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Article

Salty taste enhancement of rosemary water extract in mouse and human

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This study examined the effects of spices and herbs on aromatic amino acid decarboxylase (AroDC) activity, an enzyme that catalyzes the synthesis of the neurotransmitter serotonin in the taste cells of the oral papilla, and the effects of spices and herbs on salty taste. Among 36 types spices and herbs, rosemary (whole), dill seed, paprika, mace, and ginger showed a tendency to activate recombinant mammalian AroDC. Mice with preference for salty taste due to diuretic treatment licked 15-60 mM NaCl solution containing rosemary or paprika extracts more frequently than a solution of NaCl alone. In the sensory evaluation, the 68 mM NaCl solution containing rosemary extract was rated as having significantly higher saltiness than the NaCl solution without the extract. These results suggested that rosemary extract contains components that increase salty taste intensity. Therefore rosemary is expected to be utilized for low salt food products that do not decrease saltiness intensity.

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Introduction

The World Health Organization (WHO) guidelines on sodium intake require adults to limit their salt intake to less than 5 g/day in order to reduce the risk of diseases caused by excessive salt intake. However, according to the results of the 2019 National Health and Nutrition Survey, the average salt intake of Japanese people is 10.9 g for men and 9.3 g for women, which is about twice the amount recommended by the WHO. Considering the actual salt intake of Japanese people, the 2020 edition of the Japanese Dietary Intake Standards sets the target amount of salt intake for adults as less than 7.5 g for men and less than 6.5 g for women. Although the average salt intake of Japanese people is decreasing, excessive salt intake remains a problem and is far from achieving the target amount.

In order to achieve salt intake to the target level, it is essential to reduce salt intake and salt reduction methods are required in which good taste is maintained even with reduced salt. In recent years, various methods of salt reduction have been considered to supplement the loss of

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taste due to salt reduction, including the use of salt substitutes and salt enhancers, the use of salt localization in foods and food properties, the use of synergies and contrasting effects with other flavor components, and the use of aromas [1-3]. Potassium chloride, a common strategy for sodium reduction [4, 5], has the disadvantage of having a distinct bitterness and harshness [4], which limits the amount of potassium chloride used. There are also known methods to compensate for the lack of salt by using umami and acidity, but these methods change the taste, limiting the foods and dishes in which they can be used.

As an alternative to the conventional method, we focused on ways to enhance saltiness by enhancing taste signaling. Taste is perceived by taste buds on the epithelium of the tongue, which are composed of dozens of taste cells. Taste cells are classified into type I, II, and III taste cells and type IV basal cells [6]. It is hypothesized that the taste cells receive each of the five basic tastes separately and transmit this information to taste nerves connected to synapses, but there are many aspects that remain to be elucidated. Currently, among the five basic tastes, sweet, umami, and bitter are thought to be accepted by type II, sour by type III, and salty by type III. Type III taste cells synapse with taste nerve fibers expressing serotonin receptors, and express aromatic L-amino acid decarboxylase (AroDC) [7], which catalyzes the production of serotonin [8]. Some previous studies have suggested that serotonin released from taste buds activates serotonin receptors on taste nerve fibers to facilitate taste signaling [9, 10]. These findings suggest that increased serotonin production may promote taste signaling of Type III taste cells and salty taste might be enhanced if salty substances was accepted by type III cells.

In a previous study by Ueno et al., the salty taste enhancing effect of spices that activate glutamate decarboxylase 67 (GAD67) was investigated [11]. GAD67 is an enzyme that catalyzes the production of γ-aminobutyric acid in type III taste cells. The salty taste-enhancing effect of spice extracts, which activate GAD67, was investigated by sensory evaluation, and a positive correlation was found between the salty taste-enhancing effect of the extracts and GAD67 activity [11]. Both AroDC and GAD67 are enzymes that catalyze the production of neurotransmitters in type III taste cells, and spice extracts with components that activate AroDC are expected to have a salty taste enhancing effect, as indicated by GAD67.

In this study, spices and herbs with the potential to activate AroDC were examined for their saltiness enhancing effects by the licking behavior in mice and sensory evaluation in humans.

