Molecular and Thermodynamic Basis for EGCG-Keratin Interaction-Part II: Experimental Investigation

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In Part I, we reported all-atom, fully solvated molecular dynamics (MD) simulations of epigallocatechin-3-gallate (EGCG) binding to keratin. Herein, we report the second part of experimental investigation on EGCG binding to keratin using ultrafiltration and isothermal titration calorimetry (ITC). The thermodynamic equilibrium of EGCG binding to keratin has been quantitatively determined using ultrafiltration and high-performance liquid chromatography−UV/vis. The relationship confirms multilayer binding of EGCG to keratin which was observed in MD simulations. By combining the ultrafiltration and ITC data, the thermodynamic parameters of EGCG binding to keratin have been quantified. The obtained free energy of the first layer binding ($\Delta G = -6.37$ kcal mol^{-1}) is shown in excellent agreement with that obtained from computer simulations ($\Delta G = -6.20$ kcal mol^{-1}) presented in Part I. © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 4824–4827, 2013

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Introduction

In Part I (DOI 14220), we used molecular dynamics simulations to investigate the binding process of epigallocatechin-3-gallate (EGCG) to keratin. It has been revealed that EGCG binds to the helical and coiled coil part of type I and II human keratin. Hydrophobic assembly of aromatic interaction and hydrogen bonding are shown to be the key mechanisms governing the binding of EGCG to keratin. The binding free energy was predicted to be $\Delta G_{298} = -6.20$ kcal mol^{-1}. In part II, the thermodynamic properties of EGCG binding to keratin have been investigated experimentally. The aim is to validate the results of the fully solvated molecular dynamics (MD) simulations reported in Part I.

A variety of techniques were reported for the study of protein and phenolic compound interaction, such as equilibrium dialysis,[1,2] ultrafiltration,[3,4] fluorescence spectroscopy,[5] capillary electrophoresis,[6] and isothermal titration calorimetry (ITC).[4,7] Although most techniques are used to probe the kinetic and equilibrium parameters, the ITC method is used to determine the thermodynamic properties as well as equilibrium parameters of protein-ligand interactions. One of the challenges in using ITC is analysing the data involving complex interactions where over-fitting of parameters could be a potential problem. In this article, ultrafiltration and ITC techniques are combined to investigate the binding of EGCG to keratin extracted from sheep wool. This keratin (keratin 1) is chosen as a model protein to study the interaction with EGCG. The ultrafiltration method has been applied to quantify the thermodynamic equilibrium of EGCG binding to keratin, and the associated thermodynamic properties have been investigated by using ITC. The binding free energy determined from the ITC measurement agrees favorably with the predicted value from MD simulations reported in Part I.

Materials and Methods

Material preparations

EGCG was provided by J&K Scientific with a purity of 98%. A keratin sample was obtained from Professor Jian

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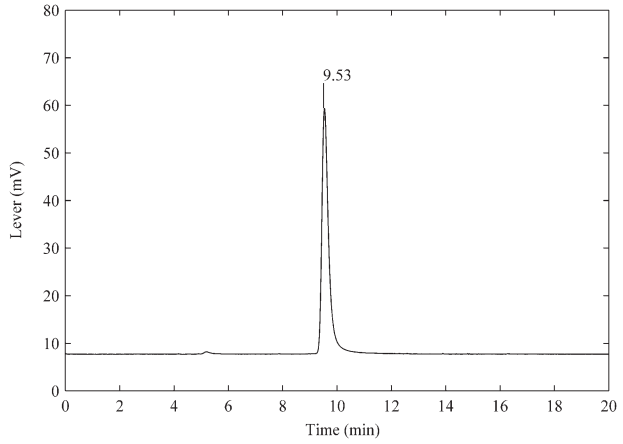

Figure 1. Chromatogram obtained analysis of EGCG (30 μg/mL) by HPLC.

Lu's group at Manchester University. The provided keratin solution (K1S) is a mixture of two main components of which the average molecular weight is 50 kDa. The samples were lyophilized and stored at −18°C in sealed bottles until use.

The keratin solution (0.0040 mM) was prepared by dissolving 5 mg lyophilized keratin powder into 25 mL 10 mM pH 6.0 Phosphate Buffered Saline (PBS) buffer. Different concentrations of EGCG solution were prepared by dissolving EGCG powder into 10 mM pH 6.0 PBS buffer and diluting to lower concentrations.

Ultrafiltration

Aliquots (2 mL) of the keratin solution (0.0040 mM) were mixed with EGCG solution (2 mL) at different concentrations at 298 K and incubated for 20 min. After incubation, each mixture (4 mL) was placed into an Amicon Ultra-4 centrifugal filter unit, of 3 kDa cutoff (Millipore, Beijing, China), and centrifuged at 4,000*g*. Control experiments were carried out using aliquots (2 mL) of ultrapure water mixed with EGCG solution (2 mL) at different concentrations. The amount of bound EGCG was calculated from the concentration difference between the control samples and the filtrate of the test samples using the high-performance liquid chromatography (HPLC) method.

