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Binding of an Oligomeric Ellagitannin Series to BSA: Analysis by Isothermal Titration Calorimetry

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1 **ABSTRACT:**

2 A unique series of oligomeric ellagitannins was used to study their interactions with bovine serum
3 albumin (BSA) by isothermal titration calorimetry. Oligomeric ellagitannins, ranging from
4 monomer up to heptamer and a mixture of octamer-undecamers, were isolated as individual pure
5 compounds. This series allowed studying the effects of oligomer size and other structural features.
6 The monomeric to trimeric ellagitannins deviated most from the overall trends. The interactions of
7 ellagitannin oligomers from tetramers to octa-undecamers with BSA revealed strong similarities. In
8 contrast to the equilibrium binding constant, enthalpy showed an increasing trend from the dimer to
9 larger oligomers. It is likely that first the macrocyclic part of the ellagitannin binds to the defined
10 binding sites on the protein surface and then the “flexible tail” of the ellagitannin coats the protein
11 surface. The results highlight the importance of molecular flexibility to maximize binding between
12 the ellagitannin and protein surfaces.

13

14 **KEYWORDS:** *Interactions, molecular size, polyphenol, protein, thermodynamics*

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18 INTRODUCTION

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20 Plants produce a variety of secondary metabolites including polyphenols in their tissues to protect
21 them for example against pathogens and insect herbivores. The most complicated polyphenol
22 structures are tannins which by definition have the ability to bind and precipitate proteins. Tannins
23 can be divided into condensed tannins, hydrolysable tannins (gallotannins and ellagitannins) in
24 addition to phlorotannins, which are found only in algae. For decades, ellagitannins were an
25 underestimated class of bioactive plant tannins.¹ However, ellagitannins are one of the most
26 promising tannin classes with potent biological activities, including antimicrobial and antioxidant
27 activities.^{1,2} More than 1000 individual ellagitannins have been identified from plants and natural
28 ellagitannins larger than pentamers were recently reported.³⁻⁵

29 Dietary tannins can affect animal nutrition and health in several ways, for example
30 through enabling a better utilization of feed proteins, generating anthelmintic effects against
31 gastrointestinal nematodes and by lowering nitrogenous and methane emissions.⁶⁻¹⁶ Tannins may
32 bind dietary proteins and thus reduce the degradation of these proteins in the rumen and may also
33 enhance the amount of protein available for digestion in the small intestine. Tannins can form
34 soluble and/or insoluble complexes with proteins and the tannin-protein interactions are both
35 tannin- and protein specific.¹⁷ Bovine serum albumin (BSA) is a well-characterized model protein
36 and it has been widely used for the investigations of tannin-protein interactions.¹⁸ Previous results
37 have shown that tannins have higher affinities to loosely structured globular proteins, such as BSA,
38 than to compact globular structures.¹⁹

39 Isothermal titration calorimetry (ITC) is a powerful technique to study the
40 thermodynamics of tannin-protein interactions. ITC has both a reference and a sample cell at a
41 constant temperature and the technique relies only on the detection of a heat effect upon binding; it
42 provides the accurate, rapid and label-free measurement of the thermodynamics of molecular

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43 interactions.^{20,21} In a single ITC experiment, the strength and stoichiometry of the interaction
44 between tannin and protein can be measured, i.e. the enthalpy (ΔH_{obs}) and stoichiometry of binding
45 (n) and the binding constant (K) can be determined. The binding of condensed tannins and
46 hydrolyzable tannins to proteins has been previously studied by ITC.^{22–26} The binding parameters
47 have been related to the structural flexibility of tannins.^{24,25} Most of the studies have been
48 conducted using condensed tannins. However, the use of condensed tannins as model compounds
49 poses particular problems as the determination of their molecular weight is not straight forward.²³
50 Oligomeric and polymeric condensed tannins cannot be chromatographically separated and
51 therefore, they are usually isolated and purified as mixtures.^{27–29} Therefore, their molecular weight
52 is an estimation based on the size distribution within a condensed tannin mixture, obtained for
53 example by acidic degradation in the presence of nucleophiles or by mass spectrometry.^{30–35} It is
54 feasible, however, to isolate ellagitannins as pure compounds^{4,36}, and this offers a unique
55 opportunity to investigate the effects of different structural features, such as molecular size and
56 monomeric units.

57 In this study, we utilized ITC to study the interaction between ellagitannins and BSA.
58 We characterized the thermodynamics of the binding of tellimagrandin I-based oligomeric
59 ellagitannins^{3,4} (Fig. 1) to bovine serum albumin BSA. The ellagitannin oligomers were a unique
60 series consisting of tellimagrandin I (monomer), oenothin B (dimer), oenothin A (trimer) plus a
61 tetramer, pentamer, hexamer and heptamer (Fig. 1) and a mixture of octamers to undecamers. These
62 ellagitannin oligomers are excellent model compounds for the ITC studies as they can be isolated as
63 individual pure compounds and are well-characterized with exact molecular weights.³ This work is
64 the very first systematic investigation of ellagitannins using an oligomeric series so that we can
65 evaluate the effect of the molecular size and decouple this from other structural features, such as
66 functional groups, which has been a problem when interactions between condensed tannins and
67 proteins were studied. The oligomers consisted of similar monomeric units, which also enabled the

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68 direct comparison of the interaction between the different oligomers based on the number of
69 monomeric tellimagrandin I units.

