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Interaction of Copper and Human Salivary Proteins

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Interaction of taste molecules with saliva is the first step in the flavor perception process. Saliva is assumed to influence copper-induced sensation by controlling the copper solubility or causing astringency via binding of proteins with copper. This study was performed to identify the nature of copper−protein interactions in relation to the sensory perception of copper. Saliva was treated with $CuSO_4 \cdot 5H_2O$ at levels of 0, 2.5, 5, 10, 20, or 40 mg/L, and changes in salivary proteins were analyzed using high performance liquid chromatography (HPLC) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Protein peaks that showed changes in HPLC were characterized with SDS−PAGE. HPLC analysis revealed that copper treatment up to 40 mg/L decreased several proteins, including the dominant peak, by 70%. This peak was composed of α-amylase, a secretory component, and basic proline-rich proteins. SDS−PAGE results showed that salivary proteins of molecular weight 29 kDa and 33 kDa precipitated when copper was added at concentrations ≥10 mg/L. This study provides biochemical information for understanding perception mechanisms of copper sensation.

KEYWORDS: Saliva; copper; proteins; astringency; HPLC; SDS−PAGE

INTRODUCTION

Sensory characteristics of metallic elements are described as metallic, bitter, sour, salty, and astringent (*1*). A recent study suggested that the metallic sensation of artificial sweeteners and metallic compounds, such as copper, iron, and zinc, is transduced by a taste receptor that is known to be activated by capsaicin, transient receptor potential vanilloid 1 (TRPV1) (*2*). Sensory stimulants are dissolved in saliva at the first stage of the perception process before further interaction with taste receptor cells (*3*). It is important, therefore, to understand the impact of saliva on delivery of sensory stimulants to taste receptors. Cuppett et al. (*4*) demonstrated that only soluble copper was readily tasted, whereas precipitated copper is poorly perceived. Hong et al. (*5*) suggested that copper interacts with salivary proteins to form insoluble complexes, leading to changes in the perception of copper by creating astringency and limiting the solubilized copper available for interaction with taste receptors.

Astringency is known to arise when salivary proteins lose their lubricating ability by precipiating upon binding with substances such as polyphenolic compounds (*3*). The mechanism of interaction between the salivary proteins and phenolic compounds has been investigated (*6*−*9*). Phenolic compounds bind to basic proline-rich proteins (PRPs) to form complexes through hydrophobic interactions or hydrogen bonding (*6*−*9*). It was observed that metallic compounds can form insoluble complexes with salivary proteins, and this binding was considered as a cause of astringency of metallic compounds (*10*). Salivary proteins, including mucin, α-amylase, PRPs, histatins, gustin, and lactoferrin, have metal-binding capacity with Ca^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{3+} (*11*−*20*). Proteins that are negatively charged at salivary pH, such as mucins and acidic PRPs, bind Ca^{2+} (*12*, *13*, *21*). Therefore, it is assumed that Cu^{2+} can bind with these proteins through electrostatic interaction following a mechanism similar to that of Ca^{2+}. α-Amylase has two metal binding sites, one is exclusive for Ca^{2+} and the other for Cu^{2+} (*11*). Histatins, a family of histidine-rich proteins, exhibit binding ability with several divalent metal ions, such as Zn^{2+}, Ni^{2+}, and Cu^{2+} (*14*−*17*). Another acidic salivary protein, gustin, strongly binds zinc (*18*, *24*). Copper and other metals could bind loosely with the second binding site of gustin (*18*). Salivary lactoferrin has two subunits that each bind one molecule of Fe^{3+} tightly ($K = 10^{20}$) (*15*), and it was reported that Cr^{3+}, Mn^{3+}, Al^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+} also can bind to lactoferrin but with much lower binding affinity than that of iron because of conformational changes in the binding site (*19*).

However, most studies have focused on the binding of calcium or metal ions produced from denture corrosion (*11*, *12*, *21*−*23*), not on protein-copper binding with regards to sensory perception. In addition, binding studies were performed with a single compound and a purified protein ligand under ideal conditions (*16*−*19*, *24*, *25*) and, therefore, do not reflect the complexity of the interactions in the oral environment.

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This research was performed to identify salivary proteins that interact with copper and that are responsible for the generation of astringency. The objectives were to (1) determine quantitative and qualitative changes in salivary proteins with HPLC; and (2) characterize salivary proteins changes by copper using gel electrophoresis.

MATERIALS AND METHODS

Sample Preparation. *Human Saliva Collection*. Unstimulated whole saliva was collected from five healthy human subjects (two males and three females) between the ages of 30 and 45 as described by Hong et al. (*5*). All subjects demonstrated an ability to detect copper in water in the previous sensory test (*26*). All subjects were reported to be nonsmoking and to have no physical conditions that may influence the results. Saliva collection was performed for 10 min each day between 10 to 11 a.m. or 2 to 4 p.m. for five consecutive days. Subjects were asked not to consume any food, beverage, and oral care products for 2 h before saliva collection. Human subjects rinsed their mouth with ultrapure water (Barnstead nanopure purification system, Barnstead International, Dubuque, IA) three times and then spit three times to prevent any dilution that might be caused by rinsing. Subjects collected saliva behind closed lips and then expectorated once per 1 or 2 min. Collected saliva from each subject was divided by 2.5 mL aliquots in 5 mL of presterilized polypropylene cryogenic vials (Corning Inc., Acton, MA) and stored at −70 °C until analyzed within two months. The saliva collection protocol was approved by the Institutional Review Board at Virginia Tech (IRB #05-368), and informed consent forms were obtained from all subjects. All procedures were performed on ice to prevent further degradation by generic hydrolytic enzymes in saliva.

