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ORIGINAL PAPER

Structure and heterogeneity of gliadin: a hydrodynamic evaluation

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Abstract A study of the heterogeneity and conformation in solution [in 70% (v/v) aq. ethanol] of gliadin proteins from wheat was undertaken based upon sedimentation velocity in the analytical ultracentrifuge, analysis of the distribution coefficients and ellipsoidal axial ratios assuming quasi-rigid particles, allowing for a range of plausible time-averaged hydration values. All classical fractions (α, γ, ω_{slow}, ω_{fast}) show three clearly resolved components. Based on the weight-average sedimentation coefficient for each fraction and a weight-average molecular weight from sedimentation equilibrium and/or cDNA sequence analysis, all the proteins are extended molecules with axial ratios ranging from $\sim$10 to 30 with α appearing the most extended and γ the least.

Introduction

The seed storage proteins (prolamins) of wheat are the major determinants of the unusual and unique (among the cereals) properties of viscosity and elasticity exhibited by wheat doughs and gluten. This combination of properties determines the technological quality of wheat, and therefore its uses, including bread making and pasta quality (Shewry and Tatham 1990). Whereas a large number of protein sequences are now available from cDNA libraries, the structures of the prolamins are poorly understood (Shewry et al. 2008).

The prolamins can be divided into two groups on the basis of their solubility: the gliadins which are soluble in aqueous alcohols and the glutenins which are soluble in aqueous alcohols on the addition of a disulphide reducing agent. Gliadins comprise about half the total prolamins of gluten, are monomeric with intramolecular disulphide bonds and contribute to the viscous nature of doughs. They have been traditionally divided into four groups on the basis of their electrophoretic mobility at acid pH into α-, β-, γ- and ω-gliadins (Woychik et al. 1961) and comprise complex heterogeneous mixtures. Comparisons of amino acid and DNA sequences show that the α- and β-gliadins are closely related and referred to as "α-type" gliadins, while the γ- and ω-gliadins are structurally distinct (Shewry and Tatham 1990). The α-type gliadins consist of

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a short N-terminal domain of five residues, a repetitive domain of about 113–134 residues and a C-terminal domain of about 144–166 residues, the latter domain containing two poly-glutamine regions. The repetitive domain consists of a repeat motif of five to eight residues of consensus sequence Pro·(Phe/Tyr)·Pro·Gln·Gln·Gln·(Gln)(Gln), and differences in the length of the repetitive domain define the differences in molecular weight of the α-gliadins, which range from about 30,000 to 34,000. The γ-type gliadins have a similar domain structure consisting of a 12-residue N-terminal domain, a repetitive domain of 78–161 residues with a consensus repeat consisting of Pro·Phe·Pro·Gln·Gln·(Gln)·Pro·Gln·Gln·(Pro·Gln·Gln), and a C-terminal domain of 135–149 residues containing a single poly-glutamine region. Differences in the length of the repetitive domain account for the variation in the molecular weight range (about 26,000–36,000) of the γ-type gliadins. There are few complete sequences available for the ω-gliadins. One consists of a short N-terminal domain of 11 residues, a repetitive domain of 238 residues, and a short C-terminal domain of 12 residues; the consensus repeat consists of 6–11 residues of Pro·Phe·Pro·Gln·(Gln)·(Gln)Pro·Gln·(Gln)·(Gln)·(Gln) and is similar to the γ-gliadin repeat (Shewry et al. 2008; Tatham and Shewry 1995; Hsia and Anderson 2001; Matsuo et al. 2005; Altenbach and Kothari 2007).

The structures and/or sequences of the gliadin repetitive domains are implicated as being the causative factors in a number of human diseases. The immunodominant activating sequences in coeliac disease (gluten intolerance) are located in repetitive domains of the ω-gliadins (and homologous proteins in barley and rye), in wheat-dependent exercise-induced anaphylaxis (WDEIA) the immunodominant protein is an ω-gliadin (Matsuo et al. 2004), and ω-gliadins are implicated in wheat hypersensitivity (Palosuo et al. 2001). The unusual structures adopted by these domains may, in part, be responsible for their association with these diseases.