70

71 MATERIALS AND METHODS

72

73 **Materials.** Acetone (technical grade) used in the collection of plant materials was
74 from VWR International (Leuven, Belgium). Acetone and methanol (analytical grade) used in the
75 Sephadex LH-20 fractionations, methanol and acetonitrile (HPLC grade) used in the preparative
76 and semipreparative HPLC were obtained from VWR International (Fontenay-Sous-Bois, France).
77 LiChroSolv® acetonitrile (hypergrade for LC-MS) was purchased from Merck KGaA (Darmstadt,
78 Germany) and formic acid (eluent additive for LC-MS) was from VWR International Ltd. (Poole,
79 England). The water was filtered through an Elgastat UHQ-PS purification system (Elga, Kaarst,
80 Germany) or with Synergy® UV water purification system (Millipore SAS, Molsheim, France).
81 BSA (purified by heat shock fractionation, pH 7, purity $\geq 98\%$, lyophilized powder, 66 kDa) was
82 purchased from Sigma-Aldrich (St. Louis, US).

83 **Isolation and Characterization of Ellagitannins.** A series of oligomeric
84 ellagitannins was purified: monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer and a
85 mixture of chromatographically non-separated octamers to undecamers.^{3,4} The monomeric
86 ellagitannin, tellimagrandin I, was isolated from meadowsweet (*Filipendula ulmaria*) inflorescences
87 and the other oligomeric ellagitannins from fireweed (*Epilobium angustifolium*) inflorescences. The
88 inflorescences were collected during summer 2011 from southwest Finland. The plant material was
89 collected and placed directly into ten bottles of 1 L, which were then immediately filled with
90 acetone, transferred to the laboratory, and stored in a cold room (+4 °C) prior the isolation of
91 ellagitannins.

92 The extraction and isolation of ellagitannins followed mainly the previously outlined
93 methods.^{3,4} However, some modifications were made in order to enhance and speed up the large-
94 scale extractions and fractionations. The acetone extracts of fireweed inflorescences were combined
95 and concentrated to 300-500 mL of water phase. The concentrated water phases were fractionated
96 twice with Sephadex LH-20 chromatography. First, a rough fractionation was performed in a
97 beaker, and then careful fractionation was performed for a selected fraction by Sephadex LH-20
98 column chromatography as previously described.³⁷ The isolation of ellagitannins from the Sephadex
99 fractions was performed by preparative HPLC. The HPLC-DAD system consisted of a Waters
100 Delta 600 liquid chromatograph, a Waters 600 Controller, a Waters 2998 Photodiode Array
101 Detector and a Waters Fraction Collector III. The column (approximately 327 mm × 33 mm) was
102 manually filled with LiChroprep RP-18 (40-63 μm) material (Merck KGaA, Darmstadt, Germany).
103 The flow rate was 8 mL min⁻¹ and the sparge rate for the helium flow 100 mL min⁻¹. Two eluents
104 were used: 1% formic acid (A) and methanol (B). The gradient was the following: 0–5 min, 100%
105 A; 5–180 min, 0-40% B in A; 180–220 min, 40–60% B in A; 220–240 min, 60-80% B in A. The
106 injection volume was 5 mL. The photodiode array detector was operating between 190–500 nm,
107 and ellagitannins were detected at 280 nm. The final purification of ellagitannins was performed by
108 semipreparative HPLC with the same HPLC-DAD system. The column was a Gemini C18 column
109 (150 × 21.2 mm, 10 μm, Phenomenex) and the eluents were 0.1 % formic acid (A) and acetonitrile
110 (B). Different gradients were used for different oligomers; for example, a typical gradient for larger
111 oligomers was as follows: 0–5 min, 17% B in A; 5–51 min, 17–47% B in A; 51–55 min, 47–70% B
112 in A. The flow rate was 8 mL min⁻¹ and the sparge rate for the helium flow 100 mL min⁻¹. The
113 injection volume was 5 mL. The photodiode array detector was operating between 190–500 nm,
114 and ellagitannins were detected at 280 nm.

115 All steps in the extraction, isolation and preparative and semipreparative purifications
116 were followed by UPLC-DAD-MS (Acquity UPLC®, Waters Corporation, Milford, USA

117 combined with Xevo® TQ, Waters Corporation, Milford, USA). Samples were filtered with a
118 syringe filter (4 mm, 0.2 μm PTFE, Thermo Fisher Scientific Inc., Waltham, USA) prior to the
119 analysis. The Acquity UPLC® BEH Phenyl column (2.1 \times 100 mm, 1.7 μm , Waters Corporation,
120 Wexford, Ireland) was used with two eluents: 0.1% formic acid (A) and acetonitrile (B). The
121 gradient was 0–0.5 min, 0.1% B in A; 0.5–5.0 min, 0.1–30.0 % B in A (linear gradient); 5.0–5.1
122 min, 30.0–90.0 % B in A (linear gradient); 5.1–8.5 min, column wash and stabilization. The flow
123 rate was 0.5 mL min⁻¹ and the injection volume 5 μL . The photodiode array detector was operating
124 between 190–500 nm, and ellagitannins were detected at 280 nm. Mass spectrometer was operated
125 in a negative ionization mode and ions at m/z 160–1200 were scanned. Capillary voltage was set at
126 3.53 kV, cone voltage ramp was used between 20–70 V, desolvation temperature was set at 650 °C,
127 and source temperature at 150 °C. Desolvation and cone gas (N₂) flow rates were 1000 L h⁻¹ and
128 100 L h⁻¹, respectively. The ellagitannins were identified based on previous work.^{3,36} Pure
129 ellagitannins were concentrated to the water-phase and freeze-dried.

