# *Protein precipitation behaviour of condensed tannins from Lotus pedunculatus and Trifolium repens with different mean degrees of polymerization*

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Accepted Version

**Protein Precipitation Behavior of Condensed Tannins from *Lotus pedunculatus* and *Trifolium repens* with Different Mean Degrees of Polymerization**

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**Abstract**

The precipitation of bovine serum albumin (BSA), lysozyme (LYS) and alfalfa leaf protein (ALF) by two large- and two medium-sized condensed tannin (CT) fractions of similar flavan-3-ol subunit composition is described. CT fractions isolated from white clover flowers and big trefoil leaves exhibited high purity profiles by 1D/2D NMR and purities >90% (determined by thiolysis). At pH 6.5, large CTs with a mean degree of polymerization (mDP) of ~18 exhibited similar protein precipitation behaviors and were significantly more effective than medium CTs (mDP ~9). Medium CTs exhibited similar capacities to precipitate ALF or BSA, but showed small but significant differences in their capacity to precipitate LYS. All CTs precipitated ALF more effectively than BSA or LYS. Aggregation of CT-protein complexes likely aided precipitation of ALF and BSA, but not LYS. This study, one of the first to use CTs of confirmed high purity, demonstrates that mDP of CTs influences protein precipitation efficacy.

Keywords: Condensed tannin, proanthocyanidin, protein precipitation, tannin-protein complexes, nuclear magnetic resonance spectroscopy, NMR, thiolysis

## Introduction

Condensed tannins (syn. proanthocyanidins) are polyphenolic secondary metabolites which are distinguished, along with hydrolysable tannins, from other plant polyphenols by their ability to precipitate proteins. Condensed tannins (CTs) are comprised of oligomeric and polymeric chains of flavan-3-ol subunits.[1,2] The most common subunits occurring in forage plants are comprised of catechin and epicatechin which give rise to procyanidin tannins (PC) and gallocatechin and epigallocatechin which give rise to prodelphinidin tannins (PD), as shown in Figure 1. The relative stereochemistry of the C-2 and C-3 substituents in the C-ring of these subunits also comprises a defining structural feature of these polyphenols with C2/C3 possessing a *cis* orientation in epicatechin and epigallocatechin and a *trans* orientation in catechin and gallocatechin. Connection of the subunits in chains of various lengths occurs most commonly through the C4-C8 interflavan linkage (classified as a B-type linkage, Figure 1) along with other less common linkages. Taken collectively, all of the structural variation possible from the combinations of different flavanol subunits, connected together with different linkage patterns and produced in varying polymer lengths, leads to millions of potential CT structures present in a CT-containing forage. The resulting CT mixtures in plants can also be characterized by their average polymer size or mean degree of polymerization (mDP). Combinations of different CTs can serve as characteristic fingerprints of different plants or accessions.[3]

The use of CT-containing forages can have a significant impact on ruminant health and productivity and is believed to primarily occur via interactions with proteins.[4-6] Interactions of CTs with proteins can generate important agricultural effects such as protecting forage protein during ensiling and rumen digestion,[7-10] reducing greenhouse gas emissions from ruminants,[11,12] reducing urea and ammonia excretion,[13] preventing bloat[14] and conferring natural anti-parasitic properties.[15] Thus, the use of CT-containing forages and other plant material containing CTs can

impact positively on farm economic and environmental sustainability. Understanding the formation of tannin-protein complexes[16] and their subsequent co-precipitation remains an ongoing endeavor in theory development of how these secondary metabolites impart these positive effects on ruminant animal production systems.[17-19]

Fundamental understanding of how CTs work in these regards remains elusive. CT-protein complex/precipitate formation is interdependent on structure of both the CT[20,21] and the protein,[21-23] pH of the medium,[24] the pI of the protein,[24,25] and the tannin-protein molar ratios.[26-28] The dependency of CTs to precipitate proteins based on molecular weights was first described by Bate-Smith[29] observing that protein precipitation increased regularly in the dimer to tetramer series and beyond. Additional studies leading to the same conclusion include inhibition of methane production parallel with molecular weight increases of CT fractions from *Leucaena* in *in vitro* rumen fermentation studies[30] and binding to BSA[31] although in these cases molecular weight did not appear to be the sole factor in determining protein binding affinity.[31,32] To add to the confusion, mixed results of CT size versus protein precipitation capability were seen even across *Leucaena* genotypes.[33] Others have reported conflicting results for protein precipitation. For example, Naumann et al.[34] reported that mDP was not a factor in methane production from *in vitro* fermentation studies or in protein precipitation studies.[35] Thus, it is clear that the question of the correlation of CT mDP on formation of CT-protein complexes and precipitates and their ramifications on biological activities remains unresolved.

Progress in this area has been hampered by the difficulty in obtaining sufficient quantities of highly pure and well characterized CT fractions. Researchers are now starting to perform the necessary purification and characterization of isolated CTs to aid in the unraveling of the intricacies of CT-protein interactions. Noteworthy in this regard is a recent report by Harbertson

et al.[36] which utilized HPLC-purified cocoa CT, consisting entirely of epicatechin (PC) subunits, into singular DP fractions of trimer to octamer oligomers. These authors reported that each individual CT fraction exhibited a linear response in the precipitation of BSA with increasing CT concentration and protein precipitation increased with DP of the CT fractions. In addition, mixtures of the trimer with pentamer through octamer oligomers generally showed an additive effect on precipitation of BSA from solution. However, the authors noted that, in some cases, a synergistic effect was observed for precipitation of BSA when a non-precipitating level of trimer was added.

Whereas HPLC purification can provide pure CTs or mixtures of CT sharing the same degree of polymerization up to DP9,[36-38] and provides insightful information on structure-activity relationships of CT-protein precipitation, the purification process is costly, labor intensive and quantity limiting. These limitations hamper scale-up preparation of CTs for executing important, next-level *in vitro* studies, such as rumen and ensiling protein degradation studies and the investigation of ammonia and methane abatement by CTs. Larger quantities of CT are required to execute these studies. At this time and at the current level of purification technology, these limitations force the use of CT mixtures isolated from plant sources containing a range of DP in each fraction to perform these *in vitro* studies.

