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Noncovalent Cross-Linking of Casein by Epigallocatechin Gallate Characterized by Single Molecule Force Microscopy

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Interaction of the tea polyphenol epigallocatechin gallate (EGCG) with β-casein in milk affects the taste of tea and also affects the stability of the tea and the antioxidant ability of the EGCG. In addition, interaction of polyphenols with the chemically similar salivary proline-rich proteins is largely responsible for the astringency of tea and red wine. With the use of single molecule force microscopy, we demonstrate that the interaction of EGCG with a single casein molecule is multivalent and leads to reduction in the persistence length of casein as calculated using the wormlike chain model and a reduction in its radius of gyration. The extra force required to stretch casein in the presence of EGCG is largely entropic, suggesting that multivalent hydrophobic interactions cause a compaction of the casein micelle.

KEYWORDS: Epigallocatechin gallate; polyphenol; casein; single molecule force microscopy; astringency; compaction

INTRODUCTION

Green tea contains a large amount of polyphenols, of which epigallocatechin gallate (EGCG, **1**) forms the largest component at approximately 9% w/w (*1*). EGCG has been suggested to have a wide range of largely beneficial effects on human health, including hypocholesterolemic, anticancer, anti-HIV, antiinflammatory, and cardiovascular (*1*−*3*). Black tea is produced from green tea by fermentation, which oxidizes many of the tea polyphenols into higher molecular weight species such as theaflavins and thearubigins. These compounds are responsible for the color of black tea and also contribute to the taste as well as to various other biological effects, particularly antioxidative effects (*4*).

EGCG binds to a wide variety of proteins, especially to nonglobular extended proteins, and particularly to proteins with a high content of proline (*5*). One such protein is β-casein, the second most abundant protein in milk. β-Casein is a 209-residue protein containing 35 prolines evenly distributed through the sequence and is phosphorylated in five positions close to the N-terminus, which gives it an amphiphilic character so that it forms micelles in milk (*6*). The binding of polyphenols to milk proteins has been suggested to reduce their accessibility and, thereby, to reduce their antioxidative potential (*4*, *7*, *8*). It has also been shown to increase the stability of the casein micelles to heat denaturation (*9*, *10*), oxidative degradation (*11*), and foaming (*8*, *12*). The relevance of casein binding to the in vivo activity of EGCG is not yet clear: there are conflicting reports on whether binding to milk (i.e., the addition of milk to tea) reduces the bioavailability of EGCG or not (*4*, *13*). A greater understanding of EGCG−casein binding would therefore be helpful.

Another biologically relevant class of extended proline-rich proteins is the salivary proline-rich proteins. These form approximately 70% of the protein content of saliva, and the main function of the basic salivary proline-rich proteins (which make up approximately 30% of salivary proteins) is apparently to bind to dietary polyphenols and prevent them becoming bioavailable (*14*). Polyphenols have the ability to bind to iron and reduce its availability; they also block receptors and channels and inhibit digestive enzymes. Therefore, the binding of salivary proteins to polyphenols is generally agreed to be beneficial in vivo, since it reduces the harmful effects of the polyphenols. By contrast, some polyphenols, such as EGCG, have well-documented health-promoting effects, as described above. This makes the binding of EGCG to proteins particularly interesting, since it clearly has both good and bad consequences. Another consequence of polyphenol binding to salivary proteins is the formation of insoluble aggregates on the surface of the mouth, which results in the sensation of astringency, usually described as a drying or puckering sensation, associated with consumption of polyphenol-rich foods and beverages, such as

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tea, red wine, and dark chocolate, and (in moderation) is usually a desirable quality in foods and beverages (*15*, *16*).

