



HAL
open science

Recombinant expression, in vitro refolding, and biophysical characterization of the N-terminal domain of T1R3 taste receptor

Elodie Maitrepierre, Maud Sigoillot, Laurence Le Pessot, Loïc Briand

► To cite this version:

Elodie Maitrepierre, Maud Sigoillot, Laurence Le Pessot, Loïc Briand. Recombinant expression, in vitro refolding, and biophysical characterization of the N-terminal domain of T1R3 taste receptor. *Protein Expression and Purification*, 2012, 83 (1), pp.75-83. 10.1016/j.pep.2012.03.006 . hal-01296425

HAL Id: hal-01296425

<https://hal.science/hal-01296425v1>

Submitted on 29 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

An efficient *Escherichia coli* expression system for the production of a functional N-terminal domain of the T1R3 taste receptor

Elodie Maîtrepierré,[†] Maud Sigoillot,[†] Laurence Le Pessot and Loïc Briand*

Centre des Sciences du Goût et de l'Alimentation; UMR-1324 INRA; UMR-6265 CNRS; Université de Bourgogne; Dijon, France

[†]These authors contributed equally to this work.

Sweet taste is mediated by a dimeric receptor composed of two distinct subunits, T1R2 and T1R3, whereas the T1R1/T1R3 receptor is involved in umami taste perception. The T1R1, T1R2, and T1R3 subunits are members of the small family of class C G protein-coupled receptors (GPCRs). The members of this family are characterized by a large N-terminal domain (NTD), which is structurally similar to bacterial periplasmic-binding proteins and contains the primary ligand-binding site. In a recent study, we described a strategy to produce a functional dimeric human T1R3-NTD. Although the protein was expressed as inclusion bodies (IBs) using the *Escherichia coli* system, the conditions for the refolding of functional hT1R3-NTD were determined using a fractional factorial screen coupled to a binding assay. Here, we report that this refolding strategy can be used to produce T1R1- and T1R2-NTDs in large quantities. We also discuss that our findings could be more generally applicable to other class C GPCR-NTDs, including the γ -aminobutyric acid type B receptor (GABA_BR), the extracellular calcium-sensing receptor (CaSR) and the large family of pheromone (V2R) orphan receptors.

Vertebrate chemosensory receptors are able to recognize diverse chemical compounds, including ions, tastants, odorants and pheromones. The first step of this detection is mostly accomplished by the activation of G protein-coupled receptors (GPCRs) by these signals. GPCRs share

a common seven transmembrane motif and are encoded by < 1% of the mammalian genes. One particular GPCR family, GPCR class C, contains receptors for sweet and amino acid taste compounds, some pheromone molecules, and the odorants in fish.^{1,2} The most studied receptors of this class are the homodimeric metabotropic glutamate receptors (mGluRs), the heterodimeric γ -aminobutyric acid type B receptor (GABA_BR), and the homodimeric extracellular calcium-sensing receptor (CaSR). Class C GPCRs share a large N-terminal domain (NTD), comprising 500–600 amino acids, which is composed of two lobes separated by a large cleft in which the agonists bind (for a review, see refs. 3 and 4). Except for GABA_BR, this domain is connected to a transmembrane heptahelical domain (HD), typical of all GPCRs, via a cysteine-rich region (CRR). Numerous studies have revealed the importance of the NTD in the functionality of class C GPCRs.^{5–7} This domain, which qualifies as an orthosteric site, is responsible for agonist recognition. Indeed, when produced as a soluble protein in insect cells, this domain was found to retain its ability to bind ligands, as demonstrated for mGluR,⁸ GABA_BR⁹ and CaSR.¹⁰

Sweet taste is mediated by a dimeric receptor composed of the two distinct subunits, T1R2 and T1R3, whereas the T1R1/T1R3 dimer is involved in the umami taste perception of L-glutamate. These receptors share the T1R3 subunit but recognize different types of taste stimuli. In addition, unlike T1R1 and T1R2, the T1R3 subunit has been suggested to

Keywords: sweet receptor, umami receptor, sugar, sweetener, GPCR, taste, recombinant protein, expression, bacteria, *Escherichia coli*