When larger quantities of CT fractions have been obtained the purities of the CT fractions were generally not determined and impurity profiles are often not known. The fact that other plant components can interfere with CT-protein complex formation, especially carbohydrates,[4] incorporates a realm of uncertainty in studies using fractions of unknown purities and may have led to confounding results. McNabb et al.[24] pointed this out, noting that their preparations should be referred to as simply “CT extracts” due to the lack of any purity assessment and their

unknown impurity profile. Thus, alternative purification protocols for producing sufficient quantities of well-characterized CTs of high purity are essential for laboratory and *in vitro* experimentation to obtain unambiguous results.

We have initiated a program directed toward isolation of highly pure and well-characterized CT fractions with the goal of utilizing information gained in laboratory and *in vitro* investigations to further our understanding of CT-protein interactions. Here we describe the protein precipitation profiles of two paired sets of purified CT fractions of similar composition against three different protein types. The CT fractions isolated from *Lotus pedunculatus* (big trefoil) leaves and *Trifolium repens* (white clover) flowers possess similar composition (PC/PD and *cis/trans* ratios) but differ two-fold in their mean degrees of polymerization (mDP ~9 versus mDP~18). We selected two commercially available proteins with different pI values, bovine serum albumin (BSA) and lysozyme (LYS) as test proteins. To provide protein material relevant to ruminant animal production systems, we selected alfalfa leaf protein extract (ALF), whose main protein is Rubisco.[39]

## Materials and Methods

**Reagents.** Lysozyme and bovine serum albumin were purchased from Sigma (St. Louis, MO), the Pierce 660 nm Protein Assay Reagent from Thermo Scientific (Rockford, IL) and Sephadex LH-20 and G-25 from GE Healthcare Biosciences (Piscataway, NJ).

**Plant Materials.** In 2013, flowers from naturalized populations of white clover were harvested from lawns in Viroqua, WI USA and in Reading, England, frozen at – 20 ºC, and subsequently freeze-dried, ground in a cyclone mill (1 mm screen), and stored frozen until used. In 2009, herbage of 45-d-old summer regrowth of big trefoil was harvested from established stands near

Prairie du Sac, WI and frozen at – 20 ºC. After freeze drying, herbage was shaken to recover leaves, which were ground with a cyclone mill (0.5 mm screen) and stored frozen until used.

**General Procedure for Purification and Analysis of Condensed Tannins.** The isolation and purification of condensed tannin fractions BT-Medium and WC-Medium were accomplished by using the previous published procedures.[40,41] . Briefly, the ground plant material was extracted with acetone/water (7:3) three times and filtered. The combined filtrates were concentrated on a rotary evaporator (<40 ºC), extracted with dichloromethane to remove non-polar components, and the resulting aqueous solution was freeze-dried. The freeze-dried extracts were dissolved in water, applied to a Sephadex LH-20 column and the column was rinsed with water to remove carbohydrates. Then CTs were eluted with acetone/water (3:7, v/v; Fraction 1) and acetone/water (1:1; Fraction 2), with fraction 2 providing CTs of high purity for the BT-Medium and WC-Medium preparations, from ground big trefoil leaves and white clover flowers, respectively, used in this study. For the isolation of CT fractions BT-Large and WC-Large, the freeze-dried acetone/water (7:3) extracts were obtained in a similar manner, but a different elution scheme was used. This scheme consisted of adsorbing the 7:3 acetone/water extraction residue onto Sephadex LH-20 in methanol/water (1:1), and then eluting the resin with the following series of solvents, first with methanol/water (1:1) and then with a series of acetone/water mixtures (1:1, 7:3, 9:1). As a representative example, details of the purification of CT from big trefoil leaves (BT-Large) are given here. Dried and ground big trefoil leaves (70.2 g) were transferred to a 1L Erlenmeyer flask, equipped with a large magnetic stir bar. The mixture was rapidly stirred with 7:3 (v/v) acetone/water for 30 min (3 x 700 mL), and filtered through a Buchner funnel. Combined extracts were concentrated on a rotary evaporator at 35 ºC to remove acetone and then washed with dichloromethane (2 x 500 mL). Traces of dichloromethane were removed by rotary

evaporation and the material was freeze-dried to yield 19.2 g of solids. The solids were transferred to an 800 mL beaker, dissolved in 1:1 methanol/water (200 mL), and then Sephadex LH-20 (60 g) was added in small portions with stirring until a slurry was formed with consistency of wet sand. The slurry was transferred to a 600 mL sintered-glass funnel equipped with a filter paper. The Sephadex LH-20 bed was sequentially suspended in the washing solvent, allowed to stand for 5-10 min, and vacuum filtered with the following series of solvents. Solvent washings were pooled into fractions as follows: Fraction 1 (BTF1), with methanol/water (1:1; 4 x 250 mL); Fraction 2 (BTF2), with methanol/water (1:1; 2 x 250 mL); Fraction 3 (BTF3), with methanol/water (1:1; 2 x 250 mL); Fraction 4 (BTF4), with acetone/water (1:1; 4 x 250 mL); Fraction 5 (BTF5), with acetone/water (7:3; 4 x 250 mL); Fraction 6 (BTF6), with acetone/water (9:1; 4 x 250 mL). After removal of volatile organics by rotary evaporation, small aliquots of all of the fractions were freeze-dried and subjected to $^{1}$H NMR analysis. Fractions deemed reasonably pure from this analysis (little or no evidence of carbohydrate signals) were forwarded to $^{1}$H-$^{13}$C HSQC NMR analysis. Inspection of the $^{1}$H-$^{13}$C HSQC NMR spectra of each fraction allowed a qualitative assessment of purity based on the absence of significant non-CT cross-peak signals. Based on these evaluations, BTF1 was discarded, BTF2 and BTF3 were set aside pending further purification, and fractions BTF4, BTF5 and BTF6 were freeze-dried. Yields of solids from fractions BTF4, BTF5 and BTF6 were 1.51 g, 0.809 g and 0.020 g, respectively. Fractions BTF4 and BTF5 were forwarded to thiolysis[3,40] for purity and compositional analysis. Herewith, fraction BTF5 is referred to as BT-Large (Tables 1 and 2 and Figures 2-4).