We have therefore studied the binding of EGCG to β-casein. We have previously used a range of biophysical techniques to probe this interaction and have shown that it causes a reduction in protein dimensions prior to aggregation and precipitation (*15*). However, it was not clear from this work whether the reduction in protein dimensions was a result of intramolecular or intermolecular cross-linking. We therefore undertook a study using single molecule force microscopy, with the aim of studying the interaction between EGCG and single molecules of β-casein attached to a surface, to characterize the interaction at a molecular level and in particular to determine whether several protein molecules interact cooperatively with one polyphenol molecule or whether a polyphenol interacts cooperatively at several sites within the same protein. We demonstrate that single protein molecules decrease in size, and become harder to extend, on addition of EGCG, therefore demonstrating the importance of multivalent cross-linking. The results demonstrate that individual protein molecules "wrap around" EGCG and have important consequences both for EGCG bioavailability in tea with milk, and for astringency.

MATERIALS AND METHODS

Bovine β-casein was donated by Unilever Research, Colworth, U.K. The five phosphoserine groups were removed by enzymatic dephosphorylation. Casein (800 mg) was treated with 24 units of acidic potato phosphatase (Sigma) while dialysing against H_2O using dialysis tubing with a molecular cutoff of 10 kDa for at least 48 h at a temperature of 4 °C, pH 7.0. The dephosphorylated protein was pure by capillary electrophoresis, with <1% mol of phosphate per mol of protein by ^{31}P NMR. EGCG was a donation from Unilever Research, Colworth and was >98% pure by NMR.

β-Casein was dissolved in water/dimethyl sulfoxide (95:5 v/v, pH 7.0 $\pm$ 0.5) at a concentration of 4.1 mg/mL or 174 μM (determined spectrophotometrically using $\epsilon = 10\,810$ mol^{-1} L cm^{-1} at 280 nm). EGCG was prepared as a 10 mM stock solution in the same solvent (for reasons of solubility and consistency with our previous studies) and added to β-casein in molar ratios of between 0.25:1 and 5:1.

A molecular force probe-stand alone (MFP-SA) (Asylum Research, Inc., Santa Barbara, CA) controlled by Igor Pro software (Wavemetrics) was used for data collection. The MFP-SA includes a two-dimensional submicrometer mechanical stage for positioning the sample as well as an inverted video microscope for viewing the sample and tip area. A V-shaped AFM tip made of Si_3N_4 (Digital Instruments) with a typical inverse optical lever sensitivity of 73.41 nm V^{-1} and a spring constant of 24.6 pN nm^{-1} was used to measure the mechanical properties of an individual protein. Each pull was of 200 nm, at a rate of 400 nm/s. The instrumental setup and method for calibration are described in (*17*). The position of the cantilever arm was determined using a laser and position-sensitive photodiode (*18*). The β-casein solution was placed on a glass microscope slide, and the cantilever was wetted prior to the measurements. All measurements were made in solution. Initial data analysis was performed using Igor Pro software, and fitting of the data to the wormlike chain model was performed using KaleidaGraph 3.09.

RESULTS AND DISCUSSION

The milk protein β-casein was dephosphorylated before use, to minimize its tendency to form micelles. The dephosphorylated β-casein in complex with different concentrations of EGCG was subjected to single molecule force microscopy measurements, in which a probe tip was lowered onto the protein and pulled off the surface. The tip adheres to the protein by nonspecific forces, and the force required to pull away from the surface was measured. **Figure 1** shows a typical force versus distance curve. Six different stages of the force curve can be distinguished

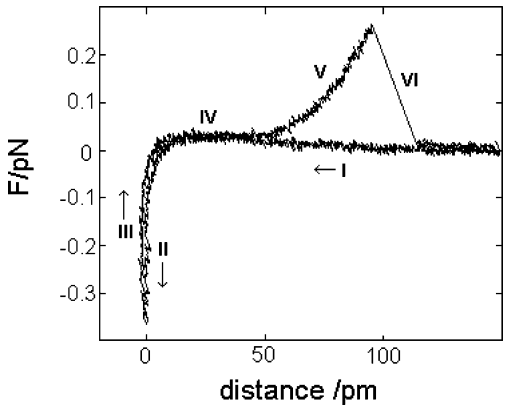

Figure 1. Typical force vs distance curve. Stages I and II represent the approaching and stages III and VI the retreating tip. The different stages are the following: I, no interaction between the tip and the glass surface; II/III, repulsive force between the tip and the glass surface; IV, attractive peak due to surface adhesion; V, entropic force due to stretching and uncoiling a single protein chain; VI, detachment of the protein.

