Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1602–1605
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Isothermal titration calorimetry study of epicatechin binding to serum albumin

Richard A. Frazier [a,*], Athina Papadopoulou [a], Rebecca J. Green [b]

[a] School of Food Biosciences, The University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, United Kingdom
[b] School of Pharmacy, The University of Reading, P.O. Box 228, Whiteknights, Reading RG6 6AJ, United Kingdom

Received 15 December 2005; received in revised form 31 January 2006; accepted 1 February 2006
Available online 7 March 2006

Abstract

The interaction of epicatechin with bovine serum albumin (BSA) was studied by isothermal titration calorimetry. The binding constant (K) and associated thermodynamic binding parameters (n, ΔH) were determined for the interaction at three solution concentrations of BSA using a binding model assuming independent binding sites. These data show weak non-covalent binding of epicatechin to BSA. The interaction energetics varied with BSA concentration in the calorimeter cell, suggesting that the binding of epicatechin induced BSA aggregation. The free energy (ΔG) remained constant within a range of 2 kJ mol^{-1} and negative entropy was observed, indicating an enthalpy driven exothermic interaction. It is concluded that the non-covalent epicatechin–BSA complex is formed by hydrogen bonding.

Keywords: Isothermal titration calorimetry; Epicatechin; Albumin; Protein–polyphenol interaction

1. Introduction

Consumption of plants and plant products that are rich in polyphenols, such as cocoa, wine, tea and berries, has been related with protective effects against cardiovascular disease and certain forms of cancer [1,2]. Polyphenols have been found to act as free radical scavengers and have been widely studied for their antioxidant activity in vitro [3–5]; however, questions remain concerning their in vivo activity, especially regarding their absorption, metabolism and bioavailability [6–11]. Current literature suggests that factors such as protein binding may impair polyphenol absorption and bioavailability and even mask their antioxidant activity [12–14]. Non-covalent protein–polyphenol association is a well known phenomenon; however, it is only relatively recently that any considerable data from modern biophysical techniques (i.e., NMR spectroscopy, mass spectrometry, etc.) has been obtained in the area of how the structure of either the protein or the polyphenol may affect the interaction [15–27].

Isothermal titration calorimetry (ITC) is an attractive approach for the study of biomolecular interactions such as the interaction of polyphenols with proteins. ITC sensitively measures changes in enthalpy during a titration experiment in which ligand is added to a protein solution in a calorimeter cell held under isothermal conditions [28]. It is a universal technique since enthalpy changes will occur during any interaction that leads to the formation of a complex, and only differential scanning calorimetry and ITC are able to directly determine interaction enthalpy. Furthermore, from a single experiment ITC allows the determination of association constant (K), stoichiometry (n), free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, and therefore provides a wealth of important data, even for low affinity interactions [29]. Principal advantages of ITC are that it can be used to characterise interactions in solution and without chemical modification or immobilisation of either interacting species. This sets the technique apart from surface plasmon resonance, which requires surface attachment, and fluorescence methods that often require labeling or are specific to proteins that contain a fluorophore that is accessible to a quencher. ITC can also be applied to systems where the complex formed is insoluble, as is often the case for protein–polyphenol systems. This is a distinct advantage over many solution based techniques, including capillary

* Corresponding author. Tel.: +44 118 3788709; fax: +44 118 9310080.
E-mail address: r.a.frazier@rdg.ac.uk (R.A. Frazier).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.02.004

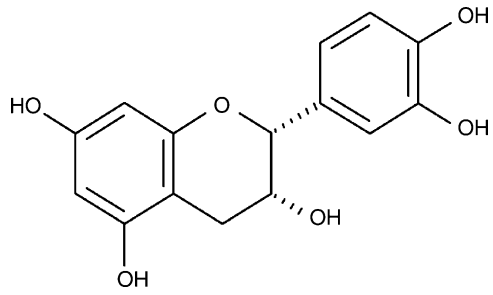

Fig. 1. Chemical structure of (−)-epicatechin.

electrophoresis (CE), where complex insolubility can be problematic [24].

