC-MS database. The retention indices (RI) were calculated using an n-alkane standard solution (C8-C20).

2.7 Statistical analysis

All the experiments were carried out in triplicates, and the mean of the yields of volatile components, essential oils, and TSC were used for the statistical analysis. Regression analysis was carried out using the EXCEL program (Microsoft Corporation, USA).

3 Results and Discussions

3.1 Optimization of the type and amount of enzymes and the shaking time

It is known that peppermint essential oil exists in cells called oil glands. We hypothesized that the yields of the essential oil could be improved by degrading the cellulose and hemicellulose constituting the oil glands using enzymes. Cellulase A “Amano” 3 from Aspergillus niger, cellubiose T “Amano” 4 from Trichoderma viride, and hemicellulase “Amano” 90 from Aspergillus niger were used in this investigation. The effects of the type and amount of enzymes and the shaking time on the extraction efficiency were investigated by quantifying the extracted l-menthol and l-menthone, which are the main components of Japanese peppermint essential oil. The effects of the type and amount of enzymes on the amount of the extracted volatile components are shown in Fig. 1(a), and the effects of the shaking time are shown in Fig. 1(b). To confirm the effects of the type and amount of enzymes, 0.1, 1.0, 2.0, 5.0, and 10.0 wt% of each enzyme were used on the dry leaves, respectively. Sowbhgya et al. investigated the enzyme-assisted extraction of essential oils from cumin seeds using cellulase and hemicellulase. They concluded that 60 min is optimal for enzymatic treatment. Therefore, the enzymatic treatment was performed for 60 min based on their study [Fig. 1(a)]. All the enzymes increased the extraction yields with the increase in the amount of enzyme, and each enzyme afforded maximum yields of both l-menthol and l-menthone at 2 wt% addition. The yields of the extraction decreased in the order of cellulase T > hemicellulase 90 > cellulase A. Although the amount of l-menthol from 1 g of dried leaves was 16.8 mg without the enzyme, 2 wt% cellulase T achieved a maximum amount of 24.2 mg. The enzyme amount of 2 wt% was found to be optimal; consequently, the shaking time was investigated next. The combined use of cellulase T and hemicellulase 90 was similarly examined. l-Menthol and l-menthone yields were investigated at 1, 3, 6, 12, and 24 h shaking time, respectively. Furthermore, increasing the shaking time improved the extraction yield, and the maximum yield was obtained in 3 h. Contrarily, both yields of l-menthol and l-menthone decreased as the shaking time increased. In this study, a centrifuge tube was used as the reaction vessel. These results were thought to be caused by the volatilization of the essential oil components. Similar to the results for the amount of the enzyme, cellulase T afforded higher yields than cellulase A and hemicellulase 90. Furthermore, the combination of cellulase T and hemicellulase 90 afforded the maximum yield of l-menthol, and the amount of the extracted l-menthol was 23.2 mg. From these results, it was found that when 2 wt% cellulase T and 2 wt% hemicellulase 90 were used in combination and shaken for 3 h, the maximum extraction amount was achieved.

3.2 Total sugar content in the soaking solution

Cellulase and hemicellulase hydrolyze cellulose and hemicellulose into monosaccharides or shorter polysaccharides and oligosaccharides. Cellulose and hemicellulose form the cell wall of plants. It appeared that the amounts of the extracted l-menthol and l-menthone increased due to the enzymatic degradation of the cell wall, which should result in an increase in the amount of sugar in the soaking
The effects of the type and amount of enzyme on the TSC in the soaking solution are shown in Fig. 2 (a), and the effects of the shaking time are shown in Fig. 2 (b). The TSC was determined by the phenol-sulfuric acid method and expressed as glucose equivalents per gram of dry weight (mg GE/g DW). All the enzymes increased the TSC in the soaking solution compared to the case without an enzyme. The TSC increased as the amount of the enzyme increased. For all the enzymes, the TSC at 10 wt% of the enzyme increased by more than ten times compared to the case without an enzyme. Conversely, the TSC increased as the shaking time was extended. The TSC decreased in the order of cellulase T > hemicellulase 90 > cellulase A. This order of enzyme was different from that in the solvent extraction of volatile components. It was considered that these results were obtained because the amount of cellulose in the plant was larger than the amount of hemicellulose. Furthermore, combining the two enzymes increased the TSC more than using a single enzyme. From these results, it was suggested that the amount of the extracted volatile components increased because the enzyme had decomposed cellulose and hemicellulose.

3.3 Total polyphenolic and flavonoid contents in the soaking solution

It appears that the amount of free polyphenolic components eluted into the soaking solution increases as the enzyme decomposes cellulose and hemicellulose. The effects of the type and amount of enzyme on the TPC and TFC in the soaking solution are shown in Fig. 3 (a), and the effects of the shaking time are shown in Fig. 3 (b). The TPC was determined by the Folin-Ciocalteu method and expressed as gallic acid equivalents per gram of dry weight (mg GAE/g DW). The TFC was quantified using aluminum chloride and expressed as quercetin equivalents per gram of dry weight (mg QE/g DW). For all the enzymes, an increase in the enzyme amount resulted in an increase in the TPC. Since a small amount of flavonoids was eluted into the soaking solution, a large change in the TFC was not observed. Contrarily, the TPC increased as the shaking time was extended in all the enzymes. The TPC decreased in the order of cellulase T > hemicellulase 90 > cellulase A. The combined use of cellulase T and hemicellulase 90 increased the TPC compared with the case, in which only a single enzyme was used. This result was in good agreement with the order of enzymes in the extraction of volatile components. Monolignols such as ferulic acid are covalently linked to hemicellulose via an ester linkage. It is considered that the TPC in the soaking solution increased because hemicellulase decomposed the hemicellulose into low molecular weight sugars.