(**Figure 1**), of which the most important here is stage V, in which the protein is being pulled away from the surface, while being attached both to the glass surface and to the tip. As the tip is retracted, it encounters a force due to uncoiling of the protein molecule. In general the protein chain is coiled to maximize its conformational freedom (entropy), and upon extending the relaxed protein, the chain generates an opposing force that arises from the reduction of the entropy of the chain. When the force to stretch the protein is equal to the sum of all adsorption forces tethering the protein chain to the tip (or the surface), the protein chain suddenly desorbs from the tip and the force returns to zero (**Figure 1** stage VI). The interaction between the tip and the protein is nonspecific, and consequently, many pulling trials are necessary to obtain a representative number of single pulling events. The majority of attempts result either in the tip not picking up any molecule, when the force trace remains at zero, or picking up more than one molecule or aggregates, when an undefined force trace or multiple break-off events are recorded. Either way, those force traces have to be rejected and only force curves that represent the stretching of an individual protein chain were accepted and subjected to further data analysis.

The selected force curves describing the force caused by the entropic elasticity of the chain were analyzed using the wormlike chain model (WLC) (*19*), which is widely used for polymers and predicts the entropy-restoring elastic force (F_{chain}) generated upon extension of a protein in terms of its persistence length p and contour length $L_{contour}$ (*20*):

$$F_{chain} = \frac{k_B T}{p}\left(0.25\left(1 - \frac{r}{L_{contour}}\right)^{-2} - 0.25 + \frac{r}{L_{contour}}\right) \quad (1)$$

where r is the measured chain end-to-end separation distance, k_B is the Boltzmann constant, T is the absolute temperature, and p is the persistence length. The contour length $L_{contour}$ is the fully extended length of the chain between the glass and the tip and is defined by

$$L_{contour} = np \quad (2)$$

where n is the number of segments of persistence length p, this being the length of a statistically straight segment in the polymer. Values for n and p were obtained by fitting force curves.

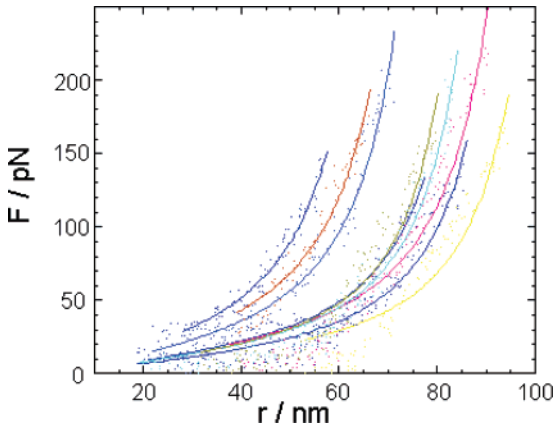

Figure 2. Family of force vs distance curves at an EGCG/β-casein ratio of 2:1. The curves were fitted to the wormlike chain model, giving the persistence length p and number of segments n. Each color represents a different pulling event.

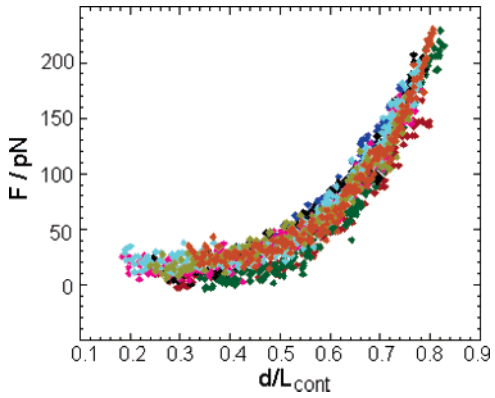

Figure 3. Master curves, for an EGCG/β-casein ratio of 2:1. Normalization of the distance by $L_{contour}$ results in an overlay of the force curves and in a final extended length of the protein of $\approx 0.85 L_{contour}$.