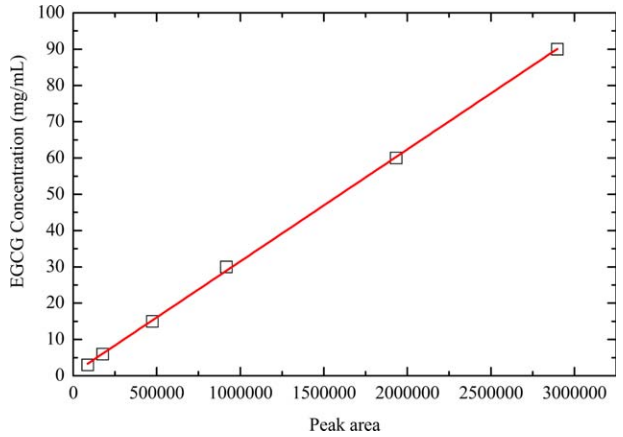

Figure 2. Standard curve used to determine the concentration of EGCG by HPLC.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

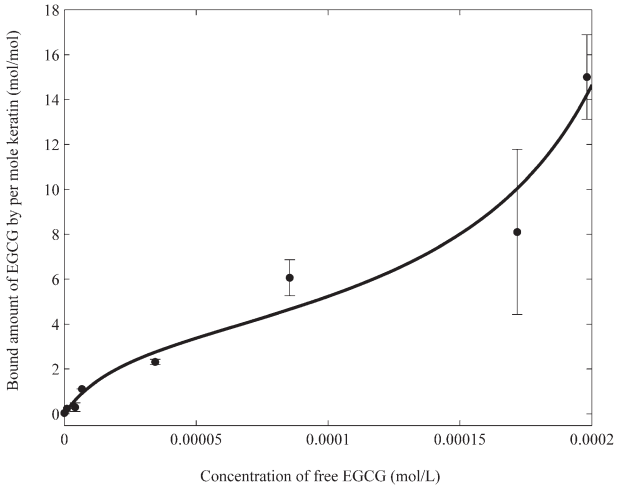

Figure 3. Ultrafiltration data of EGCG-keratin isotherm measured by ultrafiltration.

Data is best fitted by GAB equation.

Chromatographic analysis was performed on a Hitachi HPLC system equipped with a UV/vis Detector (at 280-nm wavelength). The separation was performed on a Shim-pack VP-ODS (250 × 4.6 mm Inner Diameter (ID), 5 μm) using an aqueous glacial acetic acid: (1% in water; solvent A): acetonitrile (solvent B) = 20:80 at a flow rate of 0.8 mL min^{-1}.

Isothermal titration calorimetry

For ITC measurements, keratin solution (0.0040 mM) was prepared by dissolving keratin in 10 mM PBS buffer at pH 6.0. EGCG solution (0.3027 mM) was then dissolved in the same buffer used for keratin solution. An ITC200 titration microcalorimeter (MicroCal, Beijing, China) was used at 298 K. The duration of each injection was 5s, and the time interval between injections was 180s. The solution in the cell was stirred at 500 rpm by the syringe to ensure thorough mixing. Control experiments were also carried out for the titration of EGCG solution into buffer and buffer into the keratin solution. The heat of the control experiments was subtracted from the titration experiment of EGCG and keratin titration, to remove the effects of association and dilution.

Results and Discussion

The HPLC result for EGCG

A typical chromatogram obtained in analysis of the EGCG by HPLC is shown in Figure 1. Figure 2 shows the standard curve used in determining the amount of EGCG by HPLC.

EGCG-keratin binding assay

As shown in Figure 3, the shape of the absorption equilibrium curve of EGCG and keratin binding implies multilayer binding equilibrium. The Brunauer Emmett Teller (BET) isotherm model developed by Brunauer et al.[8] is usually used to describe multilayer adsorption. However, the BET equation was developed to describe gas adsorption as a function of partial pressure. For molecular adsorption from a liquid system, the Guggenheim Anderson de Boer (GAB) equation, or the modified BET equation is used to fit the data[9]

Table 1. Fitted Isotherm Parameters of EGCG-Keratin Binding of Ultrafiltration

Parameters	GAB Fitting	95% Confidence Bounds
K_s (M^{-1})	4.62e+04	(3.46e+04, 1.27e+05)
K_L (M^{-1})	3770	(3548, 3991)
q_m (mol mol^{-1})	3.70	(1.11, 4.88)
R^2	0.9920	–

$$q = q_m \frac{K_S C_{eq}}{(1 - K_L C_{eq})(1 - K_L C_{eq} + K_s C_{eq})} \quad (1)$$

where q is the amount of ligand bound per mole of protein (mol mol^{-1}); C_{eq} is the free ligand concentration (M); q_m is the amount of monolayer binding of ligand per mole of protein (mol mol^{-1}); K_s is the monolayer binding constant (M^{-1}); and K_L is the multilayer binding constant (M^{-1}).

