The Structure and Nature of Protein-Polysaccharide Complexes

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Summary

Many food systems consist of mixtures of protein and polysaccharide and many are also thermally processed. Potential interaction phenomena in solutions of protein and polysaccharide are described using the following probes: sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation, dynamic light scattering and total intensity light scattering coupled to an on-line gel permeation chromatography system. Specifically the following mixtures were studied: (i) unheated and heated solutions containing mixtures of bovine serum albumin (BSA) and a highly characterised alginate; (ii) unheated and heated solutions of BSA with a well characterised pectin; (iii) solutions of dry heated mixtures of BSA with a dextran. In (i) a strong dependence on heating temperature is demonstrated; in (ii) no interaction is observed, rather the pectin itself appears thermally unstable; in (iii) the size of the complex appears to depend critically on the molar ratio of BSA to dextran.

1. Introduction

For many years our laboratory has been interested in the solution sizes, shapes and interactions of glycopolymers - that is glycoproteins and polysaccharides - but quite recently we have branched out into the relatively dangerous world of glycopolymer-protein interactions, and in this paper we will describe our work on three types of food protein-polysaccharide systems which we have been focussing on over the last eighteen months or so.

A fundamental complication when attempting to study possible interaction phenomena in protein-polysaccharide mixtures is that although one entity in the mixture is usually well characterised and well behaved - namely the protein component - the other, the polysaccharide, is not. Solutions of polysaccharides are usually highly non-ideal in the thermodynamic sense through exclusion volume and polyelectrolyte effects; they are also usually highly polydisperse (i.e. consist of non-interacting components of different molecular weight and density). Finally, some can also have the potential to perform self-association reactions in solution.

All these properties of polysaccharides make it very difficult to try and describe potential interactions with proteins in terms of stoichiometries and interaction constants - sometimes the best we can hope for is to semi-quantify average effects. This means that

when dealing with solutions of polysaccharides mixed with proteins we cannot use the so-called "high resolution" techniques of nmr, X-ray diffraction or computer molecular modelling but instead have to use the classical "low-resolution" solution techniques such as analytical ultracentrifugation and light scattering.

We have been looking at potential interactions in three types of mixture. First, unheated solutions of a well characterised alginate with bovine serum albumin (BSA). Secondly, mixtures of the same alginate and also pectin with BSA where the solution had previously been given various degrees of heat treatment. Finally we looked for interactions in a system where a dry mixture of a polysaccharide and a protein had been heat-treated prior to being brought into solution. It is worth pointing out that many food systems consist of mixtures of protein with polysaccharide and many are thermally processed, so seeing what happens under solution heating or dry heating conditions is, one would think, quite relevant. However we would like to stress that all the studies we have done so far on these systems have been under dilute solution conditions: it could well be the situation we see is quite different at higher concentrations.

2. Methodology

There are three principal "low-resolution" techniques we have used to study these interactions: analytical ultracentrifugation (in two formats), dynamic light scattering and finally total intensity (or classical) light scattering coupled to on-line GPC.

2.1. Analytical Ultracentrifugation

We used two types of analytical ultracentrifuge experiment. In "sedimentation velocity" the rate of movement of a sedimenting boundary is measured to obtain the sedimentation coefficient which is a function of the size, shape and interaction properties of the system. We use both absorption optics and "refractive index gradient" (or Schlieren) optics. At the much slower speeds in a "sedimentation equilibrium" experiment, which is used to measure molecular weights and - for protein systems - to quantify interaction parameters, we can record the final distribution of solute in the centrifuge cell using either Rayleigh interference optics or absorption optics (the latter is used if we are only focussing on the behaviour of the protein component of the mixture).

Three analytical ultracentrifuges were used for this work. (i) An MSE (Crawley, U.K.) Centriscan equipped with scanning absorption and scanning Schlieren optics. This is dedicated to sedimentation velocity work and is particularly useful for detecting interactions between species which have different chromophores by looking for co-sedimentation. Sedimentation coefficients were evaluated off line using a computer graphics digitising tablet interfaced to an Apple IIE microcomputer. (ii) A Beckman (Palo Alto, USA) Model E equipped with Rayleigh interference optics and a laser light source

and which is dedicated to sedimentation equilibrium measurements. Rayleigh interference solute distribution records are captured off line via an LKB laser densitometer and converted to a record of relative concentration versus radial distance via a UCSD PASCAL Fourier series PC routine ANALYSE2 [1]. These ASCII data are then passed onto a mainframe IBM 3084/Q and molecular weight analysis performed using a FORTRAN routine MSTAR (see, e.g. [2]). (iii) An Optima XL-A. This is the newest analytical ultracentrifuge from Beckman instruments [3] from whom we are privileged to have received an evaluation model. In this study, this instrument was used only for sedimentation equilibrium work with (protein) solute distributions recorded using absorption optics at 278 nm. ASCII records of solute absorbance versus distance from the interfaced PC were passed to the IBM 3084/Q and analysed as above.