The model describes a linear force versus distance curve at low extensions, where the chain behaves like an entropic Hookean spring. At moderate strains, a nonlinear and non-Gaussian force curve can be observed. At high stretches the chain has fewer and fewer configurational options and hence less entropy, and so the force rapidly increases as the contour length is approached (*21*).

Figure 2 shows a family of force versus distance curves with the corresponding fits of n and p to the WLC model for an EGCG/β-casein ratio of 2:1. The curves fit well to the model, implying the lack of any significant enthalpic transitions, which would produce a shoulder in the curve (*22*). No additional extensibility of the segments was measured, and therefore, the deformation can be assumed to be purely entropic. Each curve represents one pulling event. For each experiment the length of the bridging chain segment is variable and unknown since the interactions between the tip and the protein are unspecific, and the protein is picked up by the tip, and is attached to the glass, at random positions along the chain. Thus $L_{contour}$ varies for each measurement and hence each curve. Normalization of the distance values (x-axis of **Figure 2**) by the individual contour lengths gives the "master curve" which superimposes all of the data onto a single plot (**Figure 3**) (*19*). The data superimpose well, consistent with the recordings originating from single homogeneous polymers. **Figure 4** shows the change of the fitted value of the persistence length p upon increasing the concentration of EGCG, which is constant for low ratios of EGCG/casein and decreases after a ratio EGCG/β-casein of 0.5:1. The

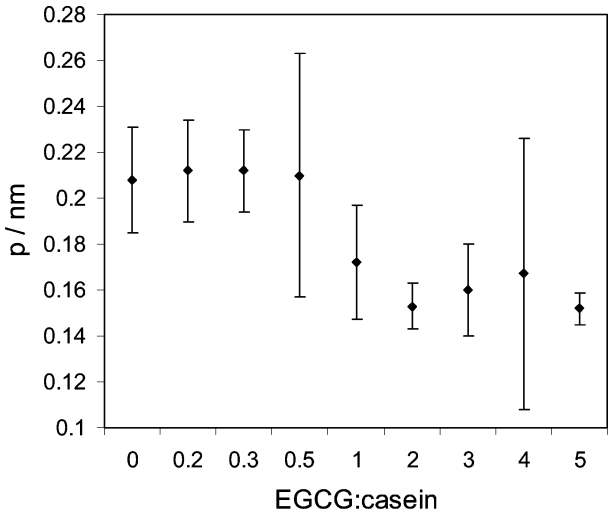

Figure 4. Persistence length p as a function of the EGCG/β-casein ratio. As EGCG is added p decreases.

persistence length is 0.15−0.2 nm, and the decrease in persistence length on addition of EGCG implies a coiling of the peptide chain. We note that this persistence length is rather shorter than the values typically found by other workers for other proteins. Persistence length has been shown to be dependent on context and solution conditions: a length of 0.3 nm seems to be typical for interdomain linkers and loops (*23*, *24*), with 0.4 nm being more typical of unfolded chains obtained by the unraveling of normally folded domains (*25*). Casein is functionally very different from most proteins investigated to date, being physiologically unstructured in micelles, which may explain its shorter persistence length. In addition, the solvent used here is pure water with the addition of 5% dimethyl sulfoxide, which is likely to have some effect on the persistence length.

