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A physiological model of tea-induced astringency

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ABSTRACT

The mechanism by which solutions containing polyphenols are perceived as astringent is not clearly understood. Salivary proline-rich proteins and histatins are products of salivary glands and rapidly bind polyphenols — thought to be the main astringent compound in such as tea and wine. However it is unclear how this interaction leads to the altered oral mouthfeel known as astringency which is characterised by a dry, puckered feeling all around the mouth. To determine the role of saliva in the perception of astringency a protocol was used to decrease the volume of saliva from the mouth (by washing with water) and then by chewing to increase the volume of saliva above resting levels. Following each of these conditions subjects tasted the same solution of black tea and were asked to rate the relative astringency. Compared to the astringency rating of black tea at rest the majority of subjects (10 out of 15) perceived an increase in astringency following washing the mouth with water. Most subjects then perceived a decrease in astringency following chewing compared to the previous state. In all subjects a reduction in salivary proteins was detected following water washout and an increase above resting levels detected following chewing although there was no change in oral mucosal wetness. A separate experiment revealed several of the proteins interacting following the water washout were salivary in origin. We conclude that salivary proteins in solution inhibit the mouthfeeling of astringency which is mediated, at least in part, by salivary proteins adhered to buccal mucosal cells.

1. Introduction

The presence of saliva is vital for the maintenance of healthy oral tissues. Three pairs of major salivary glands (parotid, sublingual and submandibular glands) secrete saliva into the mouth. Saliva is composed of many proteins that enable the oral cavity to maintain a suitable environment and to carry out certain processes, such as taste, prevention of demineralisation and speech. They are also important in covering the exposed surfaces of the mouth to maintain lubrication whilst eating and speaking [1], although salivary protein interaction with foodstuffs to allow taste perception has not been greatly studied.

Tannins, also known as polyphenols, are commonly found in plant-based food and drink and are thought to be responsible for the mouth feeling of astringency [2,3]. Astringency can be described as a rough, dry, puckering feeling of the oral mucosa [4] and the word astringency comes from the Latin phrase "*ad stringere*" meaning 'to bind' and is believed to relate to the ability of astringent substances to bind to and precipitate proteins [5]. Tannins avidly bind certain salivary proteins, especially proline-rich proteins and histatins [6] precipitating them at high concentrations as a protein–tannin complex [7]. The interaction between salivary proteins and tannins involve molecular forces, including hydrophobic forces and hydrogen bonding [7,8] causing cross-linking of polypeptides by surface exposed phenolic groups on the tannins.

How this interaction may cause the tactile sensation of astringency is unknown. Changes in lubrication of the oral mucosa [9], formation of particles [10] or loss of the mucosal wetness [7] may account for changes in perception, although how these changes are perceived as astringency, presumably by touch receptors within the mouth, is unclear.

One important function of salivary proteins is to covalently bind to the oral mucosal cells, forming a layer around the soft structures of the mouth [11]. This protein layer is known as the 'mucosal pellicle' which functions to retain moisture, provides a protective barrier and acts as a lubricant preventing frictional abrasion against the oral mucosa [12–14]. The adsorbed salivary components may be cross-linked to each other or the epithelia by epithelial cell-derived transglutaminase enzyme [15]. Using radiolabelled saliva as a substrate it was shown that this cross-linking process was selective and that the PRPs were preferentially bound to the buccal epithelial cells although other salivary proteins (Mucin Glycoprotein 1 and 2 (MG1&2), cystatins, sIgA and amylase) are known to bind.

Thus the purpose of this study was to examine the role of saliva (in a mobile phase) and saliva adhered to oral epithelial cells in the sensation of astringency following the ingestion of tea.