Submitted: 07/11/12

Revised: 08/17/12

Accepted: 08/17/12

<http://dx.doi.org/10.4161/bioe.21877>

*Correspondence to: Loïc Briand;
Email: loic.briand@dijon.inra.fr

Research Note to: Maîtrepierré E, Sigoillot M, Le Pessot L, Briand L. Recombinant expression, *in vitro* refolding, and biophysical characterization of the N-terminal domain of T1R3 taste receptor. *Protein Expr Purif* 2012; 83:75–83; PMID:22450161; <http://dx.doi.org/10.1016/j.pep.2012.03.006>

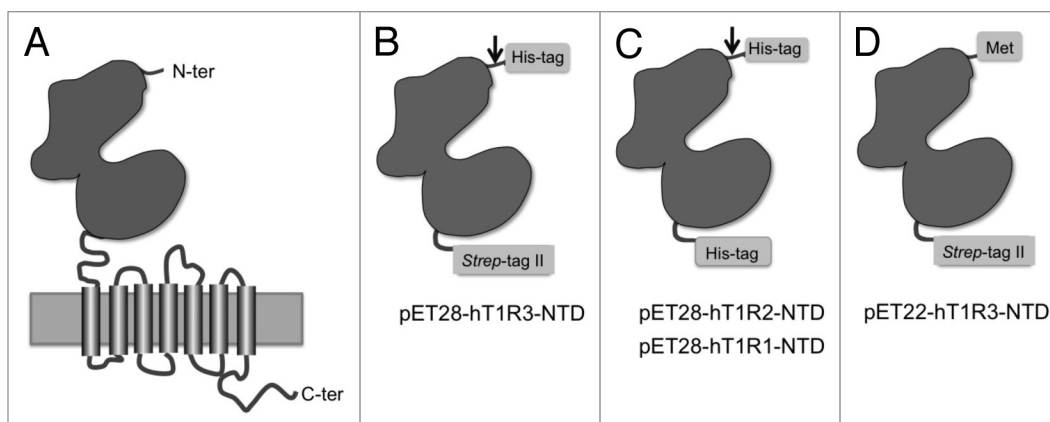


Figure 1. Expression of T1R-NTD proteins. (A) The NTDs of the T1R1, T1R2 and T1R3 proteins, minus a putative signal peptide and without the CRR, were expressed independently of the seven-transmembrane domain. (B) The construct pET28-hT1R3-NTD encodes a protein comprising an N-terminal His6-tag that can be cleaved by thrombin, followed by hT1R3-NTD and a C-terminal *Strep*-tag II. (C) The pET28-hT1R1-NTD/pET28-hT1R2-NTD plasmid encodes a fusion protein that contains an N-terminal His6-tag that can be cleaved with thrombin, followed by hT1R1- or hT1R2-NTD and a C-terminal His6-tag to facilitate the purification of the heterodimers. (D) The pET22-hT1R3-NTD plasmid encodes hT1R3-NTD with an additional N-terminal Met residue and a C-terminal *Strep*-tag II. The thrombin cleavage site is represented with an arrow.

form homodimers that are able to function as low-affinity receptors for sugars.^{11,12} The presence of multiple binding sites has been revealed for both umami and sweet taste receptors. Natural and artificial sugars (e.g., sucrose, glucose and sucralose) bind to both T1R2- and T1R3-NTDs,¹³⁻¹⁵ whereas dipeptide sweeteners (e.g., aspartame and neotame) bind only to T1R2-NTD.^{16,17} The binding sites of two other non-caloric sweeteners (cyclamate and neohesperidin dihydrochalcone) and lactisole, a human sweet-taste inhibitor, are located within the HD of human T1R3.¹⁸⁻²¹ Lastly, T1R1-NTD has been identified as the primary binding site of L-glutamate.¹⁶ Although the binding properties of T1R1- and T1R2-NTD have been demonstrated, the role of T1R3-NTD and its relative contribution to the heterodimeric receptor function remains to be elucidated.

