Shapes and sizes of food polysaccharides by sedimentation analysis—recent developments

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ABSTRACT

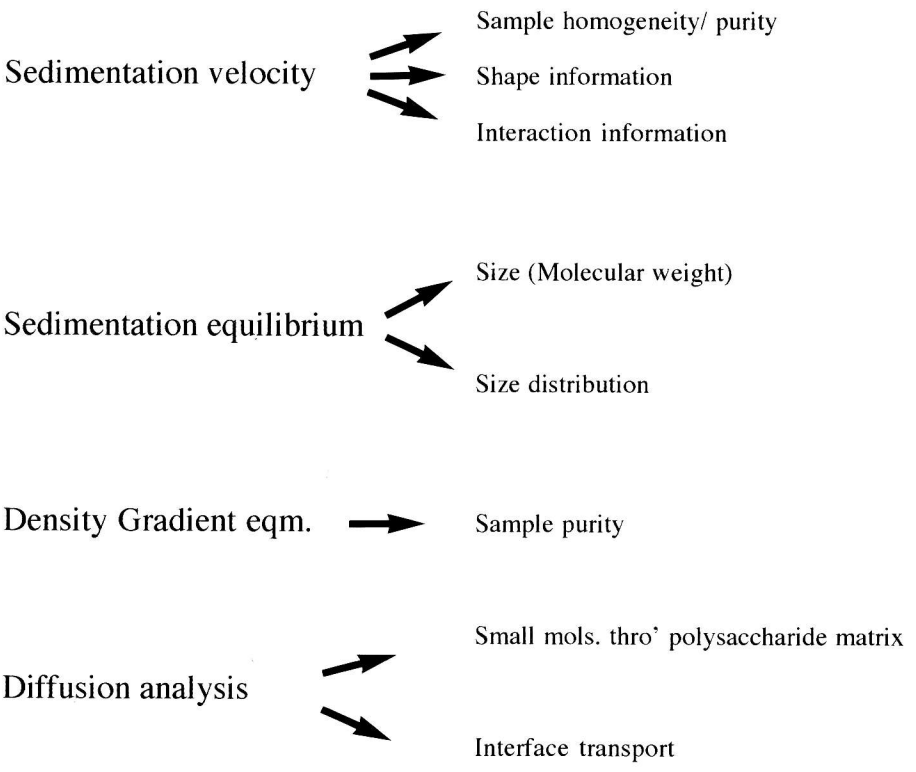
Sedimentation analysis in the analytical ultracentrifuge is currently undergoing something of a revival after many years of decline, culminating in the launch of a new instrument . This article attempts to give the general reader an overall idea of the contribution that sedimentation velocity analysis and sedimentation equilibrium analysis is making to our understanding of the shapes and sizes of a range of industrially important polysaccharides. The importance of combining information from sedimentation analysis with that from complementary techniques such as viscometry and light scattering is indicated

INTRODUCTION

Knowledge of the fundamental behaviour of food polysaccharides on a "microscopic" scale (in terms of their sizes, shapes, molecular weight distribution and interaction parameters) in dilute and concentrated solution form - both by themselves and in mixtures - is important for the proper understanding of their behaviour in food products on a "macroscopic" scale (such as gelling, thickening ability and phase separation). This information can help us choose the "right" polysaccharide with the "right" properties for a particular product.

There are several techniques currently popularly used to obtain this fundamental information (see, e.g., ref.1) but as is well known food polysaccharides are by no means easy to characterise because of their highly expanded nature in solution, their polydispersity, their variable conformations and in some cases their high charge and ability to stick together. In this article I will describe some recent developments and applications with an old (much of the technology dates back to the 1920's) and *not-so-popular* technique of sedimentation analysis in the analytical ultracentrifuge - which is now undergoing something of a revival (including the launch of a new instrument). I will describe how its absolute nature and inherent fractionation property is providing useful information - especially when used in combination with other techniques - on the

Figure 1. Ultracentrifuge methods and the potential information available



nature and behaviour of food polysaccharides, focussing on two areas - shape and size analysis. The purpose of this article is to give the general reader an overall outline of recent developments but without going into any great depth. The interested reader if he so desires can obtain a comprehensive treatment of the technique in a book that has recently appeared (2) of which a substantial part is devoted to polysaccharides and related glycopolymers.