Copper Treatment. Saliva aliquots from the five subjects were defrosted at room temperature (21 ± 2 °C) for 20 min. Saliva vials were mixed with a vortexer (Vortex-2 Genie Vortexer G-560, Scientific Industries, Bohemia, NY) for 30 s to resolubilize and resuspend any precipitate resulting from freezing−thawing (*28*). One milliliter of saliva collected from each subject was immediately transferred to a microcentrifuge tube, and copper sulfate pentahydrate (Fisher Scientific, Pittsburgh, PA) stock solution (2%, w/v) was added to saliva at the level of 0, 2.5, 5, 10, 20, and 40 mg/L as Cu. Samples were mixed using a vortexer for 5 s and then centrifuged at 13,200 rpm (16,100*g*) for 5 min (IEC Centra-M Centrifuge, Thermo Electron Co., Whaltham, MA) at room temperature to remove any insoluble material.

Determination of Total Protein Content in Saliva. Total protein content of saliva was determined using the Bradford assay (*27*). Bovine serum albumin (Sigma, St. Louis, MO) solutions at the concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 1.4 mg/mL in 0.15 M NaCl were prepared for a standard curve. Sodium chloride solution of 0.15 M was used as a blank solution. Saliva collected from each subject ($n = 5$) was analyzed in triplicate ($N = 15$). Sample means and standard errors were computed, and the effect of copper on total protein content was examined with one-way analysis of variance (ANOVA). All statistical analyses were performed using JMP IN statistical software (version 4.0, SAS, Cary, NC).

Investigation of the Effect of Copper on Salivary Proteins with High Performance Liquid Chromatography (HPLC). *Sample Preparation*. Saliva from each subject as well as pooled saliva was analyzed. For pooled saliva sample (1 mL), portions (0.2 mL) of saliva from each individual were commingled and mixed for 10 s using a vortexer and then treated with copper as described above. Supernatants obtained from centrifugation were filtered with 0.45 μm syringe filters (Fisher Scientific, Pittsburgh, PA) and then placed into HPLC autosampler vials that contained 250 μL polypropylene inserts (National Scientific, Rockwood, TN).

HPLC. Changes in protein patterns caused by copper were analyzed using an Alliance HPLC system consisting of a Waters 2695 separation module and a Waters 2487 dual wavelength absorbance detector (Waters, Milford, MA) following the method suggested by Kallithraka et al. (*29*). Analysis was performed on a C-18 reversed-phase column (Luna C18(2), 150 × 4.6 mm, particle size 5 μm, pore size 100 Å, Phenomenex, Torrance, CA) with a guard column (SecurityGuard C18, 4.0 × 3.0 mm, Phenomenex, Torrance, CA) at room temperature. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid (TFA; Pierce, Rockford, IL) in water (solvent A) and 0.1% (v/v) TFA in 65% (v/v) acetonitrile (HPLC grade, Fisher Scientific, Pittsburgh, PA) aqueous solution (solvent B). Separation was performed at a 1.0 mL/min mobile phase flow rate using the gradient conditions summarized in **Table 1**. Injection volume was 25 μL.

Table 1. Mobile Phase Gradient Conditions for HPLC Analysis of Salivary Proteins

	solvent	
retention time (min)	A[a] (%)	B[b] (%)
0	100	0
30	0	100
32	0	100
33	100	0
40	100	0

[a] 0.1% (v/v) trifluoroacetic acid (TFA) in water. [b] 0.1% (v/v) TFA in 65% (v/v) acetonitrile aqueous solution.

Signals were detected at wavelengths of 230 and 280 nm. Peaks in chromatograms were integrated using Waters Millennium chromatography manager software (version 3.20, Waters, Milford, MA). Proteins are usually detected at 210−220 nm, which is specific for peptide bonds, or at 280 nm, where aromatic amino acid residues have high absorbance. PRPs in saliva are not detected at 280 nm due to a lack of aromatic amino acid residues (*30*). If a protein peak had high absorbance at 230 nm but was not detected at 280 nm, this protein could be considered to have a high proportion of nonaromatic amino acid residues (*29*, *31*). Because of the hydrophilic nature of proline residues, PRPs are expected to be eluted early in the given mobile phase gradient condition. Therefore, if a protein peak was eluted at early RT and had high absorbance at 230 nm but no absorbance at 280 nm, the peak is assumed to be a PRP (*29*).

Statistical Analysis. The reproducibility of HPLC analysis was expressed as a mean standard deviation (SD) of peak retention times (*32*). A mean SD was calculated from 540 peaks (5 major peaks/chromatogram × 3 chromatograms/subject × 6 subjects (5 subjects and one pooled sample)/treatment × 6 treatments) for 230 and 280 nm each. All samples, including samples from individuals and pooled samples, were analyzed in triplicate. The area of each peak was subjected to ANOVA ($\alpha = 0.05$) using JMP IN to identify the effect of copper on protein peak area. Because the concentration of total and individual salivary proteins varies depending on population and collecting time within a day as well as over a long-term or a short-term period (*15*), there are significant differences in peak area among human subjects and replications. Therefore, the final statistical model included main effects of copper treatment, subject, and replication to separate variation explained by the subject and replication from the whole model. Cross-effects of these factors were not included in the final model because they were not significant. Mean values of different treatments were compared using Tukey's HSD test. Data obtained as peak area were transformed to the ratio of the mean peak area at a certain treatment to that of control for data presentation since quantification of peaks was limited by a lack of adequate reference standard materials.

Characterization of Salivary Proteins Influenced by Copper with Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). *Unfractionated Saliva Sample Preparation*. Saliva from individual subjects was pooled and treated with copper as described above. Three parts of saliva supernatant were mixed with one part of 4-fold concentrated Laemmli sample buffer (*33*). The mixture was boiled at 100 °C for 5 min and centrifuged. The upper 15 μL of supernatant was loaded on a SDS−PAGE gel.