There is a great interest in discovering new sweeteners or umami compounds. These compounds can be identified by the screening of large compound libraries for molecules that are able to bind to T1R1-, T1R2- or T1R3-NTDs, a technique requiring large quantities of functional proteins. In addition, the rational design of new tasting molecules and the elucidation of the molecular mechanisms, which are critical for detection and discrimination, relies on obtaining high-resolution X-ray crystal structures of these domains, which

also requires high-quality protein. Mouse T1R2- and T1R3-NTDs have been successfully expressed in *E. coli* as protein fusions with maltose-binding protein or the chitin-binding domain.¹⁴ However, the level of soluble protein expression was low (< 0.5 mg/L), and the presence of the fusion partner complicated the functional analyses of the ligand interactions. The baculovirus expression system has been shown to efficiently secrete a functionally active mGluR-NTD that is amenable to crystallization.⁷ In contrast, a recent study aimed at expressing mouse T1R-NTDs fused to green fluorescent protein demonstrated that the protein fusion remained almost completely in the cellular space of the insect cells, exhibiting polydisperse hydrodynamic states with large aggregated fractions and without the formation of homodimers.²²

In our previous study, we described a strategy to produce large quantities of the NTD of the human T1R3 subunit (hT1R3-NTD).¹⁵ We overexpressed hT1R3-NTD using an *E. coli* prokaryotic system because this expression system offers the advantages that include the ability to grow rapidly at high densities utilizing inexpensive substrates. The well-characterized genetics of *E. coli* and the availability of a large number of expression vectors and mutant host strains also constitute the advantages of this system. We found that large quantities

of hT1R3-NTD can be expressed in the form of inclusion bodies (IBs); using a fractional factorial screen coupled to a functional fluorescent assay, we determined conditions for the refolding of the protein. Using size-exclusion chromatography, spectroscopic techniques and microcalorimetry, we have shown that hT1R3-NTD behaves as a functional dimer that is capable of binding sucralose, a chlorodeoxysugar sweetener, with an affinity in the millimolar range. Here, we extend this previous study to report detailed strategies for obtaining other taste receptor NTDs using bacterial expression systems and discuss the benefit of using this expression system.

Construction of the Expression Plasmid

As previously described for hT1R3-NTD,¹⁵ we expressed hT1R1- and hT1R2-NTD, minus a short putative signal peptide and the CRR, independently of the seven-transmembrane domain (Fig. 1A and B). The cDNA coding for hT1R1- or hT1R2-NTD was cloned into the bacterial expression vector pET28a. The resulting expression plasmid encodes a fusion protein comprising an N-terminal His6-tag that can be cleaved with thrombin, followed by hT1R1- or hT1R2-NTD and a C-terminal His6-tag (Fig. 1C). The hT1R1- and hT1R2-NTD fusions were

produced using *E. coli* BL21 (DE3). Both constructs showed a good level of overexpression of the recombinant proteins similar to that of hT1R3-NTD as demonstrated by SDS-PAGE analysis (Fig. 2). As previously observed with hT1R3-NTD, both proteins entirely precipitated in the form of insoluble IBs in the *E. coli* cytosol. IBs were purified and solubilized in a denaturation buffer containing 6 M guanidium chloride (GuCl) as previously described.¹⁵ The quantity of purified protein was determined using UV absorbance at 276 nm. Approximately 80 and 120 mg of hT1R1- and hT1R2-NTD were obtained from 1 l of bacterial culture, respectively. A second hT1R3-NTD expression construct was also generated to evaluate the impact of the hT1R3-NTD N-terminus on the protein expression level and to avoid the use of thrombin for the proteolytic cleavage of the N-terminal His6-tag. The sequence coding for hT1R3-NTD was PCR amplified and subcloned into the *Nde*I and *Eco*R1 restriction sites of the pET22b vector (Novagen), allowing removal of the pelB leader peptide sequence encoded by the expression plasmid. This construct encodes hT1R3-NTD (Ala21-Ser497) containing an additional N-terminal Met residue and a C-terminal *Strep*-tag II to facilitate its purification (Fig. 1D). The resulting construct, named pET22-hT1R3-NTD, was sequenced (Cogenics) and expressed in *E. coli* BL21 (DE3) cells. Unfortunately, no hT1R3-NTD protein was detectable in the bacterial lysate, as demonstrated by SDS-PAGE (Fig. 3). Attempts to produce the protein using other *E. coli* strains, various isopropyl- β -D-thiogalactoside concentrations or different expression temperatures were unsuccessful. This absence of expression revealed that the N-terminal His6-tag encoded by the pET28 vector is essential to the production of a large amount of hT1R3-NTD as IBs.