130 **Isothermal Titration Calorimetry.** Titrations of ellagitannins into BSA (purity \geq
131 98%, lyophilized powder, 66 kDa; Sigma-Aldrich, St. Louis, US) were performed with a NanoITC
132 instrument (TA Instruments Ltd., Crawley, West Sussex, UK) as previously described^{18,23,24} with
133 minor modifications. All solutions were prepared in 50 mM citrate buffer adjusted to pH 6. BSA
134 solution (10, 20, 30 or 40 μM) was placed in the 950 μL sample cell of the calorimeter and 3 mM
135 ellagitannin solution was loaded into the injection syringe. The ellagitannin studied was titrated into
136 the sample cell at 298 K as a sequence of 24 injections of 10 μL . The time delay between the
137 injections was 360 s. The content of the sample cell was stirred throughout the experiment to ensure
138 comprehensive mixing. All ellagitannins were studied with three replicate titrations; the pentameric
139 and heptameric ellagitannins were studied in duplicates because of limited amounts. Control
140 experiments included the titration of ellagitannin solution into buffer, the titration of buffer into
141 BSA solution and the titration of buffer into buffer. Control experiments of buffer titrated into BSA

142 solution and buffer into buffer resulted only in small or equal enthalpy changes for each successive
143 injection of buffer, and therefore, were not considered in the data analysis.^{18,23,24} The control data of
144 ellagitannin titrated into buffer was always subtracted from the sample data as it was known that
145 ellagitannins tend to self-associate into aggregates due to hydrophobic groups; and therefore, when
146 injected from the syringe into buffer, they undergo an endothermic process of deaggregation. The
147 extent of deaggregation depends inversely on the concentration of ellagitannin already present in
148 the sample cell: therefore, successive injections of ellagitannins into buffer lead to observation of
149 progressively lower endothermic enthalpy changes as has been illustrated in earlier work.²²

150 **Data Analysis.** Raw data from isothermal titration calorimetry were obtained as plots
151 of heat (μJ) against injection number and exhibited a series of peaks for each injection. The raw
152 data were transformed using the NanoAnalyze Data Analysis software (version 2.4.1., TA
153 Instruments) to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs} , kJ mol^{-1})
154 against molar ellagitannin:protein ratio. The estimated binding parameters were obtained from ITC
155 data using the same NanoAnalyze Data Analysis software. Data fits were obtained in two different
156 ways: using a single set of multiple binding sites and a model for two independent sets of multiple
157 binding sites. The quality of fits was determined by standard deviation.

158

159 **RESULTS AND DISCUSSION**

160

161 **ITC Binding Isotherms and Data Fitting.** In this study, the interaction of seven
162 individual tellimagrandin I-based oligomeric ellagitannins (from monomer to heptamer) and a
163 mixture of larger oligomeric ellagitannins with BSA was investigated by ITC. For each
164 ellagitannin:BSA system studied, an exothermic interaction was observed. Fig. 2 shows ITC
165 binding isotherms for two of the ellagitannins studied, the trimer (oenothain A) and hexamer
166 binding to BSA. Experiments were carried out using two different concentrations of BSA, 20 μM or

167 30 μM , and this showed the good repeatability of the ellagitannin:BSA interaction. The data
168 showed that the interaction was not affected by BSA concentration suggesting no evidence of co-
169 operative binding. Upon the addition of ellagitannin, the interaction became less exothermic as the
170 binding sites of BSA became saturated. The ITC binding isotherms showed that an
171 ellagitannin:BSA molar ratio of approximately 10:1 corresponded to the inflection point for each
172 ellagitannin interaction and that there were multiple binding sites for the ellagitannin on the BSA
173 molecule.¹⁸

174 Two different binding models were used to fit the data, one assuming a single set of
175 multiple binding sites (a single-site model) and a second model assuming two independent sets of
176 multiple binding sites (a two-site model), as previously discussed in detail by Deaville et al.²⁵ In
177 Fig. 3, it can be seen that both models fit the data equally well for the ellagitannin trimer binding to
178 BSA, and thus the simpler single-site model seemed better justified as a binding model. However,
179 for many of the ellagitannins studied, both models provided an acceptable fit, but the two-site
180 model clearly exhibited a closer fit for the data. This was seen particularly at higher
181 ellagitannin:BSA molar ratios where a longer tail in the data was seen as the tannin:BSA interaction
182 was saturated. Therefore, the fit parameters for both binding models are shown in Tables 1 and 2 for
183 all ellagitannins studied. The second site showed very weak interaction with small binding
184 constants varying from 17 to $1.8 \times 10^3 \text{ M}^{-1}$ (Table 2) for all the ellagitannin:BSA interactions
185 studied. This weaker interaction might indicate a non-specific surface adsorption mechanism
186 following the more selective stronger binding of the ellagitannin, as indeed we had observed in
187 previous tannin:protein binding studies.²²⁻²⁴ This was the first study where a purified oligomeric
188 series was investigated to look at the effect of oligomerization on ellagitannin:protein binding
189 behavior. In previous studies with hydrolysable tannins, a two-binding site model had been used. In
190 those examples, the binding constants had shown to be higher than values seen here, and the two-
191 site model had been more pronounced. Interestingly, those measurements had been carried out