Materials and Methods

Sample preparation

Extracts were prepared using commercially available spices and herbs (24 types of powder and 12 types of whole). One hundred grams of water extract was prepared from 0.35 g and 7.0 g samples for enzyme assay and mouse licking, respectively. The extraction was performed separately according to the shape of the spice or herb (powder or whole), referring to the method of Kohri et al. [12]. After filtering 0.35 g or 7.0 g of the powder with 80 g of hot water above 95°C, hot water was added to reach 100 g. Whole spices were placed in 0.35g or 7.0g tea bags and immersed in 80-90 g of hot water in a thermostatic bath at 95°C for 5 minutes. The tea bag was removed and water was added to reach 100 g. In this sample preparation, the amount of water-soluble substances of spices is not consistent among samples. For comparisons

between samples regarding the effects of the components, the dry weight should be determined, from which the same weight should be dissolved in the same volume of solution.

NaCl and other chemicals used in the present study were reagent special grade. Seasoning salt was used as NaCl for sensory evaluation.

AroDC activity assay

Human AroDC cDNA provided by RIKEN BioResource Center was amplified using the following primers.

5′ - acgcccatggacgcaagtgaattccgaag

3′ - acgcctcgagctccctctctctgctcgcagca

These primers were designed to express human AroDC as an N-terminal His-tag fusion protein in pET28a.

Transformed *E. coli* BL21 (DE3) cells were grown at 37 °C in Luria-Bertani medium supplemented with chloramphenicol at the concentration of 50 mg /ml. At logarithmic growth phase, isopropyl β-D-thiogalactoside was added into the media to give a final concentration of 0.1 mM. At this stage, the incubation temperature was lowered to 25 °C. The cells were incubated for ~20 h and then collected by centrifugation and resuspended in 50 mM phosphate buffer (pH 6.8) containing 50 mM NaCl and protease inhibitor cocktail (Roche). After cell disruption by the sonication, the lysates were centrifuged at 16,000 g for 60 min. The supernatant was collected and loaded onto a histidine affinity column TALON Metal Affinity Resins (Takara Bio). Histidine–tagged protein was eluted from the column with an elution buffer (50 mM HEPES (pH 7.4), 50 mM NaCl, 150 mM imidazole). Then, the sample was loaded onto a RESOURCE™ Q column (Cytiva) that was pre-equilibrated with 10 mM HEPES (pH 7.0) containing 10 mM NaCl. The column was washed with the same buffer, and the desired protein fraction was eluted with a linear gradient of NaCl from 10 to 250 mM at a flow rate of 1 ml/ min. K_m and k_{cat} for the purified enzyme catalyzing decarboxylation with 5-hydroxytryptophan were 0.22 mM and 2.4 s^{-1}, respectively.

The AroDC activity assay mixture contained 10 µl of a test spice or herb extract, and 180 µl of enzyme solution in 100 mM potassium phosphate buffer, pH 6.8, and the reaction was initiated by adding 10 µl of 20 mM 5-hydroxytryptophan at 37 °C. After 10 min of incubation, the reaction was terminated by adding 10 µl of 60% perchloric acid. The serotonin produced in the assays was measured by injecting the aliquot of the assay mixture onto a high-performance liquid chromatography (HPLC) system equipped with a histamine Pak column (Tosoh, Tokyo, Japan). Separated serotonin was fluorometrically measured by using the *o*-phthalaldehyde method as described previously [13]. Quantification of serotonin was made based on the peak area. The AroDC activity (%) of each sample was calculated by the following equation: [(serotonin peak area of sample) - (serotonin peak area of blank)]/[(serotonin peak area of control) - (serotonin peak area of blank)] × 100.

Licking behavior test

Five 4-week-old male C57BL/6 mice were fed *ad libitum* a standard food and water for 1 week to acclimate to the environment. After learning the lick behavior in the lick counting device LKT-1 (Melquest Ltd. Toyama, Japan), mice were deprived of water for 20 hours. Then the number of licking NaCl solution were measured for 10 seconds in the cage equipped with the lick device. Considering that the total ion concentration in the body of mice, rats, and humans is approximately 150mM, that rats prefer NaCl concentrations around 150mM [14], and that the increase in salt concentration higher than 200 mM decreased the number of licks in mice [15], NaCl concentration was set from 0 to 1.0 M. The NaCl solution alone

and NaCl dissolved in each spice extract were prepared, respectively. Wilcoxon signed rank test was used for evaluating significant differences in NaCl alone and NaCl with spice extracts.