Fractionated Saliva Sample Preparation. Control and saliva sample containing 40 mg/L copper, pooled from five subjects, were injected into the HPLC system, and peaks that showed changes greater than ± 50% were collected (fractions of RT 2−4 min and RT 6−7 min). Collected peak fractions were stored at −20 °C and analyzed within one week. Fractions were defrosted in the same manner as unfractionated saliva samples. Two hundred microliter aliquots of HPLC fractions were concentrated to dryness using a Speedvac concentrator (SVC-100H, Savant, Waltham, MA). Dried samples were reconstituted by adding 12 μL of ultrapure water and 4 μL of 4-fold concentrated Laemmli buffer

to the fraction of RT 2−4 min, and 18 μL of ultrapure water and 6 μL of sample buffer to the fraction of RT 6−7 min. After adding sample buffer, tubes were mixed with a vortexer for 5 s and boiled at 100 °C for 5 min. All reconstituted fractions were combined in one tube and centrifuged at 13,200 rpm for 2 min to remove any insoluble precipitate.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using 4−20% gradient Tris-HCl gels (Ready-gel, Bio-Rad, Hercules, CA) with Mini-Protean III system (Bio-Rad, Hercules, CA) following the method of Laemmli (*33*). The dimension of gels was 7 × 8.6 cm, 1.0 mm thickness. Gel running buffer was 0.1% (w/v) SDS in 25 mM Tris base and 192 mM glycine. Unfractionated saliva samples (15 μL) were loaded with 5 μL of molecular weight standard (Mark 12 unstained wide range molecular weight standard, molecular weight range 2.5−200 kDa, Invitrogen, Carlsbad, CA) in all gels. Proteins in collected peak fractions were detected with silver staining; therefore, the molecular weight standard was diluted 20-fold, and samples were diluted so that all fractions could express similar intensity when stained with silver nitrate. Gels were run at 15 mA constant current per gel for about 2 h.

Coomassie Blue Staining. Nonfractionated saliva samples were stained with 0.1% (w/v) Coomassie brilliant blue R-350 reagent (PhastGel Blue R, Amersham Bioscience, GE Healthcare, Uppsala, Sweden) in 30% (v/v) methanol and 10% (v/v) acetic acid. Destaining was performed by the method suggested by Beeley et al. (*34*) using 10% acetic acid for 2−3 days until bands of proline-rich proteins (PRPs) developed a violet-pink color to verify those proteins.

Silver Staining. Proteins from collected peak fractions separated by HPLC were stained poorly with Coomassie brilliant blue (CBB) in a preliminary gel electrophoresis because of their low concentrations under the detection limit (0.2−0.5 μg) (*35*). Silver staining is known to be 10−100 times more sensitive than CBB (*35*). Therefore, proteins from collected peak fractions were stained with a PlusOne silver staining kit (Amersham Bioscience, GE Healthcare, Uppsala, Sweden) by the method of Heukeshoven and Dernik (*36*).

Image Processing and Data Analysis. Images of protein bands were preserved by taking a photograph using a digital camera (DSC-W5, Sony, San Diego, CA) and then drying gels at 80 °C for 30−40 min with a gel dryer (Model 583, Bio-Rad, Hercules, CA). Apparent molecular weights (M_r) of proteins were determined by comparing the migration distance of bands to that of molecular weight standards. Protein bands were further identified on the basis of molecular weight and the band patterns reported previously (*28*, *34*, *37*, *39*−*41*).

RESULTS AND DISCUSSION

Total Protein Content in Saliva. There were no significant differences in protein concentrations among different copper levels, but a slight decrease in protein content was observed in the sample treated with Cu 40 mg/L (0.63 mM) (**Table 2**). We expected to see copper treatment decrease protein content more significantly, as it was reported that copper treatment of 1.0 and 3.0 mM formed a haze in saliva, which is presumed to be insoluble copper−protein complexes (*10*). It is assumed that a decrease in protein content may not be evident because the level of copper concentration used in our study (0.04 to 0.63 mM) may be too low to induce noticeable changes. Also, a large variability in the protein content by the collection timing and population (*15*) may be another reason for the insignificant decrease in protein content.

Investigation of the Effect of Copper on Salivary Proteins with High Performance Liquid Chromatography (HPLC). General features of our chromatograms were similar to those reported by Kallithraka et al. (*29*), which could be divided into three parts according to peak eluting patterns. In our chromatograms, the first part from a retention time (RT) 0 to 9.5 min had three major peaks (peak no. 1, 2, and 4) observed from all subjects, the second part included minor peaks at RT 10−18 min that varied in their pattern and size among subjects, and the third part spanned from RT 19 to 25 min and contained three or four major peaks (peak no. 11−14) (**Figures 1** and **2**). In the report by Kallithraka et al. (*29*), two peaks at RT 2−2.5 min were the largest among all peaks, but areas of these peaks were smaller than another major peak detected at RT 6.5 min (peak no. 5) in our study. This peak with RT 6.5 had the largest peak area among all peaks detected. It was assumed that the peak of RT 6.5 min was α-amylase based on the largest peak area and its absorbance at 230 and 280 nm, because α-amylase usually shows the thickest blue band in SDS−PAGE of saliva (*37*, *39*−*41*), suggesting high concentration in whole saliva. The common peaks and their characteristics are listed in **Table 3**.

Table 2. Means and Standard Errors (SE) of Protein Concentrations of Unstimulated Whole Saliva Treated with Cu 0, 2.5, 5, 10, 20, and 40 mg/L (N = 15)

	protein concentration (mg/mL)	
copper treatment (mg/L)	mean	SE
0	1.27	0.19
2.5	1.34	0.17
5	1.30	0.18
10	1.24	0.19
20	1.23	0.19
40	1.18	0.17

Statistical analysis of peak areas showed that the actual and relative area of peaks significantly varied between individuals and replications. These differences can be explained by the large variations in protein concentrations among individuals and collection time (*15*). These variations explained only a small portion of total variation, which can be mostly explained by copper treatments. Also, an examination of a chromatogram of each individual's saliva revealed that peaks were found at distinct and repeatable retention times. A mean SD was 0.16 for the wavelength of 230 nm and 0.14 for the wavelength of 280 nm. Therefore, we concluded that HPLC results were reproducible and that protein eluting at the same retention times can be recognized as identical among subjects.