Our earlier research has demonstrated the potential for applying ITC analysis to the study of protein–polyphenol interactions through a study of the binding of two contrasting tannins, i.e., flexible tara gallotannins and more rigid ellagitannins (from myrabolan), to model proteins [21]. ITC data revealed differences in the binding data depending on the protein type, but could only be used for a qualitative study of binding due to the complexity of the tannin samples, which comprised a mixture of polyphenolic compounds of varying molecular weights. Here, we investigate the interaction of epicatechin as a model compound with bovine serum albumin (BSA). Serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions; arguably the most important of these is as a depot and transport protein for a variety of compounds. BSA has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA). (−)-Epicatechin ((2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol; Fig. 1) is a flavonoid, which are a group of natural products reported to have neuroprotective, cardioprotective and chemopreventive actions [30].

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA, ≥99% purity) and (−)-epicatechin were purchased from Sigma (Poole, Dorset, UK). All buffers were prepared with UHQ water (Elga Purelab UHQ; 1.82×10^5 Ω m at 25 °C) and all samples were degassed prior to use in the calorimeter. Sample solutions were prepared in 0.1 M phosphate buffer pH 7.4, 6.8 or 6.0, or 0.05 M citrate buffer pH 5.0, as appropriate.

2.2. Isothermal titration calorimetry

The instrument used in these studies was a CSC Nano ITC Series III (Calorimetry Sciences Corp., Lindon, UT, USA). In a typical experiment, BSA solution (0.05, 0.2 or 0.5 mM) was placed in the 1.001 cm^3 sample cell of the calorimeter and epicatechin solution (10 mM) was loaded into the injection syringe. These concentrations were chosen to obtain a complete binding isotherm, which requires the concentration in the injection syringe to be approximately 10 times the concentration of binding sites in the cell [31]; estimated from published frontal analysis CE data [24]. Epicatechin was titrated into the sample cell as a sequence of 20 injections of 10 μl aliquots. The time delay (to allow equilibration) between successive injections was 3 min. The contents of the sample cell were stirred throughout the experiment at 100 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (μJ) against injection number. These raw data were then integrated to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ mol^{-1}) against molar ratio.

Control experiments included the titration of 10 mM epicatechin into buffer, buffer into BSA and buffer into buffer; controls were repeated for each buffer system used and at each BSA concentration. The last two controls resulted in small and equal enthalpy changes for each successive injection of buffer, and therefore, were not further considered in the data analysis [31]. Corrected data refer to experimental data after subtraction of the epicatechin into buffer control data.

2.3. Data analysis

ITC data were analysed using the BindWorks™ (Version 3.1.3, Applied Thermodynamics, Hunt Valley, MD, USA) ITC data analysis program. Data fits were obtained using an independent binding model for which the analytical solution for the total heat measured (Q) is determined by the formula:

$$Q = V\Delta H\left[[\mathrm{L}] + \frac{1 + [\mathrm{M}]nK - \sqrt{(1 + [\mathrm{M}]nK - [\mathrm{L}]K)^2 + 4K[\mathrm{L}]}}{2K}\right]$$

where V is the volume of the calorimeter cell, ΔH the enthalpy, [L] the ligand concentration, [M] the macromolecule concentration, n the molar ratio of interacting species and K is the equilibrium binding constant [32]. The goodness of fit was determined by calculation of χ^2 from the following formula:

$$\chi^2 = \sum_{i=1}^{N} \frac{[y_i - f(x_i)]^2}{\sigma_i^2}$$

where N is the number of data points, y_i the actual value, $f(x_i)$ the theoretical value and σ_i is the measurement error. The data fits were acceptable in each case since the χ^2 values were less than the critical values for the appropriate degree of freedom. Free energy, ΔG, was determined from the binding constant ($\Delta G = -RT \ln K$, where R is the gas constant and T is the absolute temperature in Kelvin) and entropy, ΔS, from the second law of thermodynamics ($\Delta G = \Delta H - T\Delta S$).