Two mice with a preference for saltiness were prepared by treating a diuretic reagent referring to a previous study [16], and the number of licks of the mice for NaCl solution at low concentrations from 0 to 60 mM was counted. Briefly, two 4-week-old male C57BL/6 mice were fed *ad libitum* a standard food and water for ~ 1 month to acclimate to the environment. After learning the lick behavior in the device LKT-1, powdered diets formulated to be NaCl-free and containing 0.03% spironolactone were prepared and reared for ~ 3 weeks. The mouse was deprived of water for 20 hours and then the number of licks of NaCl solutions were measured for 10 seconds in the cage equipped with the lick device. Wilcoxon signed rank test was used for evaluating significant differences in NaCl alone and NaCl with spice extracts.

Spilanthol, an isolated compound from *Acmellaoleracea*, which was confirmed to increase the number of licks in a previous study [17], was purchased as a reagent from FUJIFILM Wako Chemicals (Tokyo, Japan). The present study was carried out according to the Ochanomizu University Animal Experimentation Regulations (approval number 23010).

Sensory evaluation

Eleven women in their 20s participated as panelists. As conditions for participation, panelists were asked to have a normal sense of taste and smell, not to eat or drink for one hour prior to the evaluation, and to refrain from using perfume or strong-smelling hairdressing products. Prior to the examination, the panelists were fully briefed on the purpose of the study, the contents and methods of the study, and the protection of data obtained in this experiment, and their written consent to participate was obtained. The test was conducted in the sensory testing room of Ochanomizu University. The temperature and humidity in the testing room were 21°C and 67%, respectively. Red lighting was used to exclude color effects, considering the possibility that the color of spices could affect saltiness sensitivity.

For concentration difference discrimination test, since the salt concentration of common soups is 140 to 170 mM, the standard salt concentration was set at 68 mM, which is a 50% reduction in salt. With reference to previous studies [12], for selection of panelists who could identify a concentration difference of 9 mM, five solutions with different salt concentrations (51, 60, 68, 77, and 86 mM) were prepared in transparent plastic cups containing 30 ml each, with labels E to I randomly attached. The panelists were asked to rearrange them in order of concentration, and their ability to discriminate differences in concentration was evaluated. Panelists were required to rinse their mouths with water before tasting each sample and spit out the sample each time.

The same concentration as in the concentration difference discrimination test, 68 mM, was used in the sensory evaluation test. This salt concentration was set to investigate the saltiness enhancing effect of spice extracts at lower salt concentrations than common soups. Samples of 68 mM NaCl solution (control) and 68 mM NaCl solution with extracts of each spice were poured in 20 ml portions into 90 ml plastic cup and presented with labels of K to M randomly attached so that the type of spice could not be identified. They first rinsed their mouths with water and tasted the control in their mouths. Then they spit out the solution and rinsed their mouths with water again. They were then asked to taste and swallow a 68 mM NaCl solution containing each spice extract in their mouth. Finally, the

samples were compared with the control to evaluate the taste intensity (-3: very weak, -2: weak, -1: slightly weak, 0: no change, +1: slightly strong, +2: strong, 3: very strong) on a 7-point scale from -3 to +3, respectively, with 0 for the control. Any order of drinking, but the number of times to drink again was limited to once to account for the fatigue effect of continuous stimulation.

The results of the concentration difference discrimination test were evaluated by using Spearman's rank correlation coefficient [18]. Mann-Whitney's U test was used for evaluating significant differences in taste intensity between controls and spice samples [19]. Sigma Plot (Systat Software Inc. CA, US) was used for statistical analysis.

Results and Discussion

When AroDC activity was examined for 36 spice and herb samples, rosemary (whole), dill seed, paprika, mace, and ginger showed a tendency toward activation (Fig. 1). In descending order of AroDC activation rate were rosemary (whole), dill seed, paprika, mace, and ginger. The average of the results after seven repetitions was 100.9% in rosemary, indicating the possibility of activation. Dill seed, paprika, mace, and ginger could inhibit AroDC activity, but the inhibition was not strong.

