Bitter Taste Receptor Antagonists Inhibit the Bitter taste of Canola Meal Extract in Chickens

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Canola meal (CM) is a commonly used feedstuff; however, it is known to be bitter, and chickens have a low preference for it. The purpose of this study was to seek clarity regarding the taste quality of CM and find methods to increase the preference for CM by chickens. We examined whether CM activates the bitter taste receptors in chickens, whether chickens show aversive responses to CM, and whether an antagonist for bitter taste receptors inhibits the bitterness of CM. Using the Ca$^{2+}$ imaging technique, we showed that CM contains bitter compounds, which activate the bitter taste receptors in chickens. Further, we showed that 6-methoxyflavanone (6-meth), an antagonist for the bitter taste receptors in chickens, inhibits the activation of these receptors by CM extract. Although chickens showed a low preference for the solution of the CM extract, their preference was improved by adding 6-meth in behavioral tests. These results suggest that the preference for CM could be improved by inhibiting the bitter taste receptors in chickens.

Key words: bitter taste receptor, canola meal, chicken

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exposure control tap water was set in a broader box, and the amount of evaporation was subtracted from the volume of water or test solution intake.

**Constructs**

From our previous research, we took the cT2R1/pDisplay and cT2R7/pDisplay (Hirose et al., 2015; Dey et al., 2017) and amplified them for this experiment. The chimeric G-protein, Gα16/gust44/pcDNA3.1(+) (Ueda et al., 2003), was gifted by Dr. Takashi Ueda (Nagoya City University).

**Cell Culture**

Human embryonic kidney (HEK)-derived 293T (HEK 293T) cells were maintained in Dulbecco’s Eagle’s medium (DMEM high glucose; FUJIFILM Wako Pure Chemical Corporation) containing 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK) and penicillin-streptomycin solution (×100) (FUJIFILM Wako Pure Chemical Corporation) at 37°C and 5% CO2.

**Measurement of the Index of Cytosolic Ca2+ Concentrations**

For the Ca2+ imaging experiments (Dey et al., 2017) HEK293T cells were transfected with either empty vector pDisplay for mock cells or the cotransfection of Gα16/gust44 /pcDNA3.1(+) with cT2R1/pDisplay or cT2R7/pDisplay using ScreenFect™A (FUJIFILM Wako Pure Chemical Corporation) on coverslips coated with poly-D-lysine (0.1 mg/mL; FUJIFILM Wako Pure Chemical Corporation). After transfection, the cells were incubated for 48 h at 37°C and 5% CO2. Further, the cells were loaded with 1.25 mM Fluo-4 AM solution for 30 min at 37°C and 5% CO2 in the dark. Fluo-4 AM solution was prepared according to the instructions of the manufacturer (Dojindo Laboratories, Kumamoto, Japan).

The coverslips were washed with the standard bath solution; Fluo-4 fluorescence was measured in the standard bath solution using a confocal laser scanning microscope (Nikon A1R; Nikon Co., Tokyo, Japan). The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a standard bath solution containing various reagents. Cell viability was confirmed by responses to 10 μM ATP.

**Statistical Analysis**

The data were expressed as means±SE. Statistical analyses were conducted using the paired t-test; differences with p-values <0.05 were considered to be significant.

**Results**

**CM Extract Increased the Index of Cytosolic Ca2+ through cT2R1 and cT2R7**

Stimuli of 0.001% and 0.01% CM extract solutions increased the relative fluorescence units (RFU) (the index of cytosolic Ca2+) in both cT2R1-expressing cells and cT2R7-expressing cells (Fig. 1A and 1B). The mock (empty vector) cells were not affected by the 0.001% and 0.01% CM extract solutions (Fig. 1C). These results suggest that the CM extract contains agonists of both cT2R1 and cT2R7.

**Behavioral Analyses Toward CM Extract in Chickens**

As the CM extract contains agonists of cT2R1 and cT2R7,
we examined whether chickens showed aversive behavior in response to the CM extract. We prepared three doses of the CM extract solution, including 0.01% and 0.001% CM extract, and an additional third dose of 0.1%. In the one-bowl drinking test, the intake volumes of 0.01% and 0.1% CM extract solutions were significantly lower than that of water. However, chickens could not detect any bitterness in 0.001% CM extract solution compared to that in water. Data are the means±SE (n=8). *p<0.05 by paired t-test.