The best fitted values of the GAB absorption parameters are listed in Table 1. When the GAB equation is used to describe the binding of two molecules in a liquid system, for example, liquid phase adsorption[9] or protein binding,[10] the binding constant is often quoted as in units of M^{-1}. However, for solid phase adsorption where the solid phase is fixed, a dimensionless binding coefficient (or distribution coefficient) is often quoted.[11] Hansen et al.[12,13] has summarized the database of small molecule binding to different keratin (bovine hoof and horn, delipidized skin, nail, hair, and wool). The dimensionless binding coefficient (K_b) to bovine hoof/horn is empirically correlated to the octanol-water distribution coefficient as follows

$$\log K_b = 1.26 + 0.34 \log D \quad (2)$$

Similar quantitative structure-property relationships were reported by Anderson and Raykar[14] and Wang et al.[15] for molecular binding to delipidized skin as well as Wang et al. for hair.[16] For EGCG at pH 6.0, both log D and log K_{ow} values were predicted to be 2.56 (calculated from EPIWEB 4.1), which leads to the logarithmic keratin binding coefficient of 2.13[12,13] for bovine hoof/horn, 1.44[14] and 1.41[15] for delipidized skin, and 1.65[16] for hair. Hence, the strength of binding to different keratin-rich biosubstrates appears to vary.[12]

From the binding constant of Table 1, the binding coefficient of EGCG to wool keratin can be also estimated as

$$K_b = \left.\frac{q}{MW_{Keratin} C_{eq}/\rho_w}\right|_{C_{eq}\to 0} = \frac{q_m K_S \rho_w}{MW_{keratin}} \quad (3)$$

where $MW_{Keratin}$ is the molecular weight of wool keratin and ρ_w is the specific density of water.

By considering the molecular weight of keratin as 50 kDa, the derived binding coefficient of EGCG to keratin is 3.53. This is about one order of magnitude higher than the value of EGCG binding to bovine hoof/horn keratin,[12,13] two orders higher than delipidized skin keratin[14,15] and hair.[16] It appears extracted and purified keratin has significantly higher binding coefficient than keratin-rich composite biosubstrates.

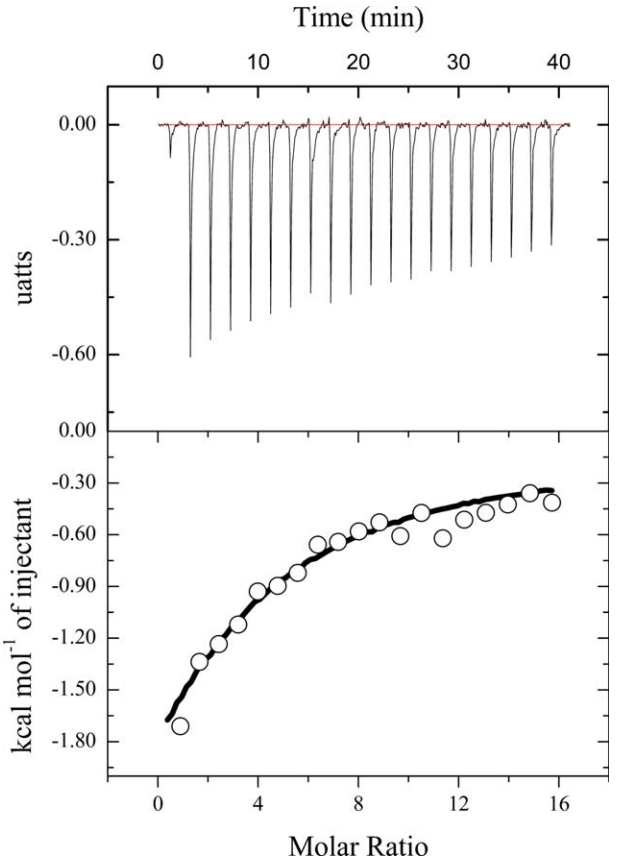

Figure 4. Titration of EGCG (0.3027 mM) to keratin (0.0040 mM) observed by ITC.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ITC measurement

ITC data of EGCG-keratin binding are plotted as a graph of heat flux against time. The peak-by-peak values are integrated and normalized to obtain the enthalpy change per mole of injectant (ΔH, kcal mol^{-1}) against the molar ratio (EGCG/keratin). Figure 4 shows the results of titration of EGCG (0.3027 mM, at pH 6.0, 298 K) into keratin (0.0040 mM, at pH 6.0, 298 K).