10

192 using lower tannin concentrations (3 mg mL^{-1} , for example approximately 2 mM for oenothain B)
193 and over a longer timescale using more injections and a higher level of protein dilution than the
194 current experiments. From our experience it was clear that we could not directly compare data from
195 previous studies, which has used different experimental conditions that could impact on the
196 interaction, due to complicating factors such as the degree of self-association of tannin molecules.

197 The two-site model indicated two independent types of binding on the protein surface.
198 The second binding site highlighted the presence of a weaker interaction in addition to a more
199 selective stronger first binding site. As seen in Table 2, the weakness of the second binding site
200 meant that it was difficult to confidently identify trends across the oligomeric series for this
201 interaction. Therefore, we have focused on the single-binding site model for our discussion of the
202 ellagitannin:BSA interaction across the oligomeric series since this interaction was likely to relate
203 to a specific molecular interaction between the tannin and protein.

204 **Monomeric, Dimeric and Trimeric Ellagitannins.** Tables 1 and 2 show trends
205 linking the oligomer size to ellagitannin:BSA interaction, that suggested that the smaller
206 ellagitannins deviated from the overall trends seen. The interaction of monomeric tellimagrandin I
207 with BSA was stronger, in terms of K_a than expected in relation to the oligomer series. The binding
208 constant ($K_a = 1.8 \times 10^4 \text{ M}^{-1}$) was higher than for the dimeric oenothain B ($5.7 \times 10^3 \text{ M}^{-1}$) and
209 trimer ($7.6 \times 10^3 \text{ M}^{-1}$) and similar to hexameric and heptameric ellagitannins ($1.7 \times 10^4 \text{ M}^{-1}$). This
210 could be due to the additional free galloyl group as the monomer contained two adjacent free
211 galloyl groups (Fig. 1A) whereas oligomeric ellagitannins contained only one free galloyl group per
212 monomeric unit. Previously, Dobreva *et al.* had reported that the strong binding site was dependent
213 on tannin flexibility and possibly also free galloyl groups.²⁴

214 The binding of the dimer, oenothain B, to BSA was also different in comparison to the
215 others. The equilibrium binding constant was the lowest ($5.7 \times 10^3 \text{ M}^{-1}$) as well as the enthalpy
216 change observed (-14 kJ mol^{-1}) indicating a relatively low affinity of oenothain B towards BSA.

217 Oenothain B was a macrocyclic, relatively rigid structure with less conformational flexibility
218 compared with all the other ellagitannins studied in this series (Fig. 1B). A previous study had
219 shown that the binding constant for the interaction with BSA was dependent on the structural
220 flexibility of the tannin molecule; and a loss of conformational freedom in the ellagitannin structure
221 impacted on its ability to bind to BSA.²⁵ The thermodynamic parameters for oenothain B (Table 1
222 and 2) were different to Dobrev et al.²⁴ although the overall conclusion linking BSA binding to
223 tannin flexibility was the same. As mentioned previously, differences in the experimental
224 procedures were likely to explain the quantitative difference in the binding interaction.²⁴ Our
225 findings suggested that the interaction between ellagitannin and protein might be dependent on the
226 ellagitannin concentration.

227 The trimeric oligomer, oenothain A, showed stronger binding to BSA than the dimer.
228 The binding constant was $7.5 \times 10^3 \text{ M}^{-1}$ and the enthalpy change was -45 kJ mol^{-1} . The trimer was
229 more flexible than the dimer as the additional monomeric unit was attached only via one bond (Fig.
230 1C). The presence of this conformationally free chain (flexible tail) allowed for a stronger
231 interaction in terms of K_a of the trimer versus the dimer and this was also seen for the larger
232 oligomers in terms of ΔH_{obs} (Fig. 4). These observations could be linked to the three-dimensional
233 structures of the oligomer series, where larger oligomers had a longer flexible chain attached to the
234 macrocycle.

235 **Larger Oligomeric Ellagitannins.** The interactions of ellagitannin oligomers from
236 trimers to octamer-undecamers with BSA revealed strong similarities. The stoichiometric number,
237 i.e. tannin to protein ratio, varied from just 9 to 12 (Table 1) and the equilibrium binding constant
238 was 10^3 - 10^4 M^{-1} for all ellagitannin-BSA interactions. The observed change in enthalpy was
239 exothermic and increased with the oligomeric size up to the heptamer. Exothermic interactions were
240 associated with hydrogen bonding or Van der Waals interactions. As the oligomeric size increased
241 the number of footholds, the points of interaction with the protein surface, increased.

