INVESTIGATING DETERMINANTS OF SWEETNESS IN SWEET MOLECULES

Synopsis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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INTRODUCTION

Understanding the mechanism of sweetness has been challenging problem. While it is necessary to understand this in order to design safer sweet molecules, for in many diseases (like) a low calorie sweetener is needed and thus a very pressing need; on the other hand, the wide range of molecules belonging to divergent chemical class taste sweet, thus posing a challenge to understand mechanism of sweetness.

Many natural sweet proteins that have been discovered are more than 4000 times sweeter as compared to sucrose. But unresolved enigma remains why they are sweet? What could be the reasons for their heightened sweetness? What do these proteins have in common, if at all? Do they have a homologous region at any level of structure be it primary or higher order? How does sweet receptor recognizes them?

Designing newer sweet molecules desires the knowledge of pharmacophores. Whereas glucophores identification for small sweet molecules has been studied exhaustively, identification of the same for sweet proteins has not been attempted because of the complexity involved. To identify these glucophores, the primary requirement is knowledge and understanding of structure of human sweet taste receptor (hSTR). hSTR having more than 800 residues, it is difficult to find a reliable structure experimentally (crystallography, NMR) or by computational modelling. Till date, most of the modelling studies are reported on amino terminal or transmembrane domain of T1R2-T1R3 subunits of hSTR.

The mechanism of sweetness remains unresolved as comparison of crystal structure of these sweet proteins yielded no similarity [1]. Also, no universal results could be obtained about determinants for sweetness from point mutation studies. Understanding their structure-function relationship can boost the performance of small molecular weight sweeteners and thus help in the design of safe sweeteners.

MOTIVATION

People affected by diseases like diabetes, obesity, hyperlipidemia, caries etc. which are linked to the consumption of carbohydrates are increasing tremendously. An estimated 346 million people worldwide are suffering from diabetes alone. Left untreated or poorly controlled, diabetes increases the risk of cardiovascular diseases, blindness, kidney failure, eye problems, nerve damage and amputations. In addition to other measures like healthy diet, regular exercise etc., decreased sugar and saturated fat intake is recommended to prevent or delay the onset of the disease. Hence search of alternate sweeteners is necessitated. Efforts in this direction led to low calorie sweeteners like aspartame, saccharin, sucralose, acesulfame-K etc that have gained wide popularity. However, there are contradictory reports regarding prolonged usage of these and associated side effects like dizziness, nausea, lung cancer, chronic respiratory disease, hallucination, hypersensitivity, bladder cancer, heart failure and brain tumor etc. [2] [3] [4] In view of above facts there is a need for alternate safer sweeteners with less side effects and which is easily metabolized. Naturally occurring sweet proteins like Brazzein[5], Thaumatin[6], Monellin[7], Mabinlin[8], Miraculin, Curculin[9] and Neoculin that are many fold sweeter than sucrose, can be an alternate[10]. Besides, they have negligible side effects and do not require insulin for their metabolism. However, in many diseases like hyperlipidemia where high protein diet cannot be administered, these proteins cannot be given. Hence there is a dire need to understand the mechanism of sweetness and the involved pharmacophores, to help design safer sweeteners.

Objective of the present work is to investigate determinants of sweetness. To achieve the above goal we have adopted two different approaches for identification of functional groups or pharmacophores responsible for sweetness – 1) ligand based and 2) receptor based approach. Former being the conventional approach widely used to find pharmacophores using ligands or in other words common substructures in sweet molecules responsible for eliciting sweetness. Since sweet molecules belong to diverse chemical class and structures we have adopted as an alternative receptor based approach to identify crucial residues of receptors responsible for sweetness. The specific objectives are