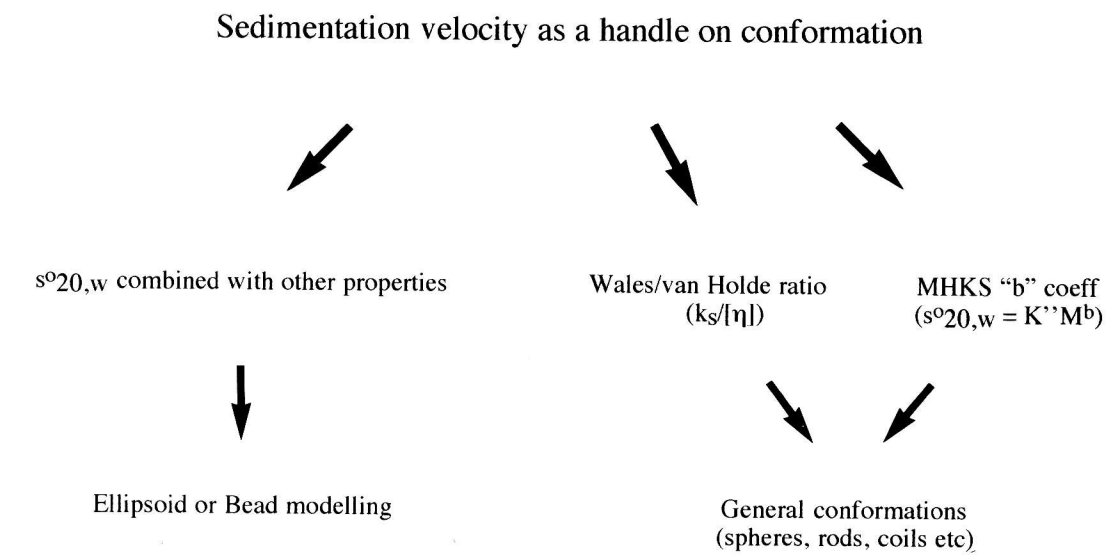
TYPES OF SEDIMENTATION ANALYSIS

What sort of information can we get from sedimentation analysis in the analytical ultracentrifuge? It depends on the type of sedimentation technique we apply - all possible with the same instrumentation (Fig. 1). *Sedimentation velocity* can provide us with information on the sample homogeneity, shape information - in some cases to surprising detail - and also interaction information by for example assaying for what we call "co-sedimentation" phenomena (i.e. species sedimenting at the same rate). At lower rotor speeds, *sedimentation equilibrium* can provide absolute size and size distribution information - in terms of molecular weight averages and molecular weight distributions. There are two other important types of analytical ultracentrifuge measurement - namely isopycnic (="constant density") *density gradient analysis*, important for assaying the purity of a polysaccharide preparation (i.e. freedom from unconjugated protein, lipid or nucleic acid) and finally *diffusion analysis* : although dynamic light scattering is now the method of choice for the measurement of translational diffusion coefficients, the optical system on the analytical ultracentrifuge is proving very useful for investigating the diffusion of molecules through matrices and towards and through interfaces between two phase systems {including aqueous two-phase polysaccharide systems (3)} However, since this article is focussed on polysaccharide shape and size analysis I am just going to describe recent developments and applications of sedimentation velocity and sedimentation equilibrium.

