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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation Studies on bioactive components from *Perilla* leaves
（シソ葉に含まれる生理活性物質に関する研究）

学位論文要約：
Dissertation Summary

Obesity and diabetes mellitus are two major lifestyle diseases in the world not only in leading countries but also in developing countries. Type 2 diabetes mellitus, a metabolic disorder characterized by insulin resistance or low insulin levels or both, is linked to inflammation and oxidative stress, and is a leading cause of cancer, cardiovascular disease and other diseases. A therapeutic approach for postprandial hyperglycemia is to control the blood glucose level by retarding carbohydrate digestion into glucose with α-amylase and α-glucosidase inhibitors. Additional, the prevalence of Type Ⅰ allergy, such as pollinosis, allergic rhinitis and allergic asthma has been increasing in the recent decades in the leading countries. Japanese cedar pollinosis occurs in spring causing the typical symptoms of seasonal allergic rhinitis, such as sneezing, rhinorrhea, nasal obstruction, nasal itching, and itching of the eyes. Allergic diseases such as rhinitis, which with the high incidence, affects social life, sleep, school and work performance, is also likely to cause a substantial economic burden on society.

Perilla frutescens (L.) Britt. (Lamiaceae) is an edible plant frequently used in some Asian countries such as China, Korea and Japan. *Perilla* leaves are used as a fresh vegetable and a food colorant in Japan and China, because of its bright red color, due to the presence of major anthocyanins, malonylshisonin and shisonin, and other related anthocyanins compounds. Natural resources of anthocyanins are urgently required for safe colorants and additives in the food industry. Moreover, phenolic compounds in *Perilla* leaves such as rosmarinic acid (RA), luteolin, apigenin, and α-linolenic acid have an antiallergic, antiinflammatory, and antioxidant properties, have a neuroprotective effect, act against tumors and suppress diabetes mellitus. RA, a typical phenolic acid in the *Lamiaceae* family,

is potentially valuable for improvement of diabetes mellitus and obesity by inhibiting digestive enzyme.

Supramolecular pigments, found in blue flower petals, such as *Commelina communis*, *Centaurea cyanus*, *Salvia patens*, *Salvia uliginosa*, and *Nemophila menziesii*, are the complex of anthocyanins, flavonoids and metals by intermolecular hydrophobic association and metal complexation of their anthocyanins. Recently, Asada et al. applied aluminum complex formation, based on supramolecular formation, to selectively purify bilberry anthocyanin 3-glycosides from the crude pigments in order to get biological active anthocyanins.

In this study, we tend to isolate some active fractions/compounds for the treatment of obesity, allergy, and other biological activities using the novel technique of supramolecular, and to improve the application value of *Perilla* leaves.

In chapter 2, we successfully applied supramolecular technology in RA extract separation from *Perilla* leaf extract. By the supramolecular complex which was formed from flavocommelin and *Perilla* leaf extract as initial materials, the supernatant containing rosmarinic acid was isolated. Rosmarinic acid extract (62.9% purity) was further and partly purified by partitioning ethyl acetate and water (Figure 1).

RA extract isolated from *Perilla* leaf extract using the supramolecular technique showed the greatest Total phenolics (TP), 433.9 ± 58.6 mg/g gallic acid equivalent (GAE) among the supramolecular products (Figure 1). The TP of standard RA (711.8 ± 5.3 mg/g GAE) was higher than RA extract. The TP of RA extract was calculated to be 61.0% of standard RA. Therefore TP of RA extract was interpreted by the purity of RA (62.9 ± 4.5%) in the extract. In addition, RA extract exhibited the highest DPPH radical scavenging activity (DRSA) with an SC_{50} value of 5.5 ± 0.2 μg/mL, which was more than two times higher DRSA than *Perilla* leaf extract (SC_{50} value, 10.8 ± 0.1 μg/mL).