Expression and Purification of hT1R3-NTD

As many biophysical studies are made possible by the availability of large amounts of pure protein, we have focused on designing strategies to recover folded and biologically active molecules from protein

overexpressed as insoluble IBs. After washing and solubilizing the IBs, approximately 110 mg of hT1R3-NTD was obtained from 1 L of bacterial culture for refolding studies. At this stage, the protein was pure to apparent homogeneity (i.e., $\approx 90\%$) by simply washing the IBs with an aqueous solution containing NaCl and urea, supplemented with Triton X-100. The IBs of hT1R3-NTD were solubilized using 6 M GuCl. In our previous work,¹⁵ we elaborated a strategy for the production of hT1R3-NTD using a refolding screen, which is outlined in Figure 4. To test a variety of refolding additives in a minimal number of experiments, we compared the effects of 12 well-known refolding reagents with 16 experiments. The screening for the optimal refolding buffer was performed with an incubation at 20°C and included a change of the pH, a variation of the pH-buffer, and the addition of varying amounts of different salts and the presence of a chaotrope (urea), ligands (sucralose and glucose) and polar additives. We also tested the impact of three detergents (non-ionic and zwitterionic) on hT1R3-NTD refolding. To evaluate the functionality of the refolded hT1R3-NTD rapidly, we measured its intrinsic tryptophan fluorescence in the absence and presence of 10 mM sucralose. Based on our analysis, we observed that the presence of a zwittergent, Zw3-14, had the largest impact on the refolding of hT1R3-NTD. We also determined the dimerization state using size-exclusion chromatography and the presence of secondary structures using circular dichroism (as shown in Fig. 5 of our previously published paper¹⁵). We determined the binding activity of hT1R3-NTD using the tryptophan fluorescence and isothermal titration calorimetry. We found that hT1R3-NTD binds sucralose with a K_d value in the low millimolar range, which is in good agreement with the value measured for mouse T1R3-NTD by Nie and coworkers.¹³ Moreover, we digested hT1R3-NTD with thrombin to evaluate further that the protein was properly refolded. As detected by SDS-PAGE, we observed the removal of the N-terminal His6-tag, giving another indication of the correct refolding of hT1R3-NTD.¹⁵ We tested hT1R1 and hT1R2-NTD proteins in our refolding strategy. Size-exclusion

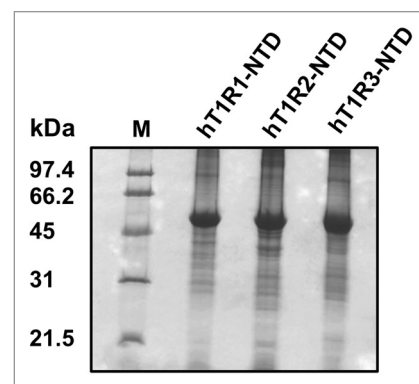


Figure 2. Purified inclusion bodies of hT1R-NTDs expressed using the pET28 vector. The proteins were separated by 12% SDS-PAGE and stained with Coomassie blue. The molecular mass markers are in lane M.

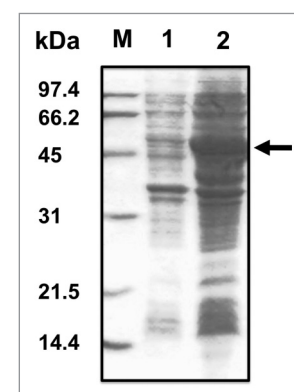


Figure 3. SDS-PAGE analysis of hT1R3-NTD expressed in *E. coli* BL21 (DE3) cells transformed with pET22-hT1R3-NTD (lane 1) and pET28-hT1R3-NTD (lane 2). The position of hT1R3-NTD is indicated with an arrow. Protein expression was induced with 1 mM IPTG for 3 h. The cell lysates were loaded onto 12% gels for SDS-PAGE, with molecular mass markers (lane M). The gel was stained with Coomassie blue.