INSTRUMENTATION

An analytical ultracentrifuge is simply an ultracentrifuge with an appropriate optical system for observing and recording solute distributions during and at the end of the sedimentation process. The technique of analytical ultracentrifugation is not new - Svedberg's inception of the technique dates from the early 1920's. {Svedberg himself published several papers on polysaccharides - mainly in the 1940's - and mostly on cellulose and cellulose derivatives}. The boom period of the technique was in the period 1940-1970 with several commercially available analytical ultracentrifuge available, most notably the famous Beckman Model E (then present in virtually every Biochemistry and Chemistry department), but for the last 20 years until very recently there has been no commercially available instrument, apart from second hand Model E's. Our laboratory at Nottingham is full of second hand equipment, such as two Beckman Model E's (these have Rayleigh interference optics dedicated to size analysis by sedimentation equilibrium). Although these are both 20-30 years old they are the hallmark of reliability and have some modern features such as laser light sources. We also have an old MSE Centriscan (this needs much more mothering) equipped with scanning Schlieren (refractive index gradient) and absorption optics and also a store full of many scrapped Model E's and Centriscans which we use for spares. Very

Figure 2


Sedimentation velocity as a handle on conformation
$s^{o}20,w$ combined with other properties
Wales/van Holde ratio
($k_S/[\eta]$)
MHKS "b" coeff
($s^{o}20,w = K''M^{b}$)
Ellipsoid or Bead modelling
General conformations
(spheres, rods, coils etc)

recently these "old-timers" were joined by the latest analytical ultracentrifuge from Beckman - the Optima XLA - with full on-line computer data capture and analysis facilities, superb drive stability (even at 1000 rev/min) and superb absorption optics. Unfortunately the current model does not have refractive index optics (either Rayleigh interference or Schlieren) and since most polysaccharides don't appreciably absorb in the visible or the "near ultra-violet" (i.e. 250-300nm) its use for these molecules is very limited; we are however exploring the application of *far* -uv absorption optics (the light source is very stable down to 200nm) and initial results - at least for sedimentation velocity work - have been encouraging. The vast majority of our polysaccharide work at the moment however is performed using the Model E's and the Centriscan.

POLYSACCHARIDE SHAPE ANALYSIS BY SEDIMENTATION VELOCITY

A typical analytical ultracentrifuge cell contains one or two sector shape channels which can take between 0.1 to 0.8 ml of solvent or solution and with an *analytical* ultracentrifuge, using the appropriate optical system you can record the position and rate of movement of the sedimenting boundary within the solution channel. With a polysaccharide we use refractive index *gradient* or Schlieren optics which records the boundary on photographic film or chart paper as a peak. The rate of movement of the boundary per unit centrifugal field gives the sedimentation coefficient, "s", which is one of our shape parameters. By correcting this using simple formulae to standard conditions - water as solvent at 20°C - and extrapolating this (or the reciprocal thereof) to zero concentration we can get from the intercept the corrected sedimentation coefficient, $s^{o}_{20,w}$ (unit = seconds or Svedbergs, S, where $1S = 10^{-13}$ sec) and from the slope we can get the sedimentation concentration regression coefficient, k_S (unit = ml/g) and then from *both parameters* our shape information.

For shape analysis there are three lines of attack (Fig. 2). If the macromolecule is fairly rigid we can combine the sedimentation coefficient with other techniques such as intrinsic viscosity, rotational diffusion (from fluorescence depolarisation or electric birefringence measurements), or the radius of gyration (from "static" light scattering or low angle x-ray scattering) to model the conformation in terms of simple ellipsoids of revolution, general triaxial ellipsoids (4) or sophisticated but beautiful bead models (5) - but this approach is really more for *fairly* rigid protein structures such as seed globulins, or, if I can quote a non-food example, antibodies. For not-so-rigid macromolecules like polysaccharides we have to use more general shapes using the "Wales/van Holde" ratio of k_S to the intrinsic viscosity, $[\eta]$ or the "Mark-Houwink-Kuhn-Sakurada" (MHKS) b coefficient which comes from the relation between $s^{o}_{20,w}$ and the molecular weight, M {similar coefficients exist for the intrinsic viscosity, the diffusion coefficient, and radius of gyration with M - see ref. 1} to permit the modelling of the conformation in terms of general shapes, between the three extremes of compact sphere, rigid rod and random coil. For this general type of modelling we employ a very useful construction known as the Haug triangle (see e.g. refs 6 or 1).