242 All oligomers contained the same macrocyclic part in their structure, i.e. dimeric
243 oenothetin B (Fig. 1B) formed by the dimerization of two tellimagrandin I monomers via two *m*-
244 DOG-type linkages. The *m*-DOG-type linkages are frequently found in ellagitannin oligomers: in
245 these linking units, the *O*-donating hydroxyl group is part of an hexahydroxydiphenoyl group and
246 the galloyl group is the acceptor.² In trimeric and larger ellagitannin oligomers, the additional
247 monomeric units were attached by one *m*-DOG-type linkage forming an elongated chain or tail to
248 this macrocyclic part (Fig. 1). It was likely that first the macrocyclic part of the ellagitannin bound
249 to the protein (to the defined binding sites on the protein surface resulting in little difference in the
250 value of *n* for each tannin) and then the “flexible tail” of the ellagitannin coated the protein surface.
251 Therefore, a trend in terms of binding affinity could be observed for this oligomeric ET series. This
252 observation differed from the previous studies of polyphenol binding to proteins where an increase
253 in the binding affinity with molecular size had been observed, but where there were also differences
254 in terms of flexibility, molecular structures and in some cases, the purity of the tannins that
255 influenced the interaction.³⁸

256 **ITC Analysis Based on Monomeric Concentrations.** In an effort to remove the
257 effect of oligomerization and molecular weight, the data were also fitted by assuming a monomeric
258 concentration for the ellagitannins as shown in Table 3 using a single-site binding model. The
259 oligomers consisted of similar monomeric units, which enabled the direct comparison of the
260 different oligomers based on the number of monomer units. By doing this, the trend seen in Table 1
261 for ΔH_{obs} was largely removed. Focusing on the dimer and larger oligomers the variation seen for
262 ΔH was small at -13.9 ± 2.6 kJ per mole of monomer. However, the number of binding sites on the
263 ellagitannin increased with the oligomer size, since the binding of the flexible chain was now
264 considered as individual molecules. We saw that the K_a was smaller per monomer compared with
265 our molecular calculations, since the interaction was now split across multiple molecules.

266 The different analyses of the data seemed to fit the concept that there were two
267 different binding events; first the binding of the rigid ring of the dimer was followed by the binding
268 of the flexible units of the tail. It was for this reason why in previous studies two-site binding
269 models had been needed and why we fitted our current data also using the two-site binding model
270 (Table 2). However, often the second binding site was a lot weaker and less specific than the first
271 one. As mentioned previously, the fits obtained using a two-site model were good fits, and
272 marginally better than the single-site model for the larger ellagitannins. In particular, oligomers
273 from tetramer to heptamer all showed good two-site binding fits to the data with nine distinct strong
274 binding sites ($n = 9$, $K_a = 10^4 - 10^5 \text{ M}^{-1}$) and a second weak binding site, a K_a of approximately 10^2
275 and a high n ranging from 35-90.

276 Previous studies had shown that the interaction of tannins with proteins could be a
277 surface phenomenon where tannins coated the surface of the protein.^{18,25,39} Our data supported this
278 observation in two ways. Firstly, the flexible elongated chain in oligomeric ellagitannins appeared
279 to coat the protein surface. Secondly, when the data were fitted using monomeric concentrations,
280 the tannin:protein binding stoichiometries increased x-fold, where x was the number for the degree
281 of oligomerization, and the ΔH and K_a values converged. We observed that there were
282 approximately nine specific binding sites on the surface of the protein, but that further interactions,
283 akin to non-specific surface adsorption, occurred allowing the flexible chain of the tannin oligomers
284 to subsequently bind to the surface.

285 In conclusion, this unique series of oligomeric ellagitannins allowed us to study the
286 effect of molecular size on the interaction between ellagitannins and BSA. The novel results
287 showed that the interactions of ellagitannin oligomers from trimers to octamer-undecamers with
288 BSA revealed strong similarities. The monomeric and dimeric ellagitannins deviated from the
289 overall trends seen. Our studies highlighted the importance of molecular flexibility to maximize
290 binding between the tannin and protein surface. This systematic investigation of ellagitannins used

14

291 an oligomeric series and was able to decouple for the first time structural features, such as
292 functional groups present and purity, from molecular weight.

293

294 ABBREVIATIONS USED

295 BSA, bovine serum albumin; HPLC-DAD, high-performance liquid chromatography diode array
296 detection; ITC, isothermal titration calorimetry; UPLC-DAD-MS, ultra-performance liquid
297 chromatography diode array detection mass spectrometry

298

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303

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424

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431 **Notes**

432 The authors declare no competing financial interest.

433

434 **FIGURE CAPTIONS**

435

436 **Figure 1.** The structures of (A) monomeric ellagitannin tellimagrandin I, (B) dimeric ellagitannin
437 oenothetin B, (C) trimeric ellagitannin oenothetin A, and (D) tetrameric tellimagrandin I-based
438 ellagitannin.

439

440 **Figure 2.** Typical ITC binding isotherms for the interaction of ellagitannin (A) trimer (oenothetin A)
441 and (B) hexamer with 20 μM BSA (\blacksquare) and 30 μM BSA in two replicate experiments (Δ and \times).

442

443 **Figure 3.** Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the
444 experimental data (\blacksquare) for the interaction of ellagitannin (A) monomer (tellimagrandin I), (B) dimer
445 (oenothetin B), (C) trimer, (D) tetramer, (E) pentamer, (F) hexamer, (G) heptamer and (H) a mixture
446 of octamer-undecamers with 30 μM BSA.

