Gastrophysics: Statistical thermodynamics of biomolecular denaturation and gelation from the Kirkwood-Buff theory towards the understanding of tofu

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**ABBREVIATIONS**

KB, Kirkwood-Buff.

**ABSTRACT**

Sugars, alcohols, or salts, when added to food, affects the heat denaturation of proteins and the sol-gel transition of macromolecules. Such an effect of cosolvents has long been known and exploited; yet understanding how they work at a molecular level has been a matter of scientific debate for decades, because of the lack of a definitive theory which can provide a microscopic explanation. Here we show that a rigorous statistical thermodynamic theory, the Kirkwood-Buff (KB) theory, provides not only a long-awaited microscopic explanation but also a clear guideline on how to analyze experimental data. KB theory synthesizes the classical Wyman-Tanford formula and partial molar volume, and enables the determination of biomolecule-water and biomolecule-cosolvent interactions solely from experimental data. Nothing beyond the materials in introductory physical chemistry or chemical thermodynamics textbooks is necessary to follow the derivations presented in this review.

**1. Statistical thermodynamics for food science: why necessary?**

Our aim is to convince the readers that statistical thermodynamics is indeed a useful tool for food science. This is especially true, when we try to understand what is really happening at a molecular scale. Molecular-based understanding is central to food science, because it attempts to elucidate the texture and taste of food based upon its microscopic behavior, i.e., the structure and interaction of the constituent molecules (de Man, 1999; Walstra, 2003; Belitz, Grosch, & Schieberle, 2009). Statistical thermodynamics, then, is indispensable, because it is the only branch of science which can provide a link between the microscopic and macroscopic worlds (Hill, 1956; Ben-Naim, 2006).

Applying statistical thermodynamics to complex systems such as food is far from being straightforward. Most commonly, two strategies have been adopted: (i) computer simulation (Barker & Grimson, 1989; Euston, Ur-Rehman, & Costello, 2007; Fundo, Quitas, & Silva, 2015) and (ii) development of simple models (van der Sman, 2016; van der Sman et al., 2013). Simulations (such as molecular dynamics and Monte Carlo) implement statistical thermodynamics numerically. Simple model-based approaches are drawn chiefly from the models of polymers, surfaces, and colloids. The crux of the both approaches lies in the elegance of approximations, aimed at grasping the essence of molecular structure and interactions out of the overwhelming complexity of food systems.

In contrast to the above, we take an alternative approach: (iii) rigorous theory as a tool to extract molecular-level information from thermodynamic data. This approach is distinct from (i) and (ii) in that certainty, credibility and clarity of interpretation are guaranteed by the rigorous nature of the theory, because the theory comes directly from the Laws of Physics (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014c; Shimizu, 2015; Stenner et al., 2016; Shimizu & Abbott, 2016).

The aim of this review is to demonstrate how useful statistical thermodynamics is. We will derive all the necessary formulae from scratch. The derivation is quite straightforward; no background knowledge is required beyond introductory chemical thermodynamics, such as Gibbs-Duhem and Clausius-Clapeyron equations (Atkins & de Paula, 2014).

**2. Thermodynamics without statistical mechanics is prone to confusion**

Our proposal to apply rigorous statistical mechanics to food science does not mean in any way that we are advocating the abolition of the current thermodynamic and calorimetric approaches. On the contrary, statistical mechanics fulfils the full potential of thermodynamic analysis, by bringing in an unprecedented interpretive clarity at a molecular level. (Such a combination of thermodynamics and statistical mechanics is commonly called statistical thermodynamics; our standpoint is to pursue food(-related) science within the framework of statistical thermodynamics.) What have instead been abolished are confusion and ambiguity caused by the lack of an explicit molecular basis (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

**Question:** Consider the addition of extra molecular component(s) − such as sugars, salts, amino acid derivatives, or macromolecules − to food. Such an addition of cosolvents affects gelation, solubility, denaturation, and aggregation. How do cosolvents modulate such equilibria? (Note that such extra components are referred to in many different names, such as cosolvents, cosolutes, additives, or solutes; “cosolvents” will be used throughout this paper.)

**A thermodynamic answer:** Consider a transition of the solute (referred to as species ), such as sol gel, solute in pure phase solute dissolved in solvent, foldedunfolded, or monomersaggregate, and the accompanying the standard Gibbs free energy . (Throughout this paper, signifies the change that accompanies a transition .) The addition of cosolvents (species ) into water (species changes the water activity, and therefore the chemical potential of water . How changes with can be expressed as a *competition* between the change in number of water and cosolvent molecules bound to the biomolecules, and (Figure 1) that accompany the transition:

 (1)

where and are bulk concentrations of water and cosolvent (Wyman, 1948; 1964; Casassa & Eisenberg, 1964; Tanford, 1968, 1969, 1970; Schellman, 1987; Timasheff, 1998, 2002a, 2002b; Parsegian, Rand & Rau, 1995, 2000). For example, in the context of protein unfolding signifies , which corresponds to the difference in the number of bound water between the unfolded state (*u*) and the folded state (*f*).

Eq. (1) is commonly referred to as the Wyman-Tanford formula, whose interpretation owes to the seminal contributions by Wyman, Eisenberg, Tanford, Schellman, Timasheff, and Parsegian (Wyman, 1948; 1964; Casassa & Eisenberg, 1964; Tanford, 1968, 1969, 1970; Schellman, 1987; Timasheff, 1998, 2002a, 2002b; Parsegian, Rand & Rau, 1995, 2000). Eq. (1) can readily be, and has indeed been, applied to interpret thermodynamic data (Baier, Decker & McClements, 2004; Miyawaki & Tatsuno, 2010; Miyawaki, Dozen & Nomura, 2013; Miyawaki, Omote & Matsuhira, 2016). All one should do is to plot against (or equivalently against where is the water activity (Atkins & de Paula, 2014)) in order to obtain (Figure 2). The food science applications of this formula include the effects of sugars, salts and alcohols on the thermal denaturation of proteins, as well as on gelation (Baier, Decker & McClements, 2004; Miyawaki & Tatsuno, 2010; Miyawaki, Dozen & Nomura, 2013; Miyawaki, Omote & Matsuhira, 2016).