Each of the three extremes of conformation has specific values for $k_S/[\eta]$ and the MHKS b coefficient, and from sedimentation velocity analysis we can easily confirm observations made by

Table 1. Polysaccharide shapes from sedimentation velocity

	ks/[η]	b	Conformation
Dextran fractions		0.44	Random coil
DIT-dextrans		0.56	Semi-flexible coil
Pullulans	1.4	0.45	Random coil
β-glucans	0.4		Extended
Amylopectin*	1.45		Spheroidal
Alginates	0.6		Extended
Citrus pectins	0.2	0.17	Rigid-rod
Mucopolysaccharide	1.5	0.4	Random coil

From ref. 7 and references cited therein
* All in aqueous solvents apart from * (90% DMSO, 10% H_2O)

other techniques that most food polysaccharides have conformations ranging from rigid rod to random coil, as can be seen by the collection of examples shown in Table 1. An exception we found was amylopectin, but that was perhaps only because a non-aqueous solvent had to be used to solubilise it.

Double logarithmic plots of $s^{o}_{20,w}$ against M which are used to obtain the MHKS b coefficient can also be used to model the *flexibility* of a polysaccharide in terms of wormlike coil models from the ratio of the contour length L to the persistence length, a: an example of the application o the latest wormlike coil theory of Garcia de la Torre and coworkers to a pectin can be found in ref 8.

POLYSACCHARIDE SIZE AND SIZE DISTRIBUTION ANALYSIS BY SEDIMENTATION EQUILIBRIUM

Whereas in a sedimentation velocity experiment at relatively high rotor speeds - for a polysaccharide say 40000-50000 rev/min - the sedimentation rate and hence sedimentation coefficient is a measure of the size and shape of the molecule, at much lower speeds, say 10000 rev/min or less in a sedimentation *equilibrium* experiment the forces of sedimentation and diffusion on the macromolecule become comparable and instead of getting a sedimenting boundar you get a steady state equilibrium distribution of macromolecule with a low concentration at the meniscus building up to a high concentration at the cell base. This final steady state pattern is a function *only* of molecular weight and related parameters (virial coefficients and association constants where appropriate) and not on molecular shape since at equilibrium there is no further movement of the macromolecule hence frictional effects through shape variation do not come into

Table 2. Molecular Weights of Food Polysaccharides {weight averages from sedimentation equilibrium analysis}. These values are normally precise to ± 5-15%.

Polysaccharide	M_W	Ref.
Pullulan P100	95000	10
Pullulan P800	76000	10
Pullulan P1200	124000	10
Dextran T500	500000	11
Guar	800000	12
Locust bean gum {Cold water soluble fraction}	340000	12
Locust bean gum {Hot water soluble fraction}	330000	12
Glucomannan	280000	13
Beta-Glucan {Barley}	160000	14
Beta-Glucan {Wheat}	230000	1
Chitosan {Protan "Sea Cure"}	162000	15
Chitosan {Trondheim "KN50-1"}	64100	15
Chitosan {Trondheim "KN50-4"}	4300	15
Xanthan {Kelco "RD"}	3,840,000	16
Alginate {Kelco "Pro-nova"}	210000	17
Pectin {Koch-Light, Citrus}	90000	
Pectin {Green Tomato}	160000	18
Pectin {Red Tomato}	96000	18
Pectin {Red Tomato - antisense polygalacturonase}	135000	19

play - so like "static" (as opposed to dynamic) light scattering it's an absolute way of getting (weight-average) molecular weight.