The effect of adding rosemary and ginger extracts on the number of times mice licked the NaCl solution was investigated. At NaCl concentrations of 600 to 1000 mM, the number of licks decreased as the concentration increased (Fig. 2). The number of licks decreased significantly in the NaCl with rosemary extract than in the NaCl alone (Fig. 2 (a)). It was inferred that the addition of rosemary extract increased the salty taste and decreased the number of licks. The addition of ginger extract significantly reduced the number of licks regardless of the concentration of NaCl (Fig. 2(b)), suggesting that ginger extract contains ingredients that prevent mice from licking.

In mice under conditions favoring aqueous NaCl solutions, licking frequency increased with increasing NaCl concentration from 0 to 60 mM (Fig. 3). Addition of spilanthol increased the number of licks at 60 mM NaCl solution significantly compared to the NaCl alone (Fig.3).

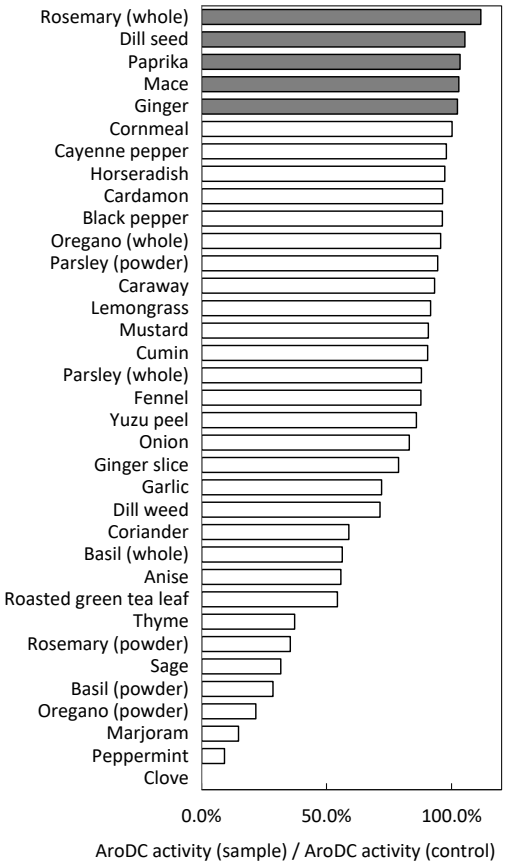

Fig. 1. AroDC activity (%) in the presence of spice and herb extract against AroDC activity in the absence of spice and herb extract. Gray bars indicate activity exceeding 100%.

By addition of rosemary extract, the number of licks increased significantly compared to the NaCl alone at 45 mM NaCl (Fig. 4(a)). Paprika extract showed a tendency to increase the number of licks compared to the NaCl alone at NaCl

concentrations of 15-60 mM, but those were not significant differences (Fig. 4 (b)). Ginger extract significantly reduced the number of licks compared to the control group, regardless of NaCl concentration (Fig. 4 (c)). In the ginger extract, as in the case of the higher NaCl concentrations discussed earlier, it was suggested

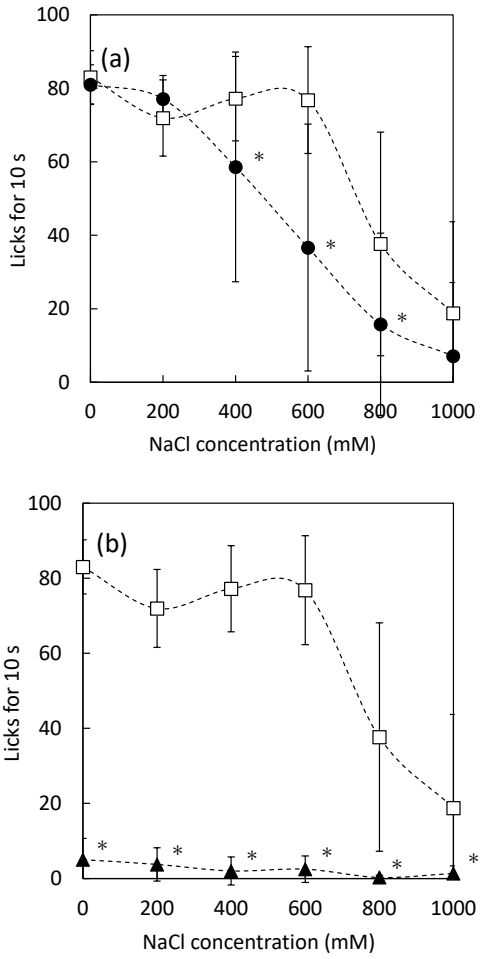

Fig. 2. Number of licks at 0-1000 mM NaCl solutions in the presence of (a) rosemary extract and (b) ginger extract against 0-1000 mM NaCl solutions. □: NaCl solutions, ●: NaCl solutions with rosemary extract, ▲: NaCl solutions with ginger extract. *p < 0.05 Wilcoxon signed rank test. n = 15