With multilayer binding, the measured change of enthalpy should be resolved into two components, one associated with the monolayer binding and the other associated with consecutive multilayer binding. Assuming GAB equilibrium of binding, it can be derived that the data presented in Figure 4 can be fitted according to the following equation for the total heat for EGCG and keratin binding

Table 2. Thermodynamic Parameters of EGCG Binding to Keratin at 298 K

Temperature (K)	q_m (mol mol^{-1})	K_s (M^{-1})	K_L (M^{-1})	ΔH_1 (kcal mol^{-1})	ΔH_2 (kcal mol^{-1})	ΔG_1 (kcal mol^{-1})	ΔG_2 (kcal mol^{-1})	ΔS_1 (kcal mol^{-1} K^{-1})	ΔS_2 (kcal mol^{-1} K^{-1})	R^2
298	3.056	46759	3917	−4.81	−3.38	−6.37	−4.90	0.0052	0.0051	0.9660

Data obtained by fitting to ITC data using GAB binding isotherm.

$$Q=M_tV\left\{q_m\frac{K_SC_{\rm eq}}{\left(1+K_SC_{\rm eq}\right)}\Delta H_1+\left[q_m\frac{K_SC_{\rm eq}}{\left(1-K_LC_{\rm eq}\right)\left(1-K_LC_{\rm eq}+K_SC_{\rm eq}\right)}-q_m\frac{K_SC_{eq}}{\left(1+K_SC_{\rm eq}\right)}\right]\Delta H_2\right\} \quad (4)$$

Here, M_t is the concentration of keratin in the cell; ΔH_1 and ΔH_2 are the molar heat of EGCG-keratin interaction of the first and consecutive layer, respectively; V is the cell volume (200 μL); and $C_{\rm eq}$ is the concentration of free (unbound) EGCG, which is calculated from the total EGCG concentration and the bound EGCG concentration in the cell as follows

$$X_t=C_{\rm eq}+M_tq_m\frac{K_SC_{\rm eq}}{\left(1-K_LC_{\rm eq}\right)\left(1-K_LC_{\rm eq}+K_SC_{\rm eq}\right)} \quad (5)$$

The measured enthalpy change of each injectant is

$$\Delta H=\left(Q(i)-Q(i-1)+\frac{dv_i}{V}\left(\frac{Q(i)-Q(i-1)}{2}\right)\right)/\left(C_{\rm egcg}\times dv_i\right) \quad (6)$$

where $C_{\rm egcg}$ is the concentration of EGCG in the syringe, 0.3027 mM; and dv_i is the volume of the ith injection.

In Eqs. 4–6, q_m, K_s, K_L, ΔH_1, and ΔH_2 are unknown parameters. The values of q_m, K_s, and K_L have been obtained from the ultrafiltration experiments. To prevent over-fitting, 95% confidence bounds of q_m, K_s, and K_L are used as boundaries. Hence, we attained the values of q_m, K_s, K_L, ΔH_1, and ΔH_2, as listed in Table 2. The free energy of the first layer ΔG is related to the equilibrium constant by the following relationship

$$\Delta G=-RT\ \ln K_S \quad (7)$$

where R is the universal gas constant (8.314 J mol^{-1} K^{-1}); and T is the temperature (K). Using Eq. 7, the free energy corresponding to the equilibrium constant of the first layer binding is determined to be $\Delta G=-6.37$ kcal mol^{-1}, which validates the value predicted by molecular dynamics simulations reported in Part I ($\Delta G=-6.20$ kcal mol^{-1}). The binding constant of the consecutive layer is an order of magnitude lower than that of the first layer and the corresponding binding free energy $\Delta G=-4.90$ kcal mol^{-1}. For both the first and consecutive multilayer, the ITC data showed $\Delta H<0$ and $\Delta S>0$. This suggests EGCG binding to keratin is governed by both hydrophobic interactions and hydrogen bonding which is consistent with the results from MD simulations. The enthalpy contributed to the majority of ΔG, indicating the binding process is enthalpy driven.

Conclusions

The equilibrium and thermodynamic properties of EGCG binding to keratin has been measured by ultrafiltration and ITC. The equilibrium data is best fitted by the GAB isotherm, suggesting multilayer EGCG binding to keratin rather than simple monolayer binding. The thermodynamic properties of EGCG binding to keratin have been characterized by ITC. The free energies calculated from both ultrafiltration and ITC are in good agreement with the result of molecular dynamic simulations reported in Part I.

Acknowledgments

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