Knowing p and n, the radius of gyration R_g of the protein molecules, free in dilute solution of a good solvent, can be approximated using eq 3.

$$R_g = \frac{p n^{3/5}}{\sqrt{6}} \tag{3}$$

Figure 5 illustrates that R_g of the β-casein molecule decreases upon complexation with EGCG, implying a compaction of the protein structure. This is consistent with our earlier results on the same system in bulk solution using analytical ultracentrifugation, small-angle X-ray scattering, dynamic laser light scattering, viscometry, and measurements of the self-diffusion constant by NMR, some of which are also indicated on **Figure 5** (*15*). The significance of the result reported here is that it proves that the protein compacts not only in bulk solution but also when it is bound to a surface as a single molecule, and to a similar degree. Interestingly, the graphs of R_g (and persistence length) and of the light scattering data versus EGCG/casein ratio have the same shape: a plateau at low EGCG/casein ratio, a fairly sharp decrease with an inflection point around 1:1, and a second plateau at high EGCG/casein ratio. Presumably both curves indicate that the coiling up of the protein around EGCG occurs most strongly at an approximate 1:1 molar ratio and that the process is similar in solution and on a surface.

Figure 6 shows the various master curves for different ratios of EGCG and β-casein (0.2:1 to 5:1). From these assemblies of master curves the force encountered by the tip as it approaches the maximum chain distance before dissociation

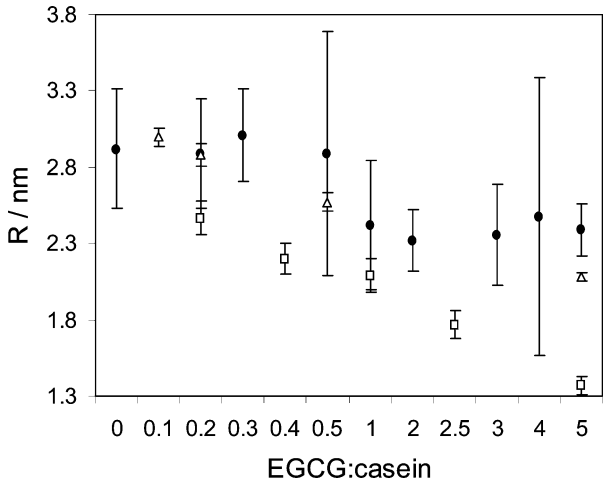

Figure 5. Radius of gyration, R_g, of β-casein on binding to EGCG, derived from fitting to eq 3 (filled circles). This is compared to values of the hydrodynamic radius, R_h, previously obtained for β-casein in solution in complex with EGCG, obtained by NMR diffusion measurements (squares) and by dynamic light scattering (triangles). The dynamic light scattering results are relative values and for comparative purposes were set to an R_g of 3.0 at 0.1 EGCG/casein.

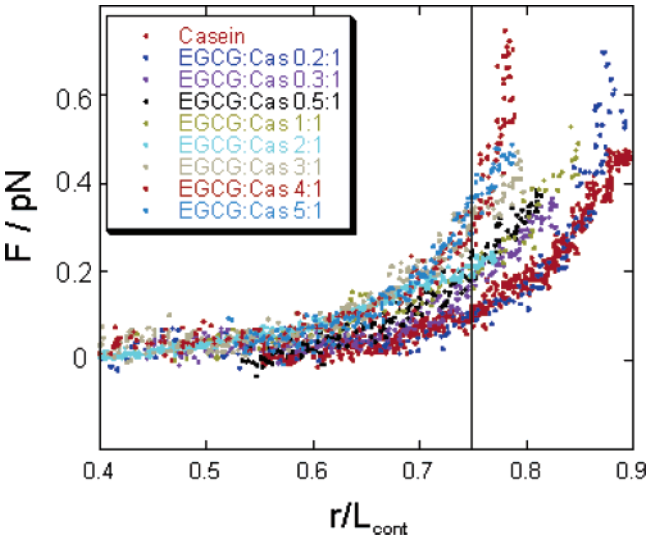

Figure 6. Master curves for the different ratios of EGCG/β-casein.