 **Further simplification:** Which is the dominant contribution to the equilibrium shift, the change of water binding () or cosolvent binding ()? The Wyman-Tanford formula (Eq. (1)) on its own does not provide a definitive answer to this question. Yet dialysis measurements show that sugars, polyols, and “kosmotropic” salts are preferentially excluded from biomolecular surfaces (Figure 1(b)) (Timasheff, 1998, 2002a, 2002b). Consequently they are not bound to biomolecules; hence the change of the bound number is zero, i.e., (Parsegian, Rand & Rau, 1995, 2000). This renders Eq. (1) a powerful tool by simplifying it to

 (2)

The change in number of bound water molecules , which accompany folding, gelation, solubilisation, aggregation, can therefore be measured directly from the Wyman-Tanford plot (Figure 2) (Parsegian, Rand & Rau, 1995, 2000).

**Controversy:** Eq. (2) was the focus of intense controversy, because of the unrealistically large numbers of water molecules have been estimated to be released from protein-ligand binding and allosteric transitions (Timasheff, 1998, 2002a). In the course of controversy, the following doubts have been raised about Eq. (1) (Timasheff, 1998, 2002a; Parsegian, Rand & Rau, 2000):

**Controversy 1:** Whether and really have a precise microscopic meaning.

**Controversy 2:** Whether and are independently determinable in principle.

**Controversy 3:** Whether preferential exclusion really signify the lack of binding.

To appreciate the depth and extent of the above controversy, one should appreciate that the cosolvent effect on biomolecular equilibria (i.e., ) have been interpreted from a number of different “perspectives” (Parsegian, 2000; Timasheff, 1998, 2002a), such as

1. Preferential solvation theory (Timasheff, 1998; 2002a) and the solvent exchange model (Schellman, 1987), i.e., Eq. (1).
2. Osmotic stress method (Parsegian, 1995, 2000), i.e., Eq. (2).
3. Macromolecular crowding, which attributes the origin of preferential exclusion of cosolvents to their large molecular size (Zhou et al., 2008; Davis-Searles et al., 2001; Sapir and Harries, 2016), i.e., Eq. (1) with the assumed dominance of .
4. The “making”/“breaking” of “water structure” by cosolvents, which enhances/weakens the hydrogen bond network of water around the hydrophobic group, thereby leading to the cosolvent-induced protein stabilization/denaturation (Frank & Franks, 1968), i.e., Eq. (1) with the assumed dominance of .

The interrelationship between these different perspectives was the deeper historical reason for the above controversy. In addition,

1. Partial molar volume measurements can also probe biomolecular hydration (Chalikian, 2003), whose estimations are very different from the osmotic stress method (Timasheff, 1998, 2002a)

What is the relationship between all these different perspectives?

**The way out.** Thermodynamics, by definition, cannot give any definitive answer to any of the above questions, because thermodynamics itself cannot give any microscopic interpretation of thermodynamic quantities. Only statistical thermodynamics (i.e., the combination of thermodynamics and statistical mechanics which can link macroscopic measurements to intermolecular interactions) can provide an answer (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a). This will be explained from scratch in Section 3

**3. Clarification comes from statistical thermodynamics**

Here we re-derive the Wyman-Tanford formula from statistical thermodynamics. Such a re-derivation clarifies what and really mean at a molecular level without any room for ambiguity. This molecular-level clarification has indeed resolved the controversy which arose from the lack of a molecular basis (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

To introduce the statistical thermodynamic theory of the cosolvent effect, there are three possible approaches. The first is the pioneering matrix inversion approach of Ben-Naim (1977, 2006), generalized later by Smith (2008), which rendered the classical Kirkwood-Buff theory (Kirkwood & Buff, 1951) a powerful and usable tool in solution chemistry (Shimizu, 2004; Shulgin & Ruckenstein, 2006; Pierce et al., 2008; Harries, & Rösgen, 2008; Ploetz & Smith, 2013). This approach, however, is mathematically lengthy. The second is our direct derivation from the first principles of statistical thermodynamics, which is much simpler yet requires some familiarity with the statistical thermodynamics (Shimizu & Matubayasi, 2014b). The third is a thermodynamic derivation pioneered by Hall (1971), which has been employed by one of us to resolve the osmotic stress controversy (Shimizu, 2004; Shimizu & Boon, 2004). This approach has the advantage of requiring only the introductory knowledge found in most undergraduate textbooks (Newman, 1994; Shimizu, 2004). One minor disadvantage of this approach, however, is that there is a small gap in derivation, which can be understood intuitively but must be accepted without proof. Yet the first, second, and third approaches derive the same equations, hence we adopt the third for its intuitive appeal (Shimizu, 2004; Shimizu, Booth & Abbott, 2013; Shimizu & Matubayasi, 2014a, 2014b).

From here, let us focus on one conformational state (either folded or unfolded state of a protein, or sol or gel state of biomolecular assemblies). The changes signified by will be introduced later. Consider a three component solution consisting of solute (), water (*i=*1), and cosolvent (*i=*2) molecules. For the purpose of deriving the Wyman-Tanford equation, here we consider a solute molecule at infinite dilution, which means we ignore solute-solute interactions. Extension to concentrated solutes is straightforward for two component systems, and is extremely cumbersome in three component systems. The theory is applicable without any modification to any principal solvent () (Hall, 1971; Newman, 1994; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b); *i =* 1 does not need to be water (Shimizu & Abbott, 2016).