**NMR Spectroscopy.** $^{1}$H, $^{13}$C and $^{1}$H-$^{13}$C HSQC NMR spectra used for screening of CT fractions were obtained on a Bruker Avance 360 instrument (Bruker Corporation, Billerica, Massachusetts) operating at 360 MHz ($^{1}$H) and 90 MHz ($^{13}$C). Spectra obtained were referenced

to the center residual signals of acetone-$d_6$ contained within the 4:1 $D_2O$/acetone-$d_6$ mixture, at 2.04 and 29.8 ppm for $^1H$ and $^{13}C$, respectively. Spectra were obtained using standard Bruker pulse programs (programs zg30, zgpg30, and invietgpsi for $^1H$, $^{13}C$ and $^1H$-$^{13}C$ HSQC NMR spectra, respectively). $^1H$, $^{13}C$ and $^1H$-$^{13}C$ HSQC NMR spectra for the CT fractions used in the precipitation studies were recorded at 27 °C on a BrukerBiospin DMX-500 ($^1H$ 500.13 MHz, $^{13}C$ 125.76 MHz) instrument equipped with TopSpin 2.1 software and a cryogenically cooled 5-mm TXI 1H/13C/15N gradient probe in inverse geometry. Spectra were recorded in DMSO-$d_6$/pyridine-$d_5$ (4:1) mixtures and were referenced to the residual signals of DMSO-$d_6$ (2.49 ppm for $^1H$ and 39.5 ppm for $^{13}C$ spectra). $^{13}C$ NMR spectra were obtained using 5K scans (acquisition time 4 h 30 min each). For $^1H$–$^{13}C$ HSQC experiments, spectra were obtained using 128 scans (acquisition time 18 h 30 min each) obtained using the standard Bruker pulse program (programs zg30, zgpg30, and hsqcegtpsi for $^1H$, $^{13}C$ and $^1H$-$^{13}C$ HSQC NMR spectra, respectively).

**Thiolysis determination of CT fraction composition and percent purity.** Thiolysis was conducted as previously described.[3,40] Briefly, approximately 8 mg of purified CT fractions were subjected to thiolysis with concentrated HCl in methanol in the presence of benzyl mercaptan providing the extender units of the CT as their benzyl mercaptan adducts and terminal units as underivatized flavan-3-ols. The resulting mixture was then analyzed by LC-MS on an HPLC Agilent 1100 series system and API-ES instrument Hewlett Packard 1100 MSD Series (Agilent Technologies, Waldbronn, Germany). Separation of mixture components was accomplished on an ACE $C_{18}$ column (3 μm; 250 x 4.6 mm; Hichrom Ltd; Theale; U.K.) fitted with a guard column at room temperature using a flow rate of 0.75 mL/ min. The HPLC eluent consisted of mixtures of 1% acetic acid in water (solvent A) and HPLC-grade methanol (solvent

B) utilizing the following gradient program: 0-52 min, 36% B; 52-60 min, 36-50% B linear; 60-65 min, 50-100% B linear; 65-73 min, 100-0% B; 73-80 min, 0% B. Chromatograms were recorded at 280 nm, and mass spectra were recorded in the negative ionization scan mode. The percent of each benzyl mercaptan adduct was calculated based on molar response factors determined relative to the internal standard taxifolin. Percent purity was calculated through summation of the total mass of all terminal and extension flavan-3-ol units divided by the initial mass of the analyzed material (g total flavan-3-ols/100 g CT fraction x 100).

**Protein Precipitation Assay.** Bovine serum albumin (BSA) and chicken egg white lysozyme (LYS) were dissolved in 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 6.5 (with NaOH) to a concentration of 10 mg/mL. Alfalfa leaf protein extract was prepared and desalted using Sephadex G-25 spin columns as described previously[42] except that the buffer used for extraction and spin column equilibration was 50 mM MES, pH 6.5. Protein concentration of the desalted alfalfa leaf extract was determined using the Pierce 660 nm Protein Assay Reagent with BSA as the standard and varied from 2.2 to 3.0 mg/mL. Small aliquots of the protein solutions (500 and 1700 μL for the pure proteins and the alfalfa extract, respectively) were flash frozen in liquid nitrogen and stored at -80 °C until needed.

A master stock solution of each CT fraction was prepared by weighing out approximately 20 mg of purified CT and dissolving to a final concentration of 10 mg/mL in ethanol. Further working stock solutions were prepared in ethanol from the master stock to give 5.00, 3.75, 2.50, 1.88, 1.25, 0.95, and 0.62 mg/mL solutions. For each protein tested, 20 μL of each CT working stock solution (or buffer control for no CT) was pipetted into 1.7 mL microfuge tubes in duplicate. A no-protein control series was also carried out for each CT fraction. The CT samples were dried down in a centrifugal vacuum concentrator (Eppendorf Concentrator 5301) and re-