We record these concentration distributions using a special type of refractive index optics known as Rayleigh Interference. In our laboratory these patterns are digitised automatically using a commercially available laser densitometer (such as of the type used primarily to scan electrophoresis gels and which you can find in most Biochemistry departments), and from a simple Fourier cosine series analysis of the digitised data we can accurately record our concentration distribution. From routines (such as "MSTAR" - see ref. 9) *almost* equivalent to getting the average slope - its not *quite* as easy as that - of plots of log concentration against distance from the centre of the rotor squared, we can get the apparent weight average molecular weight, $M_{w,app}$ which after an extrapolation (of $M_{w,app}$ or $1/M_{w,app}$) to zero concentration gives us the *weight average* molecular weight. This method has been used to get the weight average molecular weights for a range of polysaccharides - neutrals, polycationic and polyanionic - and some examples are given in Table 2. Some comments are appropriate with regards to Table 2: The first comment is that the molecular weights of *pullulans* - widely used in the polysaccharide chromatography field as standards - were all worked out *not* by static light scattering - as many people think - but by sedimentation equilibrium (10). The second comment is that if you compare in particular the values for chitosan KN50-4 and the xanthan, you can appreciate the range of sizes that can be comfortably handled by this technique. Finally with regard to the tomato pectin data of Table 2 it has been known for quite a while that when tomatoes ripen the pectin components of the cell wall become degraded, but in some recent work done with Prof. D. Grierson's group at Sutton Bonington, if you remove one of the key enzymes for degradation by scrambling the gene, the pectins remain more or less intact.

So we can obtain reliable molecular weights for food polysaccharides - and with no problems of dust or whatever you get with light scattering. We can also get reliable size or molecular weight *distribution* information out, which I suppose is more interesting to the food industry, since it's the amount of low molecular weight material festering around which tends to muck up the gelling and thickening properties, or in the case of carageenan, give rise to possible toxicity problems. There are four lines of attack here (Table 3). The simplest way ("Method 1") is to use polydispersity indices or ratios of the weight average to number average molecular weight, or, as more appropriate to sedimentation equilibrium analysis, the ratio of the z-average to weight average molecular weight. We have just considered above how we can get reliable *weight average* molecular weights from Rayleigh interference optical records of the distribution of solute at sedimentation equilibrium. What is perhaps not widely appreciated is that you can get reliable *z-average* information directly if you use instead the Schlieren optical system. This latter system records the concentration *gradient* against distance as opposed to *concentration* against distance which we get from Rayleigh optics, and we have a 3rd Beckman Model E dedicated to this optical system.

With Method II (Table 3) you can model the log concentration versus distance squared plot directly, but this method which came out about 9 years ago (20) has only be applied to relatively simple two- or three-component systems because of the severe demands this method puts on computational resources. A much easier method - so called Method III - is to model the

Table 3. Molecular Weight Distributions {by sedimentation equilibrium analysis}

Method	Type of Analysis
I	Polydispersity indices (M_Z/M_W etc)
II	Modelling the concentration distribution: Non-ideal polydisperse fit
III	Modelling the concentration distribution: Equivalent self association fit
IV	Coupling to gel permeation chromatography

concentration distribution as if the system were self-associating (even though it may not be!). This is not phoney but uses the principle of indistinguishability in a single sedimentation equilibrium experiment between the solute distribution for a system where the species of different molecular weight are in chemical equilibrium (i.e. a "self-associating" system) from a system in which they are not (i.e. a "polydisperse" one). Using this principle its possible to use the much easier to handle equations for a (non-ideal) association (as opposed to a non-ideal polydisperse distribution) to work out an *effective* association constant which then defines the distribution of molecular weight at zero centrifugal field, no matter what the cause of that distribution was (by a self-association, or in our case, by straight polydispersity). Although this has been successfully applied to mucopolysaccharides (21) - giving a distribution apparently in agreement with a distribution evaluated from electron microscopy (22) - and appears promising it has not yet been applied to food polysaccharides.

The final method - so called Method IV (23) - and which is currently our method of choice - is to use sedimentation equilibrium in conjunction with gel permeation chromatography, giving the latter an absolute basis, and this has been applied to alginate, dextran (24) and citrus pectin (8). With the latter, good agreement with a similar procedure of Dr. G. Berth (Potsdam) using light scattering coupled to gel permeation chromatography has been obtained.