Our central interest here is the effect of cosolvents on the solvation of a solute molecule. To focus exclusively on the interaction between the solute and water, we conceptually fix the centre-of-mass position of the solute molecule, which means that we adopt Ben-Naim’s standard state (Ben-Naim, 2006). We then divide the solution into two parts: the first part (called the “solute’s vicinity”) contains a fixed solute molecule, the other part (called the “bulk”) is far away from the solute (Figure 3) (Hall, 1971; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b).

Simply put, what we want to describe here is how the solute molecule changes the concentration of the solvent species around it. If the cosolvent interacts favourably with the solute, increased cosolvent concentration is observed in solute’s vicinity compared to the bulk (Figure 1a). If, on the other hand, the cosolvent interacts unfavourably to the solute, decreased cosolvent concentration follows compared to the bulk phase (Figure 1b). Thus the key of the KB theory, in a nutshell, is the concentration difference between solute’s vicinity and the bulk solution (Hall, 1971; Newman, 1994; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b).

Our goal, therefore, is to express theoretically the vicinity-bulk concentration difference. To achieve this, the Gibbs–Duhem equations from introductory chemical thermodynamics textbooks will be demonstrated to be powerful. Under the constant temperature (i.e., ) the Gibbs-Duhem equation for the bulk solution is

 (3)

where and respectively express the number and chemical potential of species , is the volume, is the pressure. Likewise, the Gibbs-Duhem equation for the vicinity is

 (4)

where “” indicates the number and volume of the vicinity, and “” indicates that the solute’s centre of mass is fixed. Note that and are the same in the local and bulk systems due to phase equilibrium condition (Hall, 1971; Newman, 1994; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b).

Since the local-bulk *concentration* difference is our goal, we introduce the vicinity and bulk concentrations, and , which transform Eqs. (3) and (4) respectively into

 (5)

 (6)

Note the existence of in the definition of for the introduction of vicinity (local) concentration. The Gibbs-Duhem equations can now be expressed in terms of the vicinity-bulk concentration difference, , by subtracting Eq. (5) from Eq. (6):

 (7)

Now we introduce the key concept, *excess solvation numbers*, , defined as the vicinity-bulk concentration change per solute molecule

 (8)

with the help of which Eq. (7) can be rewritten as

 (9)

Eq. (9) is the fundamental relationship in the Kirkwood-Buff theory, which links the hydration free energy to the excess solvation numbers (Hall, 1971; Newman, 1994; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b). Here, it is important to note the value of and does not depend on the choice of as long as it is sufficiently large (Hall, 1971).

Now the Wyman-Tanford formula can be obtained straightforwardly from Eq. (9). Firstly, differentiating Eq. (9) with respect to yields

 (10)

Combining Eq. (10) with , which comes straightforwardly from Eq. (5) under the isobaric condition , we finally obtain

 (11)

which is essentially the Wyman-Tanford formula. More precisely, Eq. (11) is for one conformational state of the biomolecule; Eq. (1), which refers to the change , can straightforwardly be derived by subtracting Eq. (11) for the state from that for (Hall, 1971; Shimizu, 2004; Shimizu et al., 2013; Shimizu & Matubayasi, 2014a, 2014b). Eq. (11) can also be derived from other approaches (Smith, 2004, 2008; Shulgin & Ruckenstein, 2006; Pearce et al., 2008).

So far we have derived everything based purely on chemical thermodynamics through the use of the Gibbs-Duhem equations. The same results can be obtained statistical thermodynamically. The only, yet crucial, additional insight that statistical mechanics can bring is the rigorous microscopic definition of the excess solvation numbers in terms of the radial distribution function, (Figure 4),

 (12)

where *r* is the distance between the centers of the molecular species *i* and *j*. (Note that can be measurable experimentally by X-ray and neutron scattering experiments.)

Now we show that the resolution to Controversy 1 in Section 2 comes from Eq. (12). As shown in Figure 4, and have been defined microscopically in terms of the radial distribution functions (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a); represents a net excess of the molecules of species *i* around the solute molecules compared to the bulk solution. Figure 4 shows that there are positive and negative contributions to (Figure 4, “Contact”). The attraction between the solute and the species *i* gives rise to a peak of , which contributes positively to , which has been appreciated by the previous models. What was not clearly realised before our KB-based theory was the *negative* contribution to arising from the excluded volume effect, namely the contribution from the impossible molecular overlap configurations (Figure 4, “Excluded volume”). Since such configurations with small distance between solute and the species *i* do not exist, ; such *r* contributes as density depletion compared to the bulk, and contributes negatively to . Such a negative contribution forced a reconsideration of the interpretation of the osmotic effect, which will be discussed in the subsequent sections.

This clarification leads also to the resolution to Controversies 2 and 3, which will be explained in Section 4.

**4. Statistical thermodynamics (the Kirkwood-Buff theory) completes and fulfils the Wyman-Tanford formula**

What is the point of re-deriving the classical Wyman-Tanford formula yet again in a different way as in Section 3? The re-derivation unlocks a full interpretative potential at a microscopic level, which is a marked departure from the age-old assumptions, limitations and consequent confusions that had long plagued the Wyman-Tanford formula (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

First we show that and can both be determined independently from experimental data, thereby resolving Controversy 2 in Section 2. To determine the two unknowns ( and ), two independent equations are necessary; we need an additional relationship that supplements the Wyman-Tanford formula. Fortunately, the partial molar volume can fit the bill

 (13)

where is the isothermal compressibility, which only makes a negligible contribution. and can be determined by solving the simultaneous equations (Eq. (11) and (13)). (Here, is the chemical potential of the solute, whereas is the chemical potential for a solute whose centre of mass is fixed in position; they are related by (Ben-Naim, 2006; Shimizu & Matubayasi, 2014b)). Likewise, the changes accompanying the conformational change , namely and , can be determined by supplementing the Wyman-Tanford formula (Eq. (1)) with the following relationship for the partial molar volume change arising directly from Eq. (13):

 (14)

Both and are determinable by solving the simultaneous equations consisting of Eqs. (1) and (14). This has been made possible only by the help of statistical thermodynamics (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu et al., 2006; Shimizu & Matubayasi, 2014a). This has led to an understanding as to why the osmotic stress method had grossly overestimated hydration by neglecting the exclusion of osmolytes from biomolecular surfaces. For the details of this resolution, see Shimizu (2004) and Shimizu & Matubayasi (2014a).