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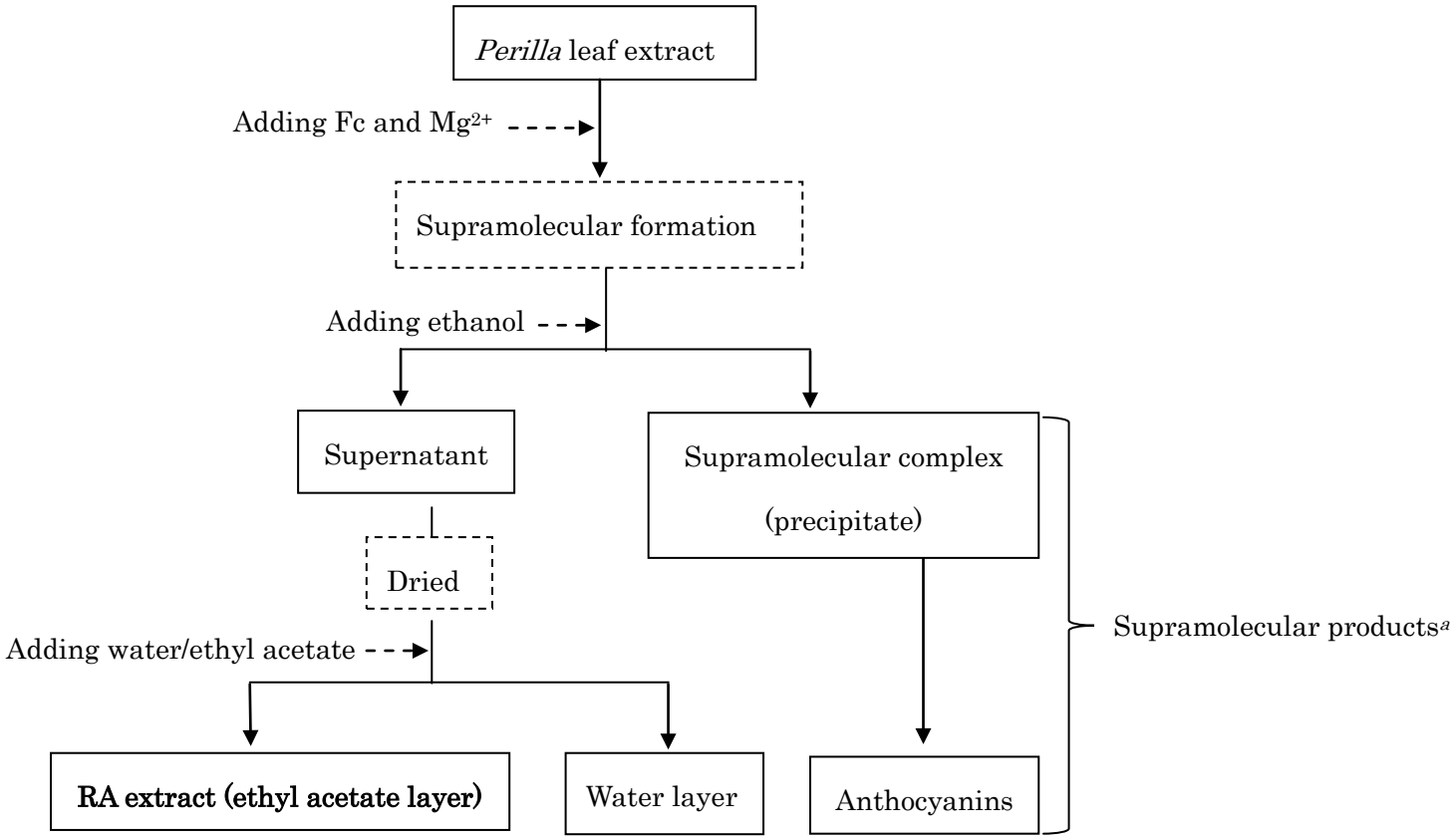

Figure 1

Rosmarinic acid extract and anthocyanins isolated from *Perilla* leaf extract by supramolecular formation. [a]Supramolecular products are supernatant and supramolecular complex obtained by supramolecluar formation, and the related extracts such as RA extract, water layer and anthocyanins described above.

For antiallergic activity, RA extract (52.9 ± 6.7 μg/mL) suppressed *β*-hexosaminidase release 3.9 times and 2.6 times more than *Perilla* leaf extract and standard RA, respectively, and exhibited the highest antiallergic activity. Also, it is suggested that other compounds as well as RA in RA extract may contribute to antiallergic activity. Rosmarinic acid methyl ester (RA-me) was found as a minor component in RA extract suppressed *β*-hexosaminidase release with IC_{50} of 9.9 ± 0.8 μg/mL. At IC_{50} of RA extract (52.9 μg/mL), it was calculated as 33.3 μg/mL for RA and 3.7 μg/mL for RA-me, respectively, which contributed to 43% *β*-hexosaminidase release inhibition as an additive effect. Therefore, RA-me, as a component in RA extract, contributed to high *β*-hexosaminidase release inhibitory activity besides RA. We found the greatest activity of RA-me in RA extract and supernatant as a minor component.