A number of studies have reported the shape of gliadins. Krejci and Svedberg (1935) used analytical ultracentrifugation to analyse the gliadin fraction of wheat extracted with aqueous ethanol. This study first demonstrated the heterogenous nature of wheat gliadins, although they identified a principal component with a molecular weight of approximately 34,500 g/mol and calculated the dissymmetry factor which indicated the non-globular nature of these proteins. Lamm and Poulsen (1936) and Entrikin (1941) analysed the shapes of gliadins using translational diffusion and dielectric dispersion measurements (in terms of translational and rotational frictional properties respectively); both studies showed asymmetric molecules with axial ratios between 8:1 and 13:1. Later measurements based on intrinsic viscosity, however, indicated more globular structures (Taylor and Cluskey 1962; Wu and

Dimler 1964; Cole et al. 1984), although Field et al. (1986) determined the intrinsic viscosity of C-hordein (the ω-gliadin homologue from barley) and described a rod-shaped molecule. Thomson et al. (1999) used small-angle X-ray scattering to study the size and shape of α-, γ- and ω-gliadins and described prolate ellipsoids of differing axial ratio. Both intrinsic viscosity and X-ray scattering require relatively high concentrations of protein in contrast to analytical ultracentrifugation. At higher concentrations, aggregation can become problematic and may, in part, account for the apparent disparity in the results. In this study of the solution conformation of the gliadins, an assessment of the oligomeric state under the conditions employed was also undertaken.

By contrast, advantage can be taken of recent developments in analytical ultracentrifugation procedures for the study of the size and shape of the different gliadins in dilute solution conditions. Although the principles of both sedimentation velocity and sedimentation equilibrium methodology in the ultracentrifuge are essentially the same as at the time of Krejci and Svedberg (1935), the instrumentation, data capture and analysis software have advanced enormously (see for example Scott and Schuck 2005).

Materials and methods

Gliadin sample preparation

Total gliadins were extracted from chloroform-defatted wheat flour cv. *Mercia* with 70% (v/v) aqueous ethanol and then dialysed against 1% (v/v) acetic acid and freeze-dried. Gliadins were then separated by ion exchange chromatography on carboxymethyl cellulose (CM) according to the procedure of Booth and Ewart (1969) using 3 M urea, 0.01 M glycine acetate buffer pH 4.6 and eluted with a linear gradient of salt. The gliadin fractions were dialysed against 1% (v/v) acetic acid prior to freeze drying. Gliadin fractions were identified and assayed for purity by acid-PAGE (Clements 1987) and SDS-PAGE (Laemmli 1970). The four fractions were taken to correspond to α-, γ-, $ω_{slow}$- and $ω_{fast}$-type gliadins.

Instrumentation

Sedimentation experiments were performed on a Beckman Optima XL-A (Palo Alto, CA, USA) analytical ultracentrifuge, equipped with UV absorption optics (280 nm). A four-hole titanium rotor was used with reference for the calibration of radial distance. Ultracentrifuge cells of 12-mm optical path length were used, with aluminium alloy type double sector centrepieces containing the sample

and reference solvent channels. Cell windows were of optical grade quartz.

Sedimentation velocity

Whole gliadin and gliadin fractions (α, γ, ω_{slow} and ω_{fast}) were prepared at different concentrations (0.25–2.0 mg/mL; 390 μL) and injected into the sample channel of the cell; the reference channel was filled with 70% (v/v) aq. ethanol reference solvent (400 μL). Samples were centrifuged at 50,000 rpm at 20.0°C. Concentration profiles and the movement of the sedimenting boundary in the analytical ultracentrifuge cell were recorded using the UV absorption optical system and converted to concentration versus radial position. The data were then analysed using the "$c(s)$ model" incorporated into the SEDFIT (Version 9.4b) program (Schuck 1998). This software based on the numerical solutions to the Lamm equation follows the changes in the concentration profiles with radial position and time and generates a distribution of sedimentation coefficients in the form of $c(s)$ versus $s_{T,b}$ (Schuck 1998).