Thus the simultaneous equations (Eqs. (1) and (14)) not only enabled a determination of both and but also a synthesis of the osmotic (the Tanford-Wyman formula) and volumetric measurements, which had hitherto been conducted separately and independently. What we propose here is to carry out the Wyman-Tanford and volumetric experiments simultaneously to obtain a full picture, the simultaneous determination of biomolecule-water and biomolecule-cosolvent interactions (Gekko, 1989; Chanasattru, Decker & McClements, 2008). This will further develop fruitful applications of volume and density measurements in food science.

Statistical thermodynamics thus brought a molecular-based clarification to the Wyman-Tanford formula, the foundation of the cosolvent effect on food biomolecular folding, aggregation, and gelation. What remains to be explained is the statistical thermodynamic resolution to Controversy 3, which can be best demonstrated through the analysis of experimental data in Section 7.

**5. The Wyman-Tanford formula can be simplified when cosolvents are dilute**

With an additional insight from the KB theory, the Wyman-Tanford formula can be simplified further. Instead of the excess numbers (Eq. (12)), it is more convenient to use the following KB integral (not to be confused with the Gibbs free energy!)

 (15)

which measures the interaction per bulk density. Using Eq. (15), Eqs. (1) and (14) can be rewritten as

 (16)

 (17)

since from Eqs. (12) and (14) the relation can be obtained. Eq. (16) has a clearer meaning: the cosolvent effect on is due to the competition between solute-water and solute-cosolvent interactions expressed in terms of the KB integrals (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

When the cosolvent concentration is dilute, Eqs. (16) and (17) can be simplified even further. Leaving a more rigorous derivation to the literature (Shimizu et al., 2013), we present here an intuitive approach. When the cosolvent is dilute, Raoult’s Law, (in its differential form), holds true, where is the mole fraction of water. Since , , where is the concentration (density) of pure water. Using this, Eq. (16) can be simplified at as follows:

 (18)

Eq. (17) can be simplified also by noting (Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014c):

 (19)

To summarise, when the cosolvents are dilute, we can use Eqs. (18) and (19) to facilitate the analysis of the experimental data significantly (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

**6. The Clausius-Clapeyron equations fill the remaining theory-experiment gap**

We have thus established a rigorous theory and a method to analyse experimental data to reveal how biomolecule-water and biomolecule-cosolvent interactions competitively contribute to the cosolvent-induced changes on protein denaturation and aggregation, solubilisation, and gelation. The general theory (applicable to any cosolvent concentration) requires and (Eqs. (1) and (14)), and the special theory valid for dilute cosolvents () requires and (Eqs. (18) and (19)). The goal here is to link these quantities to the convention of measurements undertaken in food chemistry.

 Let us take the gel sol transition as an example (see Appendix A for why the theory can be used for gel sol transition); the theory below can straightforwardly be applied to other transitions. What is important here is that as long as the thermodynamic quantities relating to a transition is available, we can calculate the KB integrals. Let us summarise the conclusions first, before justification. What is readily measurable is the gel sol transition temperature (), and how it is dependent on cosolvent concentration (), from which and can be calculated by the following formulae:

 (20)

 (21)

where is the entropy change which accompanies gel sol transition, which can be obtained directly from calorimetry by , where refers to the enthalpy change. Likewise, the volume change can either be obtained directly from dilatometry, or by the hydrostatic pressure dependence of in the following manner (Gekko & Koga, 1983; Gekko & Kasuya, 1985; Gekko et al., 1987; Shimizu, 2011b):

 (22)

Deriving Eqs. (20) -(22) is straightforward. In the following, the outline of derivation is presented, while a more detailed discussion can be found in Appendix B. Let us focus here on Eqs. (20) and (22), because Eq. (21) has already been derived in our previous paper (Shimizu & Matubayasi, 2014c). Suppose that, along the sol-gel coexistence, the gelsol transition temperature rises from to , when the chemical potential increases from to or when the pressure is changed from to . (Here we have used for temperature only to emphasise that we are dealing with the transition temperature.) Along the sol-gel coexistence,

 (23)

holds true. Note that (** = *s* or *g*) in this equation is the total chemical potential, not the pseudochemical potential, so that the superscript \* is not attached. It is remarked furthermore that is the chemical potential per biopolymer in the gel state, not per aggregate particle. Using Eq. (B4) for the changes along the sol-gel coexistence, , and , we have

 (24)

Hence leads to Eq. (20). Under *n*2 = 0, Eq. (22) follows (Shimizu & Matubayasi, 2014c).

Thus we have made it possible to analyze transition temperature measurements in terms of the KB theory.

**7. The Kirkwood-Buff theory can readily be applied to food science: tofu as an example**

Here we demonstrate that the KB theory can indeed be applied to food systems quite straightforwardly. (For other, already published examples, see: Shimizu & Matubayasi, 2014c; Shimizu, 2015; Stenner et al., 2016). As an example for this review, we have chosen tofu, which is an important class of food gels, which has not been analyzed previously through the use of statistical thermodynamics. The gap in the available experimental data, which will be revealed along the process of data analysis, will hopefully be filled, in order to achieve a full, molecular-based understanding of food gelation.