Peak nos. 2, 3, 5, 6, 7, and 9 (**Table 3**) showed significant differences between control and copper treatments. They were eluted before 15 min. Because the mobile phase was mainly composed of water (**Table 1**) during this elution time, partitioning of hydrophilic compounds was facilitated by the reverse-phased column. This indicates that salivary proteins that interact with copper were hydrophilic.

When 10−40 mg/L of copper was added to saliva, areas of peaks that showed significant differences were decreased by 40−60% compared to those of the control. Low levels of copper concentration, 2.5 mg/L and 5 mg/L, did not cause significant changes in peak areas. Decreases in peak areas corresponded to a slight decrease in total protein content in the sample treated with Cu 40 mg/L (**Table 2**), although the change in total protein content was not statistically significant.

Figure 3 showed the pattern of decrease in area of the peaks at different copper concentrations. Different patterns of change in peak area suggested that each peak may have different mechanisms of interaction with copper. A new peak (peak 3 in **Table 3**), which appeared at RT 3.0 min, increased linearly (R^2 = 0.75) (**Figure 4**). This peak can be identified as a PRP on the basis of the rationale of Kaillithraka et al. (*29*) since it was detected only at 230 nm. Increase in the area of peak 3 was accompanied with decrease in the adjacent peak, peak 2 (RT 2.9 min). This implies that peak 3 may be a product of the interaction between copper and peak 2. The other possible explanation is that peak 2 may be an interaction product between copper and one of the proteins

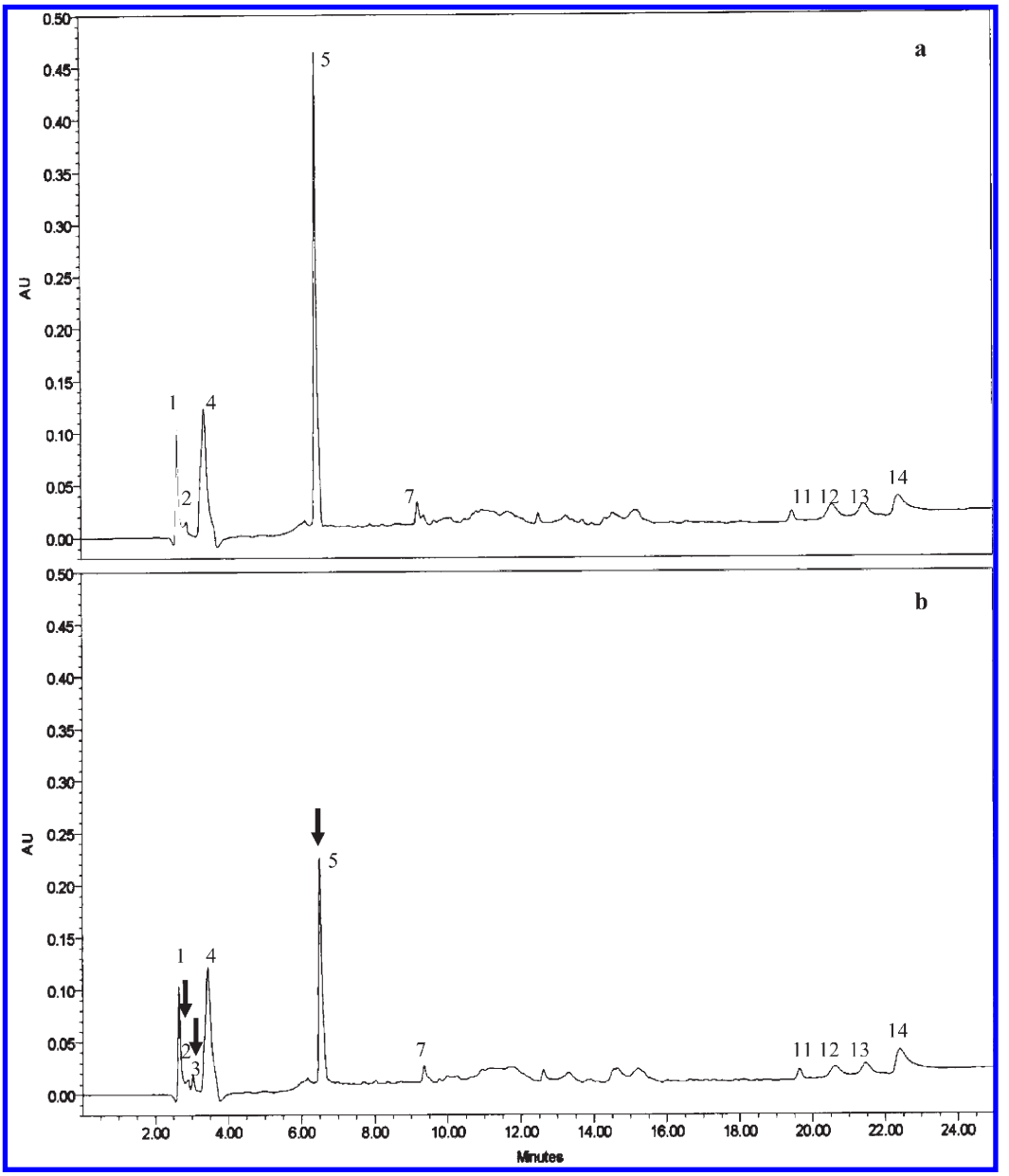

Figure 1. Representative HPLC chromatograms of pooled human saliva detected at the wavelength of 230 nm. (**a**) Combined human saliva containing no copper; (**b**) combined human saliva containing 40 mg/L of copper. An arrow mark indicates significant change in peak area caused by the addition of copper. Peak numbers 6 and 8−10 are shown in **Figure 2**. Peaks are identified by number in **Table 3**. The results obtained from pooled saliva were used to demonstrate the representative pattern of chromatograms as areas of protein peaks in the chromatograms of pooled saliva were not significantly different from mean peak areas obtained from individual saliva samples.