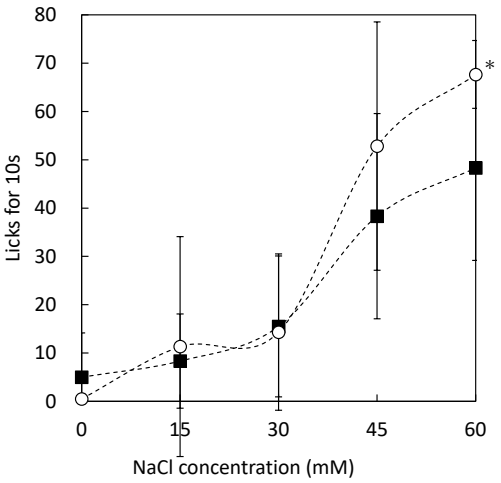

Fig. 3. Number of licks at 0-60 mM NaCl solutions in the absence (■) or presence (○) of 30 ppm spilanthol. Mice with a preference for saltiness were prepared by treating a diuretic reagent. *p < 0.05 Wilcoxon signed rank test. n = 6

that some components were present that caused licking to be avoided.

In the lick test on mice, salty-taste enhancing effect was observed for rosemary extract. In mice, at low NaCl concentrations, saltiness is thought to be transmitted via the ion channel inhibitor amiloride-sensitive (AS) receptor pathway, and at high NaCl concentrations above 150 mM, saltiness is thought to be transmitted via the amiloride-insensitive (AI) receptor pathway [20-22]. In the present study, salty-taste enhancing effects were observed for both low and high concentrations of NaCl, suggesting that rosemary extract affected both the AS and AI receptor pathways. AI-sensitive saltiness has been reported to be mediated by a type III taste cell in mice [23]. Although AI receptors have not yet been identified and the detailed reception mechanism of salty substances in taste cells is unclear, it is clear that the perception of saltiness mediated by type III taste cells requires signal transduction to the gustatory nerve fibers that connect to type III taste cells outside the cell. Serotonin has been