chromatography indicated that both proteins were mainly produced as aggregates with a low proportion of properly refolded protein (data not shown). Extension of folding screens for improving the refolding yields of hT1R1- and hT1R2-NTDs by the inclusion of other factors such as receptor ligands (glutamate or sweeteners), other detergents or additives, are in progress. Our produced proteins can be also used to generate antibodies against individual refolded proteins. Therefore, because commercial antibodies against hT1R1 are of poor quality, we recently

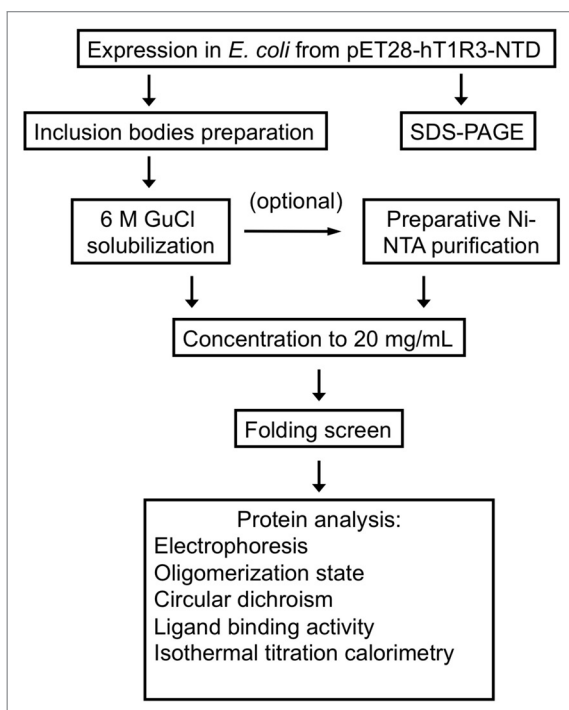


Figure 4. Outline of the strategy for the production of hT1R3-NTD.

developed rabbit polyclonal antibodies raised against refolded hT1R1-NTD, which specifically recognized the corresponding receptor protein.²³

Conclusion

In this paper, we provide additional information about the production of recombinant hT1R3-NTD. We show that the presence of a His6-tag in the N-terminal position is essential to obtain a high level of expression of our protein of interest in *E. coli*. Our data indicate that our developed strategy is applicable for other protein members of this family, such as hT1R1- and hT1R2-NTD.

Owing to this high amount of functional hT1R3-NTD protein, biochemical

tests and the screening of large compound libraries are now possible. In addition, the rational design of sweeteners and elucidation of the molecular mechanisms, which are critical for detection and discrimination, rely on obtaining high-resolution X-ray crystal structures of T1R2- and T1R3-NTD, a technique that also requires high-quality protein. Current experiments with the *in vitro* refolded hT1R3-NTD include crystallization trials and NMR spectroscopic analyses to gather the desired structural information for hT1R3-NTD in the near future. Certain detergents, such as maltosides and glucosides, have been used more often than others for crystallization studies, and, due to ion-exchange or affinity chromatography, the zwitterionic

detergent (Zw3-14) that we used for the protein refolding could be exchanged with *n*-dodecyl- β -D-maltopyranoside or *n*-octyl- β -D-glucopyranoside detergents. Our approach should also offer a method for the reconstruction of the heterodimeric T1R2/T1R3 and T1R1/T1R3 taste receptors at a quality that is suitable for the detailed study of its structure, dynamics, and interactions with taste molecules.

It has been shown that the activation of the human T1R2/T1R3 receptor by the sweet-tasting protein brazzein is dependent on the CRR.²⁴ Our approach should allow the production of the entire extracellular region (i.e., comprising the CRR). The availability of large amounts of recombinant hT1R3-NTD will enable detailed studies of its interactions with sweet-tasting proteins, such as brazzein, monellin or thaumatin and gurmamin,²⁵ a sweet-taste-suppressing protein.