2.2. Dynamic Light Scattering

For the dynamic light scattering we use a Malvern 4700 instrument with a 25 mW laser (see e.g. [4]) at a scattering angle of 90°. The data are analysed only to give simple apparent diffusion coefficient measurements at a temperature of 25°C from the slope of the log of the normalised autocorrelation function against delay time (or equivalently channel number). This gives a rough measure of particle size (without correction of the apparent translational diffusion coefficient to zero angle, zero sample time or zero concentration) and, from comparison of the goodness of linear and quadratic fits, a rough idea of sample polydispersity via the so-called "polydispersity factor" (see, e.g., [5]).

2.3. Total Intensity Light Scattering (TILS)/ Gel Permeation Chromatography (GPC)

The other light scattering instrument which we have used is a total intensity (or classical) light scattering photometer from Wyatt instruments (see, e.g., [6]). A cleverly constructed array of detectors allows simultaneous intensity measurements for a range of angles. More significant is the provision of a flow cell which allows the instrument to be coupled on-line to gel permeation chromatography - we use this total set-up as a second independent technique from sedimentation equilibrium for molecular weight work. The idea of a scattering cell as a flow cell coupled directly on-line to GPC separation columns and associated filters is in our opinion a superb one for (i) circumventing the well-known but, these days, ill-reported dust problem of light scattering and (ii) fractionating a heterogeneous system directly prior to molecular weight analysis. It appears to give reliable results - as checked by sedimentation equilibrium - but with only a fraction of the time and effort, as will be seen below.

3. Solution Heated Mixtures of BSA with an Alginate

We will first consider mixed solutions of a well-characterised protein - BSA - with a well characterised, highly purified and fractionated alginate polysaccharide called Pronova (Protan Ltd., Drammen, Norway). This is an alginate extensively studied by groups at Trondheim and Trieste [7] using light scattering and osmometry and by ourselves [8,9] using analytical ultracentrifugation and total intensity light scattering/gel permeation chromatography. We have a consensus molecular weight of just over ~200,000 for this substance. Solutions (each of concentration 2 mg/ml) of BSA (Sigma, Poole, UK) and alginate dispersed in a phosphate chloride buffer (pH=6.8, I=0.1) were mixed together in a 1:1 ratio and then studied either as made up or after being heated for 30 minutes at various temperatures. The aim was to search for changes in apparent molecular size, sedimentation coefficient or molecular weight and from these to obtain a qualitative idea of the strength of any interaction as a function of temperature. All the *measurements* were done at a temperature of 25.0°C.

3.1. Mean size

Mean size was estimated in terms of the effective hydrodynamic diameter from dynamic light scattering measurements as described above.

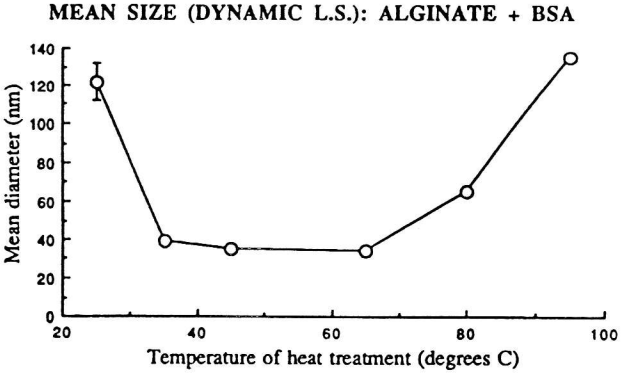

Fig. 1.
Effective hydrodynamic diameter of solution heated mixtures of BSA with pro-nova alginate. All measurements performed at a temperature of 25.0°C.

