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Sweetness characterization of recombinant human lysozyme

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ABSTRACT

Lysozyme, a bacteriolytic enzyme, is widely distributed in nature and is a component of the innate immune system. It is established that chicken egg lysozyme elicits sweetness. However, the sweetness of human milk lysozyme, which is vital for combating microbial infections of the gastrointestinal tract of breast-fed infants, has not been characterized. This study aimed to assess the elicitation of sweetness using recombinant mammalian lysozymes expressed in *Pichia pastoris*. Recombinant human lysozyme (h-LZ) and other mammalian lysozymes of mouse, dog, cat and bovine milk elicited similar sweetness as determined using a sensory test, whereas bovine stomach lysozyme (bs-LZ) did not. Assays of cell cultures showed that h-LZ activated the human sweet taste receptor hT1R2/hT1R3, whereas bs-LZ did not. Point mutations confirmed that the sweetness of h-LZ was independent of enzyme activity and substrate-binding sites, although acidic amino acid residues of bs-LZ played a significant role in diminishing sweetness. Therefore, we conclude that elicitation of sweetness is a ubiquitous function among all lysozymes including mammalian lysozymes. These findings may provide novel insights into the biological implications of T1R2/T1R3-activation by mammalian lysozyme in the oral cavity and gastrointestinal tract. However, the function of lysozyme within species lacking the functional sweet taste receptor gene, such as cat, is currently unknown.

1. Introduction

Lysozyme (EC 3.2.1.17) is a bacteriolytic enzyme that catalyzes the hydrolysis of the β-1-4-glycosidic bond in peptidoglycan heteropolymers of the bacterial cell wall, thereby accounting for its main biological function of protecting the host from bacterial infection. Lysozyme acts as an antimicrobial agent (Ibrahim et al., 2001), anti-inflammatory agent (Ogundele, 1998), and anti-HIV agent (Lee-Huang et al., 1999) and possesses tumoricidal activity (Le Marbre et al., 1981). Chicken egg-white lysozyme reportedly elicits a sweet taste (Masuda et al., 2001).

Sweetness is a pleasant sensation for most animals and is believed to be a signal for high caloric substances in foods because sugars are representative of natural sweet compounds. However, there are numerous structurally diverse compounds that elicit sweetness other than sugars. Some proteins such as monellin, thaumatin, and brazzein that are present in tropical fruits of West Africa elicit intense sweetness (Kant, 2005). Because they elicit 2000–3000-fold more sweetness by weight than sucrose, these compounds may replace low-calorie artificial sweeteners (Kant, 2005). The nutritional value of these specific proteins is yet unknown because proteins are not high-calorie substances. Monellin is a member of the cystatin superfamily (Murzin, 1993), whereas thaumatin is structurally and evolutionarily related to thaumatin-like proteins that are involved in plant defense (Menu-Bouaouiche et al., 2003), although neither cystatin nor thaumatin-like proteins elicit sweetness. These limited examples for sweet proteins of plant origin; however, lysozyme is the only sweet protein of animal origin.

We purified various avian and reptilian lysozymes from egg white, and using a human sensory test, we demonstrated a 20-fold increase by weight in the sweetness of lysozyme isolated from chicken, turkey, quail, guinea-fowl, and soft-shelled turtle egg whites compared with that of sucrose (Maehashi and Udaka, 1998). Although the sweetness of chicken egg-white lysozyme has been extensively studied (Masuda et al., 2001), there has been no report on the sweetness of lysozyme originating from other sources, particularly mammals. This may be due to the cumbersome process required to purify adequate amounts of lysozyme from animals. Microbial production of recombinant proteins using the *Pichia pastoris* system is well validated for this purpose. We have previously used this expression system to characterize a novel lysozyme in emu egg white (Maehashi et al., 2012) and have successfully obtained *adequate* protein *levels*.

In mammals, lysozyme is present in the breast milk and most other body fluids (Hankiewicz and Swierczek, 1974). In addition, lysozyme occurs in excess in the human colostrum (Chandan et al., 1964) and contributes to the reduction of microbial infections in the gastrointestinal tract of breast-fed infants (Maga et al., 2006). Milk provides a nutritious diet for mammalian infants, and lysozyme is present as a nonspecific host defense factor, regardless of the animal species.

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Therefore, the definition of the novel nutritional function of mammalian lysozymes may be facilitated by investigations on the sensory attributes of mammalian lysozymes. In the present study, we characterized the sweetness of recombinant lysozymes generated using the *P. pastoris* expression system.

2. Materials and methods

2.1. Molecular cloning of genes encoding lysozyme

Genes encoding human (*Homo sapiens*) lysozyme (h-LZ, GenBank: M19045), mouse (*Mus musculus*) lysozyme M (GenBank: NM_017372), and bovine (*Bos taurus*) stomach lysozyme C2 (bs-LZ, GenBank: NM_180999) were amplified using PCR with Platinum *Pfx* DNA polymerase (Invitrogen, San Diego, CA) and gene-specific primers (Supplementary File 1) from QUICK-clone cDNAs of human placenta and mouse spleen (Takara Bio, Inc., Otsu, Japan) and from cDNA of Bovine Normal Stomach Tissue (Biochain Institute, Inc., Newark, CA), respectively. Genes encoding the lysozyme of cat (*Felis catus*; GenBank: XM_003989032), dog milk (*Canis lupus* familiaris; GenBank: XM_846220), bovine milk (GenBank: NM_001077829) and bovine tracheal (GenBank: NM_001080336) and bovine intestinal (GenBank: NM_001007805) lysozymes were amplified using PCR with gene-specific primers (Supplementary File 1) from their respective genomic DNAs (Novagen, Milwaukee, WI, for cat and dog, and Biochain Institute for bovine). Amplified fragments of exons were assembled using overlapping PCR.