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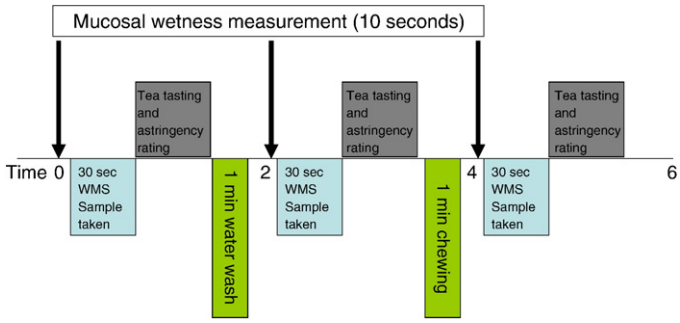
2. Materials and methods

2.1. Whole mouth saliva (WMS) collection experimental protocol

WMS was collected from 15 healthy individuals (8 men and 7 women; mean age 25 years, range 21–34 years) who had no history of oral complaints and were not taking any medication. Saliva samples were taken between 10 and 12 am at least 1 h after consuming any food.

WMS was collected by gentle spitting for 30 s, under the following conditions:

1) Unstimulated at rest.
2) After rinsing the mouth out with water for 1 min.
3) After chewing on paraffin film (10 cm by 5 cm) for 1 min (saliva collected whilst not chewing).



Schematic to show time (mins) points of WMS, mucosal wetness and tea tasting after either water washout of the mouth or following chewing.

Total volume of saliva collected was measured and salivary flow rate expressed in ml/min. Following centrifugation of samples (at 10,000RPM for 5 min) the supernatant was separated from the residue, and total protein content of saliva samples (diluted down to 1:100 concentration) was assayed by absorbance at 215 nm, using double distilled H_2O as a reference value and a protein standard (albumin and IgG) to construct the standard curve.

2.2. Residual saliva collection — mucosal wetness

To determine amounts of saliva adhered to the oral mucosal surface samples were taken on filter paper and volumes estimated by electrical conductance. At the same time as whole mouth saliva collection, (following the 3 different conditions; rest, water washout and chewing) residual saliva was collected using Sialopaper (Oraflow Inc., Plainview, NY) filter paper strips from the site of the lower labial mucosa for 10 s, as described (Pramanik et al., submitted for publication). Periotron® 8000 (Oraflow Inc) micro-moisture meter was used to quantify the minute volumes of fluid samples from the strips.

2.3. Preparation of tea solution

A single teabag (containing approximately 3 g of strong tea; Marks & Spencer Ltd., London, UK) was added to 200 ml of boiling water, left to brew for 5 min, after which the teabag was removed and the solution left to settle and cool for a further 10 min.

2.4. Astringency perception

Following each WMS and sialopaper collection the subject sampled the tea solution and rated the perception of astringency from a scale of slight, medium, strong and extra strong. These procedures were undertaken according to a protocol approved by King's College London research ethics committee.

2.5. Protein analysis of salivary samples

SDS-PAGE of samples was carried out (NuPAGE Novex Bis–Tris 10% gel, Invitrogen, Paisley, UK) and resolved proteins were stained using a solution of 0.2% w/v Coomassie Brilliant Blue-R250 (Sigma, Poole, Dorset, UK) in 25% methanol and 10% acetic acid for 1 h. Gels were then destained in 10% acetic acid overnight. Alternatively proteins separated by SDS-PAGE were stained with periodic acid Schiff's reagent (PAS) to detect highly glycosylated proteins within the