Fig. 1 shows an interesting trend of a drop in size after mild heat treatment compared with its value when no heat treatment was applied. Between treatment temperatures of 70 and 80°C the size increases again to over three times the previous size at a treatment

temperature of 95°C. The alginate control shows no change in effective diameter {~(70+10) nm} whereas the BSA control shows an increase from about 10 nm to about 50 nm beyond its denaturing temperature (~55°C). It would appear therefore *on the basis of this data alone* that we have an interaction with no heat treatment, destroyed by mild treatment - the bottom plateau level appears to be approximately a weighted average of the alginate and BSA species -until beyond the thermal denaturation temperature of the protein the interaction process becomes significant again.

3.2. Sedimentation velocity

Now you can never trust one technique in isolation in this business -especially if it's a light scattering one. Sedimentation velocity however appears to confirm the above observations. Consider for example a Schlieren diagram for the non-heat treated mixture (Fig. 2) showing two clear components, one sedimenting at 1.5 S, presumably unbound alginate, the other moving down much faster at 10.6 S, far faster than even dimeric BSA: and this is a complex. Indeed the sedimentation coefficient of the faster component measured at 25°C as a function of treatment temperature appears to reproduce the trend observed with the dynamic light scattering data, with a drop in s value for mild heat treatment and a steady increase beyond the thermal denaturation temperature of the BSA when presumably more potentially interactive groups are exposed.

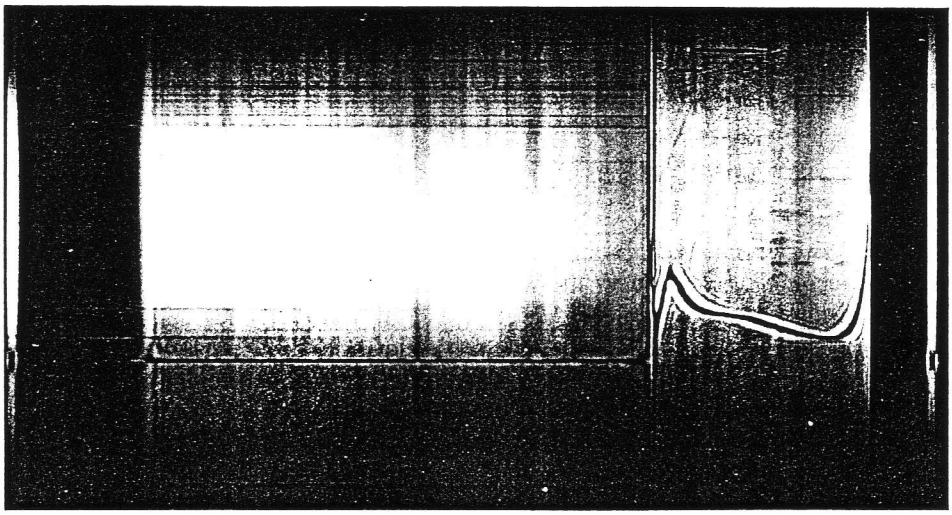

Fig. 2.
Sedimentation diagram for a solution containing 1 mg/ml BSA and 1 mg/ml pro-nova alginate, after no prior heat treatment.
Rotor speed 34790 rev/min. Direction of sedimentation is from left to right.

In fact, if we combine the sedimentation data with the dynamic light scattering diffusion data, then, notwithstanding the fact that we haven't corrected our results properly to standard solvent conditions or extrapolated to zero solute concentration, we can get a *relative* estimate of the weight average molecular weight of the mixtures' components. This is shown in Fig. 3 with values hovering around the unbound alginate value of ~200000 between temperatures of 35°C to 55°C reaching up to 10 times this value at either extreme.

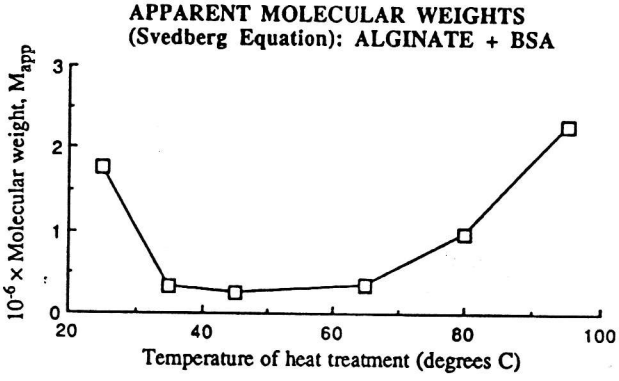

Fig. 3.
Apparent molecular weights of solution heated mixtures of BSA with pro-nova alginate evaluated from the Svedberg equation. All measurements performed at a temperature of 25.0°C.