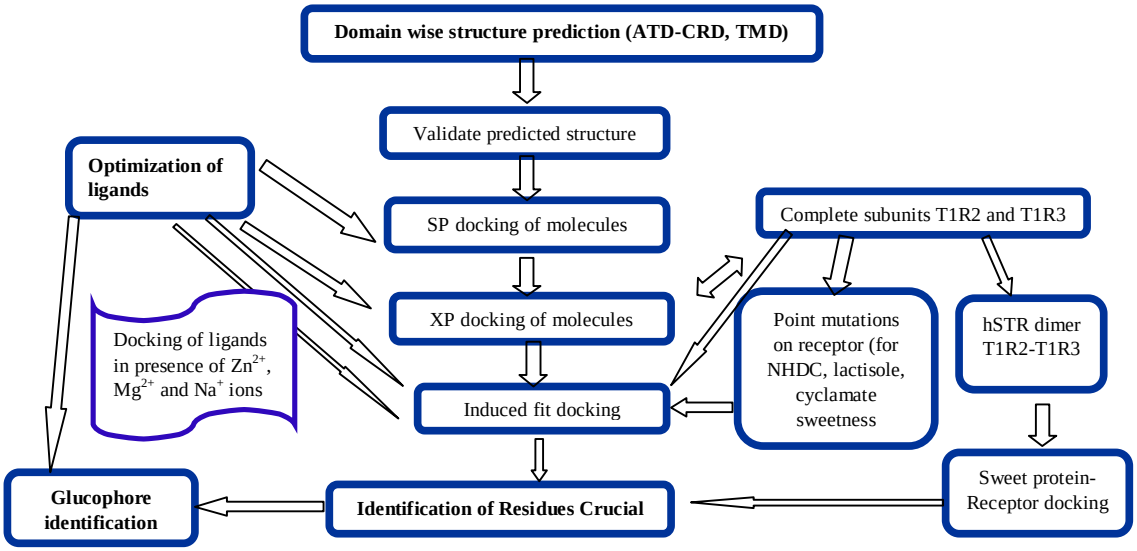
1) Classification of available sweet molecules based on active site(s) of interaction.

2) Identification of crucial/essential residues of the receptor involved in interaction.

3) If possible pinpoint the source of stability and specificity.

Thesis is structured as Introduction (Chapter 1) followed by Review of Literature (Chapter 2) and the Methodology (Chapter 3) used in the present work and finally Results and Discussion is presented in Chapter 4. Thesis is concluded with presentation of important results and summary in Chapter 5 Conclusion. Plan or design of study is presented below.

Plan of Study



Chapter 1 presents the perspective, motivation or need of the present study in view of the lacunae of the existing artificial sweeteners. This is followed by **Chapter 2** presenting a detailed **review of the literature** of artificial (synthetic) as well as natural sweeteners, their features and associated controversies. In addition to this, experimental studies involving point mutations and chimeras (human and mouse sweet taste receptor chimeras) [11] [12], and effect of inorganic salts on sweetness [13] of sweet molecules and proteins and computational studies to identify pharmacophores [14] have been reviewed.

Chapter 3 gives a brief description of the **methodology** followed by us to identify substructures in sweet molecules as well as in human sweet taste receptor (hSTR) responsible for conferring sweetness. The strategy followed in the present work can be

subdivided into four major sections – 1) Optimization of structure of sweet molecules and sweet inhibitors, 2) Pharmacophore analysis, 3) Structure prediction of hSTR and 4) Docking of sweet molecules and proteins with hSTR for identification of site of interaction of sweet molecules, crucial residues involved in interaction and responsible for eliciting sweetness and stability to the receptor structure.

Small sweet molecules were optimized by density functional theoretic approach using Gaussian 03 software. Sweet molecules belonging to wide variety of sweeteners with sufficient representative molecules from each category - sacchrides (sucrose, glucose), nonsacchriferrous inorganic salts (saccharine, cyclamate etc.) and organic salts (acesulfame, aspartame etc.) have been used for the study. 13 sweet molecules (Aspartame, neotame, cyclamate, NHDC, acesulfame, sucrose, saccharin, sorbitol, sucralose, glucose, fructose, tryptophane, perillartin) and four non sweet molecules (lactisole, melatonin, perillaldehyde and Indole 3 glycerolphosphate) were selected and optimized. In **hard sphere approach** glucophores were identified for all optimized sweet molecules on the basis of AH-B-X theory (AH is proton donor group, B is proton acceptor group and X is a polar group). **Soft sphere approach** was used to confirm whether the identified glucophores are same as hard sphere docking or not. **Phase** (module of First discovery software) was also used for glucophore identification of the sweet molecules.