447

448 **Figure 4.** Plots of (A) ΔH and (B) K_a vs degree of oligomerization for the interaction of
449 ellagitannin oligomers with BSA fitted by single-site binding model.

450

451

21

Table 1. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Single-site Binding Model^a

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer-	undecamers
K_{a1}^b	18403 ± 5052	5739 ± 794	7552 ± 136	26810 ± 2708	23200	16925 ± 551	16925		35520
ΔH_1^c	-24 ± 3	-14 ± 4	-45 ± 5	-69 ± 3	-80	-88 ± 3	-93		-93
n_1	6 ± 1	12 ± 2	11 ± 1	9 ± 1	9	10 ± 1	10		9
SD	13	11	11	71		94			

^a SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer to undecamer n = 2 due to the limited amount of individual oligomers. ^b K_{a1} (M^{-1}) is the equilibrium binding constant for the single set of multiple binding sites. ^c ΔH_1 ($kJ\ mol^{-1}$) is the corresponding enthalpy.

22

Table 2. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Two-site Binding Model^a

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer-undecamer
K_{a1}^b	22188 ± 6280	9786 ± 1597	11863 ± 1090	72560 ± 10124	76620	55890 ± 5419	35280	42940
ΔH_1^c	-20 ± 3	-14 ± 1	-33 ± 3	-52 ± 4	-56	-63 ± 5	-73	-88
n_1	6 ± 2	6 ± 1	11 ± 1	9 ± 1	9	9 ± 1	9	9
K_{a2}^b	1828 ± 1815	1138 ± 998	350 ± 93	184 ± 19	262	115 ± 11	174	17
ΔH_2^c	-10 ± 9	-7 ± 3	-9 ± 3	-19 ± 3	-44	-59 ± 2	-27	-24
n_2	4 ± 1	36 ± 12	32 ± 14	90 ± 11	35	59 ± 1	84	83
SD	12	7	14	50		73		

^a SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer-undecamer n = 2 due to the limited amount of individual oligomers. ^b K_{a1} and K_{a2} (M^{-1}) are the equilibrium binding constants for the two set of multiple binding sites. ^c ΔH_1 and ΔH_2 ($kJ mol^{-1}$) are the corresponding enthalpies.

23

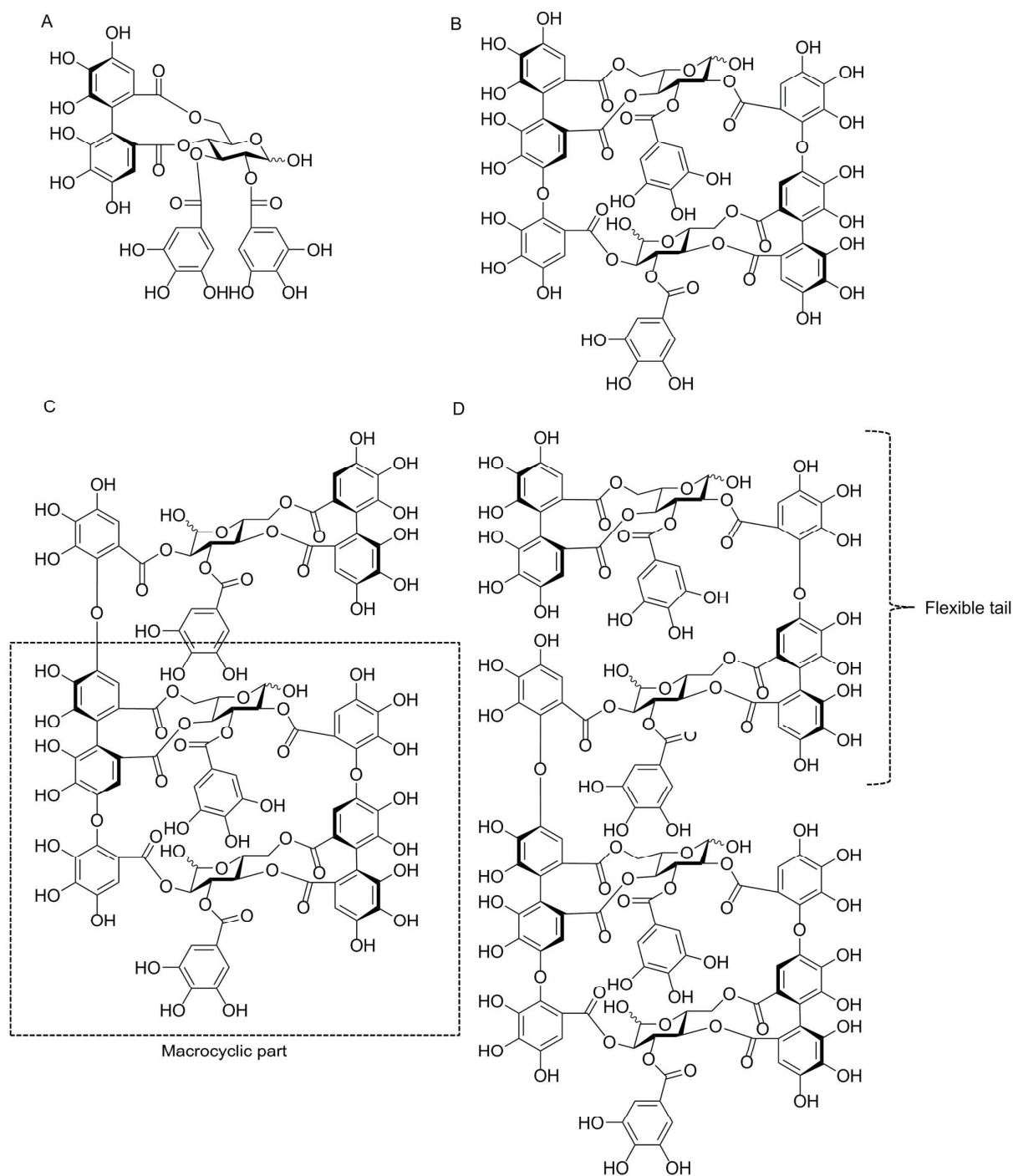
Table 3. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Single-site Binding Model. The Concentrations of Oligomers Have Been Set to the Corresponding Monomeric Concentrations in Order to Remove the Impact of Molecular Weight^a