4. Solution Heated Mixtures of BSA with a Pectin

If we replace the alginate by a highly characterised pectin { "GENU" pectin with a degree of esterification ~70% [10] } we get quite a different picture. The apparent mean size from dynamic light scattering drops and continues to drop with increasing temperature of heat treatment (Fig. 4a). We believe this proves that the interaction of BSA is more specific to alginate and that the pectin itself is thermally unstable as shown in the control of Fig. 4b. Perhaps a native pectin molecule is not a simple covalent entity but made up of smaller non-covalent basic units. This view appears to be confirmed by measurement of (apparent) molecular weights (at a loading concentration of ~0.5 mg/ml) as determined directly by low speed sedimentation equilibrium (Fig. 4c).

This marks the present extent of our work on heated solution mixtures. Obviously we need to look at a range of concentrations and for a range of solvent conditions (pH, ionic strength etc.) to understand better what exactly is happening.

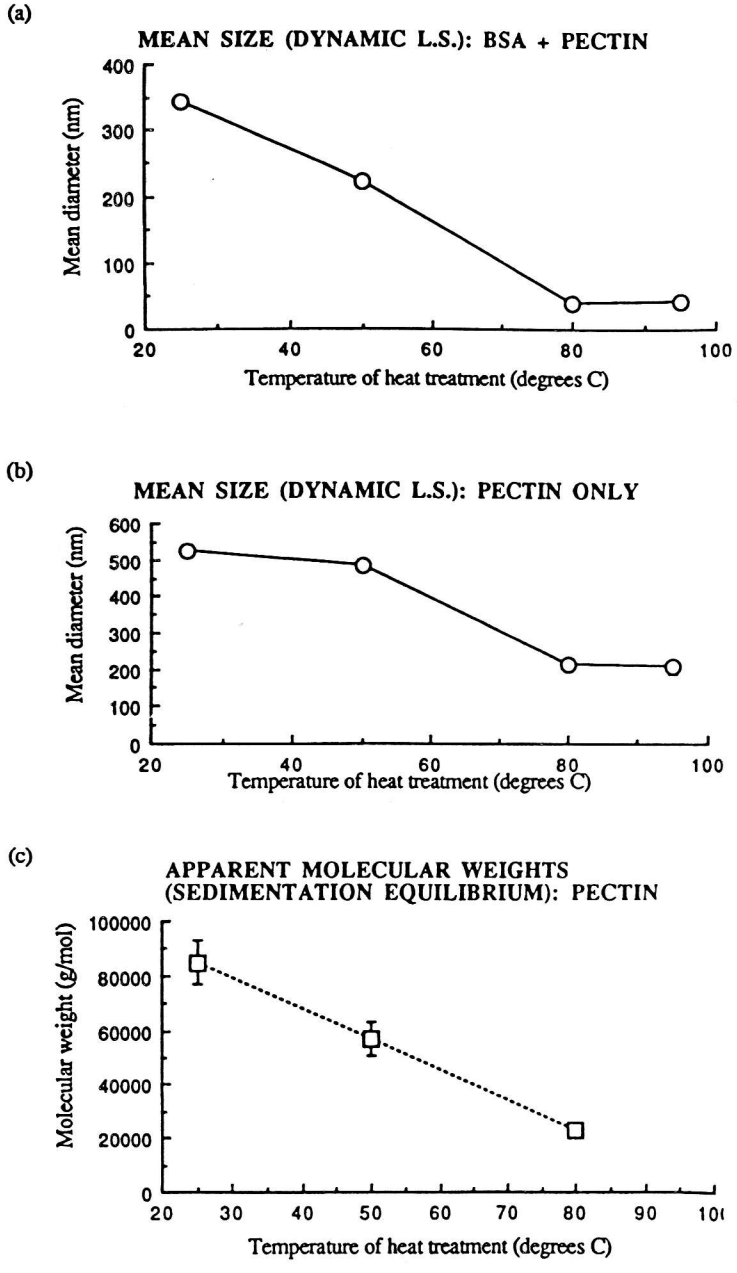

Fig. 4.
Effective hydrodynamic diameter of (a) solution heated mixtures of BSA with GENU pectin and (b) solution heated GENU pectin. (c) Apparent molecular weights (at a loading concentration of 0.5 mg/ml) for GENU pectin. All measurements performed at a temperature of 25.0°C.