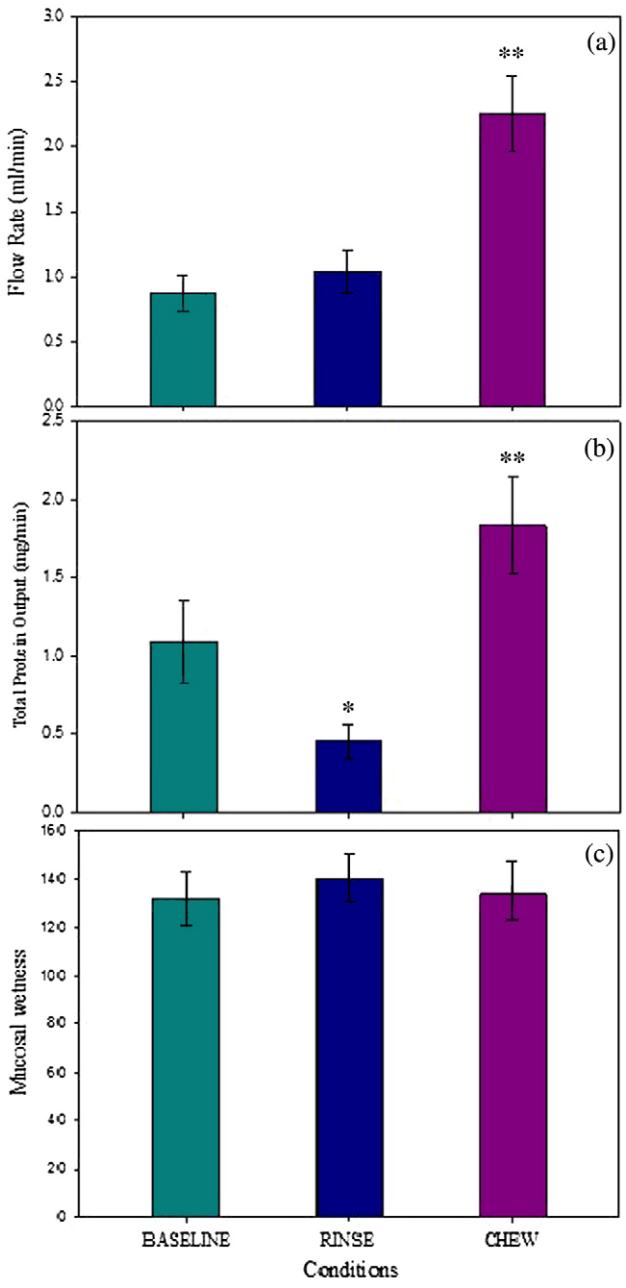


Fig. 1. (a) Average salivary flow rate (±SEM) at baseline (resting) and after rinsing or after chewing conditions. $^{**}P<0.001$ comparing baseline state to after chew ($n=15$). (b) Average total protein output in the same samples as for (a) $^{*}P<0.05$ and $^{**}P<0.001$ comparing baseline state to after rinse, and rinse state to after chew ($n=15$). (c) Mucosal wetness in the three states as assessed by Periotron readings of filter paper applied to the buccal surface.

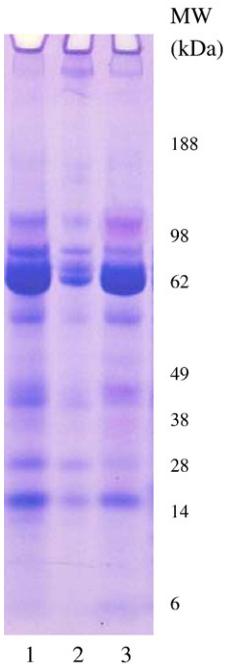


Fig. 2. SDS-PAGE and Coomassie Brilliant Blue (CBB R250) staining of WMS proteins from a single subject under 3 different conditions; baseline-rest (1), after rinsing (2) and after chewing (3).

samples. Gels were left in a solution of 25 ml methanol, 10 ml acetic acid in 65 ml of de-ionised water and left with gentle agitation for 1 h. Gels were washed in de-ionised water for 20 min and then oxidised in 2% (w/v) periodic acid for 15 min, and stained in Schiff's reagent until bands appeared. To increase band intensity gels were washed in de-ionised water then tap water.