3. Results and discussion

The interaction of epicatechin with BSA was studied with varying pH (5, 6.0, 6.8 and 7.4) and BSA concentration in the calorimeter cell (0.05, 0.2 and 0.5 mM). The resulting data at pH 7.4 are plotted as observed molar enthalpy change (ΔH_{obs}) against molar ratio (epicatechin:BSA) in Fig. 2. The data (not shown) at the other pH values studied showed no effects of

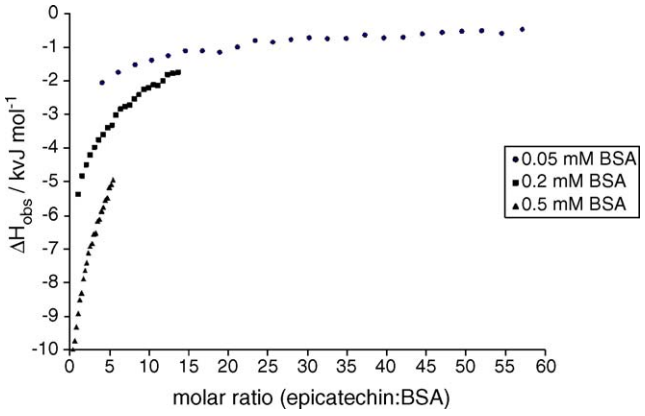

Fig. 2. Integrated plots of molar enthalpy change (ΔH_{obs}, kJ mol^{-1}) against molar ratio (epicatechin:BSA) at pH 7.4: (●) 0.05 mM BSA; (■) 0.2 mM BSA; (▲) 0.5 mM BSA.

pH on the interaction. This is supported by earlier data on polyphenol–protein complexation by Papadopoulou et al. [27] and by Charlton et al. [17], and suggests that electrostatic interactions are not a major factor in forming the epicatechin–BSA complex.

It is apparent from Fig. 2 that the interaction between epicatechin and BSA is exothermic and, from the shape of the binding isotherms, that the interaction is weak [29]. A marked effect of BSA concentration on the magnitude of ΔH_{obs} is observed, although the general shapes of the plots are similar. The magnitude of ΔH_{obs} was clearly greater at higher BSA concentration at any given molar ratio, although it does appear that it would be possible to extrapolate the binding isotherms to a common saturation point. However, although the titration plot at the lowest concentration of BSA (0.05 mM) approaches zero enthalpy at high molar ratios, the actual binding saturation was not reached within the experiment. Similar isotherms have been exhibited for binding of gallotannins and ellagitannins to BSA [21], surfactant interactions with hydrophobically modified polymers [33] and for the binding of non-ionic surfactants to BSA [34]. It is therefore our hypothesis, especially in view of the apparently high molar ratio required for binding saturation (>60 epicatechin molecules per molecule of BSA), that epicatechin "non-specifically" adsorbs to the surface of the BSA rather than binding to specific receptor sites on the protein.

The increasing exothermicity of the interaction with increasing BSA concentration in the calorimeter cell suggests that ligand-induced aggregation of BSA occurs [35]. This inference would support current hypotheses which suggest a surface adsorption mechanism leading to protein aggregation and eventual precipitation [17,25,26]. These models suggest that the protein becomes coated with polyphenol until a stage at which inter-complex polyphenol bridges form to induce aggregation of the non-covalent polyphenol–protein complexes.