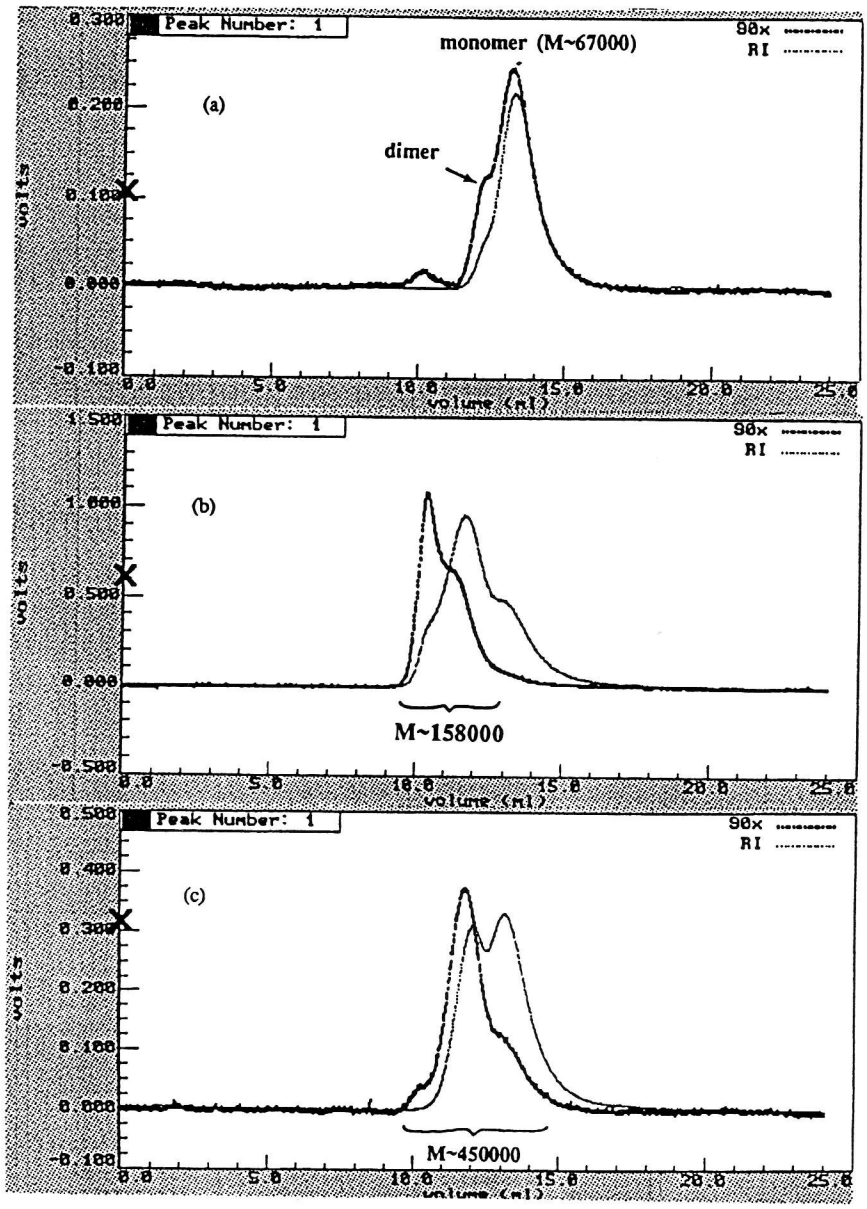

Fig. 5.
Total intensity light scattering/gel permeation chromatography 90° scattering angle intensity (solid lines) and refractive index (lighter lines) *versus* elution volume profiles for solutions of (a) native BSA (b) dry heated BSA
(c) dry heated mixture of BSA (T-40 dextran at a molar mixing ratio of 2:1.

5. Dry Heated Mixtures of BSA with T-40 Dextran

We will conclude this paper with a summary of our work on dry heated mixtures of BSA with another fairly well characterised but neutral polysaccharide called T-40 dextran. (The "T-40" means the commercial manufacturer thinks its molecular weight is ~40000.) This work is part of a collaboration now under way with Professor Eric Dickinson's group at Leeds [11,12] which is investigating the proposal originally made by Kato and co-workers (see, e.g., [13]) that by dry heating a protein with a polysaccharide such as a dextran its emulsification properties can be enhanced considerably. The procedure for dry heating is to incubate the mixture at various molar ratios for a period of three weeks at 60°C with the goal of finding the mixing ratio which gives the biggest complex, and seeing if this corresponds to the mixing ratio which gives the optimum emulsification properties as being investigated by Eric's group. All solution measurements were performed at 25°C in solvent of ionic strength 0.1 and pH 6.8 as above.