Since crystal structure of hSTR is not available till date, we predicted the structure of hSTR, a heterodimer of T1R2-T1R3, following homology modeling as well as threading based approach. Each of the subunits T1R2 and T1R3 being greater than 800 amino acids long [15], it is difficult to predict the complete structure with good confidence level. The fact that T1R2 and T1R3 have three domain – amino terminal domain (ATD), cysteine rich domain (CRD) and transmembrane domain (TMD) was exploited for domain wise structure prediction followed by building complete subunits from individual domains. TMpred, HMM Top, TMHMM tools were used to predict the topology of TMD region. Homology modeling was done using Swiss model, ESyPred3D, CPH models, Geno3D, SWISS MODEL - GPCR and PRIME while for threading based model prediction Phyre, HHpred, LOOPP and ITASSER software were used. ITASSER was the only software that could predict complete subunit structure other software could predict only partial structures of ATD+CRD [16] or TMD domain.[17] The models predicted from each of

the software were then validated in terms of stereochemical quality, packing quality etc. using structure assessment and validation tools at ADIT and Swiss model server. This was followed by refinement of the structures and revalidation of the same. Finally, each subunit was built from various combinations of the ATD+CRD and TMD domain structures predicted from different software and revalidated as discussed above. Evaluation of the final structures was done based on docking of five molecules (Aspartame, neotame [18], NHDC [19], sodium cyclamate[20] and lactisole[21]) having experimentally known active site. After evaluation, best structure was chosen for identification of site of interaction of all the sweet molecules.

Three different modes were elected for the **docking** of sweet molecules- rigid docking, flexible docking and induced fit docking. Hex (rigid docking), AutoDock (flexible docking), Lead IT (flexible docking), and Schrodinger (flexible docking and induced fit docking) software. To identify the residues important for stability and specificity of the receptor, we took two different approaches first is mutational study and another one is docking of sweet molecules in presence of ions based on corresponding experimental studies. **Single point mutations** were done to identify the changes occurring on the receptor due to mutation, affecting sweetness. In another study, sweet molecules were docked in presence of ions [Zn^{2+}, Na^{+} and Mg^{2+}] and the **effect of ions on the activity of molecules** was evaluated.

Active site identification for sweet proteins Brazzein, Monellin, Thaumatin, Mabinlin, Curculin and taste modifying protein Miraculin was done using protein-protein docking software – Hex, GRAMM-X and Patch Dock.

Results and Discussion is presented in **Chapter 4**.

Ligand based Pharmacophore/Glucophore Analysis

Small sweet molecules were optimized using density functional theoretic approach. These optimized sweet molecules were analyzed for the presence of AH-B-X glucophores as proposed by Kier et al. [1] We found that not all sweet molecules obey the reported values for the glucophores, i.e. - AH-B (2.6 Å), B-X (5.5 Å), AH-X (3.4 Å). These sweet molecules can be placed into two groups on the basis of their identified AH-B-X distances. Sucrose, Sucralose, Glucose, Sorbitol, Acesulfame, Fructose, NHDC,

Aspartame and Tryptophan that follow AH-B-X glucophores while lactisole, perillartin, saccharin, cyclamate and neotame do not follow the reported glucophoretic distances. Experimentally it is known that lactisole and cyclamate have an overlapping site of interaction in TMD region of T1R3 while aspartame and neotame are reported to bind in ATD region of T1R2. This implies that TMD binding ligands probably have different pharmacophores. These results were further verified after soft sphere docking studies and analysis of glucophores after docking of sweet molecules with the hSTR.

Glucophore identification for small sweet molecules was also attempted using quantitative structure activity relationship (QSAR) analysis through **Phase** software**.** 13 sweet molecules (Aspartame, neotame, cyclamate, NHDC, acesulfame, sucrose, saccharin, sorbitol, sucralose, glucose, fructose, tryptophane, perillartin) and four non sweet molecules (lactisole, melatonin, perillaldehyde and Indole 3 glycerolphosphate) were used in QSAR study. However, attempts to identify pharmacophore failed and gave no conclusive results.

Receptor Based Pharmacophore/Glucophore Analysis

Glucophore identification through receptor based approach requires knowledge of structure of receptor as well as sweet molecules. Glucophores are identified based on docking studies followed by identification of residues involved in interaction of sweet molecules with the receptor and finally identifying crucial residues for sweetness and stability of the receptor.