The binding isotherms were further analysed by fitting the data to a binding model to yield the binding constant, K, and the binding parameters, n and ΔH. The results of this analysis of the data are shown in Table 1 along with the derived parameters, ΔG and ΔS. These data showed agreement in the values of K and n with previously published data on this interaction from frontal CE analysis [24]. It can be seen that it was not possible to fit the same binding model to the data at different BSA concentrations, with the value of K decreasing with increasing BSA concentration and ΔH increasing exothermically with increasing BSA concentration. However, the free energy (ΔG) was similar at each concentration and was negative, which is a requirement for a spontaneous biomolecular interaction [31]. In each case, the entropy term (ΔS) was negative, indicating that the interaction was driven by enthalpy as opposed to entropy. Negative entropy indicates an increase in molecular order, which would occur upon aggregation and may also imply a role for hydrogen bonding in the formation of the complex. Indeed, Kríž et al. [36] also reported negative entropy from ITC data for the complexation of catechin by β-cyclodextrin, which involves hydrogen bond formation. The binding enthalpies imply that the interaction was non-covalent since the enthalpies are too low for covalent bond formation to have occurred (200–400 kJ mol^{-1}).

Table 1
Binding and thermodynamic parameters for the interaction of epicatechin with BSA at three different BSA solution concentrations

	0.05 mM BSA	0.2 mM BSA	0.5 mM BSA
n	4.0 ± 0.4	3.3 ± 0.3	3.7 ± 0.4
K (M^{-1})	291 ± 15	213 ± 11	107 ± 6
ΔH (kJ mol^{-1})	-37 ± 2	-45 ± 2	-61 ± 3
ΔG (kJ mol^{-1})	-14 ± 1	-13 ± 1	-12 ± 1
ΔS (J mol^{-1} K^{-1})	-78 ± 4	-105 ± 5	-166 ± 8
χ^2	5.9	4.8	4.1

In conclusion, it is noteworthy that although our data for the interaction of epicatechin with BSA is in agreement with our findings for the interactions of higher molecular weight gallotannins and ellagitannins with BSA, we did not observe any interaction with gelatin by ITC (data not shown), which was shown to have a stronger interaction than BSA with gallotannins and ellagitannins [21]. The absence of any interaction with gelatin, which is rich in hydrophobic proline residues, suggests that epicatechin is not driven to interact with proteins by hydrophobic forces. It is therefore more likely that hydrogen bonding is important, which is implied by our data and has been suggested by earlier literature for the binding of more polar polyphenols with BSA [37].

Acknowledgement

We thank the University of Reading's Research Endowment Trust Fund for financing the studentship for A.P. and the purchase of the ITC instrument.

References

[1] L. Bravo, Nutr. Rev. 56 (1998) 317–333.
[2] G.G. Duthie, S.J. Duthie, J.A.M. Kyle, Nutr. Res. Rev. 13 (2000) 79–106.
[3] C. Rice-Evans, N.J. Miller, G. Paganga, Free Radical Biol. Med. 20 (1996) 933–956.
[4] O.V. Brenna, E. Pagliarini, J. Agric. Food Chem. 49 (2001) 4841–4844.
[5] C. Counet, S. Collin, J. Agric. Food Chem. 51 (2003) 6816–6822.