2.2. Construction of vectors that express mammalian lysozymes

The amplified sequences comprising the complete coding region of each lysozyme were cloned into the pPIC9 expression vector (Invitrogen Co. Carlsbad, CA) using appropriate restriction enzymes. The expression vector was constructed using an α-factor signal peptide according to the instructions of the manufacturer. Because the prepro sequence of the α-factor dramatically increases the secretion of h-LZ by *P. pastoris* (Oka et al., 1999), a four-residue spacer sequence (Glu-Ala-Glu-Ala) was inserted between the signal cleavage site and the N-terminus of h-LZ to facilitate the processing of the expressed protein. Site-directed mutagenesis was performed using overlapping PCR with synthetic oligonucleotide primers containing the desired mutation (Supplemental Table 1) and pPIC9-h-LZ or pPIC9-bs-LZ as templates to construct 7 mutants of h-LZ and 15 mutants of bs-LZ. The mutant lysozymes were inserted into pPIC9, and their sequences were validated using DNA sequencing (Macrogen Japan Co., Tokyo, Japan).

2.3. Expression of recombinant lysozymes

Transformants with mammalian lysozyme genes constructed into pPIC9 vector were obtained and cultured to produce recombinant lysozymes following the method described in our previous article (Maehashi et al., 2012). *P. pastoris* transformed with the gene-encoding chicken lysozyme (Mine et al., 1999) was a kind gift from Dr. Taiji Imoto (Sojo University, Kumamoto, Japan). Expression of lysozymes in *P. pastoris* was performed in 3 L of buffered minimal methanol medium using a jar fermenter (MDL-4CR; BE Marubishi Co., Ltd., Tokyo, Japan) as described previously (Maehashi et al., 2012).

2.4. Purification of recombinant lysozymes

The culture supernatant was filtered through a 1-μm membrane filter and diluted 5-fold with water. The pH was adjusted as follows: pH 7.0, human wild-type, chicken, cat, dog, and bovine milk LZs; pH 5.0, human mutants, mouse, bovine tracheal, and intestinal LZs; and pH 4.5, bovine stomach wild-type and mutant LZs. Secreted lysozyme was isolated using a two-step cation-exchange chromatography method using a CM-Sepharose Fast Flow column (ϕ 50 mm × 50 mm; GE Healthcare Bio-Sciences Corp., NJ, USA). The supernatant of the diluted culture was applied to the column, followed by washing with 20 mM phosphate buffer (pH 7.0), 50 mM of pH 5.0 acetate buffer, or 50 mM of pH 4.5 acetate buffer. A gradient of wash buffer and buffer containing 0.5 M NaCl was used for the first elution. The eluted fraction was diluted to <0.1 M NaCl and applied to the column (ϕ 25 mm × 300 mm). The adsorbed lysozyme was eluted using a linear salt gradient of 0.1–0.35 M NaCl in wash buffer. The fractions containing the purified lysozymes were desalted by ultrafiltration using a regenerated cellulose membrane NMWL: 500 (Millipore Corp., Billerica, MA) and were lyophilized. The lyophilized powder was dissolved in distilled water.

The purified lysozymes were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA). The bands were excised, and N-terminal amino acid sequences were determined using a protein sequencer, PPSQ-21 (Shimadzu Co., Ltd., Kyoto, Japan).

2.5. Measurement of enzyme activity

The lytic activity of lysozyme was determined by measuring the lysis of a *Micrococcus luteus* suspension (lytic activity), and its hydrolytic activity was measured using ethylene glycol chitin as a substrate. Lysis of the bacterial cell wall by lysozymes was measured as described previously (Maehashi et al., 2012), with the minor modification of using 66 mM phosphate buffer (pH 6.24). A 0.02% suspension of *M. luteus* (Sigma-Aldrich, Japan) prepared in phosphate buffer (pH 6.24) was added to the lysozyme solution, and the decrease in turbidity was measured at 30 °C at 450 nm.

The chitinase activity of lysozyme was measured as described elsewhere (Imoto and Yagishita, 1971). Ethylene glycol chitin (0.05% w/v; Wako Pure Chemical Industries Co.) dissolved in 0.1 M acetate buffer (pH 4.5) served as substrate and was incubated for 30 min at 40 °C. After stopping the reaction with 0.05% potassium ferricyanide solution, the absorbance at 420 nm was measured. The enzyme activity of lysozyme is expressed as a percentage of that of commercial chicken lysozyme (Seikagaku Kogyo, Tokyo, Japan).

2.6. Fluorescence spectra

The fluorescence spectra of mutant h-LZ and bs-LZ lysozymes in 10 mM sodium acetate buffer (pH 5.5) at 25 °C were determined using a fluorescence spectrophotometer (F-2700, Hitachi Ltd., Tokyo, Japan) at excitation and emission wavelengths of 280 and 300–400 nm, respectively. All samples were adjusted to a final protein concentration of 2.5 μM.

2.7. Native PAGE

Native PAGE was performed according to a method described by Reisfeld et al. (1962). Separating and stacking gels were prepared using 20% (pH 4.3) and 4% polyacrylamide (pH 6.8), respectively. Electrophoresis was performed at 20 mA for the stacking gel and at 40 mA for the separating gel for 5 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

2.8. Sensory analysis of lysozyme

Lyophilized lysozyme samples were dissolved in distilled water, and the sweetness threshold was determined using an unbiased triangle test with 0.5 mL of the solution at room temperature. Four well-trained panel members (one female and three males, average age 29 ± 5 y) were asked to point out the cup with the sweetest solution. The sample solutions were provided to the panel members in decreasing order of concentration. After each evaluation, panelists were asked to spit out the sample solution and repeatedly rinse their mouths with distilled water