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer- undecamer
K_{a1}^b	18403 ± 8750	2391 ± 1364	2517 ± 79	6703 ± 1182	4645 ± 368	3082 ± 372	2420 ± 135	5119 ± 2760
ΔH_1^c	-24 ± 6	-12 ± 11	-15 ± 3	-17 ± 1	-16 ± 1	-15 ± 1	-13 ± 1	-9 ± 1
n_1	6 ± 1	21 ± 13	30 ± 7	37 ± 4	46	57 ± 3	67	85 ± 8
SD	13	9	11	71		94		39

^a SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer to undecamer n = 2 due to the limited amount of individual oligomers. ^b K_{a1} (M^{-1}) is the equilibrium binding constant for the single set of multiple binding sites. ^c ΔH_1 ($kJ\ mol^{-1}$) is the corresponding enthalpy.

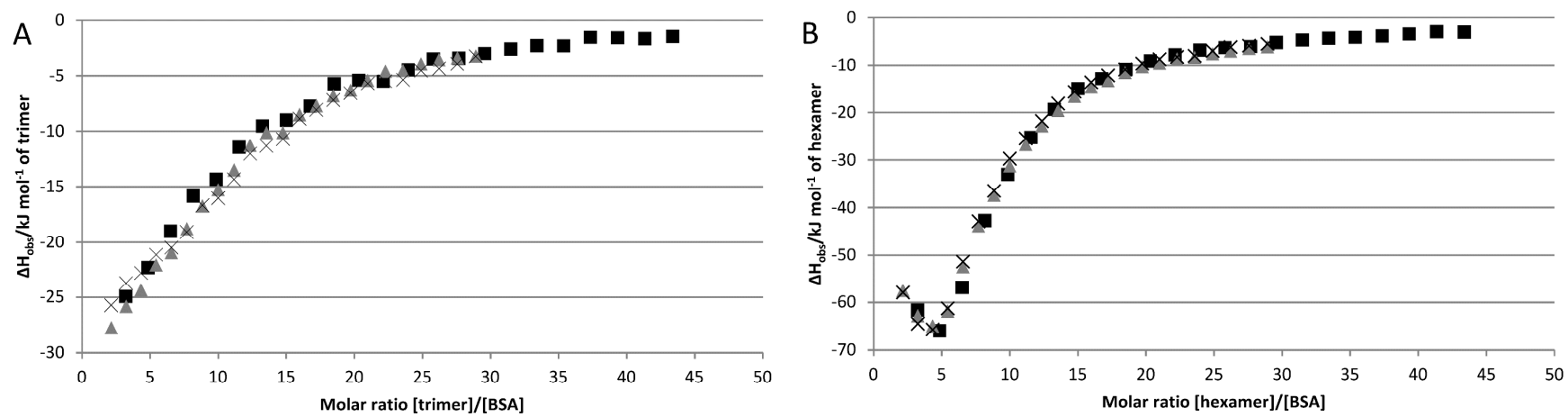
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Figure 1.