and that peak 3 reflects proteins dissociated from peak 2. We hypothesized that this could occur because a protein−copper complex is dissociated due to the decrease in copper solubility as acetonitrile in the mobile phase increased. Further investigation is needed to examine the interaction between copper and salivary proteins from the HPLC results. Peaks showing large changes, peaks at RT 2−4 min (peak nos. 1, 2, 3, and 4) and RT 6.5 min (peak no. 5), were collected and analyzed by gel electrophoresis for further identification.

Characterization of Salivary Proteins Influenced by Copper Using Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). *SDS−PAGE of Unfractionated Saliva.* SDS−PAGE was performed to examine the effect of copper on salivary proteins. A typical electrophoretic pattern of unfractionated saliva on the 4−20% gradient gel is shown in **Figure 5**. Protein bands were characterized by comparing the estimated apparent molecular weight (M_r) of each protein band and gel eluting pattern with those reported in the literature. PRPs can be identified easily because PRPs are stained into violet-pinkish color with CBB when destained with 10% acetic acid due to the phenomenon called metachromasia, a shift of the spectrum resulting from dye interaction with proline residues (*37*). Reported molecular weight values for any salivary protein vary in the literature when gel electrophoresis was performed on different duct saliva under different conditions (*34*, *37*−*42*, *45*). Electrophoretic patterns of salivary proteins, especially PRPs, are different among individuals and exhibit a complex nature such as double-bonded patterns and variations in molecular size due to genetic polymorphisms (*15*, *43*). Thus, band characterization based on literature review is tentative, and verification would require additional identification methods such as immunoblotting.

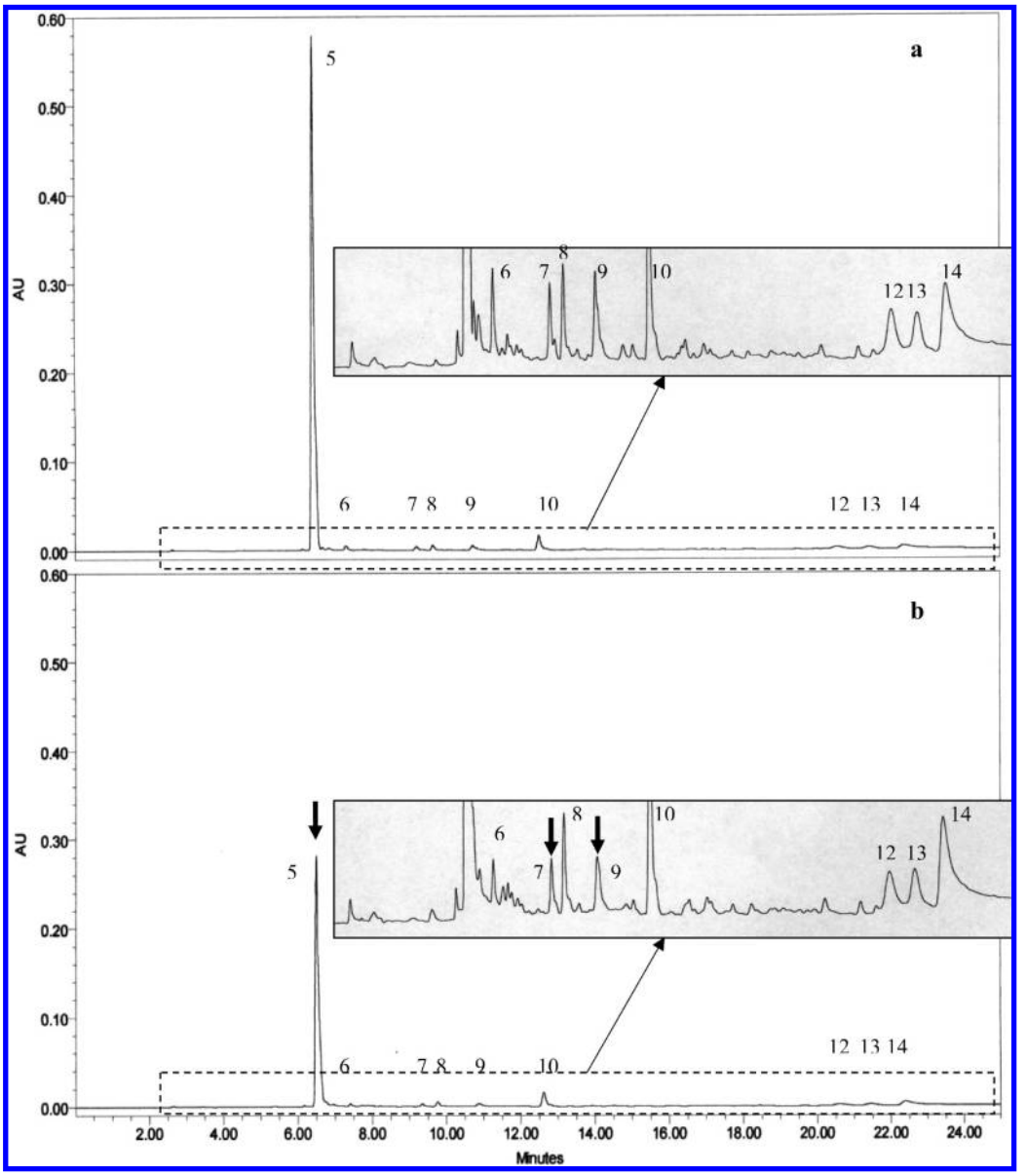

Figure 2. Representative HPLC chromatograms of pooled human saliva detected at the wavelength of 280 nm. (**a**) Combined human saliva containing no copper; (**b**) combined human saliva containing 40 mg/L of copper. An arrow mark indicates significant change in peak area caused by the addition of copper. Peak numbers 1−4 are shown in **Figure 1**. Peaks are identified by number in **Table 3**. The results obtained from pooled saliva were used to demonstrate the representative pattern of chromatograms as areas of protein peaks in the chromatograms of pooled saliva were not significantly different from mean peak areas obtained from individual saliva samples.