**7.1 Heat denaturation of soy protein isolates in the presence of salts**

Heat-induced denaturation of soy proteins is the first step in tofu-making, prior to gelation. This process has been known to be strongly affected by the presence of salts (Kohyama & Nishinari, 1993; Banerjee & Bhattacharya, 2012; Nishinari et al., 2014). How at a molecular level do salts affect heat denaturation? Here we demonstrate that a straightforward analysis of the experimental data, which stems from the KB theory, can tell a correct mechanism from other.

Why can some salts enhance the thermal denaturation of soy protein whereas the others can prevent it? There have been two different hypotheses proposed so far

(**i) Hydration/water structure changes hypothesis** stems from the classical view that the hydrophobic effect is the driving force of protein folding and that the hydrophobic effect is caused by the enhanced hydrogen bonding network of water (called the “iceberg”) around the hydrophobic solute (Frank & Evans, 1945). Cosolvents either promote (“structure makers”) or interrupt (“structure breakers”) the iceberg indirectly (Frank & Franks, 1968). This hypothesis has been used to account for i) the effects of various anions (SO42-, Cl-, Br-, SCN-) on the gelling and rheological behaviour of soy proteins (Babajimopoulos, Damodaran, Rizvi & Kinsella, 1983), and ii) the interactions between soy proteins and cations, such as Ca2+ (Kohyama, Sano & Doi, 1995; Puppo & Anon, 1998; Sakakibara & Noguchi, 1977; Zhao, Li, Qin & Chen, 2016) and Na+ (Anon, Lamballerie & Speroni, 2011; Puppo & Anon, 1998).

(ii) **Preferential solvation hypothesis,** namely the competition between protein-water and protein-cosolvent interactions. If protein-cosolvent interaction is stronger than protein-water (Figure 1(a)), then, as we increase cosolvent concentration, equilibrium shifts towards the increased exposure of protein surface, to which cosolvents can be bound; this will promote protein denaturation. On the contrary, if protein-cosolvent interaction is unfavourable compared to protein-water (Figure 1(b)), the increase in cosolvent concentration shift the equilibrium towards compactness. This hypothesis has been employed to account the effects of Ca2+ on soy protein isolates (Canabady-Rochelle, Sanchez, Mellema & Banon, 2009; Scilingo & Anon, 1996; Yuan, Velev, Chen, Campbell, Kaler & Lenhoff, 2002).

Which of the above two hypotheses is correct? The KB theory can not only answer this question but also eliminate the ambiguity through the individual determination of protein-water and protein-cosolvent interactions.

The KB-analysis of the protein denaturation data is straightforward. We consider the transition from the native state (denoted as *n*) to the denatured state (*d*). Here we focus on the dilute salt limit; extension to higher salt concentrations can be done (which requires more thermodynamic data – See section 7.2). At this limit, all we need are: (how the denaturation temperature depends on cosolvent concentration ), (dependence of on pressure ), and (the entropy change that accompanies denaturation at zero cosolvent concentration). Combining Eqs. (18), (19), (21) and (22), a straightforward algebra yields

 (25)

 (26)

In carrying out the data analysis based on Eqs. (25) and (26), care must be taken for a correct unit conversion. Table 1 shows the data analysis all the way from the raw experimental data to the KB integrals. What is crucial to the calculation of is the large magnitude of the gradient , which causes at low salt concentrations (15 mM, or even at 5mM) significantly different from those in the absence of salts, which are large Note that for soy proteins have not been measured experimentally to the best of our knowledge. We therefore have to employ the empirical formula to estimate (Chalikian & Filfil, 2003)). The empirical formula by Chalikian and Filfil (2003) may seem upon first inspection to contain many parameters. However, thanks to their extensive correlational analysis using experimental volumetric and structural data, the required input parameters have been reduced to two: molecular weight of the protein, and the degree of unfolding. The molecular weight data used in the analysis have been summarized in Tables 1 and 2. There is no other way than to assume the degree of unfolding as being 1 (fully unfolded), which may be debatable. Fortunately, the contribution from is estimated to be much smaller in magnitude than (Shimizu, 2011a); so the error in does not affect the analysis. The resultant and are able to judge whether the above hypotheses are valid.

**The water structure hypothesis**, in the language of Kirkwood-Buff theory, translates to the dominance of over () i.e. change in the water structure/hydration is the driving force behind protein denaturation. However, for salt-soy protein systems (Table 1), the opposite is true: the changes in hydration/water structure indeed contribute negligibly to the denaturation free energy change (), hence cannot account for the salt-induced changes in the thermal stability. Even though this conclusion has been drawn, relying upon the estimated , it is consistent with other proteins (Shimizu et al., 2006; Shimizu, 2011a; Shimizu & Matubayasi, 2014a).

**The preferential solvation hypothesis** is consistent with the KB theory, which provides a rigorous link between the competitive solvation to the cosolvent-induced equilibrium changes. This conclusion was reached purely theoretically. Nevertheless, what is important and insightful here is that the KB analysis can clarify what “preferential solvation” really means. The concept of preferential solvation is inseparable from the Wyman-Tanford formula (Eq (1)), in which and (or and ) are not independent; hence only the difference, (or ) could be extracted from experimental data (Timasheff, 1998, 2002a). The KB theory, on the contrary, has enabled the indivisual calculation of and (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a). For salts (Table 1), the dominance of over (i.e. ) shows that the widely varying magnitude of the protein-salt interaction is the cause for the strongly cosolvent-dependent thermal denaturation temperature changes. The large, negative shows in conjunction with Eq. (15) that the salts are more excluded from the denatured state than from the native state (Shimizu & Boon, 2004; Shimizu et al., 2006; Shimizu 2011a; Shimizu & Matubayasi, 2014a).