2.6. Western blotting

Proteins resolved by electrophoresis were electroblotted onto 0·45 μm nitrocellulose membranes (Anderman & Co., Kingston Upon Thames, UK) for 60 min using a 'wet-blot' apparatus (Invitrogen) set at 30 V and with a current of 1 A. Blotted proteins were detected using FITC as previously described [16]. Primary antibodies against Muc 1, Muc 5b, Muc 7a, secretory component and statherin were diluted 1 in 200 with Tris-buffered saline containing 0·1% Tween 20. These antibodies were subsequently detected using a secondary antibody of biotin labelled goat-anti-mouse (Universal Biologicals Ltd, Stroud, UK) followed by avidin–biotin complex (Vector Laboratories Ltd, Peterborough, UK). Antibody binding bands were detected using enhanced chemiluminescence (ECL, Amersham International) recorded photographically on X-ray film (Hyperfilm, Amersham International) as previously described [16].

2.7. Statistics

Following analysis by one-way ANOVA student's *t*-test was used to analyse the differences between the baseline resting, after rinsing and after chewing conditions of saliva and mucosal wetness, where $P<0.05$ was considered significant. The Chi-square test (χ^2) was used to test the association between the astringency perception between two variables of a set of frequencies, in this case before rinsing the mouth and after stimulation upon chewing.

2.8. Tannin interactions with the mucosal layer

Within a subset of 3 volunteers, the mouth was washed out three times with water (10 ml) for 1 min and removed. Ten milliliters of tea solution (as previously described) was added to the mouth, vigorously mixed for 1 min and then expectorated into a pre-weighed tube. Tubes were weighed and centrifuged (30 000 *g* for 5 min). The supernatant was discarded and the any precipitate formed homogenized in 1 ml dd H_2O. To an aliquot (100 μl) of homogenized pellet an equal volume of 40% trichloroacetic acid (20% TCA w/v final) was added, the precipitate collected by centrifugation and re-suspended in 100 μl of water.

3. Results

3.1. Salivary results

Fig. 1 shows the average WMS salivary flow rates (for $n=15$) at rest and immediately following rinsing the mouth with water and chewing on paraffin film. Only following chewing was there an increase in flow ($P<0.001$) compared to rest. The total protein output (salivary flow rate × protein concentration) in the same samples decreased greatly after rinsing the mouth with water ($P<0.05$) and increased greatly after chewing on paraffin film ($P<0.001$) (Fig. 1(b)). These changes were consistent within all subjects. An electrophoretic analysis with CBB staining from a single subject under 3 different conditions confirms that rinsing with water has reduced the number of salivary