As with the solution heated mixtures it is important to perform the appropriate controls to determine the individual behaviour of BSA and dextran. Fig. 5a shows two traces from the Dawn-F total intensity light scattering-GPC for unheated BSA as a function of elution volume. One is the intensity of light scattered at an angle of 90° and the other is the corresponding refractive index (concentration) profile. Most of the BSA appears as monomer (with a molecular weight extrapolated from the angular intensity envelope of ~67000) with a smaller proportion of dimer. The same experiment on BSA that had been dry heated before being made up into dilute solution shows, as with the solution heated material before, a tendency to dimerise or form high molecular weight n-mers (Fig. 5b). The weight average molecular weight over the macromolecular distribution eluting from the columns (PSS Hema Biolinear and Hema Bio40 in series) comes out to 2-3 times the monomer value. Similar experiments on the T-40 dextran control yield weight average molecular weights of just under 40000 and no tendency to aggregate upon dry heating. Dry heated mixtures however show a clear disposition to aggregate over and beyond the small increase for the BSA, and this is shown in Fig. 5c for a 2: 1 molar ratio of BSA to dextran with the weight average molecular weight coming over the macromolecular components coming out as ~450000, averaged over several runs.

In fact the size of the complex appears to depend critically on the mixing ratio (rather like antigen-antibody interactions) with the 2: 1 ratio of BSA to dextran giving by far the largest size (Fig. 6) as based on the total-intensity light scattering / GPC data. This seems to be reproducible. Because of this rather remarkable value we've tried to check this using independent sedimentation methods. First of all using sedimentation equilibrium performed using the new Optima XL-A, we find a value for the apparent weight average molecular weight from the M* procedure [14] of ~330000 for the 2: 1 complex and ~130000 for heat treated BSA, both in good agreement with the light scattering results (bearing in mind that the sedimentation equilibrium results correspond to a loading

concentration of ~0.5 mg/ml and not corrected for thermodynamic non-ideality).

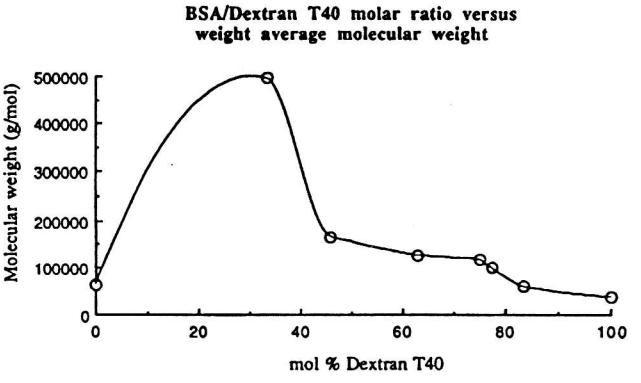

Fig. 6.
Molecular weight of BSAIT-40 complex as a function of molar mixing ratio.

This concept of a strong interaction at the mixing ratio of 2: 1 was confirmed by co-sedimentation experiments (see, e.g., [15]) using sedimentation velocity and a mixture of Schlieren and absorption optics. Finally, Table 1 summarises the clear difference in size between the 2:1 dry heated mixture compared with native and heat treated BSA and native and with heat treated T-40 dextran as supported by two independent techniques, namely light scattering and analytical ultracentrifugation.

With that philosophical statement of the importance of the virtue of combining results from independent techniques together when handling tricky systems such as these, we close this paper.

Table 1. Dry heated BSA dextran T-40 mixture - molecular weights and sedimentation coefficients.

Sample	M_w (g/mol) TILS/GPC	M_w (g/mol) Sed. eqm.	s_{20} (S)	$s_{20,w}$ (S)
BSA				
native	62700 ± 5000	66700[a]	3.93 ± 0.1	4.04 ± 0.1
head treated	158000 ± 10000	130000 ± 10000	5.43 ± 0.1	5.59 ± 0.1
BSA/T40	450000 ± 20000	330000 ± 20000	4.85 ± 0.1	4.98 ± 0.1
2:1 ratio	37100 ± 3000	-	~2.0	~2.0
T40	37300 ± 3000	-	-	-
native				
heat treated				

[a] Value from Physical Biochemistry, K.E. van Holde, Prentice-Hall, 1971;
[b] For 3 weeks at 60°C.

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