Identifying the dark blue band with 60 kDa with α-amylase is valid since the eluting pattern and molecular weight of α-amylase were consistent with literature values (*6*−*8*, *15*, *28*, *34*, *37*−*40*, *45*). A protein band of M_r = 90 kDa was tentatively identified as lactoferrin, on the basis of the result of Beeley et al. (*37*). A blue band of 14.3 kDa was determined as lysozyme because the apparent molecular weight of this band was identical to the value reported in the literature (*28*, *37*, *39*, *40*). Several violet bands of M_r = 9−25 kDa were characterized as PRPs, which have a wide molecular weight range (*37*, *39*, *42*). The violet band set of M_r = 9−14.3 kDa was characterized as basic PRPs according to reported apparent molecular weight. Two minor blue bands are shown with PRPs of 9−14.3 kDa, suggesting the existence of non-PRPs. Protein bands between α-amylase and PRPs were identified as isoforms of basic PRPs (37−43 kDa) or glycosylated PRPs (35 kDa) in other studies (*37*, *39*, *42*), but protein bands in our study did not develop the characteristic violet-pink color. They may be non-PRP proteins of 40−50 kDa, such as gustin. Gustin is related with taste acuity by controlling homeostasis of salivary zinc (*43*, *44*). Gustin has a strong zinc-binding ability ($K_d = 4.5 \times 10^{-11}$ M at pH 7.2). One mole of zinc is bound to 1 mol of the protein, but a second zinc can loosely bind to gustin in the presence of excess zinc. The second Zn binding site was reported to coordinate loosely with Cu, Ni, Fe, or Mn, but it was unknown whether copper on the second binding site had physiological functions (*18*).

A faint blue unresolved region observed alongside the 2.5−3.5 kDa molecular weight standard was assumed to be histatins on the basis of a comparison to the results of Beeley et al. (*37*), who observed the binding capacity of this band with ^{65}Zn. It is not surprising that histatins were not detected well because (1) the gel pore size is not small enough to resolve this peptide; (2) the level of acidic PRPs and histatins is considerably reduced in whole saliva compared to that in parotid saliva (*34*). It was initially assumed that histatins would show interaction with copper as they are reported to have high binding affinity to copper at pH 7.4

Table 3. Identification of Protein Peaks on the HPLC Chromatogram of Pooled Saliva Samples Containing 0, 2.5, 5, 10, 20, and 40 mg/L Copper

peak no.	typical retention time (min)	A_{230}[a]	A_{280}[b]	tentative identification[c]	significant changes[d]
1	2.6	Y[e]	N[f]	proline-rich proteins (PRPs)	N
2	2.9	Y	N	PRPs	decrease[g]
3	3.0	Y	N	new peak for saliva + Cu	increase[h]
4	3.5	Y	N	PRP	N
5	6.5	Y	Y	α-amylase	decrease
6	7.4	N	Y	non-PRP	decrease
7	9.5	Y	Y	non-PRPs	decrease
8	9.7	N	Y	non-PRPs	N
9	10.9	N	Y	non-PRPs	decrease
10	12.5	N	Y	non-PRPs	N
11	19.5	Y	N	PRPs with hydrophobic regions	N
12	20.5	Y	Y	Non-PRPs	N
13	21.5	Y	Y	Non-PRPs	N
14	22.5	Y	Y	Non-PRPs	N

[a] Absorbance at 230 nm. [b] Absorbance at 280 nm. [c] Peaks were identified based on retention time and absorbance at 230 and 280 nm. If a peak is detected at 230 nm but not at 280 nm, this peak is assumed to be a proline-rich protein. [d] Significant decrease or increase in protein peaks by the addition of different amount of copper in pooled human saliva ($\alpha = 0.05$). [e] Observed. [f] Not observed. [g] The peak was significantly decreased by copper treatment. [h] The peak was significantly increased by copper treatment.

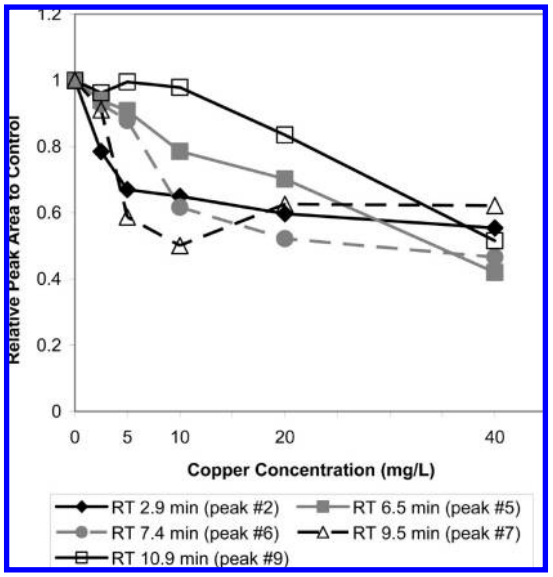

Figure 3. Pattern of decrease in salivary protein peaks from pooled human saliva treated with 0, 2.5, 5, 10, 20, and 40 mg/L separated by reversed-phase HPLC.