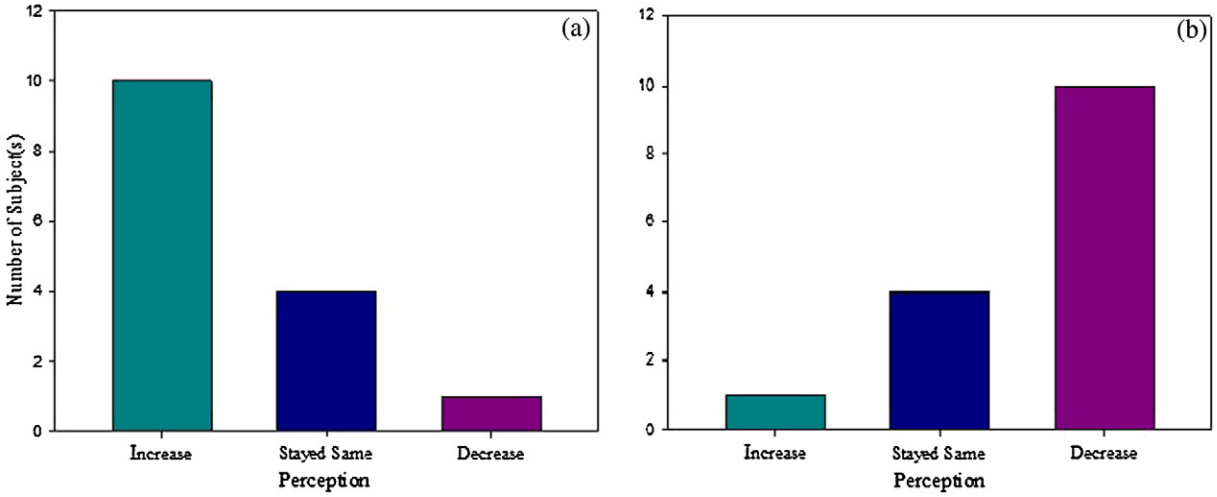


Fig. 3. Change in astringency perception of a tea solution from (a) baseline to after rinsing conditions ($n=15$) and (b) after rinsing to after chewing conditions ($n=15$).

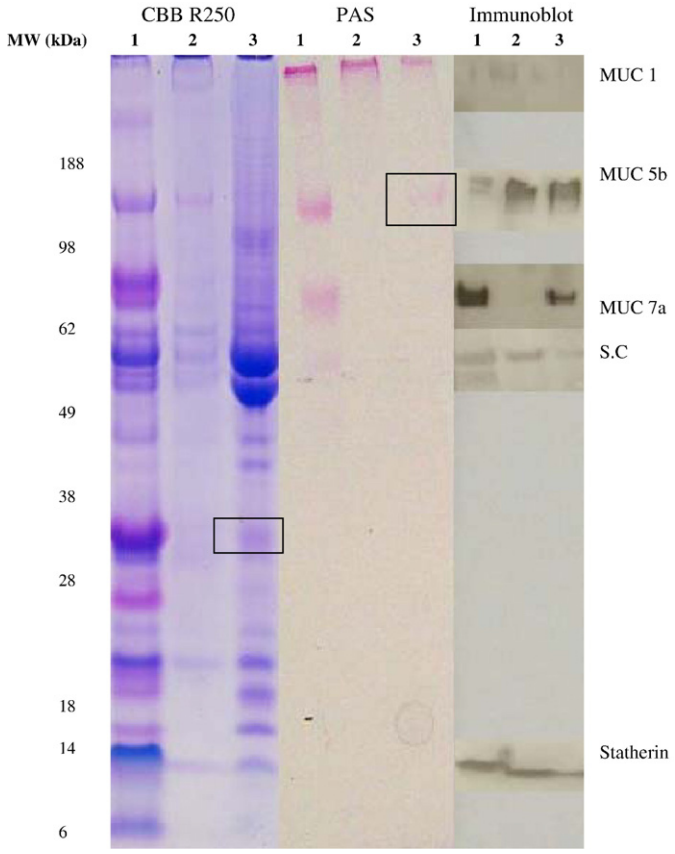


Fig. 4. CBB R250 (panel 1), PAS staining (panel 2) and immunostaining (panel 3) of resting whole mouth saliva (lane 1), tea–saliva precipitate (lane 2) and tea–saliva precipitate treated with TCA (lane 3). Square boxes indicate in panel 1 a metachromatic staining PRP in lane 3, and MUC 5b in panel 2 (lane 3). Following Western blotting of a similar gel to panels 1 and 2 antibodies to MUC1, MUC5b, MUC7a, Secretory component (S.C.) and Statherin were used. The third panel is a montage of each X-ray film.

proteins present, whereas a return of salivary proteins occurred following chewing (Fig. 2). Average mucosal wetness as measured by the Periotron (Fig. 1(c)) for the 3 conditions (rest, post water washout, post chew) indicated little variation between the 3 conditions, with no statistical difference ($P>0.05$).