dissolved in 50 mM MES, pH 6.5 (80 μL for BSA and LYS precipitation assays, 10-20 μL for ALF assays depending on the protein concentration of the extract). Previously frozen protein stock solutions were thawed and added to the CT in each tube to give a final reaction volume of 100 μL and a final protein concentration of 2 mg/mL (20 μL for BSA or LYS, 80 to 90 μL for ALF, depending on the protein concentration of the extract). The precipitation reactions were incubated for 10 min on ice then centrifuged for 5 min at 20,000 x g. A sample of each resulting supernatant (20 μL) was added to 30 μL of 50 mM MES (pH 6.5). Protein present in the supernatant samples was determined by adding 750 μL Pierce 660 nM Protein Assay Reagent and measuring absorbance at 660 nm using a Beckman DU800 spectrophotometer (Beckman-Coulter, Brea, CA). In pilot experiments we found that the Pierce 660 Protein Assay Reagent was linear over the range of protein concentrations being examined and showed virtually no reactivity with the tannin preparations tested. The duplicate data points were averaged and data for each tannin/protein combination were analyzed by fitting a curve to the data using Prism 5. Because in pilot experiments protein could be quantitatively precipitated by tannins, an inhibitor dose-response curve (log [tannin concentration] versus normalized response with variable slope) was the non-linear regression curve fitting model selected. Fitted curves had r-squared values of >0.95, except for BSA with medium mDP CT, which had r-squared values of >0.87 and >0.89 for big trefoil and white clover tannins, respectively. This approach allowed determination of an IC50 (in this case the tannin concentration at which half the protein was precipitated, hereafter referred to as PP50). The entire experiment was replicated (for $N = 2$) using independently prepared master stock solutions of each tannin.

**Statistical Analysis.** The replicated PP50 values were subjected to a mixed model analysis in a randomized complete block design by running PROC MIXED (SAS, 2014). Protein, CT, and

their two-way interactions were considered fixed effects, while replicates ($N = 2$) from two independent experiments and their interactions with fixed effects were considered random effects. If $F$-tests were significant ($P \leq 0.01$), then least square means of fixed effects were compared at $P = 0.01$ using a $t$-tests performed by a SAS pdmix800 macro.[43] Unless noted otherwise, treatment differences described in the text were significant at $P = 0.01$.

## Results and Discussion

**Determinination of Purity and Composition of CT Fractions by NMR and Thiolysis.** In previous studies, CT bound to Sephadex LH-20 or other resins were typically washed with selected polar protic solvent mixtures, eluted with acetone-water, and apparently used in protein precipitation studies without any assessment of purity. Unknown variations in CT purity may therefore account for the conflicting results from previous studies examining the effects of CT structure on protein precipitation. When using such isolation procedures, we have found that acetone-water fractions contain between 30 and 80% CT (unpublished data) with the balance comprised mainly of carbohydrate impurities. As carbohydrates do not possess chromophoric entities, they would not be observed if commonly employed UV detectors were used to track CT elution from chromatography columns. In addition to introducing errors through hitherto unaccounted for variations in CT purity, carbohydrates and carbohydrate derivatives can also bias CT-protein precipitation results by competing with proteins during CT complexation.[44-46] Thus, the need for highly pure CT fractions, free of significant carbohydrate impurities, seems a prerequisite for obtaining meaningful results from CT-protein precipitation studies. Hence, for our protein precipitation studies, we opted to use only CT fractions with purities exceeding 90% as determined by thiolysis. The results of thiolysis analysis for PC/PD and *cis/trans* ratios, and

mDP of the four CT fractions used in the protein precipitation studies are presented in Table 1. The percent purity provided by thiolysis for the CT fractions were: BT-Medium (91.3 ±0.4); BT-Large (BTF5, 108.0 ±0.1); WC-Medium (106.6 ±5.1); and WC-Large (120.6 ±0.9). Occasionally, purity determined by the thiolysis method exceeds 100% especially when analyzing samples of very high purity. This is one identifiable flaw in the method and may be due to the cumulative errors in the summation of multiple HPLC peaks. The presence of co-eluting, undetected compounds is unlikely as NMR analysis confirmed the high purity of all fractions. Figure 3 provides the $^{1}H$-$^{13}C$ HSQC NMR spectra for the CT fractions used in the precipitation studies. The absence of any significant non-CT related cross-peak signals imply high purity of these samples. Cross-peak signals (see Figure 2 for assignments) for H/C-4, H/C-6,8, H/C-2',6' (PD), H/C-2',5' (PC) and H/C-6' (PC) appear as compact contours. Clusters of cross-peaks are seen for C-2 and C-3 signals and are not the result of impurities present. These clusters arise from different H/C-2 and H/C-3 chemical shifts for *cis/trans* isomers, interflavan (C4-6 vs. C4-8) bond isomers and from terminal flavanol subunits. A comprehensive analysis of these spectra is currently underway in an effort to assign specific structural features to these cross-peak signals. Based on thiolysis, both plant species yielded a medium-sized CT (mDP ~9) and a large-sized CT (mDP ~18). Overall, the compositionally matched pairs of PD-based CTs with mDPs of ~9 and ~18 appear ideal for testing whether medium- and large-sized CTs differ in their capacity to precipitate protein.

**Protein Precipitation Studies.** The three protein preparations used in our precipitation studies were selected because their interaction with CT has direct relevance to protein utilization by ruminants or because they represent proteins with contrasting chemical and structural properties. Alfalfa leaf protein extract (ALF) represents the main group of soluble proteins that react with

CT in forage crops consumed by ruminants. The proteins of ALF are composed of approximately 70% Rubisco,[39] which is a large, loose globular protein with an acidic pI ~ 4.6.[47] The reaction of soluble proteins such as those in ALF with CT limits their degradation by plant proteases and by proteases produced by rumen microflora, and this potentially increases the amount of forage protein digested in the gastrointestinal tract for use in animal growth and production.[48,49] Like Rubisco, bovine serum albumin is a large, loose globular protein with an acidic pI ~ 4.7[50] that is commonly used in CT precipitation assays, in part because it is readily available from commercial sources. In contrast to ALF and BSA, LYS is a small basic protein with a pI =11.35[51] and has a compact, globular topology. We included LYS in the study to gain some insight into whether the relative reactivity of medium vs large sized CTs is markedly affected by the charge, size and structure of protein.