The conversion of the $s_{T,b}$ value to standard solvent conditions (that of the density and viscosity of water at 20°C) gives $s_{20,w}$ (see for example van Holde 1985):

$$s_{20,w} = s_{T,b} \left[\frac{(1 - \bar{v}\rho_{20,w})\eta_{T,b}}{(1 - \bar{v}\rho_{T,b})\eta_{20,w}} \right] \qquad (1)$$

$\eta_{T,b}$ and $\rho_{T,b}$ are the viscosity and density of the experimental solvent [70% (v/v) aq. ethanol] at the experimental temperature (20.0°C), and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20.0°C.

The partial specific volume ($\bar{v}$) was calculated from the amino acid composition of the gliadins using the "Traube rule" principle as encoded in the SEDNTERP algorithm (Laue et al. 1992). The partial specific volumes for α, γ and ω-gliadins were found to be 0.729, 0.724 and 0.723 mL/g respectively. To eliminate effects of solution non-ideality, the corrected $s_{20,w}$ values were then plotted against concentration to obtain the sedimentation coefficient at infinite dilution ($s_{20,w}^0$), from the linear extrapolation to zero concentration using the Gralén (1944) equation:

$$s_{20,w} = s_{20,w}^0 (1 - k_s c) \qquad (2)$$

where k_s is the Gralén concentration-dependent parameter (mL/g).

Sedimentation equilibrium

The sample solution (100 μL) and the reference solvent (105 μL) of 70% (v/v) aq. ethanol were injected into the relevant sectors of double sector 12-mm optical path length cells. The sedimentation equilibrium runs were performed at 20,000 rpm and 10.0°C (10.0°C was used in order to minimise potential sample degradation). Scans were recorded every 4 h. After equilibrium was attained, the sample was run for a further 4 h at over-speed 55,000 rpm to give an optical baseline (total run time ~36 h).

Concentration distributions at equilibrium (recorded as a function of radial displacement from the centre of rotation) were analysed using the MSTARA (MSTARA is the version of the MSTAR programme for use with UV absorption data) programme (Cölfen and Harding 1997), which provides model-independent evaluation of sedimentation equilibrium data using the M^* function (Cölfen and Harding 1997). In brief, MSTAR allows the evaluation of the apparent molecular weight, $M_{w,app}$, over the whole distribution (from meniscus to cell base) and also the point average molecular weight, $M_{w,app}$, as a function of radial position, r, in the cell and also as a function of concentration $c(r)$ [expressed in terms of absorbance $A(r)$]. The function $M^*(r)$, at a given radial position, when extrapolated to the cell base, gives the M (over the whole distribution). Apparent weight-average molecular weights are calculated at different concentrations and extrapolated to zero concentration to eliminate effects of thermodynamic non-ideality to give 'ideal' weight-average molecular weight, M_w.

$$\frac{1}{M_{w,app}} = \frac{1}{M_w} + 2Bc \qquad (3)$$

cDNA analysis

Molecular weights for the gliadins were obtained from the NCBI GenBank sequence database (accessed December 2008) (with the omission, where necessary, of the signal sequences) using a search of all databases and the specific gliadin. Putative sequences, partial sequences and sequences containing stop codons were omitted.

Results and discussion

Heterogeneity and sedimentation coefficient distributions of gliadin

The $c(s)$ profile of whole gliadin (Fig. 1; Table 1) shows three resolved peaks: a major component at 0.7 S (66%) and two minor components at 1.2 S (15%) and 1.4 S (19%).