To summarise, a simple KB-based analysis can not only show that water structure hypothesis cannot account for the cosolvent effect on thermal denaturation but also identify that protein-cosolvent interaction is the true cause. The latter identification could not be done by the Wyman-Tanford formula alone, which could not separate proein-cosolvent interaction from protein-water interaction; the separation between the two could be achieved only by the KB theory (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a). The effect of salts at higher concentrations (Kohyama & Nishinari, 1993; Banerjee & Bhattacharya, 2012; Nishinari et al., 2014) can be analyzed using Eqs. (1) and (14), instead of Eqs. (18) and (19).

**7.2. Effects of polyols on the thermal denaturation of soy proteins**

Here we turn to the glycerol and ethylene glycol, which have been known to act weakly on protein stability. The reason why we turn to such model cosolvents (which are not used for setting tofu) is because of the important insights into the mechanism of cosolvent-induced change of gelation, as has been revealed by Gekko et al. (1999) through the study of such cosolvents.

Sorbitol and glycerol have been known to stabilize the native structures of proteins. Their stabilization effect is indeed weaker than MgCl2 and CaCl2 from Section 7.1, as well as than sucrose, trehalose and polyethelene glycol (Shimizu & Boon, 2004; Shimizu, 2011a; Shimizu & Matubayasi, 2014a). Table 2 shows that sorbitol and glycerol indeed acts as weak stabilizers for glycinin and β-conglycinin (Gekko et al., 1999).

Even for weak stabilizers like sorbitol and glycerol, the water structure dominance hypothesis () does not apply (Table 2). Yet the relationship , valid for many stronger osmolytes and salts (Shimizu, 2011a; Shimizu & Matubayasi, 2014a), does not hold true for sorbitol and glycerol. Indeed, for glycerol and sorbitol are much smaller in magnitude than salts, even in the same order of magnitude as , suggesting that and both contribute competitively to the cosolvent-induced change of denaturation. The exclusion of sorbitol and glycerol from protein surfaces is therefore only very mild.

The exclusion of ethylene glycol from the protein surface is even weaker, as can be shown by its much smaller magnitude of (Table 2). Even though ethylene glycol is generally known as weak osmolyte, it acts as a weak denaturant for soy proteins, lowering the denaturation temperature (Gekko et al., 1999). This is because is so small in magnitude that wins over (Table 2).

Based upon the appreciation of a weak exclusion of glycerol and an even weaker exclusion of ethylene glycol, let us move onto how these cosolvents act on tofu gelation.

**7.3. Effects of polyols on the gelation of tofu**

Setting tofu, namely the gelation of soy bean proteins, requires the addition of gelling agents. Three common gelling agents are MgCl2, CaSO4 and Glucono-δ-lactone (Kohyama & Nishinari, 1993; Banerjee & Bhattacharya, 2012; Nishinari et al., 2014) Unfortunately, to the best of our knowledge, systematic experimental data on tofu gelation in the presence of these cosolvents, which can readily be applied to the analysis based upon the KB theory, have not been found in the literature. Instead, the most systematic thermodynamic study has been done by Gekko and coworkers, who have measured the effect of model cosolvents, i.e., polyols, on the gelation of soy protein extract, together with the pressure dependence (Gekko, 1993; Gekko, Li, & Makino, 1999). Hence, in this section, we study the effect of polyols on tofu gelation, in order to illustrate the applicability of our theory.

What can our statistical thermodynamic analysis say about the mechanism on the enhancement of tofu gelation by cosolvents? Three different hypotheses have been proposed: (i) the enhancement of the water structure around the protein, and the concurrent change in protein hydration, induced by the cosolvent (Babajimopoulos et al., 1983); (ii) exclusion of cosolvents from protein surfaces in the sol phase (Canabady-Rochelle et al., 2009; Scilingo & Anon, 1996; Yuan et al., 2002); (iii) binding between cosolvents and proteins in the gel phase (Arii & Takenaka, 2013, 2014). Which of the above hypotheses is/are likely to be correct? To answer this question, we shall again analyze , and obtained from experiments, which are not limited to very dilute concentrations of cosolvents. This is why we have to solve the simultaneous equations (Eqs. (1) and (14)), in combination to Eq. (20), we obtain:

 (27)

 (28)

where at has been used in the derivation. Now we evaluate both KB integrals. The key quantity is calculated from the Wyman-Tanford plot, which is shown in Figure 5. Density data at has been used to calculate , , and . (Gelation data in the presence of sorbitol could not be analysed because of the lack of density and osmotic data). was available only at , and assumed to remain constant irrespective of ; the small magnitude of makes this a good approximation.

Figure 6 shows that for both glycerol and ethylene glycol the dominant contribution is , and that the hydration change negligibly small relative to . This shows straightway that **Water structure hypothesis (i)** is unlikely, because of the negligibly small contribution from the hydration change . But more importantly, for gelation is in stark contrast to how the same cosolvents acted on soy protein denaturation in Section 7.2; glycerol was weakly excluded from soy proteins, and even weaker exclusion was observed for ethylene glycol. What can this stark difference between denaturation and gelation teach us about how glycerol and ethylene glycol interact with sol and gel?

To answer this question, let us start by understanding the origin of the large, positive and dominant contribution is for glycerol-tofu (Figure 6), which is defined as

 (29)

where and respectively represent the KB integrals for gel and sol states (Stenner et al., 2016). Note that sol state consists of denatured soy proteins. According to Eq. (29), for to be large and positive, . A strong attraction between glycerol and the denatured soy protein would make more positive. But this is not the case; we already know from Table 2 that glycerol is excluded weakly from protein surfaces. Hence the only way that can take place is to make larger negative. This takes place when glycerol is excluded more from the gel phase than from the sol phase.