For *α*-glucosidase inhibitory activity, RA extract inhibited 4.1 times and 1.8 times higher than standard RA (IC_{50} value, 0.95 ± 0.01 mg/mL) and *Perilla* leaf extract (IC_{50} value, 0.42 ± 0.01 mg/mL), respectively, although RA is reported to be a better research target on *α*-glucosidase inhibitory activity. It is found that IC_{50} values of caffeic acid and RA-me as minor components in RA extract were 3.46 ± 0.18 mg/mL and 0.44 ± 0.01 mg/mL, respectively. It showed that RA-me exhibited greater α-glucosidase inhibitory activity than standard RA. We concluded that RA extract containing RA-me and other minor components as unknown compounds besides RA contributed to high α-glucosidase inhibitory activity, especially unknown compounds might highly contribute to the activity.

Through supramolecular formation, RA-containing supernatant was excluded by supramolecular complex and RA extract (62.9 ± 4.5% purity) with potent biological activities was obtained. The obtained RA extract had DPPH radical scavenging activity, antiallergic activity and *α*-glucosidase inhibitory activity. RA extract exhibited higher antiallergic activity and *α*-glucosidase inhibitory activity than standard RA. This study shows that RA and other minor components in RA extract contributed antiallergic activity and *α*-glucosidase inhibitory activity. We will discuss molecular mechanisms of the activities in detail by linking with the interaction of biologically important chemicals after identification of these active compounds. In conclusion, the RA extract isolated from *Perilla* leaf extract by supramolecular formation and solvent extraction has potential applications in managing diabetes mellitus and other chronic and degenerative diseases.

As rosmarinic acid-rich extract with high *α*-glucosidase inhibitory activity was isolated on a large

scale using a supramolecular separation method and the contribution of rosmarinic acid methyl ester, a minor component in the extract contributed to *α*-glucosidase inhibitory activity more than double that of rosmarinic acid, in Chapter 3, we investigated the esterification of rosmarinic acid and its related compounds as an approach to optimize *α*-glucosidase inhibitory activity as a leading compound to explore potentially attractive therapeutic reagents for diabetes mellitus. Methyl, propyl and hexyl esters of rosmarinic acid (RA), caffeic acid (CA) and *p*-coumaric acid (Cou) synthesized were assessed *α*-glucosidase inhibitory activities.

Rosmarinic, caffeic and *p*-coumaric acids esterified with methyl, propyl and hexyl chains showed great *α*-glucosidase inhibitory activity. Rosmarinic acid hexyl ester exhibited the greatest *α*-glucosidase inhibitory activity with IC_{50} values of 0.05 mM for *B. stearothermophilus* source, and 0.61 mM for *S. cerevisiae* source. The *α*-glucosidase inhibitory activity of these esters was greatly related to alkyl chain length of each ester series, and the correlation between Log*P* (octanol-water partition coefficient) and pIC_{50} values ($-\log IC_{50}$) of all these compounds was observed as positive correlation coefficients, 0.80 for α-glucosidase of *B. stearothermophilus* source ($p<0.001$) and 0.46 for *S. cerevisiae* source ($p<0.05$). Furthermore, the inhibition kinetics and interaction mechanism of rosmarinic acid hexyl ester was investigated. It was found that rosmarinic acid hexyl ester was a mixed inhibition type, with the inhibition constants, K_i of 0.24 mM (for the binding with the free enzyme), and K_i' of 0.80 mM (for the binding with the substrate-enzyme complex). It is suggested that rosmarinic acid hexyl ester has a strong binding affinity with the free enzyme. Additionally, the molecular docking result shows that rosmarinic acid hexyl ester was highly fitting to the active site pocket by the hydrophobic interaction and hydrogen bonding of 3,4-dihydroxyphenyl group with amino acids surrounded.