The $c(s)$ profiles of the gliadin fractions (α, γ, ω_{slow} and ω_{fast}) also show three components (Fig. 2; Table 1). In each case the major component has the lowest sedimentation coefficient (0.8, 1.2, 1.2 and 0.9 S for α, γ, ω_{slow} and ω_{fast}-gliadin fractions respectively). This leads us to estimate that the three components we see in the whole gliadin fraction are likely to be due to the four "major"
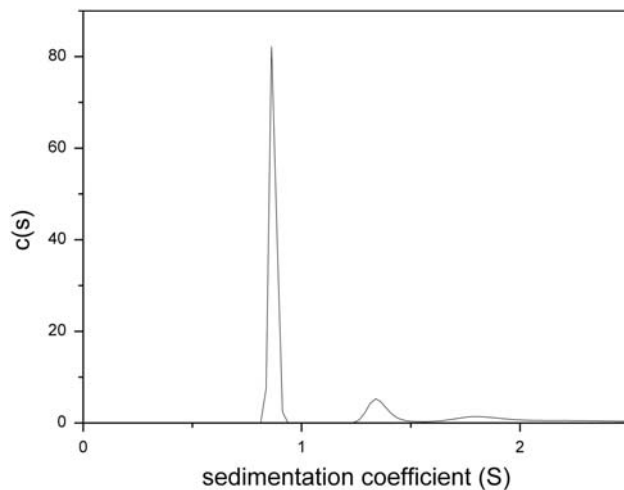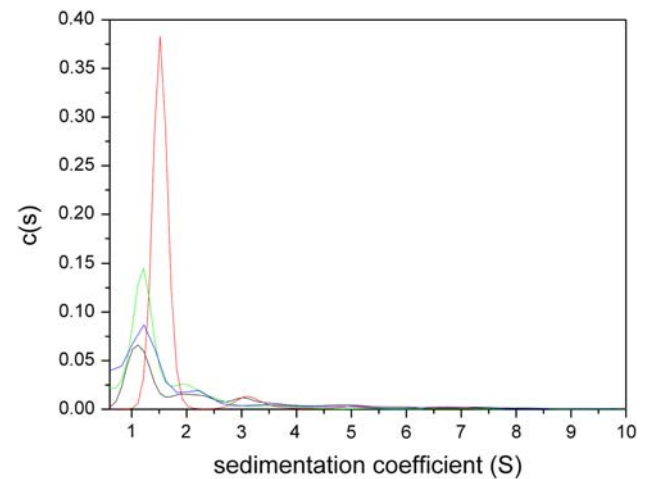
Fig. 1 The $c(s)$ profile for whole gliadin at a nominal total loading concentration of 2.0 mg/mL

Table 1 Sedimentation coefficient $s^0_{20,w}$ values (in Svedberg units, S) for whole, α-, γ-, ω-gliadins and approximate percentage by weight

Gliadin fraction	Subfraction	$s^0_{20,w}$ (S)	Proportion in fraction (%)	$s^0_{20,w}$ (weight-average)
Whole gliadin	F_1	0.7	66	0.9
	F_2	1.2	15	
	F_3	1.4	19	
α	α_1	0.8	62	1.3
	α_2	1.9	18	
	α_3	2.5	20	
γ	γ_1	1.2	83	1.6
	γ_2	2.8	13	
	γ_3	4.6	5	
ω_s	ω_{s1}	1.2	65	1.6
	ω_{s2}	1.8	27	
	ω_{s3}	4.2	8	
ω_f	ω_{f1}	0.9	75	2.1
	ω_{f2}	2.1	12	
	ω_{f3}	9.1	13	

Fig. 2 The $c(s)$ profiles for gliadin fractions: α-gliadin (*black*), γ-gliadin (*red*), ω_s-gliadin (*green*) and ω_f-gliadin (*blue*) at nominal total loading concentrations of 0.25, 0.25, 0.25 and 0.75 mg/mL respectively

Table 2 Weight-average molecular weights (M_w), polypeptide chain molecular weight (M_1), translational frictional ratio (f/f_o), Perrin shape parameter (P), and estimated axial ratio (a/b) for differing plausible hydrations (δ), for α-, γ- and ω-gliadins in 70% (v/v) aq. ethanol solutions

Gliadin fraction	M^a_w (g/mol)	M^b_1 (g/mol)	f/f_o^c	P^d	a/b^d
α	$33,400 \pm 1,000$	30–36,000	2.9	2.2–2.5	25–34
γ	$24,600 \pm 1,000$	27–32,000	2.0	1.5–1.7	9–13
ω_s		30–43,000[e]	2.6	1.9–2.3	18–28
ω_f		52,000	2.5	1.8–2.2	15–25

[a] From sedimentation equilibrium

[b] From cDNA sequences

[c] Calculated from sedimentation equilibrium values where possible

[d] Range based on (time-averaged) hydration values δ ranging from 0.35–1.0 g/g

[e] Mean value of 36,500 g/mol used for the estimation of f/f_0

components of each fraction, although upon extrapolation to infinite dilution, the absolute values are slightly different, and we are, therefore, unable to assign components directly.