REFERENCES

1. Harding, S.E., Vårum, K.M., Stokke, B.T. and Smidsrød, O. (1991) Adv. Carbohydrate Analysis 1, 63-144
2. Harding, S.E., Rowe, A.J. and Horton, J.C. eds (1992) *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (629pp) Royal Society of Chemistry, Cambridge UK
3. Harding, S.E. and Tombs, M.P.(1989) in *Interactions in Protein Systems* (Schwenke, K.D., Raab, B. eds.) pp65-72, Abhandlungen der Akademie der Wissenschaften der DDR, Akademie-Verlag, Berlin
4. Harding, S.E. (1989) in *Dynamic Properties of Biomolecular Assemblies* (Harding, S.E. & Rowe, A.J. eds) pp32-56, Royal Society of Chemistry, Cambridge, UK
5. Garcia de la Torre, J. (1989) in *Dynamic Properties of Biomolecular Assemblies* (Harding, S.E. & Rowe, A.J. eds) pp3-31, Royal Society of Chemistry, Cambridge, UK
6. Smidsrød, O and Andresen, I.-L. (1979) *Biopolymerkjemi* (383pp) Tapir, Trondheim
7. Harding, S.E. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S.E., Rowe, A.J. and Horton, J.C. eds) pp495-516, Royal Society of Chemistry, Cambridge UK
8. Harding, S.E., Berth, G., Ball, A., Mitchell, J.R. and Garcia de la Torre, J. (1991) Carbohyd. Polym. 16, 1-15
9. Harding, S.E., Horton, J.C. and Morgan, P.J. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S.E., Rowe, A.J. and Horton, J.C. eds) pp275-294, Royal Society of Chemistry, Cambridge, UK
10. Kawahawa, K., Ohta, K., Miyamoto, H. and Nakamura, S. (1984) Carbohyd. Polym. 4, 335
11. Edmond, E., Farquhar, S., Dunstone, J.R. and Ogston, A.G. (1968) Biochem. J. 108, 755
12. Gaisford, S.E., Harding, S.E., Mitchell, J.R. and Bradley, T.D. (1986) Carbohyd. Polym. 6, 423-442
13. Winwood, R. (1986) PhD Dissertation, University of Nottingham, UK
14. Woodward, J.R., Phillips, D.R. and Fincher, G.B. (1983) Carbohyd. Polym. 3, 143
15. Errington, N., Harding, S.E., Vårum, K.M. and Illum, L. (1993) Int. J. Biol. Macromol. 15, 113-117
16. Dhami, R., Harding, S.E, Mitchell, J.R, Hunt, T. and Jones, T (1993) mss. in preparation
17. Horton, J.C., Harding, S.E., Mitchell, J.R. and Morton-Holmes, D.F. (1991) Food Hydrocolloids, 5, 125-127
18. Seymour, G.B. and Harding, S.E. (1987) Biochem.J. 245, 463-466
19. Smith, C.J., Watson, C.F., Morris, P.C., Bird, C.R., Seymour, G.B., Gray, J.E., Arnold, C., Tucker, G.A., Schuch, W., Harding, S.E. and Grierson, D.

(1990) Plant Mol. Biol. 14, 369-379
20. Harding, S.E. (1985) Biophys. J. 47, 247-250
21. Creeth, J.M. and Cooper, B. (1984) Biochem. Soc. Trans. 12, 618-621
22. Sheehan, J.K., Oates, K. and Carlstedt, I. (1986) Biochem.J. 239, 147-153
23. Ball, A., Harding, S.E. and Mitchell, J.R. (1988) Int. J. Biol. Macromol. 10, 259-264
24. Ball, A., Harding, S.E. and Simpkin, N.J. (1990) in (Phillips, G.O., Williams, P.A. and Wedlock, D.J.eds) *Gums and Stabilisers for the Food Industry* Vol 5, pp447-450, IRL Press, Oxford UK