6-meth Inhibited the Cytosolic Ca^{2+} Responses to CM Extract

We previously reported that 6-meth is an antagonist for the functional bitter taste receptors (cT2R1 and cT2R7) in chickens (Dey et al., 2017). In this research, we used the same compound to analyze whether 6-meth blocked the responses to CM extract via both cT2R1 and cT2R7. In Ca^{2+} imaging tests, although the RFU slightly increased for the first stimuli of the mixture (0.01% CM extract solution and 50μM 6-meth), the RFU of the second stimuli (0.01% CM extract) increased more than that of the first stimuli (Fig. 3A and 3C). There were significant differences between the peak values of the first and second stimuli (Fig. 3B and 3D). However, in mock cells there were no responses to these solutions (Fig. 3E). These results suggest that 50μM 6-meth can partially inhibit the responses to CM-extract-mediated cT2R1 and cT2R7.

6-meth Inhibited the Bitterness of CM Extract in Chickens

In the cell-based assays, 50μM 6-meth inhibited the activities of 0.01% CM extract-solution-mediated cT2R1 and cT2R7. Thus, we examined whether 6-meth would inhibit the bitterness of the solution containing CM extract in vivo. In a one-bowl drinking test, addition of 50μM 6-meth to 0.01% CM extract solution resulted in a significantly higher intake of the solution than that observed using 0.01% CM extract solution alone (Fig. 4). However, the addition of a lower concentration of 6-meth (25μM) to 0.01% CM extract did not lead to any significant differences in intake during the 10-min drinking test, although the intake volume slightly increased by the addition of 25μM 6-meth (p=0.064 by paired t-test) (Fig. 4). These results suggest that 6-meth can inhibit the bitterness of CM extract at the in vivo level.

Discussion

In this study, we found that CM contains agonists of both cT2R1 and cT2R7 by using both cell-based assays and be-
Behavioral experiments. Further, we found that the bitterness of CM is inhibited by 6-meth, which was previously identified as an antagonist for the functional bitter taste receptors of chickens (Dey et al., 2017). We identified why the inclusion of high levels of CM in the diet reduces feed intake in chickens. Since 6-meth can improve the reduction of feed intake caused by the presence of CM, the present information will be useful to improve feedstuffs containing CM.

Chickens drank less CM extract solution than water in a 10 min drinking test. Since 10 min was insufficient time for the solution to be fully absorbed in the gastrointestinal tract, it is thought that chickens could detect the bitterness of the CM extract solution.
bitter inhibition effects of 6-meth was dose-dependent. As a second stimuli. In the behavioral tests, we confirmed the antagonistic effects of 6-meth were partial. However, the antagonistic activities of 6-meth were identical with those of our previous reports, which showed that cT2R1 activities were compatible with behavioral sensitivity to bitterness (Hirose et al., 2015), and the dose-dependencies of 6-meth in the behavioral tests almost matched those in the cell-based assay (Dey et al., 2017). Further, we confirmed that chickens did not show any aversion to 50 μM 6-meth itself compared to normal drinking water (Dey et al., 2017). So, 6-meth is not a substance that chickens can taste, and it can be used in the chicken feed industry to block the bitter compounds in nutritionally potential ingredients.

In the present study, we used both male and female chicks randomly; thus, we did not confirm the effects of sex on the sensitivity of 6-meth. Females are industrially important as egg layers; thus further studies are required to understand whether sex differences affect the taste sensitivities for CM and the effects of 6-meth in chicks. In this study, we prepared the CM extract by using DMSO; other compounds, which were not extracted by DMSO were not examined. The main bitter compounds of CM, such as tannin, sinapine, and glucosinolates are organic compounds, thus, it was considered that most of the main bitter compounds was extracted by DMSO. However, since CM might contain unknown water-soluble bitter compounds, it is important to examine these compounds in future. Moreover, we did not confirm whether 6-meth can inhibit the bitterness of CM itself. Further studies are also needed to confirm that for developing poultry industry.

In summary, we have shown that that CM contains agonists for the functional bitter taste receptors in chickens and that 6-meth inhibited the bitterness of CM. These findings will be very useful in the development of new feedstuffs for chickens derived from CM. Further, as 6-meth is only one of the antagonists for the bitter taste receptors of chickens, it is necessary to explore other antagonists that may be economical and easier to use.

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Conflicts of Interest

The authors declare no conflict of interest.

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