Structure prediction of hSTR- I-TASSER was the only software that could predict the structure of complete T1R2 and T1R3 subunit while all other homology based (Swiss model, ESyPred3D, CPH models, Geno3D, SWISS MODEL - GPCR and PRIME) as well as threading based (Phyre, HHpred, LOOPP, PRIME and ITASSER) tools could predict only ATD+CRD and/or TMD domain. Swiss model GPCR tool was used for structure prediction of TMD regions followed by building loops between different helix using Prime module. These domain structures were used as a unit for **complete structure prediction**. We generated six models using different combinations of domain structure.

Validation of structures after refinement of predicted models shows more than 90% residues in allowed region and less than 5% in disallowed region in Ramachandran plot.

EsyPred, LOOP, HHPred, Phyre, Prime, gives less than 2% residues in disallowed region for T1R2, and CPH, EsyPred, Swiss, LOOP, HHPred, Phyre, shows less than 2% residues in disallowed region for T1R3 ATD-CRD. These Ramachandran values suggested that predicted models are reliable.

Comparative analysis of packing quality and stereochemical quality using Anolea and Gromos shows models predicted from Phyre, 3-D Jigsaw, HHpred, EsyPred 3D and LOOPP had greater than 50% of residues in energetically less favourable environment. While models predicted by I-TASSER, Prime, CPHmodels and SWISS-MODEL were of acceptable quality with more than 95% residues having energetically favourable environment. Complete T1R2 and T1R3 subunit structure prepared through different domain structure shows more than 95% residues in energetically favourable region where as complete subunit structure predicted through ITASSER give 90% residues in energetically favourable environment.

After structure validation these were further **evaluated** through docking of four sweet molecule (NHDC, Aspartame, Neotame and Cyclamate) and one sweet taste inhibitor Lactisole with the predicted receptor structure. After docking we have identified the site of interaction and compared it with experimental results. Domain structures predicted through CPH, Swiss, Schrodinger and ITASSER for T1R2-T1R3 gave same interaction site for Lactisole, Cyclamate, Aspartame, Neotame and NHDC as reported in the literature. In complete subunit structure out of the six models only one model i.e. hSTR model-6 could dock all the five molecules in conformity with experimental results. Final selected model was T1R2: ATD-CRD (SWISS-MODEL) + TMD (I-TASSER), T1R3: ATD-CRD (CPHmodels) + TMD (I-TASSER). The energy of complete subunit model for T1R2 is -28159.3 Kcal/mol while that of T1R3 subunit is -27384.8 Kcal/mol.

Docking Analysis of sweet molecules was performed using Hex, Lead IT, AutoDock and Prime. No reproducible results were obtained on rigid body docking with **Hex**. Ligand docking with the same centroid atom gives different E-value. Docking of sweet molecules with **Lead IT** gave same active site for Aspartame and Neotame however, the site of interaction predicted for cyclamate, NHDC and lactisole were different from known active sites. Active site of these three molecules was identified from experimental results as T1R3 TMD but Lead IT predicted T1R2 ATD for cyclamate and NHDC and T1R2

TMD for Lactisole. Hence docking results of Lead IT were also not pursued further for identification of crucial residues.

Docking studies performed using **PRIME** standard precision **SP docking** was followed by **PRIME** extra precision **XP docking** (see Fig 1a)**.** Tryptophan, Acesulfame, Neotame, Aspartame, Fructose, Sucralose, and Cyclamate interacting on T1R2 site 1(ATD) while Glucose, Sucrose, Sorbitol on T1R2 site 5 (ATD). Acesulfame had interaction on two different active sites with approx same glide score (-6.67) on T1R2 site1 and (-6.53) on T1R2 site5 and energy -22.9kcal/mol and -22.59 kcal/mol respectively. T1R3 site 1(TMD) was the predicted active site for the activity of NHDC, saccharin and lactisole and T1R3 site 6 (TMD) for Cyclamate and Perillartin. However, experimentally it has been found that cyclamate and lactisole (sweetness inhibitor) share common active site and a residue. Finally **PRIME induced fit docking** (see Fig 1b) was performed and it was found that five molecules interacting on T1R2 site1 (ATD) were Aspartame, Neotame, Fructose, Sucralose and Tryptophan. T1R2 site5 (ATD) is identified as important for the activity of Sorbitol, Sucrose, Saccharin and Glucose. Acesulfame and NHDC show interaction on T1R2 site7 (ATD-CRD) and T1R3 site1 (TMD) respectively. Cyclamate and Perillartin interacts on T1R3 site6 (TMD). Predicted results were in agreement with experimental results in terms of site of interaction as well the residues. Acceptability of the predicted model and induced fit docking was based on the fact that few of the important residues identified experimentally matched well with docking results for NHDC (Gln637, Ser640, His641) for cyclamate (Ser640, Trp775, Tyr771, Leu782, Val779, Phe778**)** and for lactisole (Ser640, Phe778, Leu782).