from the surface ($d/L_{contour} = 0.75$) was determined. In **Figure 7** the force is plotted against the EGCG/β-casein ratio, showing that a larger force is required to stretch the protein when it is bound to EGCG. Since these data derive from single protein molecules, they must indicate multivalent cross-linking of a protein strand by the polyphenol. This conclusion is consistent with the compaction shown in **Figure 5**, implying a wrapping of the protein around the multidentate EGCG. The force encountered can be attributed to various contributions: First, there is an entropic component caused by the stretching of the protein chain, the concomitant distortion of its configuration, and reduction in the possible number of conformations available for the segments of the chain in the stretched state. The energy of the chain rises with the square of the extension, as is expected for Hookean springs (*26*). Second, upon progressive stretching of the protein chain, the hydrophobic bonds formed between β-casein and EGCG will be gradually lost. Hydrophobic interactions constitute the transfer of a nonpolar moiety from water to a nonaqueous environment and are characterized by a positive entropy change (*27*). When this process is reversed,

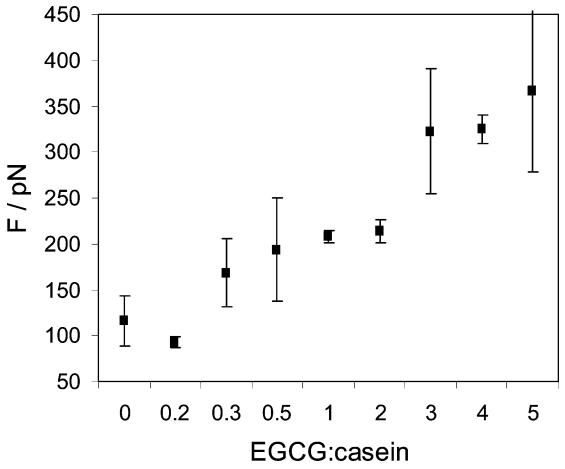

Figure 7. Force exerted by the β-casein molecule on the AFM tip at a distance of $d/L_{contour} = 0.75$. As the protein binds to EGCG a higher force is required to uncoil and stretch the chain apart.

i.e., the bonds are broken, the entropy change becomes negative and the free energy of the system increases. Third, there may be an enthalpic component, since the formation of hydrogen bonds (often postulated for protein−polyphenol interactions (*16*, *28*, *29*)) causes a negative enthalpy change (ΔH). However, no enthalpic effects were observed, as noted above. Hence, hydrogen bonding must be a relatively weak effect in comparison to the hydrophobic interactions, in agreement with our earlier conclusions (*30*, *31*), and the two entropic contributions determine the overall force required to unravel the protein.

In conclusion, our results have demonstrated that the β-casein molecule wraps itself around EGCG, becoming stiffer and more tightly packed. They therefore support the view that the binding reduces the availability of EGCG and stabilizes the casein structure (*11*). The addition of milk to tea (and coffee) is therefore likely to have a significant impact on its physiological effects. Astringency is known to be a result of the interaction between salivary proline-rich proteins and dietary polyphenols. Astringency is detected on the surface of the mouth, particularly on the hard palate, which is normally coated by a flowing mucous layer of salivary protein (*32*). Our earlier results in bulk solvent showed that the interaction resulted in compaction of the protein. Here we have demonstrated (with the chemically similar protein β-casein) that the compaction also occurs when the protein is attached to a surface and that it occurs at the level of single protein molecules, implying wrapping of a single protein around a multidentate ligand. The sensation of dryness that is perceived as astringency occurs when mucosal wetting is insufficient. We therefore suggest that a substance is astringent because it reduces the lubricating power of the saliva by precipitating salivary proteins. Because lubricating proteins achieve their function mainly by creating a hydrophilic hydrated intermolecular layer, this is equivalent to saying that an astringent substance leads to dewetting of the mucosal surface. As a consequence friction in the mouth increases. This hypothesis explains why astringency increases with repeated consumption (*33*), in contrast to the more familiar sweet, salt, bitter, and sour tastes that reduce with repeated exposure. The hypothesis also fits well with the duration of the astringent sensation, which fades typically 1 min after ingestion of the astringent. Considering that the whole saliva volume in the mouth amounts to 1−2 mL (*34*) and that the saliva flow rate after stimulation is approximately 1 mL/min (*35*), it can be concluded that the loss of the astringent sensation is due to replacement of the oral protein layer by fresh protein.