[6] S. Baba, N. Osakabe, M. Nastume, Y. Muto, T. Takizawa, J. Terao, J. Agric. Food Chem. 49 (2001) 6050–6056.
[7] K.E. Heim, A.R. Tagliaferro, D.J. Bobilya, J. Nutr. Biochem. 13 (2002) 572–584.
[8] G. Mazza, C.D. Kay, T. Cottrell, B.J. Holub, J. Agric. Food Chem. 50 (2002) 7731–7737.
[9] T.K. McGhie, G.D. Ainge, L.E. Barnett, J.M. Cooney, D.J. Jensen, J. Agric. Food Chem. 51 (2003) 4539–4548.
[10] Y. Nakamura, Y. Tonogai, J. Agric. Food Chem. 51 (2003) 7215–7225.
[11] K.A. O'Leary, A.J. Day, P.W. Needs, F.A. Mellon, N.M. O'Brien, G. Williamson, Biochem. Pharmacol. 65 (2003) 479–491.
[12] M. Serafini, A. Ghiselli, A. Ferro-Luzzi, Eur. J. Clin. Nutr. 50 (1996) 28–32.
[13] K.M. Riedl, A.E. Hagerman, J. Agric. Food Chem. 49 (2001) 4917–4923.
[14] M.J.T.J. Arts, G.R.M.M. Haenen, L.C. Wilms, S.A.J.N. Beetstra, C.G.M. Heijnen, H.-P. Voss, A. Bast, J. Agric. Food Chem. 50 (2002) 1184–1187.
[15] N.J. Baxter, T.H. Lilley, E. Haslam, M.P. Williamson, Biochemistry 36 (1997) 5566–5577.
[16] K. Wroblewski, R. Muhandiram, A. Chakrabartty, A. Bennick, Eur. J. Biochem. 268 (2001) 4384–4397.
[17] A.J. Charlton, N.J. Baxter, M.L. Khan, A.J.G. Moir, E. Haslam, A.P. Davies, M.P. Williamson, J. Agric. Food Chem. 50 (2002) 1593–1601.
[18] A.J. Charlton, E. Haslam, M.P. Williamson, J. Am. Chem. Soc. 124 (2002) 9899–9905.
[19] F. Zsila, Z. Bikadi, M. Simonyi, Biochem. Pharmacol. 65 (2003) 447–456.
[20] S.V.E. Pringent, H. Gruppen, A.J.W.G. Visser, G.A. van Koningsveld, G.A.H. de Jong, A.G.J. Voragen, J. Agric. Food Chem. 51 (2003) 5088–5095.
[21] R.A. Frazier, A. Papadopoulou, I. Mueller-Harvey, D. Kissoon, R.J. Green, J. Agric. Food Chem. 51 (2003) 5189–5195.
[22] C. Simon, K. Barathieu, M. Laguerre, J.-M. Schmitter, E. Fouquet, I. Pianet, E.J. Dufourc, Biochemistry 42 (2003) 10385–10395.
[23] X.-L. Chen, L.-B. Qu, T. Zhang, H. Liu, F. Yu, Y.-Z. Yu, Y.-F. Zhao, Supramol. Chem. 16 (2004) 67–75.
[24] A. Papadopoulou, R.A. Frazier, Trends Food Sci. Technol. 15 (2004) 186–190.
[25] E. Jobstl, J. O'Connell, J.P.A. Fairclough, M.P. Williamson, Biomacromolecules 5 (2004) 942–949.
[26] Y. Chen, A.E. Hagerman, J. Agric. Food Chem. 52 (2004) 4008–4011.
[27] A. Papadopoulou, R.J. Green, R.A. Frazier, J. Agric. Food Chem. 53 (2005) 158–163.
[28] A. Cooper, Curr. Opin. Chem. Biol. 3 (1999) 557–563.
[29] W.B. Turnbill, A.H. Daranas, J. Am. Chem. Soc. (2003) 14859–14866.
[30] R.J. Williams, J.P.E. Spencer, C. Rice-Evans, Free Radical Biol. Med. 36 (2004) 838–849.
[31] R. O'Brien, J.E. Ladbury, B.Z. Chowdhry, in: S.E. Harding, B.Z. Chowdhry (Eds.), Protein–Ligand Interactions: Hydrodynamics and Calorimetry, Oxford University Press, Oxford, 2001, pp. 263–286.
[32] E. Freire, O.L. Mayorga, M. Straume, Anal. Chem. 62 (1990) 950A–958A.
[33] J. Kevelam, J.F.L. van Breemen, W. Blokzijl, J.B.F.N. Engberts, Langmuir 12 (1996) 4709–4717.
[34] A.D. Nielsen, K. Borch, P. Westh, Biochim. Biophys. Acta 1479 (2000) 321–331.
[35] A. Cooper, K.E. McAuley-Hecht, Philos. Trans. R. Soc. Lond. A 345 (1993) 23–35.
[36] Z. Kríž, J. Koča, A. Imberty, A. Charlot, R. Auzély-Velty, Org. Biomol. Chem. 1 (2003) 2590–2595.
[37] A.E. Hagerman, M.E. Rice, N.T. Ritchard, J. Agric. Food Chem. 46 (1998) 2590–2595.