The large, negative and dominant contribution for ethylene glycol-tofu can be attributed likewise to . This can be achieved by (1) a very strong exclusion of ethylene glycol from the denatured (sol) proteins or (2) the binding of ethylene glycol to the gel. We know that (1) is in contradiction to our observation in Section 7.2 that ethylene glycol is excluded only very weakly from proteins. Hence ethylene glycol should bind the gel phase more strongly than the sol phase.

Taken all together, the KB theory reveals that ethylene glycol, which is the smaller cosolvent, is embedded into the gel phase, whereas glycerol, which is larger, tends to be excluded from the gel network.

Thus gel melting temperature data were sufficient to judge the validity of the classical hypotheses, when analyzed in the framework of statistical thermodynamic theory. Statistical thermodynamics can therefore make a significant contribution to food science through its power of definitive clarification at a molecular scale. Towards this goal, systematic soy protein gelation data in the presence of the common gelling agents are badly needed.

**7.4. What the Kirkwood-Buff theory cannot do in its present form**

Analysing the thermodynamic data on protein denaturation and gel melting was sufficient to dispel some of the age-long hypotheses on the mechanism on the cosolvent effect on protein denaturation and gelation. This was enabled because the KB theory is rigorous, which stems directly from the basic principles of statistical thermodynamics (Kirkwood & Buff, 1951; Hall, 1971; Ben-Naim, 1977; Newman, 1994; Shimizu, 2004; Shimizu & Matubayasi, 2014a).

Note, however, that the basis of interpretation, the KB integrals, are statistical averages that refer to the overall deviation of the radial distribution from their bulk values. Hence it is often difficult to identify the specific atomic groups or protein residues that are responsible for cosolvent-protein binding or the exclusion of cosolvents (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu, 2011a, 2011b). For example, the KB theory has identified the binding (or embedment) of polyols as the important driving force of gelation. We expect (subject to the availability of experimental data) that the same would be the case for salts; yet the KB theory on its own cannot comment, for example, on the electrostatic effect of the bound ions on crosslinks. To address such questions at an atomistic basis, carrying out simulations would be necessary. Simulations, on the other hand, cannot have the ability of giving the definitive answer as the KB theory has due to the model approximations involved (force field, system size, and statistical procedures); yet the accuracy of simulations can be validated by a comparison to the KB integrals obtained directly from experiments (Pierce et al., 2008; Ploetz & Smith, 2013). Hence combining models and the rigorous theory would prove fruitful in revealing the mechanism of food processes at atomistic detail.

**8. Conclusion**

Statistical thermodynamics is useful in understanding how cosolvents affect protein heat denaturation, biomolecular gelation, and aggregation. When analyzing thermodynamic data, statistical thermodynamics provides an unambiguous method to calculate both the biomolecule-water and biomolecule-cosolvent interactions. To do so, all one needs to know is how transition temperature depends on water activity (or cosolvent density) and pressure, together with the bulk density data of water-cosolvent mixture (Shimizu, 2004; Shimizu & Boon, 2004; Shulgin & Ruckenstein, 2006; Pierce et al., 2008; Harries, & Rösgen, 2008; Ploetz & Smith, 2013; Shimizu & Matubayasi, 2014a).

Before the statistical thermodynamic refurbishment, the study of the cosolvent effects was plagued with confusion and controversies. This is because thermodynamics on its own cannot provide an unambiguous link between thermodynamic data and molecular behaviour. Statistical thermodynamics therefore provides a clear interpretive tool, fruitful for the analysis of thermodynamic data on food (Shimizu, 2004; Shimizu & Boon, 2004; Shimizu & Matubayasi, 2014a).

What statistical thermodynamics has revealed is somewhat different from the previous thermodynamic expectations. The classical view, that cosolvents induce changes in biomolecular hydration, which drives the shift in denaturation, gelation, and aggregation, is not supported by statistical thermodynamics. Biomolecule-cosolvent interaction (whether attractive or net exclusive) is instead the dominant driving force (Shimizu & Matubayasi, 2014c; Shimizu, 2015; Stenner et al., 2016).

 Statistical thermodynamics thus provides a useful guideline, clearly showing what kind of measurements should be undertaken for understanding the cosolvent action at a molecular level. An attempt in this review to apply the KB theory to soy proteins revealed that there still are crucial gaps in the available thermodynamic data, which are waiting to be filled.

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**Appendix A**

Here we discuss further how to treat the gel state within the framework of biomolecules in dilution, following Shimizu & Matubayasi (2014c). The crux of the matter is that the protein molecules can make linked network structure in the gel state, yet are dilute enough so that protein-protein interactions other than the formation of link can be ignored. Such an approach has been adopted, albeit implicitly, in the thermodynamic study on the effect solvation on binding and aggregation (Shimizu & Matubayasi, 2014c). Hence we consider dilute biopolymer aggregate (of aggregation number ) as the gel state, for which (Eq. (11)) can be rewritten as

 (A1)

where is the pseudochemical potential of the aggregate of the biopolymers at the gel state. This quantity, introduced per aggregate particle, can be converted to the chemical potential per biopolymer through the division by the aggregation number.

Here we show that Eq. (B1) can be simplified further in terms of the KB integral *per biopolymer.* To do so, we have to exploit the following: (1) The biopolymer-biopolymer interactions except for the small region in which the interaction takes place can be ignored; (2) The biopolymer is much larger in size than the water and cosolvent molecules. It follows from (1) and (2) that, since biopolymers are large and dilute, we can safely assume that each water and cosolvent molecules, which are not in the bulk, interact only with its nearest biopolymer.