3.2. Astringency perception

Immediately following each condition (rest, water washout and post chew), subjects were asked to sample the same preparation of tea and the astringency perception was identified per person as a change in perception from the previous mouth condition; from resting to after rinsing and from after rinsing to after chewing. The perception of astringency was noticeably increased after rinsing the mouth with water compared to baseline in 10 out of 15 subjects ($P<0.05$, Fig. 3). After chewing on paraffin film the perception of astringency had significantly decreased in 10 out of 15 subjects, compared to after rinsing the mouth with water ($P<0.05$, see Fig. 3).

3.3. Analysis of saliva–tea precipitate

In a separate series of experiments after rinsing the mouth with 10 ml of water 3 times a visible precipitate formed following rinsing with tea solution. This precipitate was not visible if the prior rinsing with water was not performed. Furthermore a precipitate did not occur in a tea solution left to stand. Initial results indicated few resolved proteins in the saliva–tea precipitate, suggesting that proteins could not be solubilised by SDS. If re-precipitated by the addition of an equal volume of 40% TCA then analysed by SDS-PAGE several proteins were resolved which were not apparent if the tea–saliva precipitate was not treated with TCA (compare lanes 2 and 3 in Fig. 4). CBB staining suggests that the saliva–tea precipitate includes Gl-PRP, amylase, PRPs and statherin (as judged by metachromatic staining and comparable molecular weights). PAS glycoprotein staining shows high molecular weight glycoproteins (MG 1) in WMS control (lane 1), saliva–tea sample (lane 2) and TCA precipitate. Further investigation by immunoblotting (Fig. 8) reveals: MUC 1, MUC 5b, MUC 7a, secretory component and statherin. The solubilising effect of TCA was particularly well illustrated by MUC 7a bands which appeared only in WMS lane and TCA precipitate of saliva–tea precipitate sample; no bands were found to be present in saliva–tea precipitate lane.

4. Discussion

Saliva plays an important role in the mouth feeling of astringency. A reduced salivary protein content within the mouth increases the perception of astringency whereas an increased salivary protein content inhibits the feeling of astringency. Therefore the mobile phase of saliva (that can be washed away by water) modulates the feeling of astringency by inhibition. These results agree with a previous study [9] but more carefully determine the oral environment and the effect of decreasing salivary content of the mouth. The method used to alter the oral conditions were efficient since after rinsing the mouth with water, the amount of proteins within saliva significantly decreased ($P<0.001$) whereas protein content increased to greater than baseline resting value after stimulation by chewing on paraffin film ($P<0.001$). Although water is known not to stimulate salivary flow [17] the mechanical stimulation of swirling water in the mouth [18] or the thermal effect [19] may be thought to stimulate saliva. This did not occur within this study; since salivary flow was measured and found to be unchanged and indeed salivary protein content reduced immediately following the water rinse, confirming little if any stimulation of saliva had occurred. Interestingly despite reducing the mobile phase of saliva by washing out with water results from the Periotron 8000 moisture meter, which reflects the mucosal wetness, didn't show any variation between the moisture of the oral mucosa after rinsing with water and after chewing. Thus the rinsing with water did not remove the surface associated layer of saliva and furthermore chewing did not increase it. The use of the Periotron to measure mucosal wetness has already shown variation in thickness around the mouth [20] and can be used to show variations in protein profiles (Pramanik et al., submitted for publication).

Most previous studies of the interaction of saliva with tannins or polyphenols have considered saliva to be a single entity. This is an oversimplification since saliva exists in the mouth as a thin dynamic film, varying in thickness and speed at different parts of the mouth [21] and as an absorbed layer of proteins on hard and soft tissues in the mouth. On teeth it is known as the enamel pellicle [22] where it functions to lubricate [23] and maintain a calcium enriched layer [24] although it may be the cause of tooth-staining [25]. On soft tissues it forms a layer called the “mucosal pellicle” [11] in which proteins are probably cross-linked by transglutaminase to buccal epithelial cells [15]. In our study the action of washing the mouth out with water seems to remove the mobile salivary film but leave the mucosal pellicle intact (as judged by mucosal wetness readings). This allows a greater interaction with tannins in tea causing an increased perception of astringency and in a separate experiment, the precipitation of proteins probably from the mucosal pellicle. The mobile film presumably forms a barrier to tea interacting with oral epithelial cells probably because it is more viscoelastic [24,26] than tea. Tannins and salivary proteins are likely to form ever larger precipitates [27] which may affect the lubricity of saliva [28] or may be detected as particles [10,29] however these interactions must occur on the surface of the mouth and not in solution since this study has shown