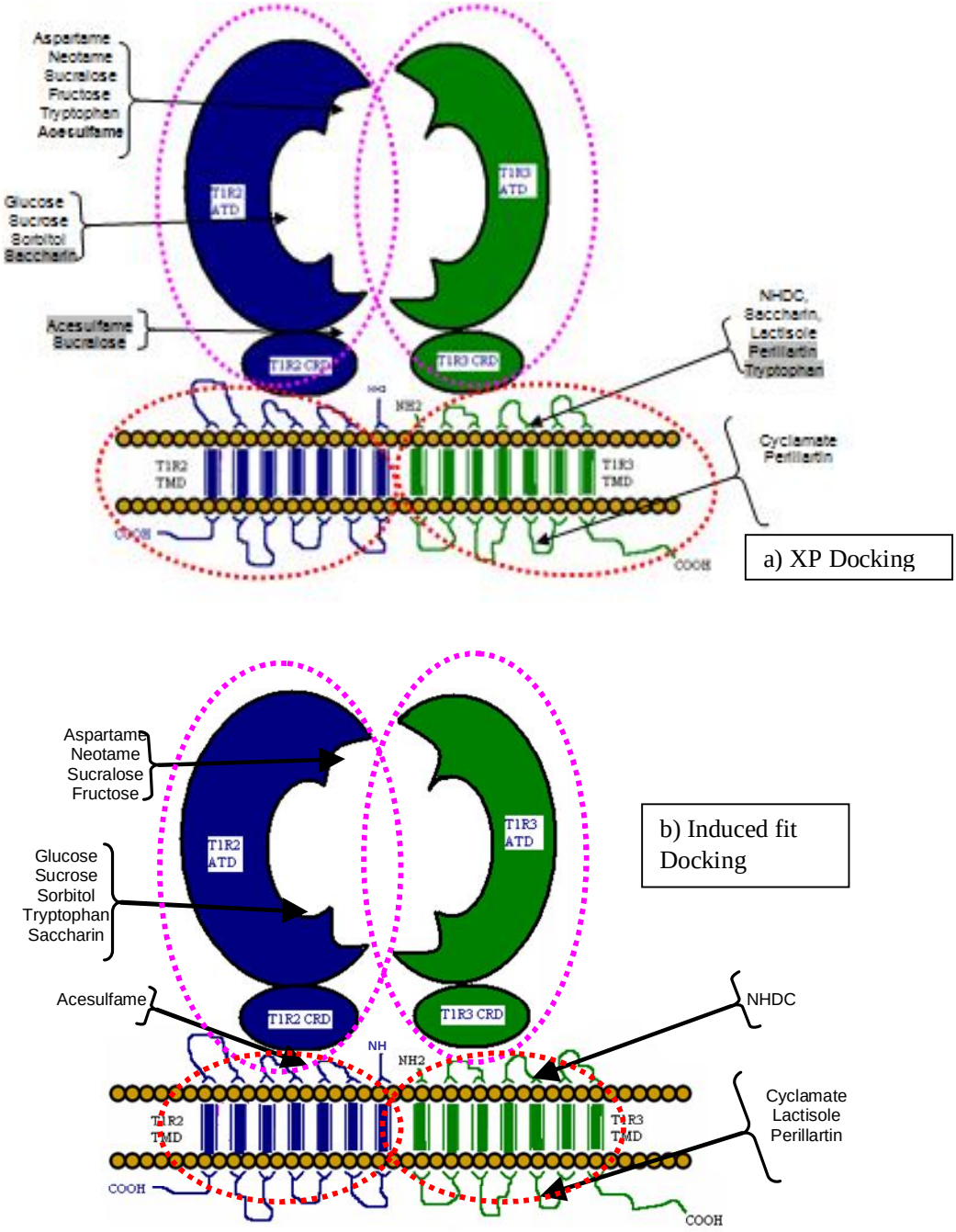


Fig. 1: Site of interaction of sweet molecules and inhibitor lactisole a) XP docking b) induced fit docking