The class C GPCR family consists of 22 human proteins, including the receptor for GABA, CaSR, GPRC6A and seven orphan receptors, which may constitute drug targets. In addition, the mouse genome contains approximately 120 genes coding orphan pheromone (V2R) receptors that seem to be activated by peptides or proteins.²⁶ We anticipate that our method will also be more generally applicable to all of these class C GPCR-NTDs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by a grant from Agence Nationale de la Recherche (ANR-09-ALIA-010). The work was supported by INRA and Burgundy council (Région Bourgogne) grants to E.M. and M.S.

References

1. Bockaert J, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* 1999; 18:1723-9; PMID:10202136; <http://dx.doi.org/10.1093/emboj/18.7.1723>.
2. Pin JP, Galvez T, Prézeau L. Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. *Pharmacol Ther* 2003; 98:325-54; PMID:12782243; [http://dx.doi.org/10.1016/S0163-7258\(03\)00038-X](http://dx.doi.org/10.1016/S0163-7258(03)00038-X).
3. Wellendorph P, Johansen LD, Bräuner-Osborne H. Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients. *Mol Pharmacol* 2009; 76:453-65; PMID:19487246; <http://dx.doi.org/10.1124/mol.109.055244>.
4. Conigrave AD, Hampson DR. Broad-spectrum amino acid-sensing class C G-protein coupled receptors: molecular mechanisms, physiological significance and options for drug development. *Pharmacol Ther* 2010; 127:252-60; PMID:20451554; <http://dx.doi.org/10.1016/j.pharmthera.2010.04.007>.
5. Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, et al. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* 2000; 407:971-7; PMID:11069170; <http://dx.doi.org/10.1038/35039564>.
6. Tsuchiya D, Kunishima N, Kamiya N, Jingami H, Morikawa K. Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Gd³⁺. *Proc Natl Acad Sci U S A* 2002; 99:2660-5; PMID:11867751; <http://dx.doi.org/10.1073/pnas.052708599>.