Precipitation studies were carried out in aqueous buffered solutions at a pH of 6.5, value that approximates the pH of freshly chopped or macerated alfalfa[52] and of rumen contents where excessive proteolysis of alfalfa protein commonly occurs. In the assay, proteins were incubated with various quantities of CT and protein precipitation was estimated by analyzing the supernatants with a protein assay reagent that does not react with CTs. The overall ability of CT to precipitate protein was determined by fitting the data to a dose-response curve to estimate PP50 (similar to previously described *b* values),[31,33] which we defined as the amount of CT required to precipitate 50% of the protein from a 2 mg/mL solution.

Overall, PP50 values suggested protein precipitation was significantly more effective with large CTs (mDP ~ 18) than with medium CTs (mDP ~ 9), and with ALF than with BSA or lysozyme (Table 2). The quantity of CT required to precipitate protein was, however, influenced by a highly significant ($P < 0.001$) protein X CT interaction. Within each protein type, large CTs

prepared from white clover or big trefoil had similar capacities to precipitate protein, but responses to medium CT from these plant sources differed with protein type. Medium CTs from both plant sources had a similar capacity to precipitate ALF or BSA, but medium CTs from big trefoil were more effective than those from WC for precipitating LYS. The mechanism responsible for these differing protein precipitation responses to medium CT is currently unknown.

All CTs precipitated ALF much more effectively than BSA or LYS, and among the proteins examined, ALF exhibited a relatively small albeit significant difference in PP50 for medium- vs. large CTs (Table 2). It has been previously shown that leaf protein extracts from white clover precipitate at lower CT concentrations than BSA.[24] The precipitation profiles of ALF in response to increasing CT concentrations were somewhat sigmoidal (Figure 3), suggesting that aggregation of CT-protein complexes may have slightly accelerated the precipitation process. This aggregation effect on CT-protein precipitation has been noted in previous studies.[53,54] Compared to BSA and LYS, Rubisco, the major component of ALF, is relatively unstable and disruption of its quaternary structure can cause its large subunit to precipitate out of solution even in the absence of tannins.[55] Thus, precipitation of ALF proteins by CT may be facilitated by the disruption of the quaternary structure of Rubisco, as was recently observed with the gallotannin pentagalloylglucose.[56] While needing confirmation, the results with ALF might also suggest that CTs aggregate and precipitate a mixed population of proteins more readily than individual proteins.

Differences in the PP50 values of BSA and LYS were quite modest considering the greatly differing pI and structural characteristics of these proteins. These two proteins, however, had markedly different precipitation profiles in response to increasing CT concentrations (Figure

3). The precipitation profiles of BSA were strongly biphasic or sigmoidal, suggesting that aggregation of CT-protein complexes accelerated the precipitation process. The precipitation profile of LYS, however, exhibited a linear response ($R^2 = 0.99$) to increasing CT concentrations. Examination of the amino acid sequences for existing lysozyme crystal structures from the Protein Data Bank[57] shows a high ratio of basic/acidic (>2) amino acid residues, translating to a high positive charge density of the resulting CT-lysozyme complex, which is bound to inhibit aggregation. Thus, using lysozyme in protein precipitation studies at pH 6.5 may remove complications in analysis due to aggregation. We did not pursue precipitation studies at higher pH ranges because CT fractions tend to oxidize at pH >8 in aqueous solutions.[58] Because LYS-CT complexes do not appear to undergo accelerated precipitation due to aggregation, CT precipitation studies with LYS might provide a way to more clearly distinguish how CT structural features such as PC/PD ratios, *cis*/*trans* ratios, and different inter-flavanol linkage types affect the formation of CT-protein complexes. For this reason, additional studies with LYS along with those of ALF and BSA are underway to further investigate how structural features of CT affect protein precipitation.

Coupled with results from Harbertson et al.[36] these studies clearly show that CT with higher mDP more readily precipitate soluble proteins. The synergy described for protein precipitation using well defined mixtures of cocoa CTs[36] may indicate that a combination of CT of varying lengths, as present in all CT-containing forages, facilitate precipitation through cooperative complexation and more readily promotes aggregation with the soluble forage proteins. This phenomenon was clearly observed in the current study as non-linear precipitation profiles for BSA and ALF, and is in contrast to the lower DP cocoa CTs' linear precipitation

profiles. These results may explain, to some extent, that having mixtures of large and small CTs provide a more powerful combination for generating insoluble protein complexes.

In conclusion, we have demonstrated that BSA, ALF and LYS proteins are more readily precipitated at pH 6.5 by larger-sized CTs (mDP of ~18) than by medium sized CTs (mDP of ~9) prepared from white clover and big trefoil. The confounding effects of other CT properties on protein precipitation were largely eliminated by using CT pairs that had comparable PD/PC and *cis/trans* ratios of subunits and high purity as determined $^{1}$H-$^{13}$C HSQC NMR and thiolytic degradation with benzyl mercaptan. Based on PP50 values, all CTs were very efficient at precipitating ALF proteins, possibly because the large subunit of Rubisco has a propensity to dissociate and self-aggregate. Such a dissociation could be facilitated by interactions with CT. The presence of other types of proteins in ALF might further promote aggregation and precipitation by CT. Compared to ALF, the precipitation of BSA and LYS were affected to a larger extent by the mDP of CTs and both proteins had relatively high PP50 values. Based on the biphasic or sigmoidal response to increasing CT concentrations, the precipitation of ALF and BSA was likely accelerated by aggregation of CT-protein complexes. By contrast, the linear response of LYS to increasing CT concentration suggests that aggregation was inhibited by the positive charge of this protein at pH 6.5. Studies with high purity CT exhibiting a wider range of mDP, PD/PC ratios and *cis/trans* ratios will be pursued to more thoroughly define how compositional and structural features of CT affect their ability to precipitate of various types of proteins under differing pH conditions.