The weight-average molecular weights (the weight-averages over all components in that fraction) obtained for the α- and γ-gliadin fractions using the MSTARA programme (Cölfen and Harding 1997) are shown in Table 2. It is seen that the weight-average molecular weights are in reasonable agreement with the cDNA sequence data values and offer little evidence of associative behaviour in 70%

(v/v) aq. ethanol solutions. Due to lack of sufficient sample material, we were unable to perform sedimentation equilibrium experiments on the ω-gliadin fractions and, therefore, the cDNA sequence data values have been used.

Estimation of shape

Estimates for shape (molecular asymmetry) of the gliadin fractions can, in principle, be obtained by combining their $s^0_{20,w}$ values with their molecular weights (where possible the weight-average molecular weights from sedimentation equilibrium were used) by determining the translational frictional ratio f/f_o. In order to be certain that we are comparing like-for-like, we have used the weight-average

sedimentation coefficient. After assigning hydration (in terms of grams of physically bound or entrained solvent per gram of protein) values (0.35, 0.5 and 1.0 g/g), an estimate of the axial ratio of the equivalent prolate ellipsoid, commonly used to represent the average solution conformation of protein, can be obtained using the procedure ELLIPS1 (Harding et al. 1997; Harding et al. 2005).

The translational frictional ratio (ratio of the frictional coefficient of the gliadin molecule to the frictional coefficient of a spherical particle of the same anhydrous mass) was obtained from M_w and $s_{20,w}^0$ via (see Tanford 1961):

$$\frac{f}{f_0} = \frac{M_w(1 - \bar{v}\rho_{20,w})}{(N_A 6\pi\eta_{20,w}s_{20,w}^0)} \left(\frac{4\pi N_A}{3\bar{v}M_w}\right)^{1/3} \quad (4)$$

This depends on the shape and molecular hydration (chemically bound and physically entrained solvent associated with the protein). The Perrin shape parameter, P (or 'frictional ratio due to shape'; Tanford 1961), can then be calculated from f/f_o by assigning a hydration value, δ, using the expression:

$$P = \left(\frac{f}{f_0}\right) \left[1 + \left(\frac{\delta}{\bar{v}\rho_{20,w}}\right)\right]^{-1/3} \quad (5)$$

Therefore a greater (time-averaged) hydration will result in a lower value of the Perrin shape parameter and hence a lower axial ratio.

Two factors have to be considered in interpreting ultracentrifuge data. Firstly, the assignment of the molecular weight for the subfractions must be considered—the sedimentation equilibrium values give only the weight-average M_w for the subfractions of a given gliadin fraction. Secondly, the assignment of a value for the (time-averaged) molecular hydration parameter δ has been the subject of considerable discussion (see Harding 2001; Squire and Himmel 1979). For proteins with little or no glycosylation, values between 0.35 and 0.5 are typical in aqueous solution, whilst 1.0 is an extreme estimate; these values were used, although we should keep in mind that the solution was 70% (v/v) aq. ethanol, not a pure aqueous system.

Since δ is not known, a range of plausible values (from 0.35 to 1.0) (Harding 2001; Squire and Himmel 1979) were used to specify a range of P values for each (Table 2). Corresponding (prolate) ellipsoidal axial ratios were calculated using the ELLIPS1 routine (Harding et al. 1997; Harding et al. 2005) and visualised (Fig. 3) using Ellips-draw (Harding et al. 2005).