Elongation of alky chain of rosmarinic acid, caffeic acid and *p*-coumaric acid esters in accompany with an increase of hydrophobicity, enhanced α-glucosidase inhibitory activity. Rosmarinic acid hexyl ester with great inhibitory activity is supposed to be a high binding affinity with the enzyme pocket. The molecular docking shows that hydroxyl groups forming hydrogen bonding with the important amino acid residues, surrounding by the hydrophobic amino acids, fitting to the active site pocket of α-glucosidase, which may lead to great inhibitory activity of rosmarinic acid hexyl ester on *α*-glucosidase. The inhibition kinetics and molecular docking of rosmarinic acid hexyl ester facilitate

its inhibition mechanism evaluation. The further study of rosmarinic acid hexyl ester on its toxicity, *in vivo* test and food/medicinal application should be deeply investigated.

In chapter 4, methyl, propyl and hexyl esters of rosmarinic, caffeic and *p*-coumaric acids were tested for antiallergic activity using *β*-hexosaminidase release suppression. Among all free acids and esters, rosmarinic acid propyl ester (RA-pro, IC_{50}, 23.7 μM) exhibited the greatest suppression on *β*-hexosaminidase release, followed by rosmarinic acid hexyl ester (RA-hex, IC_{50}, 34.5 μM), caffeic acid propyl ester (CA-pro, IC_{50}, 55.3 μM) and rosmarinic acid methyl ester (RA-me, IC_{50}, 57.3 μM).The activity of rosmarinic acid propyl ester was only 9.1 times lower than luteolin (IC_{50}, 2.6 μM), a flavonoid with high antiallergic activity that was tested in a parallel experiment of our laboratory, but increased 55.4 times compared to the activity of rosmarinic acid.

Furthermore, strong quadratic correlations were observed between Log*P* and pIC_{50} ($-\log IC_{50}$) for each of the acid-esters series, with correlation coefficients equal to 0.94 ($p < 0.05$) for the rosmarinic acid series, 0.98 ($p < 0.01$) for the caffeic acid series and 1.00 ($p < 0.01$) for the *p*-coumaric acid ester series. The quadratic equations reflect the lowering of antiallergic effects of hexyl esters after the very strong effects of shorter esters. Our results showed that the hydrophobic propyl esters of RA, CA and Cou strongly suppressed *β*-hexosaminidase release, thus it is possible that propyl esters have higher affinity to the cross-linked IgE/Fc*ε*RI receptor while the bulky hexyl esters would bind less efficiently.

Esterification of rosmarinic, caffeic and *p*-coumaric acids with short alkyl chains influences their antiallergic activity. Propyl esters exhibited the highest antiallergic activity. Hydrophobicity and steric bulkiness of the alkyl chain are likely to be modulating the interaction between phenolic acid/esters and the binding sites in the IgE/Fc*ε*RI receptor. Furthermore, our data indicates that the 3,4-dihydroxyphenyl moieties in phenolic ester backbone could be essential for the inhibitor-binding site interaction. The elucidation of mechanism underlying the interactions between phenolic acids/esters warrants further investigations.

In conclusion, rosmarinic acid extract with high biological activities including DPPH radical scavenging activity, antiallergic activity and *α*-glucosidase inhibitory activity, can be easily extracted from *Perilla* leaves using a supramolecular technique with a newly-established isolation method. Rosmarinic acid extract shows high potential for diabetes mellitus and allergy treatments by inhibiting *α*-glucosidase activity and measuring *β*-hexosaminidase, related to life-style disease. Esters of

rosmarinic acid and its related compounds exhibited enhanced *α*-glucosidase inhibitory activity and antiallergic activity. Elongation of alky chain of rosmarinic acid, caffeic acid and *p*-coumaric acid esters in accompany with an increase of hydrophobicity, enhanced *α*-glucosidase inhibitory activity. Rosmarinic acid hexyl ester with great inhibitory activity is supposed to be a high binding affinity with the enzyme pocket. The antiallergic activity of these compounds is modulated by their hydrophobicity and bulkiness of alkyl chain and rosmarinic acid propyl ester exhibited the greatest *β*-hexosaminidase release suppression. The further study of the active rosmarinic acid esters on its toxicity, *in vivo* test and food/medicinal application should be deeply investigated.

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