**Acknowledgements**

This work was funded in part by a USDA-ARS specific cooperative agreement #58-3655-0-155F with the University of Reading, UK and was supported by a European Union Marie Curie Initial Training Network (PITN-GA-2011-289377, ('Legume-Plus'). The authors would like to acknowledge the technical assistance of Nicholas Sampalis and Abert Vang, and Jane Marita for assistance with NMR experiments. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

## Supplementary Materials

$^{1}$H and $^{1}$H-$^{13}$C HSQC NMR spectra (360/90 MHz) for residues from purified CT fractions BTF1 through BTF5 are provided in the supplementary materials. This information is available free of charge via the Internet at http: //pubs.acs.org.

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Figure 1. Structures of major flavanol subunits which occur in condensed tannins (left). A condensed tannin tetramer (right) showing a terminal unit with different extender units connected by C4-C8 (B-Type) linkages.

Figure 2. Signal assignments for the $^{1}H$-$^{13}C$ HSQC NMR spectrum (360/90 MHz, $D_2O$/acetone-$d_6$, 4:1) of a large-sized condensed tannin fraction purified from big trefoil (BT-Large). The purity of the fraction was 91.3% by thiolysis.

Figure 3. $^{1}H$-$^{13}C$ HSQC NMR spectra (500/125 MHz, DMSO-$d_6$/pyridine-$d_5$, 4:1) of large- and medium-sized condensed tannin fractions purified from white clover (WC) and big trefoil (BT).

Figure 4. Precipitation of bovine serum albumin (BSA), alfalfa leaf protein extract (ALF), or lysozyme (LYS) by two large- and two medium-sized condensed tannins (CT) isolated from big trefoil and white clover; big trefoil-medium -□-, big trefoil-large (BT-Large) -■-, white clover-medium -○-, and white clover-large -●-. The concentrations of CT are expressed on a mg per mL basis (left) and a Log [CT] basis (right). Graphs depict data from one of two experimental replicates.

Table 1. Composition of Large- and Medium-Sized Condensed Tannin Fractions Purified from White Clover (WC) and Big Trefoil (BT) as Determined by Thiolysis with Benzyl Mercaptan.

| **CT fraction** | mDP | SD | PC/PD ratio | SD | *cis/trans* ratio | SD |
|---|---|---|---|---|---|---|
| **WC-Large** | 17.4 | ±0.42 | 0.8/99.2 | ±0.01 | 69.2/30.8 | ±0.07 |
| **WC-Medium** | 9.30 | ±0.13 | 0.9/99.1 | ±0.04 | 58.3/41.7 | ±0.24 |
| **BT-Large** | 18.2 | ±0.33 | 16.0/84.0 | ±0.07 | 81.8/18.2 | ±0.22 |
| **BT-Medium** | 9.80 | ±0.01 | 26.0/74.0 | ±0.3 | 78.7/21.3 | ±0.23 |

mDP = mean degree of polymerization; PC/PD = procyanidin/prodelphinidin ratio; *cis/trans* = ratio of *cis* (epicatechin and epigallocatechin) and *trans* (catechin and gallocatechin) flavanols.

Table 2. PP50 Values* of Large- and Medium-Sized Condensed Tannin Fractions Purified from White Clover (WC) and Big Trefoil (BT) for Precipitation of Alfalfa Leaf Protein Extract (ALF), Bovine Serum Albumin (BSA), and Lysozyme (LYS).

| Protein | WC-Large (mDP 17.4) | WC-Medium (mDP 9.3) | BT-Large (mDP 18.2) | BT-Medium (mDP 9.8) | SE |
|---|---|---|---|---|---|
| | ----------mg CT per mg protein---------- | | | | |
| ALF | 0.254 b z | 0.305 a z | 0.253 b z | 0.322 a z | 0.0034 |
| BSA | 0.433 b y | 0.698 a y | 0.447 b y | 0.731 a y | 0.0116 |
| LYS | 0.500 c x | 0.744 a y | 0.490 c x | 0.600 b y | 0.0131 |
| SE | 0.0046 | 0.0145 | 0.0022 | 0.0138 | |

[a-c]Within rows, means with unlike letters differ at $P = 0.01$.

[x-z]Within columns, means with unlike letters differ at $P = 0.01$.

SE, Standard error of the mean

[*]PP50 values are defined as the amount of CT required to precipitate 50% of the protein from a 2 mg/mL solution.

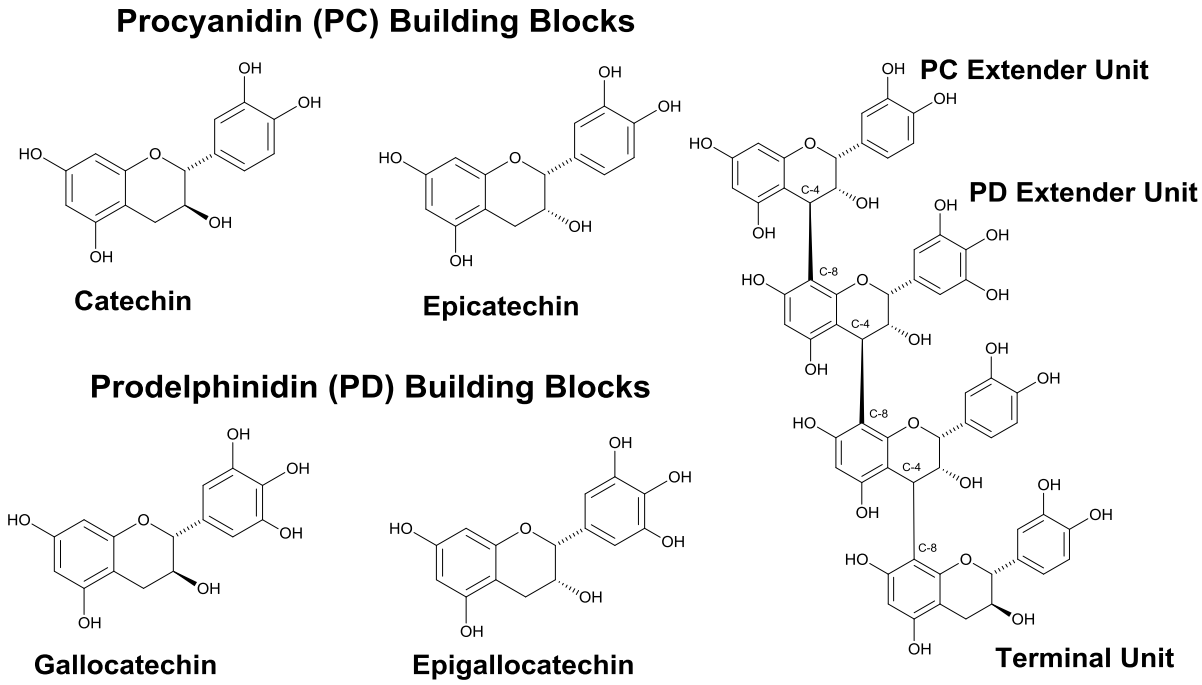
Procyanidin (PC) Building Blocks
Catechin
Epicatechin
PC Extender Unit
PD Extender Unit
Prodelphinidin (PD) Building Blocks
Gallocatechin
Epigallocatechin
Terminal Unit