7. Muto T, Tsuchiya D, Morikawa K, Jingami H. Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. *Proc Natl Acad Sci U S A* 2007; 104:3759-64; PMID:17360426; <http://dx.doi.org/10.1073/pnas.0611577104>.
8. Okamoto T, Sekiyama N, Otsu M, Shimada Y, Sato A, Nakanishi S, et al. Expression and purification of the extracellular ligand binding region of metabotropic glutamate receptor subtype 1. *J Biol Chem* 1998; 273:13089-96; PMID:9582347; <http://dx.doi.org/10.1074/jbc.273.21.13089>.
9. Malitschek B, Schweizer C, Keir M, Heid J, Froestl W, Mosbacher J, et al. The N-terminal domain of gamma-aminobutyric Acid(B) receptors is sufficient to specify agonist and antagonist binding. *Mol Pharmacol* 1999; 56:448-54; PMID:10419566.
10. Bräuner-Osborne H, Jensen AA, Sheppard PO, O'Hara P, Krogsgaard-Larsen P. The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. *J Biol Chem* 1999; 274:18382-6; PMID:10373443; <http://dx.doi.org/10.1074/jbc.274.26.18382>.
11. Ariyasu T, Matsumoto S, Kyono F, Hanaya T, Arai S, Ikeda M, et al. Taste receptor T1R3 is an essential molecule for the cellular recognition of the disaccharide trehalose. *In Vitro Cell Dev Biol Anim* 2003; 39:80-8; PMID:12892531; [http://dx.doi.org/10.1290/1543-706X\(2003\)039<0080:TRTIAE>2.0.CO;2](http://dx.doi.org/10.1290/1543-706X(2003)039<0080:TRTIAE>2.0.CO;2).
12. Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, et al. The receptors for mammalian sweet and umami taste. *Cell* 2003; 115:255-66; PMID:14636554; [http://dx.doi.org/10.1016/S0092-8674\(03\)00844-4](http://dx.doi.org/10.1016/S0092-8674(03)00844-4).
13. Nie Y, Vignes S, Hobbs JR, Conn GL, Munger SD. Distinct contributions of T1R2 and T1R3 taste receptor subunits to the detection of sweet stimuli. *Curr Biol* 2005; 15:1948-52; PMID:16271873; <http://dx.doi.org/10.1016/j.cub.2005.09.037>.
14. Nie Y, Hobbs JR, Vignes S, Olson WJ, Conn GL, Munger SD. Expression and purification of functional ligand-binding domains of T1R3 taste receptors. *Chem Senses* 2006; 31:505-13; PMID:16621970; <http://dx.doi.org/10.1093/chemse/bjj053>.
15. Maîtrepière E, Sigoillot M, Le Pessot L, Briand L. Recombinant expression, *in vitro* refolding, and biophysical characterization of the N-terminal domain of T1R3 taste receptor. *Protein Expr Purif* 2012; 83:75-83; PMID:22450161; <http://dx.doi.org/10.1016/j.pep.2012.03.006>.
16. Xu H, Staszewski L, Tang H, Adler E, Zoller M, Li X. Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc Natl Acad Sci U S A* 2004; 101:14258-63; PMID:15353592; <http://dx.doi.org/10.1073/pnas.0404384101>.
17. Masuda K, Koizumi A, Nakajima K, Tanaka T, Abe K, Misaka T, et al. Characterization of the modes of binding between human sweet taste receptor and low-molecular-weight sweet compounds. *PLoS One* 2012; 7:e35380; PMID:22536376; <http://dx.doi.org/10.1371/journal.pone.0035380>.
18. Jiang P, Cui M, Zhao B, Liu Z, Snyder LA, Benard LM, et al. Lactisole interacts with the transmembrane domains of human T1R3 to inhibit sweet taste. *J Biol Chem* 2005; 280:15238-46; PMID:15668251; <http://dx.doi.org/10.1074/jbc.M414287200>.
19. Jiang P, Cui M, Zhao B, Snyder LA, Benard LM, Osman R, et al. Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit T1R3. *J Biol Chem* 2005; 280:34296-305; PMID:16076846; <http://dx.doi.org/10.1074/jbc.M505255200>.
20. Winnig M, Bufer B, Meyerhof W. Valine 738 and lysine 735 in the fifth transmembrane domain of *rTas1r3* mediate insensitivity towards lactisole of the rat sweet taste receptor. *BMC Neurosci* 2005; 6:22; PMID:15817126; <http://dx.doi.org/10.1186/1471-2202-6-22>.
21. Winnig M, Bufer B, Kratochwil NA, Slack JP, Meyerhof W. The binding site for neohesperidin dihydrochalcone at the human sweet taste receptor. *BMC Struct Biol* 2007; 7:7; PMID:17313689.
22. Ashikawa Y, Ihara M, Matsuura N, Fukunaga Y, Kusakabe Y, Yamashita A. GFP-based evaluation system of recombinant expression through the secretory pathway in insect cells and its application to the extracellular domains of class C GPCRs. *Protein Sci* 2011; 20:1720-34; PMID:21805523; <http://dx.doi.org/10.1002/pro.707>.
23. Raliou M, Grauso M, Hoffmann B, Schlegel-Le-Poupon C, Débat H, et al. Human genetic polymorphisms in T1R1 and T1R3 taste receptor subunits affect their function. *Chem Senses* 2011; 36:527-37; PMID:21422378; <http://dx.doi.org/10.1093/chemse/bjr014>.
24. Jiang P, Ji Q, Liu Z, Snyder LA, Benard LM, Margolske RF, et al. The cysteine-rich region of T1R3 determines responses to intensely sweet proteins. *J Biol Chem* 2004; 279:45068-75; PMID:15299024; <http://dx.doi.org/10.1074/jbc.M406779200>.
25. Sigoillot M, Brockhoff A, Lescop E, Poirier N, Meyerhof W, Briand L. Optimization of the production of gurmardin, a sweet-taste-suppressing protein, secreted by the methylotrophic yeast *Pichia pastoris*. *Appl Microbiol Biotechnol* 2012; In press; PMID:22307499; <http://dx.doi.org/10.1007/s00253-012-3897-3>.
26. Young JM, Trask BJ. V2R gene families degenerated in primates, dog and cow, but expanded in opossum. *Trends Genet* 2007; 23:212-5; PMID:17382427; <http://dx.doi.org/10.1016/j.tig.2007.03.004>.