Mutational analysis: Experimental studies of point mutation on receptor and its effect on sweetness of NHDC, cyclamate and lactisole were exploited to do a similar computational study and identify the changes in interacting residues. Generally it is expected that if some changes lead to increase in sweetness should be reflected in computational study in terms of decrease energy of the docked complex or in other words better binding is taking place or vice versa if some mutation leads to decreased sweetness intensity. However, no conclusive results on the basis of their energies were obtained on the basis of energies.

We have analyzed the interacting residues (hydrogen bond interaction and hydrophobic interaction) before and after mutations. Sweet molecules interacting with the same active site should show similar variation in critical residues i.e. loss of interaction with important residues in case of sweetness unfavorable mutations and no change in interaction of important residues in case of favorable mutation.

These mutational analysis were further corroborated with docking studies in presence of Zn^{2+}, Na^{+} and Mg^{2+} ions. Since sweetness was not inhibited by Na^{+} and Mg^{2+} ions while Zn^{2+} inhibited the sweetness of all except cyclamate [13], interacting residues maintained in Na^{+} and Mg^{2+}case of while absence of the same in case of Zn^{2+} was indicative of crucial residues for sweetness.

Based on the above analysis Asn312, Ser356, Arg378 were found to be important hydrogen bond interaction for the activity of molecules (Aspartame, sucralose) having active sites on T1R2 ATD site1. **Cys499** seems to be important interaction for sorbitol, glucose, sucrose, tryptophan and saccharin interacting on T1R2 site5. **Gln654, Asn755, Glu758, and Ala668** must be important for the activity of **acesulfame** on T1R2 site7. **Ser577, Gly603, Leu642** was identified as important for receptor activity for NHDC. **Tyr771 and Thr736** was found to be crucial for the sweetness of Cyclamate and Perillartin**.**

Hydrophobic interactions– **Pro57, Val309** hydrophobic interactions play essential role in sweetness of aspartame, fructose and sucralose**. Pro308, Asn312, Leu377, Tyr357, Ser356** must play an important role for sweetness of neotame**. His528** seems as important for the sweetness of sorbitol, glucose and saccharin**.** Arg498 interaction must play an important role in sweetness of sucrose. **Gly603, Gln637, Pro638, Leu639, His641, Ser640, Tyr771, Cys613,** are hydrophobic interactions seems as important interactions

for NHDC activity on T1R3 TMD site1. **Ser640, Thr736, Tyr771, Trp775 and Leu782** is interactions identified as important for the sweetness of perillartin and cyclamate.

Protein-Protein docking. Small sweet molecules docking study was carried out using individual T1R2 and T1R3 subunits and comparing the docked complex energies to assign specific site of interaction. The assumption that dimeric structure of hSTR (T1R2-T1R3) would not cause much of steric hinderance in case of small molecules and hence individual subunit docking studies would be a reasonable choice. However, sweet proteins being large macromolecules needs due consideration of steric affects. Hence we modelled the dimer of T1R2-T1R3 from individual subunits before doing docking studies of sweet proteins. Docking of sweet protein with hSTR was done using Hex, Patch Dock and GRAMM-X software. Analysis of results showed that sweet proteins dock through a large surface and their active site is not confined to CRD domain as known for brazzein. It was observed that brazzein binds to T1R2 ATD and T1R3 ATD CRD and curculin interacts with T1R2 ATD-CRD and T1R3 ATD-CRD. While Mabinlin and Monellin both interact with transmembrane domains only i.e, T1R2 TMD and T1R3 TMD. Miraculin the sweet taste modifying protein binds to T1R3 ATD –CRD only with no interacting residues with T1R2 subunit. There are certain common interacting residues of hSTR between pairs of sweet proteins (brazzein-curculin; monellin-mabinlin), however, there is no generalization in the sense that certain residues are essential for sweetness. This explains structurally different sweet proteins with insignificant sequence or structural similarity have enormous sweetness as compared to sucrose.