Figure 1.

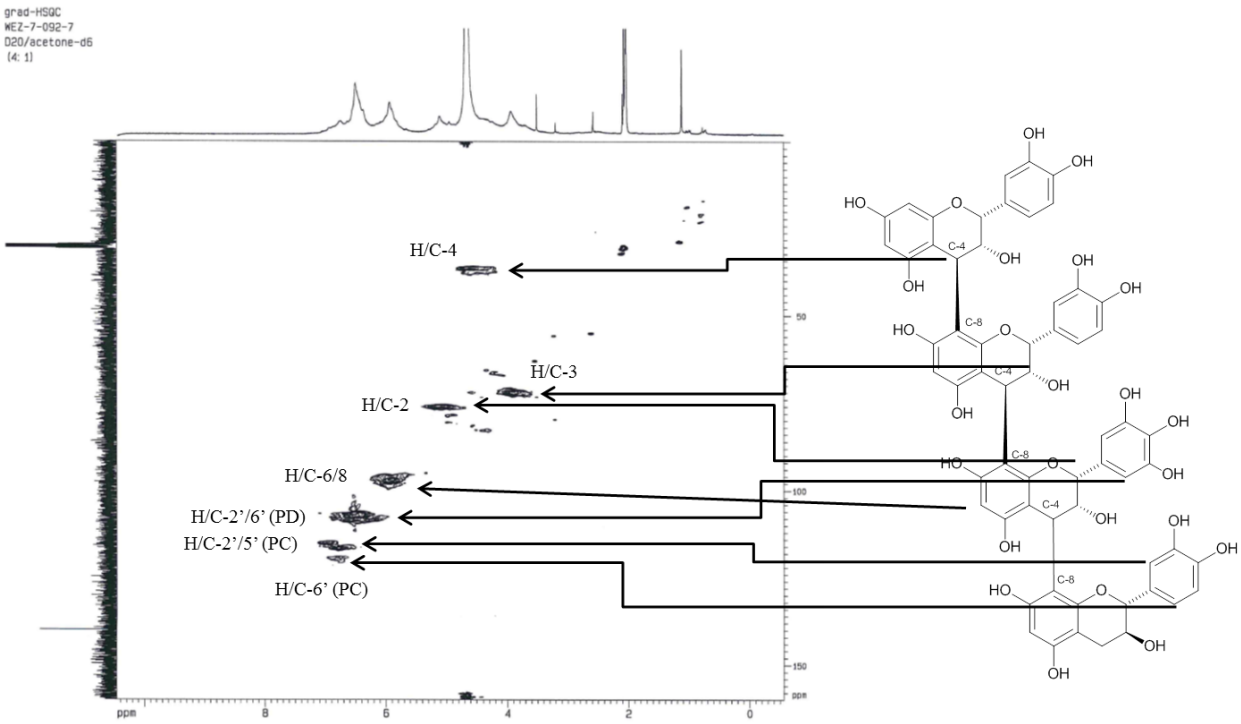
grad-HSQC
WEZ-7-092-7
D20/acetone-d6
(4: 1)
H/C-4
H/C-3
H/C-2
H/C-6/8
H/C-2'/6' (PD)
H/C-2'/5' (PC)
H/C-6' (PC)
ppm
8
6
4
2
0
50
100
150
OH
HO
O
C-4
C-8

Figure 2.