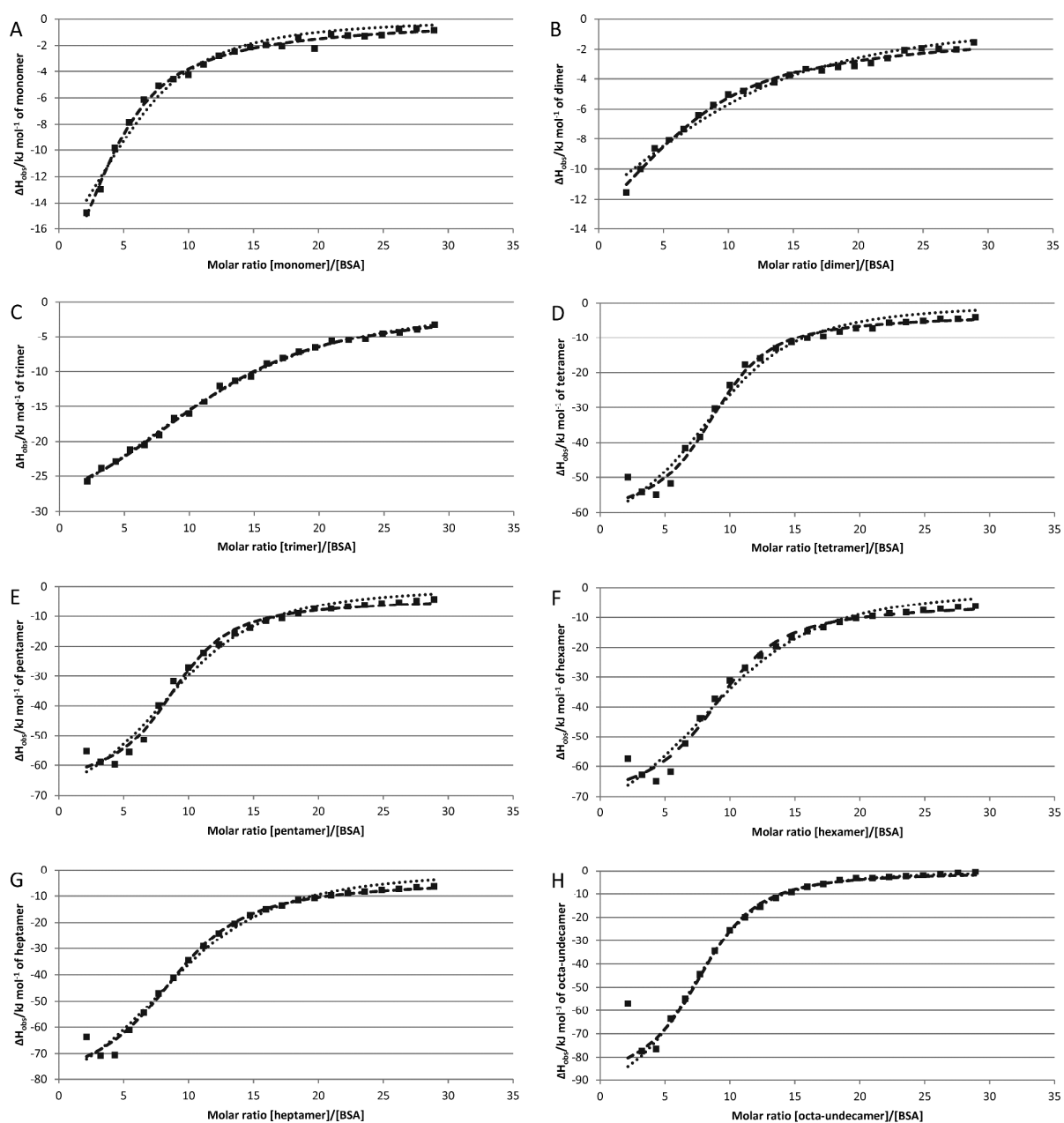
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Figure 2.



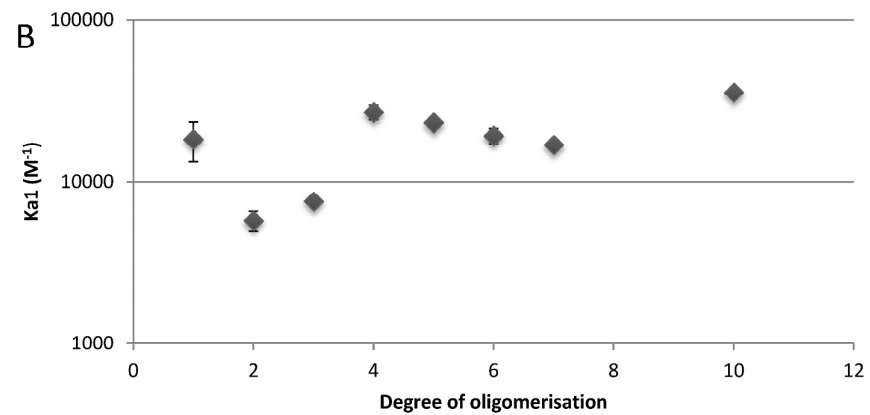
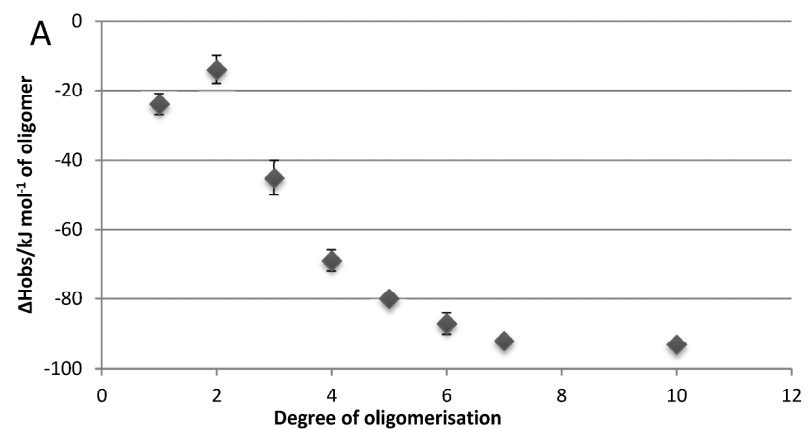
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Figure 3.



27

Figure 4.



Graphic for table of contents