All classical fractions (α, γ, ω_{slow}, ω_{fast}) show three clearly resolved components. Based on the weight-average sedimentation coefficient for each fraction and a weight-average molecular weight from sedimentation equilibrium
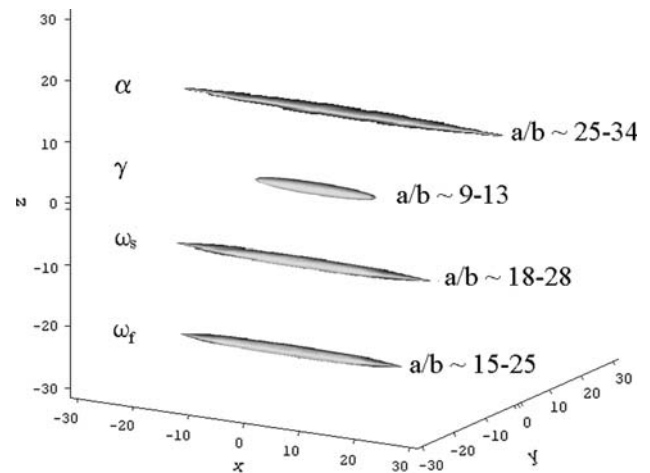
Fig. 3 Schematic representation for gliadin fractions: α-gliadin, γ-gliadin, ω_s-gliadin and ω_f-gliadin in terms of prolate ellipsoids (x, y and z represent the orthogonal axes in which the ellipsoid lies and a, b and c are ellipsoid semi-axes ($a \geq b \geq c$) in the x, y and z directions with $c = a$ for an oblate ellipsoid and $c = b$ for a prolate ellipsoid). The axial ratio shown is the median value from Table 2 ($a/b \sim 30$, 11, 23 and 20 respectively)

and/or cDNA sequence analysis, all the proteins are extended molecules with axial ratios ranging from ~ 10 to 30 with α appearing the most extended and γ the least (Fig. 3). The treatment of the data does not however exclude the possibility of the gliadin molecules adopting other extended or flexible conformations in solution (e.g. rods or stiff coils).

Conclusions

The α-, γ- and ω-gliadins, some of the main determinants of the baking quality of wheat, consist of at least three discernible subfractions. Assigning solution conformations for these subfractions is, however, problematic due to difficulties in assigning the appropriate molecular weights for each. Sedimentation equilibrium gives only the weight-average for a particular fraction; to overcome this we used the weight-average sedimentation coefficient. All four gliadin fractions were found to be highly asymmetric with axial ratios ranging from approximately 10 to 30 depending on the estimate of the time-averaged hydration of these substances. The maximum hydration estimate of 1.0 (g/g) is high for a typical globular protein but is quite conservative for macromolecules which appear to be polysaccharide-like in their conformation. Therefore if their hydrations are higher than 1.0 g/g, the values of the Perrin shape parameters and axial ratios will be lower. This is interesting since small-angle X-ray scattering (SAXS) studies on α-, γ- and ω-gliadins have also suggested an extended structure but with lower axial ratio (Thomson

et al. 1999). Other reported structural studies of the gliadins are however limited. Structural prediction and circular dichroism studies indicate that the repetitive domains consist of a mixture of poly-L-proline II and β-reverse turn structures and that the non-repetitive domains are rich in α-helical structure (Shewry et al. 2008; Tatham and Shewry 1995; Hsia and Anderson 2001; Matsuo et al. 2005; Altenbach and Kothari 2007). Limited studies of the ω-gliadins and homologous C hordeins from barley indicate a mixture of β-reverse turn and poly-L-proline II structures forming an extended rod-like structure in solution, consistent with the results of this study (I'Anson et al. 1992).

Non-covalent interactions between the repetitive domains, predominantly hydrogen bonding, molecular entanglement, van der Waals etc., contribute to the viscous nature of gluten, hydrated ω-gliadins forming highly viscous materials (Wellner et al. 1996). Extended rod-like structures would allow extensive hydrogen bonding and non-covalent interactions between protein molecules, contributing to gluten viscosity. Within the elastic-polymeric glutenin network, homologous proteins to the gliadins are found, with additional cysteine residues allowing the formation of disulphide-bonded polymers (Shewry and Tatham 1997). The repetitive domains of the gliadins and polymeric glutenins could interact and, in part, contribute to the viscoelastic behaviour associated with wheat flours. Although the precise molecular bases for the viscoelastic properties of gluten are unknown, highly asymmetric repetitive protein domains would provide higher levels of contact between protein molecule surfaces than, for example, globular or ellipsoidal molecules. Whatever the precise bases for the properties are, they are doubtless related to the molecular structure and interactions between the constituent proteins (Shewry and Tatham 1997).

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