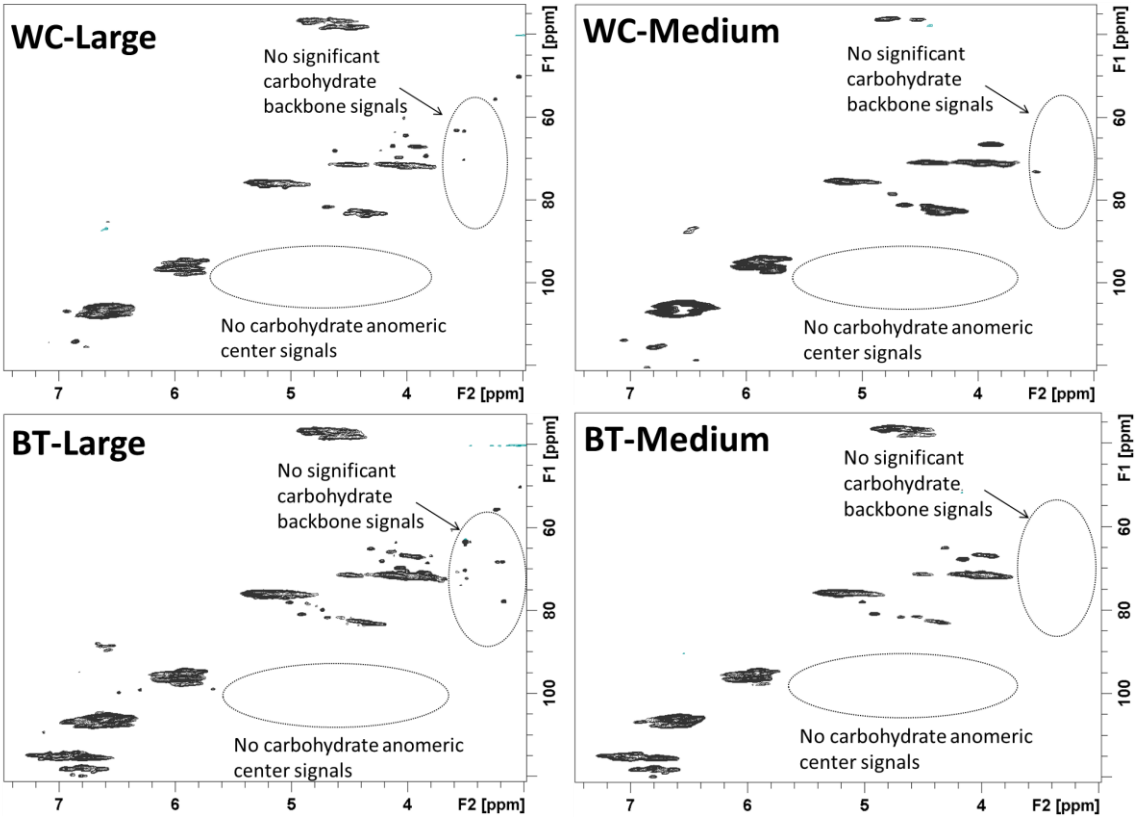
WC-Large
No significant carbohydrate backbone signals
No carbohydrate anomeric center signals
WC-Medium
No significant carbohydrate backbone signals
No carbohydrate anomeric center signals
BT-Large
No significant carbohydrate backbone signals
No carbohydrate anomeric center signals
BT-Medium
No significant carbohydrate backbone signals
No carbohydrate anomeric center signals
F1 [ppm]
F2 [ppm]
60
80
100
7
6
5
4

Figure 3.

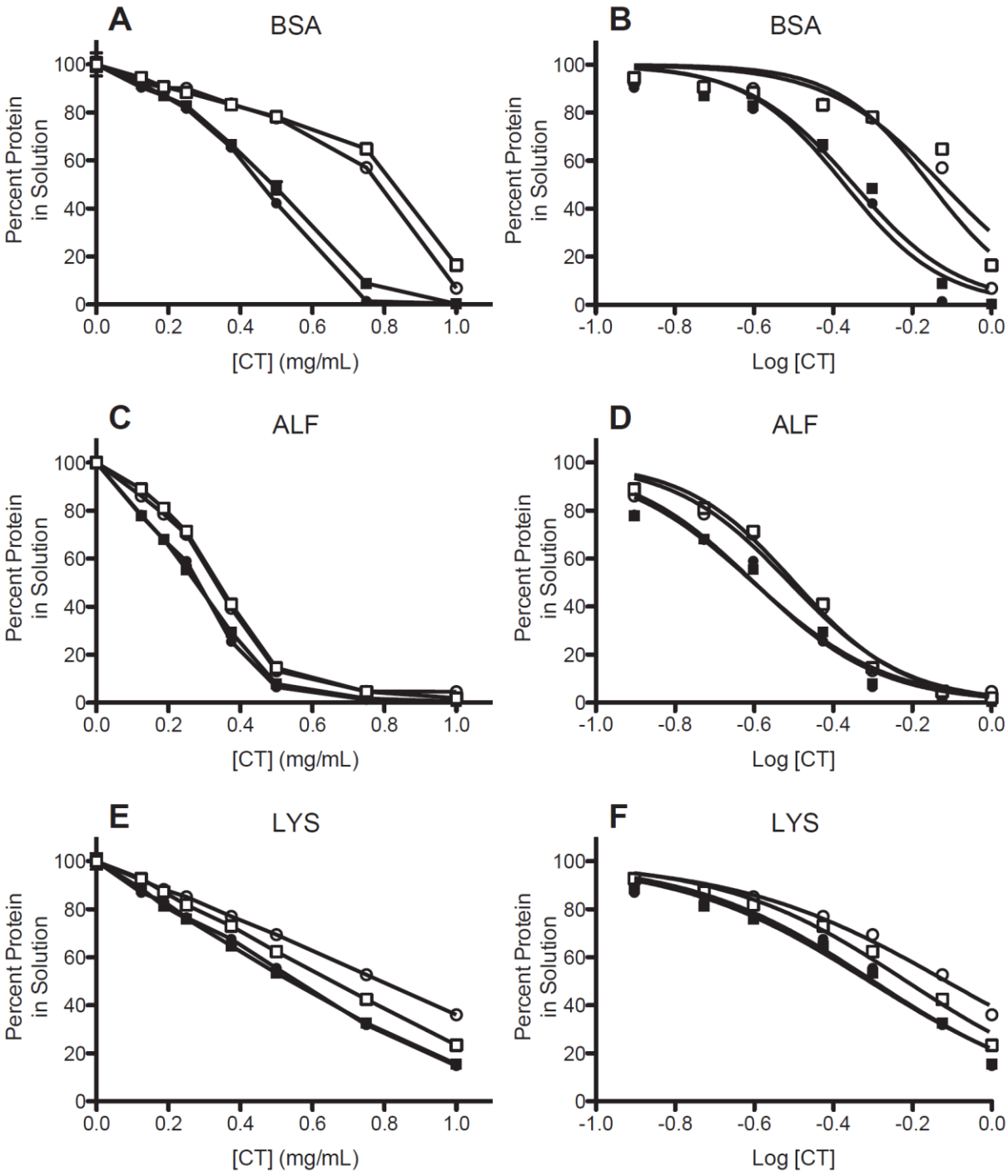
A
BSA
Percent Protein in Solution
[CT] (mg/mL)
B
BSA
Log [CT]
C
ALF
D
ALF
E
LYS
F
LYS

Figure 4.

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