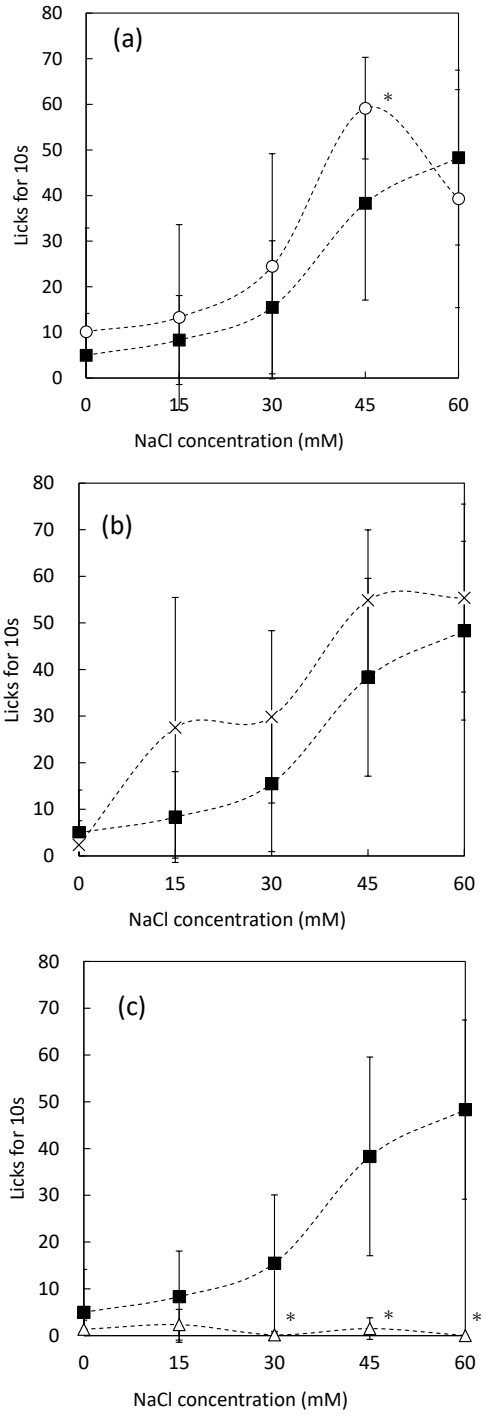

Fig. 4. Number of licks at 0-60 mM NaCl solutions in the absence or presence of (a) rosemary, (b) paprika, and (c) ginger extracts. Mice with a preference for saltiness were prepared by treating a diuretic reagent. ■: NaCl solutions, ○: NaCl solutions with rosemary extract, ×: NaCl solutions with paprika extract, △: NaCl solutions with ginger extract. *p < 0.05 Wilcoxon signed rank test. n = 6

reported to be one of neurotransmitter from type III cells to nerves [9, 10]. The rosemary extract used in this study activated AroDC *in vitro,* as shown in Figure 1. If AroDC was activated in type III cells, serotonin production would have been enhanced and serotonin-mediated signaling could have been enhanced. This could have resulted in increased salt sensitivity. Another possible interpretation of the effect of rosemary observed in this study is odor-induced saltiness enhancement, which has been reported in humans [24, 25], but the reports examining the effect of odor-induced saltiness enhancement in mice could not be found.

Table 1. Saltiness of 68 mM NaCl solution with rosemary, paprika and ginger extract compared to 68 mM NaCl solution.

**p < 0.01 Mann-Whitney's U test

		Saltiness intensity (n = 11)		
Score		Rosemary**	Paprika	Ginger
+3	Very strong	0	0	0
+2	Strong	1	1	3
+1	Slightly strong	5	5	3
0	No change	5	3	3
-1	Slightly weak	0	2	2
-2	Weak	0	0	0
-3	Very weak	0	0	0

In the sensory evaluation, a panel of 11 people who were able to discriminate concentration differences of NaCl were asked about the intensity of the salty taste of the 68 mM NaCl solution with and without spice component. A panel of eleven was selected from the twelve who were evaluated as being able to identify concentration differences by Spearman's rank correlation coefficient. (data not shown). The NaCl solution with rosemary showed significant saltiness enhancement at $p < 0.01$ (Table 1).

Paprika and ginger showed a trend toward saltiness enhancement, but not significantly (Table 1).

In the previous studies, saltiness enhancing effect of rosemary extract was confirmed[12] and aroma was perceived at the experimental condition and thus saltiness enhancement was considered to be due to the aroma. In the present study, sensory evaluation was conducted with a concentration of 0.01% of the spice extract in order to eliminate the influence of aroma. Therefore, the saltiness enhancement shown in this study was most likely caused by non-aroma components. Since the AI receptor pathway has been reported to be predominant over the AS receptor pathway at all NaCl concentrations in humans [26, 27], one possible interpretation is the activation of salty taste signaling through type III taste cells by the rosemary components, as described in the section on lick studies in mice. However, five of the 11 participants in the study indicated that they could taste some flavor. The scent thresholds are known to be different individually, and it is thought that panels with smaller thresholds perceived even small amounts of scent. In order to completely exclude the influence of scent, future studies will need to use the nose clip method.

In this study, the effects of 36 spices on AroDC activity were investigated, from which rosemary, paprika, and ginger were selected for mouse lick test and sensory evaluation. The results showed that rosemary had a salty taste enhancing effect.; AroDC screening might be useful in selecting spices that exhibit saltiness enhancing effects. However, yuzu peel, anise, and cumin, which exhibit GAD67 activation and saltiness enhancing effects [11], were not selected. Although speculative, it is possible that sorting by AroDC activation or GAD67 activation may select samples with different salty taste enhancing mechanisms.

In conclusion, rosemary exhibited a salty taste enhancing effect in the mouse and human in the present study. Rosemary was considered to contain a saltiness-enhancing component. This study was conducted at very low concentrations to prevent aroma effects, and it is unlikely that the components that enhance salty taste are aroma components. It is necessary to perform sensory evaluation in an environment that excludes the influence of scent in future work. Additionally, experiments will be conducted to identify salty taste-enhancing compounds derived from rosemary.