 Let us translate this into the language of the radial distribution function (RDF). The RDF between the gel aggregate and the species at the position can be expressed as

 (A2)

where is RDF between the th biopolymer in the gel and the species . The basis of Eq. (A2) is for all the biopolymers except for the one nearest to the solvent. This means that the gel aggregate-solvent KB integral

 (A3)

can be expressed in terms of the KB integral *per biopolymer* in the gel state, . We have thus generalized the Wyman-Tanford formula for sol-gel transition.

**Appendix B**

Here we present a detailed derivation of Eqs. (20), (21) from Eq. (24), and of Eq. (22).

**Derivation of Eq. (24).** Using basic calculus, the total differential at the state can be expressed in terms of the change of the variables () in the following manner:

 (B1)

where the last two partial differentials have the physical interpretations respectively as and , namely the partial molar entropy and volume of at the state . Hence for the sol () and gel () states, Eq. (B1) can be rewritten as

 (B2)

 (B3)

Subtracting Eq. (B3) from Eq. (B2) yields

 (B4)

in which has been introduced. Eq. (24) can be obtained as the special case of Eq. (B4), i.e., because of phase equilibrium (Eq. (23)), and around the transition temperature, .

**Derivation of Eq. (20).** Here we consider how solgel equilibrium is affected by when is kept constant, as has been required by the Wyman-Tanford formula. Hence we consider Eq. (24) under the isobaric condition, . This leads to

 (B5)

from which Eq. (20) can be derived straightforwardly through algebraic manipulation. Note that we have used and instead of and , in order to emphasise that experimental values have been used to calculate .

**Derivation of Eq. (21).** Eq. (21) is a special case of Eq. (20) at the dilute cosolvent concentration limit, . Just as in the derivation of Eq. (18), we use Raoult’s Law, which yields

 (B6)

Combining Eq. (B6) with the l.h.s. of Eq. (20), one obtains,

 (B7)

and with the r.h.s. of Eq. (20), one obtains,

 (B8)

Combining Eqs. (20), (B7) and (B8) yields Eq. (21).

**Derivation of Eq. (22).** Now we consider the change of pressure when the cosolvent is absent. Going back to the total differential at the state , note that the variables here are (), because there is no change in the solvent composition. Hence Eq. (B1) under this condition should be rewritten as

 (B9)

Repeating the same argument, one arrive at the following two-component equivalent of Eq. (24):

 (B10)

Eq. (22) can be derived straightforwardly from Eq. (B10).

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**Figure and Table Captions**

**Table 1:** The effect of salts on the thermal denaturation of soy proteins analyzed with the Kirkwood-Buff theory.

aExperimental data taken from Liu et al. (2010); bEstimated using the empirical formula by Chalikian and Filfil (2003), in which 360 and 190 kDa (Zhang & Zeng, 2008) have been used as the upper-limit molecular weights of glycinin and β-conglycinin, respectively; cCalculated at the low concentration limit using the data at 0 and 5mM salt concentrations.

**Table 2:** The effect of polyols (ethylene glycol (EG), glycerol and sorbitol) on the thermal denaturation of soy proteins analyzed with the Kirkwood-Buff theory.

aExperimental data taken from Gekko et al. (1999); bEstimated using the empirical formula by Chalikian and Filfil (2003), in which 360 and 190 kDa (Zhang & Zeng, 2008) have been used as the upper-limit molecular weights of glycinin and β-conglycinin, respectively.

**Figure 1:** Schematic representations of preferential interactions: (a) preferential interaction of cosolvents (dark red) with the biomolecule (black) compared to that of water (blue) with the biomolecule; (b) preferential water-biomolecule interaction over water-cosolvent interaction.

**Figure 2:** Wyman-Tanford analysis of the cosolvent effect. See Eq. (1).

**Figure 3:** Intuitive derivation of the Kirkwood-Buff theory. The first step is to divide the solution into the “local” (vicinity of the solute) and “bulk” (far away from the solute). The goal is to evaluate the net excess/deficiency of water (blue) and cosolvent (dark red) molecules in the “local” region relative to the bulk.

**Figure 4:** The microscopic interpretation of the excess cosolvent number, (Eq. (12)). **“Excluded volume”**: Due to steric repulsion (excluded volume effect) the distance between the solute (black) and the cosolvent (dark red) cannot be shorter than the contact distance; this, in the language of the radial distribution function, corresponds to . Such distance ranges contribute negatively to the excess number due to the depletion of cosolvent density. **“Contact”**: At the distance of solute-cosolvent contact, an attractive interaction would increase the occurrence of such a configuration, making peak higher, thereby contributing positively to the excess number. **“Bulk”**: As becomes larger, the cosolvent density approaches to that of the bulk.

**Figure 5.** Wyman-Tanford plot for the gel sol transition of the soy protein isolate in the presence of glycerol (black) and ethylene glycol (red). The experimental data (circle and square) are taken from Gekko et al. (1999). The source of experimental data used for analysis are the following: density of ethylene glycol-water mixture (Azfal et al., 2009); vapor pressure of ethylene glycol-water mixture (Horstmann et al., 2009); density and vapor pressure of glycerol-water mixture (Glycerin producer’s association, 1963). Lines representing fitting are: glycerol () and ethylene glycol (). These fitting lines are used for differentiation (Eq. (21)). Multiplying 5.5 J K-1 (mole crosslinks)-1 (Gekko, 1999) can convert this figure to a Wyman-Tanford plot.

**Figure 6.** Kirkwood-Buff integrals for the gel → sol transition of soy bean isolate protein in the presence of glycerol and ethylene glycol, calculated using Figure 5 and the KB theory. Note that the KB integrals have been calculated approximately at 353 K for glycerol and ethylene glycol. Contribution from has been assumed to be constant (which is indeed negligible for larger for glycerol and smaller for ethylene glycol) for which the pure water value from Gekko (1993) have been used in the absence of data in the presence of cosolvents. The KB integral changes are expressed in the units of cm3 per mole of cross links.