that soluble interactions inhibits the mouthfeeling of astringency. We believe that by thoroughly rinsing the mouth with water (3 times 1 min washes) the mobile salivary film was removed allowing the saliva–tea precipitate to form. It was observed that not incorporating the prior rinsing step did not produce a saliva–tea precipitate, suggesting that the mobile phase of saliva prevents tannins in tea from interacting with the mucosal pellicle.

As astringency is a perception that can vary between individuals, a possible complication was the absence of a standard. In a control study 4 out of 6 subjects experienced an increase in astringency perception whilst drinking the same tea sample several times without a change in oral conditions confirming that unlike the basic tastes astringency increases with increased exposure [9]. Also particular attention was paid to inform the volunteers to observe the changes in perceived astringency rather than any bitterness in an attempt to determine if the tactile (mouthfeel) component changed instead of the associated taste (bitterness) component of astringency [5].

The formation of a large visible precipitate of tea–saliva after thoroughly rinsing out with water was a surprise. We attempted performed an electrophoretic analysis to determine whether this was just a precipitation of tea or there were any salivary proteins that might cause the precipitation. Initial analysis of the saliva–tea precipitate by SDS-PAGE, PAS and Western blot analysis, indicated few proteins present — only high molecular weight proteins, suggesting that proteins were still complexed to the tannins, which are likely to be present in tea. Separation of the complex by TCA disrupted this interaction and allowed separation of mucins MUC 1, 5a from 7b; Statherin and Secretory component, which are also components found within the mucosal [20] and enamel pellicles [30]. This sample also contained proteins found in buccal cell scrapings (results not shown) suggesting that some buccal cells had also sloughed off during the interaction with tea. However we do not consider this to be an important effect since large numbers of buccal cells may slough following rinsing with water which does not cause an astringent feeling.

Therefore we conclude that whole mouth saliva inhibits the feeling of astringency which is probably mediated by the interaction of tannins/polyphenols with salivary proteins within the mucosal pellicle adhered to the buccal cell surface.