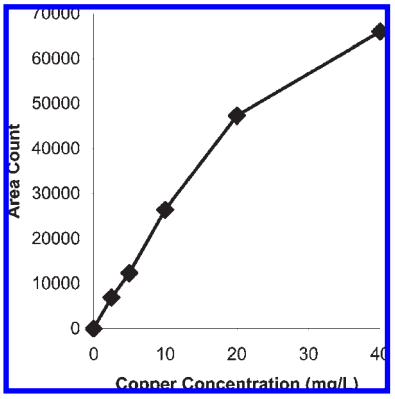

Figure 4. Pattern of increase in the salivary protein peak at RT 3.0 (peak 3 in **Table 3**) from pooled human saliva treated with 0, 2.5, 5, 10, 20, and 40 mg/L separated by reversed-phase HPLC.

(a binding constant $2.6 \times 10^7\ M^{-1}$). However, changes in histatins were not clearly identified. It is possible that histatins would not contribute to the perception of astringency in the mouth because the level of histatins in whole saliva is very low (*34*).

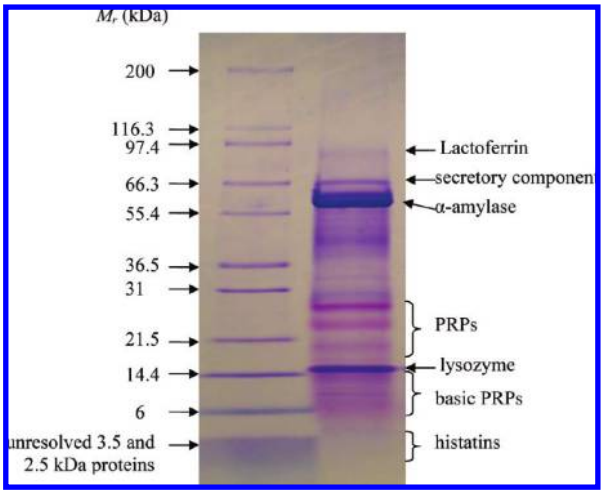

Figure 5. SDS−PAGE of human whole saliva pooled from five subjects containing no copper (protein load = 15 μg per a lane). Proteins were detected using Coomassie blue staining. The molecular weight standard is on the left side of the gel. Protein bands were characterized on the basis of molecular weight and previous SDS−PAGE studies for tentative identification (*6*−*8*, *15*, *28*, *34*, *37*−*41*, *42*, *45*).

Electrophoretic patterns of salivary proteins changed as copper concentration increased (**Figure 6**). Two protein bands with M_r = 33 kDa and 29 kDa showed changes at higher copper concentrations (10−40 mg/L). These protein bands became blurred upon the addition of 10 mg/L copper. The protein band of M_r = 33 kDa totally disappeared in 40 mg/L copper. Saliva containing 2.5 mg/L copper and 5 mg/L copper did not exhibit differences in electrophoretic patterns from that of control (data not shown). Molecular weights of these proteins indicate that they are likely to be PRPs, but the CBB staining result did not confirm this because these two bands were not stained purple. It is also possible that these protein bands are composed of several proteins of the same molecular weight due to separation mechanism of SDS−PAGE. More advanced techniques such as LC-MS are expected to give more accurate characterization of these bands.

One of the hypotheses regarding the mechanism of astringency caused by copper would be the same as that of polyphenolic compounds, which is delubrication resulting from the precipitation of PRPs. PRPs are proline-rich proteins and are classified into acidic (pI 3.5−4.5), basic (pI > 8.0), and glycosylated (pI > 8.0) proteins (*34*). Polyphenolic compounds are known to bind to

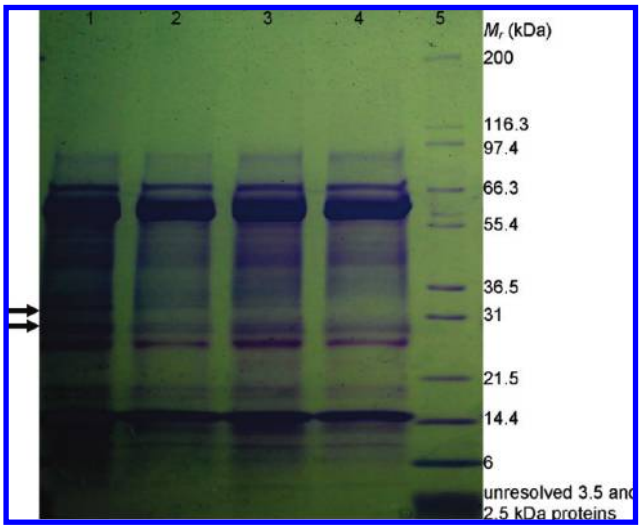

Figure 6. SDS−PAGE of combined human whole saliva treated with 0, 10, 20, and 40 mg/L copper (protein load = 15 μg per a lane). Proteins were detected using Coomassie blue staining. The bold arrow on the left designates the bands changed by the addition of copper (lane 1, control; lane 2, 10 mg/L Cu; lane 3, 20 mg/L Cu; lane 4, 40 mg/L Cu; lane 5, molecular weight standard).

basic PRPs by hydrophobic interaction or hydrogen bonding. More specifically, the hydrophobic faces of the aromatic rings of polyphenols stack onto the pyrrol ring of prolines in basic PRPs and result in the soluble protein−tannin complex. As more phenolics are bound to proteins, phenolics on the surface of proteins provide intermolecular bridges between proteins, and then proteins become polymerized and precipitate (*6*−*9*). A recent study reported that glycosylated PRPs also formed soluble complexes with tannin at low tannin concentrations (*46*). For metallic compounds, acidic PRPs may be a potential candidate to participate in interaction with metals. Acidic PRPs are known to bind Ca^{2+} due to negatively charged amino residues such as aspartate and glutamate. If the disappearing bands are acidic PRPs, electrostatic interactions between Cu^{2+} and negatively charged amino acid residues of acidic PRPs would likely be a major binding mechanism. The same mechanism was observed in the binding of Ca^{2+} and acidic PRPs in dental pellicle formation (*15*). Precipitation may occur by neutralization of protein charge via binding with Cu^{2+} or polymerization of PRPs by forming Cu^{2+} bridges between PRP molecules as seen in Ca^{2+} cross-linking between mucin molecules (*12*, *21*).