References

[1] Kawai, K., (2017) *J. Brew. Soc. Jpn.* **112**, 22-28.

[2] Ishida, M., Tezuka, H., Hasegawa, T., Cao, L., Imada, T., Kimura, E., Matsumoto, H., Kawano, R., Arai, H., (2011) *J. Jpn, Soc. Nutr. Food Sci.* **64**, 305-311.

[3] Ogasawara, Y., Yoshida, T., Okada, C., Sakamoto, M., Akano, H., Hatae, K., (2009) *J. Cookery Sci., Jpn.* **42**, 238-243.

[4] Sinopoli, D.A., Lawless, H.T., (2012) *J Food Sci*. **77**, S319-322.

[5] *Taste and flavor roles of sodium in Foods*, in *Strategies to Reduce Sodium Intake in the United States*, J.E. Henney, C.L. Taylor, and C.S. Boon, Editors. 2010: Washington (DC). p. 67-90.

[6] Farbman, A.I., (1965) *J Ultrastruct Res*. **12**, 328-350.

[7] Seta, Y., Kataoka, S., Toyono, T., Toyoshima, K., (2007) *Histochem Cell Biol*. **127**, 415-422.

[8] Pan, H.R., Tian, M., Xue, J.B., Li, S.M., Luo, X.C., Huang, X., Chen, Z.H., Huang, L., (2018) *Front Cell Neurosci*.

12, 461.

[9] Stratford, J.M., Larson, E.D., Yang, R., Salcedo, E., Finger, T.E., (2017) *J Comp Neurol*. **525**, 2358-2375.

[10] Larson, E.D., Vandenbeuch, A., Voigt, A., Meyerhof, W., Kinnamon, S.C., Finger, T.E., (2015) *J Neurosci*. **35**, 15984-95.

[11] Hisaki, K., Hamano, K., Ueno, H., (2014) *J. Biol. Macromol.* **14**, 3-14.

[12] Kohri, T., Kira, R., Myojin, C., Kawanishi, M., Tomotake, H., (2020) *J Nutr Sci Vitaminol (Tokyo)*. **66**, 325-330.

[13] Nitta, Y., Kikuzaki, H., Ueno, H., (2007) *J Agric Food Chem*. **55**, 299-304.

[14] Kawamura, Y., Okamoto, J., Funakoshi, M., (1968) *Physiol. Behav.* **3**, 537-542.

[15] Kawai, T., Kusakabe, Y., (2007) *Jpn. J. Taste Smell Res.* **14**, 305-308.

[16] Kawai, T., Ookura, T., Kusakabe, Y., (2008) *Jpn. J. Taste Smell Res.* **15**, 489-492.

[17] Miyazawa, T., (2020) *J. Cookery Sci., Jpn.* **53**, 295-297.

[18] Spearman, C., (1904) *Am. J. Psychol.* **15**, 72–101.

[19] Mann, H.B., Whitney, D.R., (1947) *Ann. Math. Statist.* . **18**, 50-60.

[20] Nomura, K., Nakanishi, M., Ishidate, F., Iwata, K., Taruno, A., (2020) *Neuron*. **106**, 816-829 e6.

[21] Yoshida, R., Horio, N., Murata, Y., Yasumatsu, K., Shigemura, N., Ninomiya, Y., (2009) *Neuroscience*. **159**, 795-803.

[22] Chandrashekar, J., Kuhn, C., Oka, Y., Yarmolinsky, D.A., Hummler, E., Ryba, N.J., Zuker, C.S., (2010) *Nature*. **464**, 297-301.

[23] Lewandowski, B.C., Sukumaran, S.K., Margolskee, R.F., Bachmanov, A.A., (2016) *J Neurosci*. **36**, 1942-53.

[24] Djordjevic, J., Zatorre, R.J., Jones-Gotman, M., (2004) *Exp Brain Res*. **159**, 405-408.

[25] Shimoda, M., (2007) *Jpn. J. Taste Smell Res.* **14**, 3-8.

[26] Feldman, G.M., Mogyorosi, A., Heck, G.L., Desimone, J.A., Santos, C.R., Clary, R.A., Lyall, V., (2003) *J Neurophysiol*. **90**, 2060-2064.

[27] Halpern, B.P., (1998) *Neurosci Biobehav Rev*. **23**, 5-47.

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