References

[1] Nieuw Amerongen AV, Veerman ECI. Saliva — the defender of the oral cavity. Oral Diseases 2002:12–22.
[2] Noble AC. Astringency and bitterness of flavonoid phenols. Chemistry of Taste. Mech Behav Mimics 2002:192–201.
[3] Vidal S, Francis L, Noble A, Kwiatkowski M, Cheynier V, Waters E. Taste and mouthfeel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. Anal Chim Acta 2004:57–65.
[4] Prinz JF, Lucas PW. Saliva tannin interactions. J Oral Rehabil 2000:991–4.
[5] Lyman BJ, Green BG. Oral astringency — effects of repeated exposure and interactions with sweeteners. Chem Senses 1990:151–64.
[6] Bennick A. Interaction of plant polyphenols with salivary proteins. Crit Rev Oral Biol Med 2002:184–96.
[7] Jobstl E, O'Connell J, Fairclough JPA, Williamson MP. Molecular model for astringency produced by polyphenol/protein interactions. Biomacromolecules 2004:942–9.
[8] Baxter NJ, Lilley TH, Haslam E, Williamson MP. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 1997:5566–77.
[9] Breslin PAS, Gilmore MM, Beauchamp GK, Green BG. Psychophysical evidence that oral astringency is a tactile sensation. Chem Senses 1993:405–17.
[10] de Wijk RA, Prinz JF. Mechanisms underlying the role of friction in oral texture. J Texture Stud 2006:413–27.
[11] Bradway SD, Bergey EJ, Jones PC, Levine MJ. Oral mucosal pellicle — adsorption and transpeptidation of salivary components to buccal epithelial-cells. Biochem J 1989:887–96.
[12] Gibbons RJ, Qureshi JV. Selective binding of blood group reactive salivary mucins by streptococcus-mutans and other oral organisms. Infect Immun 1978:665–71.
[13] Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary mucins in the protection of the oral cavity. J Oral Pathol Med 1982:1–17.
[14] Hatton MN, Loomis RE, Levine MJ, Tabak LA. Masticatory lubrication — the role of carbohydrate in the lubricating property of a salivary glycoprotein albumin complex. Biochem J 1985:817–20.
[15] Bradway SD, Bergey EJ, Scannapieco FA, Ramasubbu N, Zawacki S, Levine MJ. Formation of salivary-mucosal pellicle — the role of transglutaminase. Biochem J 1992:557–64.
[16] Carpenter GH, Proctor GB, Pankhurst CL, Linden RW, Shori DK, Zhang XS. Glycoproteins in human parotid saliva assessed by lectin probes after resolution by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis 1996:91–7.
[17] Hodson NA, Linden RWA. The effect of monosodium glutamate on parotid salivary flow in comparison to the response to representatives of the other four basic tastes. Physiol Behav 2006:711–7.
[18] Scott BJJ, Bajaj J, Linden RWA. The contribution of mechanoreceptive neurones in the gingival tissues to the masticatory-parotid salivary reflex in man. J Oral Rehabil 1999:791–7.
[19] Lee A, Guest S, Essick G. Thermally evoked parotid salivation. Physiol Behav 2006:757–64.
[20] Won SH, Kho HS, Kim YK, Chung SC, Lee SW. Analysis of residual saliva and minor salivary gland secretions. Arch Oral Biol 2001:619–24.
[21] Sas R, Dawes C. The intra-oral distribution of unstimulated and chewing-gum-stimulated parotid saliva. Arch Oral Biol 1997:469–74.
[22] Dawes C, Jenkins S, Tonge CH. The nomenclature of the integuments of the enamel surface of teeth. Br Dent J 1963:65–8.
[23] Douglas WH, Reeh ES, Ramasubbu N, Raj PA, Bhandary KK, Levine MJ. Statherin — a major boundary lubricant of human saliva. Biochem Biophys Res Commun 1991:91–7.
[24] Proctor GB, Hamdan S, Carpenter GH, Wilde P. A statherin and calcium enriched layer at the air interface of human parotid saliva. Biochem J 2005:111–6.
[25] Proctor GB, Pramanik R, Carpenter GH, Rees GD. Salivary proteins interact with dietary constituents to modulate tooth staining. J Dent Res 2005:73–8.
[26] Stokes JR, Davies GD. Viscoelasticity of human whole saliva collected after acid and mechanical stimulation. Biorheology 2007:141–60.
[27] Kay BK, Williamson MP, Sudol P. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J 2000:231–41.
[28] Rossetti D, Yakubov GE, Stokes JR, Williamson A-M, Fuller GG. Interaction of human whole saliva and astringent dietary compounds investigated by interfacial shear rheology. Food Hydrocoll 2008;22(6):1068–78.
[29] Pascal C, Poncet-Legrand C, Imberty A, Gautier C, Sarni-Manchado P, Cheynier V, Vernhet A. Interactions between a non glycosylated human proline-rich protein and flavan-3-ols are affected by protein concentration and polyphenol/protein ratio. J Agric Food Chem 2007:4895–901.
[30] Yao Y, Lamkin MS, Oppenheim FG. Pellicle precursor protein crosslinking: characterization of an adduct between acidic proline-rich protein (PRP-1) and statherin generated by transglutaminase. J Dent Res 2000:930–8.