SDS−*PAGE of Fractionated Saliva.* The peaks influenced by copper treatment in HPLC analysis were characterized with SDS−PAGE for further identification (**Figure 7**). Two fractions, the early eluting peaks collected at RT 2-4 min (peak nos. 1, 2, 3, and 4) and the largest peak at RT 6.5 min (peak no. 5), were loaded. Because the volume of reconstituted samples was very limited, final protein concentrations of fractions were not determined. The loading amount of each fraction was adjusted so that all fractions could express similar intensity when stained with silver nitrate. The concentration ratio of each fraction was not the same since the amount of protein eluted and total elution time were different among fractions. Therefore, it is noteworthy that the SDS−PAGE result should not be interpreted quantitatively.

Unfractionated saliva in lane 1 showed slight changes in gel electrophoretic pattern compared to that obtained from CBB staining. PRPs that showed distinctive violet-pink color in the CBB stained gel were not detected well in silver stained gel because of their amino acid composition. Silver staining is more sensitive than CBB staining, but its reactivity varies by protein compositions.

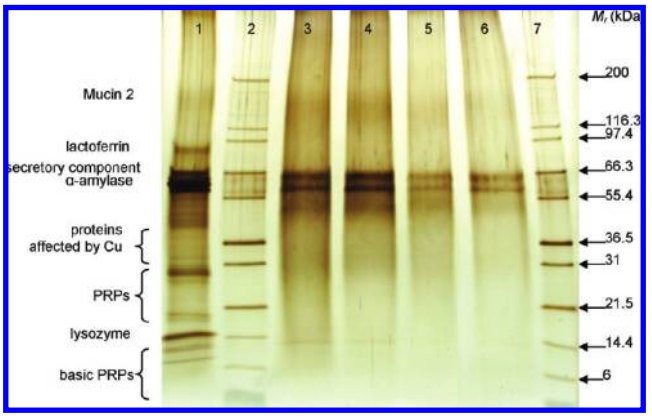

Figure 7. SDS−PAGE of peak fractions collected from HPLC. Proteins were detected using silver staining. The protein load may be different in each lane [lane 1, unfractionated saliva with no Cu, protein load 0.75 μg; lanes 2 and 7, molecular weight standard; lane 3, fraction of RT 2−4 min with no Cu (×1 dilution); lane 4, fraction of RT 6.5 min with no Cu (×5 dilution); lane 5, fraction of RT 2−4 min with 40 mg/L Cu (×1 dilution); lane 6, fraction of RT 6.5 min with 40 mg/L Cu (×15 dilution)].

Silver staining has more sensitivity to basic residues and sulfur-containing residues, such as cysteine (*35*), but less sensitivity to acidic residues. It is postulated that the silver-stained bands found in the region of PRPs are non-PRP proteins that were minor in CBB staining. Bands that were not seen in the CBB stained gel appeared in the silver-stained gel, especially one wide band of MW 168 kDa. This band is thought to be low-molecular mucin because it has the same electrophoretic pattern as the result of immunoblotting with mucin antibody reported by Carlen et al. (*41*).

Lanes 3 and 4 are the fractions with no copper from HPLC; each of the peaks eluted in RT 2−4 min, and the peak eluted in RT 6.5 min. Several protein bands were detected in the fraction of RT 6.5 min (lane 4), which had appeared as just one peak in the HPLC chromatogram (**Figure 1**). Lane 4 contained α-amylase, secretory component, proteins of 45 kDa (either basic PRP or gustin), and basic PRPs. A shadowy area was detected in the region where the intense PRPs had appeared in the CBB stained gel and basic PRPs showed up as a very faint band under lysozyme. Our initial expectation was to observe more bands in the region of PRPs, as these peaks were tentatively assumed to contain PRPs based on our HPLC result. However, the fraction of RT 2−4 min (lane 3) was not distinctively different from the fraction of RT 6.5 min.

Also, we expected to see a new band corresponding to the peak at RT 3.0 in HPLC chromatograms, which was a peak detected only in saliva samples, treated with Cu. However, the comparison between the fractions of control (lane 3 and 4) and the fraction with Cu (lane 5 and 6) did not exhibit any differences in a band pattern. This is probably due to the minute amount of proteins loaded below the detection limit of silver staining. It is also possible that copper−protein complexes are not stable at treatments for SDS−PAGE, such as mixing with SDS and heating. More specific information on a product of copper−salivary protein interaction will require a different approach.

Another interesting fact found in the SDS−PAGE of the HPLC fractions is that the proteins in the fractions did not match the protein bands that disappeared in the CBB stained gel (**Figure 6**). It was expected that the fraction of RT 6.5 would consist of proteins of 29 and 33 kDa because the peak with RT 6.5 was reduced by the largest amount in the HPLC. Instead, the major compounds of the peak at RT 6.5 were the proteins of higher molecular weight. The proteins of 29 and 33 kDa might be

precipitated when copper was added, and centrifugation had removed these precipitated proteins before injection to the HPLC. Thus, changes in peak area in the HPLC chromatograms are related to several other proteins such as α-amylase and mucin.

In summary, copper incorporated in the mouth is shown to interact with salivary proteins that have molecular weights of 29 kDa and 33 kDa. These proteins were not clearly identified but were assumed to be either acidic PRPs or other non-PRPs. HPLC and SDS−PAGE of saliva fractionated by HPLC showed that salivary proteins such as low molecular weight mucin, α-amylase, and basic PRPs also seem to interact with copper.

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