ABBREVIATIONS USED

EGCG, epigallocatechin gallate; WLC, wormlike chain.

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LITERATURE CITED

(1) Yang, T. T. C.; Koo, M. W. L. Hypocholesterolemic effects of Chinese tea. *Pharmacol. Res.* **1997**, *35*, 505−512.
(2) Chung, K. T.; Wong, T. Y.; Wei, C. I.; Huang, Y. W.; Lin, Y. Tannins and human health: A review. *Crit. Rev. Food Sci. Nutr.* **1998**, *38*, 421−464.
(3) Fujiki, H.; Suganuma, M.; Imai, K.; Nakachi, K. Green tea: Cancer preventive beverage and/or drug. *Cancer Lett.* **2002**, *188*, 9−13.
(4) Serafini, M.; Ghiselli, A.; Ferro-Luzzi, A. In vivo antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996**, *50*, 28−32.
(5) Haslam, E. *Plant Polyphenols: Vegetable Tannins Revisited*; Cambridge University Press: Cambridge, U.K., 1989; p 230.
(6) Ribadeau-Dumas, B.; Mercier, J. C.; Grosclaude, F. Amino acid composition and sequence of bovine α_{s1}- and β-caseins. *Ned. Melk Zuiveltijdschr.* **1973**, *27*, 304−12.
(7) Kilmartin, P. A.; Hsu, C. F. Characterisation of polyphenols in green, oolong, and black teas, and in coffee, using cyclic voltammetry. *Food Chem.* **2003**, *82*, 501−512.
(8) O'Connell, J. E.; Fox, P. F. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. *Int. Dairy J.* **2001**, *11*, 103−120.
(9) O'Connell, J. E.; Fox, P. D.; Tan-Kintia, R.; Fox, P. F. Effects of tea, coffee and cocoa extracts on the colloidal stability of milk and concentrated milk. *Int. Dairy J.* **1998**, *8*, 689−693.
(10) O'Connell, J. E.; Fox, P. F. Proposed mechanism for the effect of polyphenols on the heat stability of milk. *Int. Dairy J.* **1999**, *9*, 523−536.
(11) Rosenthal, I.; Bernstein, S.; Nakimbugwe, D. N. Effects of tea solids on milk. *Milchwissenschaft* **1999**, *54*, 149−152.
(12) Sausse, P.; Aguie-Beghin, V.; Douillard, R. Effects of epigallocatechin gallate on β-casein adsorption at the air/water interface. *Langmuir* **2003**, *19*, 737−743.
(13) van het Hof, K. H.; Kivits, G. A. A.; Weststrate, J. A.; Tijburg, L. B. M. Bioavailability of catechins from tea: the effect of milk. *Eur. J. Clin. Nutr.* **1998**, *52*, 356−359.
(14) Bennick, A. Interaction of plant polyphenols with salivary proteins. *Crit. Rev. Oral Biol. Med.* **2002**, *13*, 184−96.
(15) Jöbstl, E.; O'Connell, J.; Fairclough, J. P. A.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5*, 942−949.
(16) Luck, G.; Liao, H.; Murray, N. J.; Grimmer, H. R.; Warminski, E. E.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Polyphenols, astringency and proline-rich proteins. *Phytochemistry* **1994**, *37*, 357−71.
(17) Seog, J.; Dean, D.; Plaas, A. H. K.; Wong-Palms, S.; Grodzinsky, A. J.; Ortiz, C. Direct measurement of glycosaminoglycan intermolecular interactions via high-resolution force spectroscopy. *Macromolecules* **2002**, *35*, 5601−5615.
(18) Zlatanova, J.; Lindsay, S. M.; Leuba, S. H. Single molecule force spectroscopy in biology using the atomic force microscope. *Prog. Biophys. Mol. Biol.* **2000**, *74*, 37−61.