3.4 Hydrodistillation with enzymatic treatment

Currently, the extraction of peppermint oil is carried out by steam distillation in Japan. To confirm that the optimum conditions determined by the solvent extraction method are practical, peppermint oil was extracted by steam distillation from dried leaves by enzymatic treatment. For com-
Enzyme-assisted Extraction of Japanese Peppermint Oil

Fig. 2 Effect of (a) enzyme amount and (b) shaking time on total sugar content. Bars represent the standard deviation. Data are shown as mean ± SD (n = 3).

Fig. 3 Effect of (a) enzyme amount and (b) shaking time on TPC (symbols filled) and TFC (symbols emptied). Bars represent the standard deviation. Data are shown as mean ± SD (n = 3).
Y. Shimotori, T. Watanabe, Y. Kohari et al.

The essential oil was also extracted from dried leaves without enzymatic treatment. Steam distillation was performed three times under both conditions. Enzyme-assisted extraction was carried out with the combined use of 2 wt% cellulase T and 2 wt% hemicellulase 90, which is the optimum condition described above. Peppermint oil (2.2 mL) was extracted from 50 g of dried leaves without enzymatic treatment. Enzyme-assisted extraction yielded 3.0 mL of essential oil. As with n-hexane extraction, the enzymatic treatment caused an increase in the amount of extracted peppermint essential oil. Furthermore, there was no significant difference in the components of both essential oils from GC analysis (Table 1). From these results, we succeeded in increasing the yield of peppermint essential oil from peppermint dried leaves without deteriorating the quality, by enzyme-assisted extraction.

### Table 1 Chemical components of the essential oils from Mentha arvensis L. ‘Hokuto’ with and without enzyme.

| Compounds         | Retention Time (min) | Retention Index | Area (%)       |
|-------------------|----------------------|----------------|----------------|
|                   |                      |                | No enzyme      | Combination use of cellulase T and hemicellulase 90 |
| α-Pinene          | 13.4                 | 1017           | 0.03 ± 0.02    | 0.11 ± 0.03 |
| β-Pinene          | 17.1                 | 1100           | 0.02 ± 0.00    | 0.03 ± 0.00 |
| Sabinene          | 18.1                 | 1115           | 0.02 ± 0.01    | 0.01 ± 0.00 |
| β-Myrcene         | 19.8                 | 1160           | 0.08 ± 0.04    | 0.23 ± 0.08 |
| α-Limonene        | 21.3                 | 1191           | 0.22 ± 0.06    | 0.30 ± 0.05 |
| (Z)-β-Ocimene     | 22.8                 | 1230           | 0.05 ± 0.02    | 0.12 ± 0.02 |
| (E)-β-Ocimene     | 23.5                 | 1247           | 0.05 ± 0.02    | 0.11 ± 0.02 |
| 3-Octanol         | 28.5                 | 1386           | 0.30 ± 0.02    | 0.64 ± 0.03 |
| l-Menthone        | 31.3                 | 1468           | 14.29 ± 0.25   | 13.87 ± 0.15 |
| Isomenthone       | 32.2                 | 1493           | 2.63 ± 0.12    | 3.77 ± 0.06 |
| Menthy acetate    | 34.2                 | 1565           | 1.75 ± 0.14    | 1.68 ± 0.31 |
| Neomenthol        | 35.0                 | 1594           | 1.54 ± 0.05    | 1.50 ± 0.06 |
| Caryophyllene     | 35.5                 | 1608           | 1.08 ± 0.08    | 0.62 ± 0.11 |
| l-Menthol         | 36.4                 | 1650           | 75.05 ± 0.47   | 74.70 ± 0.45 |
| α-Terpineol       | 38.7                 | 1695           | 0.79 ± 0.07    | 0.29 ± 0.05 |
| Piperitone         | 39.1                 | 1736           | 2.10 ± 0.06    | 2.02 ± 0.06 |

a) Identified and determined by GC-MS and GC-FID, respectively.

4 Conclusions

Enzyme-assisted extraction using polysaccharide-degrading enzymes, such as cellulase and hemicellulase, caused an increase in the yields of the essential oil extracted from dried Japanese peppermint leaves. The yields of the extracted essential oil decreased in the order of cellulase T ”Amano” 4 > hemicellulase ”Amano” 90 > cellulase A ”Amano” 3. The yields were further increased by the combined use of cellulase T and hemicellulase 90 compared with the use of a single enzyme. Conversely, all the enzymatic treatments increased the TSC in the soaking solution. It was confirmed that the yields of the extracted essential oil components were increased through the enzymatic degradation of cellulose and hemicellulose. The hydrodistillation of the dried leaves treated with a combination of 2 wt% cellulase T and 2 wt% hemicellulase 90 afforded peppermint essential oil in excellent yield, and it increased from 2.2 mL to 3.0 mL compared with the yield for the extraction without enzymatic treatment. Furthermore, although the enzymatic treatment caused an increase in the amount of the extracted essential oil, the quality did not deteriorate. These results showed that the enzyme-assisted extraction improves the extraction efficiency of the Japanese peppermint essential oil.

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Enzyme-assisted Extraction of Japanese Peppermint Oil

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