Chapter 5 Conclusion

- Evaluation of the predicted complete T1R2 and T1R3 subunit structure prepared by using ATD-CRD model predicted by Swiss model and CPH respectively and TMD domain predicted through ITASSER and Swiss GPCR + Prime was of acceptable quality since the active site of interaction and experimentally identified important residues were identified through our computational docking study as well. The important residues identified experimentally that matched well with docking results are – for NHDC (Gln637, Ser640, His641) for cyclamate (Ser640, Trp775, Tyr771, Leu782, Val779, Phe778) and for lactisole (Leu782, Phe778, Ser640).

- There are multiple binding sites on receptor suitable for different types of molecules. The sweet molecules can be grouped together based on their active site of interaction as -

Site of Interaction	Sweet Molecules
T1R2 site 1 (ATD)	Neotame, Fructose, Acesulfame, Aspartame, Sucralose
T1R2 site 5 (ATD)	Glucose, Sucrose, Sorbitol, Saccharin, Tryptophan
T1R2 site 7 (ATD-CRD)	Acesulfame
T1R3 site 1 (TMD)	NHDC
T1R3 site 6 (TMD)	Cyclamate, Lactisole, Perillartin, Fructose

- Crucial residues for eliciting sweetness for each group of molecules are

Site of Interaction	Sweet Molecules	Crucial Residues	
		H-Bond Interaction	**Hydrophobic Interaction**
T1R2 site 1(ATD)	Neotame	Ser356, Arg378	Pro308, Asn312, Ser356, Tyr357, Leu377
	Aspartame, Sucralose	Asn312, Ser356, Arg378	Pro57,Val309, Asn312,
	Fructose	Ser356, Arg378	Pro57, Val309, Ser356, Leu377
T1R2 site 5 (ATD)	Saccharin	Cys499, Glu532	His528, Met492, Met494
	Tryptophan	Cys499, Glu532	-
	Glucose, Sorbitol	Cys499	His528
	Sucrose	Cys499	Arg498, Leu793
T1R2 site 7 (CRD-TMD)	Acesulfame	Gln654,Ala668, Asn755, Glu758	-
T1R3 site 1 (TMD)	NHDC	Ser577, Gly603, Leu642	Gly603, Cys613, Gln637, Pro638, Leu639, Ser640, His641, Tyr771
T1R3 site 6 (TMD)	Cyclamate, Perillartin	Thr736, Tyr771	Ser640, Thr736, Tyr771, Trp775, Leu782

- Sweet proteins were found to interact through a much larger surface. There are certain common interacting residues of hSTR between pairs of sweet proteins (brazzein-curculin; monellin-mabinlin), however, there is no generalization in the sense that certain residues are essential for sweetness

- Our study indicates that there are no common pharmacophores as such. This explains how a wide variety of molecules – different in chemical nature, (even proteins having no sequence or conformational similarity) can elicit sweet response**.** Group of molecules having common active sites of interaction, even they do not have any common pharmacophore

- Hydrophobic interactions play a major role both in specificity and stability of interaction of hSTR – sweet molecule**.** Residues responsible for stability of the receptor structures were Leu-644, Thr-645, Tyr-771, Gln-794, Ile-805

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LIST OF PUBLICATIONS DURING Ph.D THESIS WORK

International Journals

1. **Aditi Shrivastav**, Nidhi Gupta and Sudha Srivastava. Is AH-B-X glucophore sufficient condition for sweetness? World Journal of Science and Technology 1(10): 21-29, 2011
2. **Aditi Shrivastav** and Sudha Srivastava Interaction of Sweet Proteins with Human Sweet Taste Receptor J Pharmaceutical Science (accepted for publication in March 2013 issue)
3. **Aditi Shrivastav** and Sudha Srivastava Human Sweet Taste Receptor : Complete Structure Prediction and Evaluation (submitted to Int. J of Chemical and Analytical Science)