(19) Ortiz, C.; Hadziioannou, G. Entropic elasticity of single polymer chains of poly(methacrylic acid) measured by atomic force microscopy. *Macromolecules* **1999**, *32*, 780−787.
(20) Marko, J. F.; Siggia, E. D. Statistical mechanics of supercoiled DNA. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdisc. Top.* **1995**, *52*, 2912−38.
(21) Hugel, T.; Grosholz, M.; Clausen-Schaumann, H.; Pfau, A.; Gaub, H.; Seitz, M. Elasticity of single polyelectrolyte chains and their desorption from solid supports studied by AFM based single molecule force spectroscopy. *Macromolecules* **2001**, *34*, 1039−1047.
(22) Marszalek, P. E.; Oberhauser, A. F.; Li, H. B.; Fernandez, J. M. The force-driven conformations of heparin studied with single molecule force microscopy. *Biophys. J.* **2003**, *85*, 2696−2704.
(23) Zhou, H. X. Loops in proteins can be modeled as wormlike chains. *J. Phys. Chem. B* **2001**, *105*, 6763−6766.
(24) Carrion-Vazquez, M.; Marszalek, P. E.; Oberhauser, A. F.; Fernandez, J. M. Atomic force microscopy captures length phenotypes in single proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11288−11292.
(25) Schwaiger, I.; Sattler, C.; Hostetter, D. R.; Rief, M. The myosin coiled-coil is a truly elastic protein structure. *Nat. Mater.* **2002,** *1*, 232−235.
(26) Atkins, P. W. *Physical Chemistry*, 4th ed.; Oxford University Press: Oxford, 1993; Part 1.
(27) Lavialle, F.; Rogard, M.; Alfsen, A. Calorimetric determination of the enthalpy and heat capacity changes for the association of haptoglobin with hemoglobin. I. Demonstration of two interacting systems. *Biochemistry* **1974**, *13*, 2231−4.
(28) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4 → 8) catechin (procyanidin). *J. Agric. Food Chem.* **1998**, *46*, 2590−2595.
(29) Vergé, S.; Richard, T.; Moreau, S.; Nurich, A.; Merillon, J. M.; Vercauteren, J.; Monti, J. P. First observation of solution structures of bradykinin-penta-O-galloyl-D-glucopyranose complexes as determined by NMR and simulated annealing. *Biochim. Biophys. Acta* **2002**, *1571*, 89−101.
(30) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.
(31) Charlton, A. J.; Haslam, E.; Williamson, M. P. Multiple conformations of the proline-rich protein/epigallocatechin gallate complex determined by time-averaged nuclear Overhauser effects. *J. Am. Chem. Soc.* **2002**, *124*, 9899−9905.
(32) Levine, M. J.; Reddy, M. S.; Tabak, L. A.; Loomis, R. E.; Bergey, E. J.; Jones, P. C.; Cohen, R. E.; Stinson, M. W.; Al-Hashimi, I. Structural aspects of salivary glycoproteins. *J. Dent. Res.* **1987**, *66*, 436−41.
(33) Noble, A. C. Astringency and bitterness of flavonoid phenols. *ACS Symp. Ser.* **2002**, *825*, 192−201.
(34) Weatherell, J. A.; Strong, M.; Robinson, C.; Nattress, B. R.; Nakagaki, H.; Main, D. M. G. Determination of volumes of fluid in the mouth by fluoride dilution. *Caries Res.* **1992**, *26*, 94−97.
(35) Fawcett, D. W. *A Textbook of Histology*, 12th ed.; Chapman & Hall: New York, 1994.

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