

## **The Conformation of Wheat Gluten Proteins. The Secondary Structures and Thermal Stabilities of $\alpha$ -, $\beta$ -, $\gamma$ - and $\omega$ -Gliadins**

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The secondary structures of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins were studied by circular dichroism spectroscopy. The  $\omega$ -gliadins contained no detectable  $\alpha$ -helix or  $\beta$ -sheet, but were rich in  $\beta$ -turns. Increasing the temperature of a solution in 70% (v/v) aqueous ethanol from 20 to 80°C resulted in a conformational change, with an increase in class B  $\beta$ -turns. In contrast, the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins contained  $\alpha$ -helix (30–35%) and  $\beta$ -sheet (about 10% in  $\alpha$ -gliadins). Heating resulted in an increase in aperiodic structure, with partial loss of the  $\alpha$ -helical content. These results indicate that whereas the  $\omega$ -gliadins are stabilised by strong hydrophobic interactions, the main stabilising forces in the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins are covalent disulphide bonds and non-covalent hydrogen bonds.

### **Introduction**

Wheat gluten is the visco-elastic mass that remains when dough is washed to remove starch and components soluble in water and dilute solutions of salt. It is composed mainly of proteins (typically about 70%), which are usually classified into two groups. The gliadins are readily soluble in aqueous alcohols, and are monomers associated by hydrogen bonding and hydrophobic interactions. Although they are classically separated into four groups ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins) on the basis of their mobilities on electrophoresis at low pH<sup>1</sup>, more recent studies indicate that a more valid classification is into two groups: the S-poor  $\omega$ -gliadins and the S-rich  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins<sup>2</sup>.

The glutenins are less easy to define and less well understood. Although they are strongly aggregated into complexes of very high molecular weight, even in the presence of strongly dissociating solvents, there has been some dispute about the nature of the stabilising forces. Whereas Bernadin<sup>3</sup> argued that they are non-covalent, most workers now agree on the importance of disulphide bonds<sup>4</sup>. The glutenin aggregates may not be soluble in aqueous alcohols, and the individual subunits, after reduction, only soluble at low pH<sup>5</sup>. Two major groups of subunits are present: the low molecular weight and high molecular weight subunits.

The gluten proteins are largely responsible for the visco-elastic properties of wheat dough, which, in turn, determine the suitability of the dough for breadmaking<sup>4</sup>. Thus,

Abbreviations used: cd = circular dichroism; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulphate.

it is important for an understanding of gluten functionality to study not only the amino acid sequences of the gluten proteins, but also their conformations and how they interact and aggregate to form whole gluten. This information is also of value in evaluating new uses in the food industry for gluten produced by industrial separation processes.

We present in this paper a study of the conformations of the  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins. Circular dichroism (cd) spectroscopy was used to determine the secondary structures of purified fractions, while the nature and strength of the stabilising forces were studied by comparing the conformations at different temperatures in the reduced and native states.

## Experimental

### *Purification of gliadins*

Whole untreated grains of wheat (cv. Maris Butler) were milled to pass a 0.7 mm sieve. Milled grain (200 g) was stirred for 1 h with 2000 ml of 70% (v/v) aqueous ethanol at 20 °C. The supernatant, after centrifugation, was mixed with 2 vols of 1.5 M NaCl, allowed to stand for 18 h at 4 °C and the precipitated gliadin removed by centrifugation. The precipitated gliadin (1 g) was dissolved in 6 M urea, 0.01 M acetic acid, and separated into aggregates and monomers by gel filtration in the same solvent on a 90 × 4.4 cm column of Sephacryl S-300. Fractions containing monomers were bulked, dialysed against water and freeze-dried. The monomer fraction (1 g) was dissolved in 0.1 M acetic acid and separated by gel filtration in the same solvent on a 90 × 4.4 cm column of Biogel P100. Fractions containing  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins were bulked, freeze-dried and separated by ion exchange chromatography on CM cellulose in 10 mM glycine/acetate buffer, pH 4.6, containing 3 M urea, and eluted with a linear gradient of 0–0.25 M NaCl. To prepare  $\omega$ -gliadins the crude gliadin preparation was separated by ion-exchange chromatography on CM cellulose, at pH 3.6 (Kasarda *et al.*<sup>6</sup>).

### *Polyacrylamide gel electrophoresis*

The purified fractions were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a modified Laemmli system; polyacrylamide gel electrophoresis at pH 3.1 (lactate-PAGE) was as described by Lafiandra and Kasarda<sup>7</sup>. Total seed proteins for SDS-PAGE and gliadins for lactate-PAGE were extracted from single seeds as described by Payne *et al.*<sup>8</sup>.

### *Circular dichroism measurements*

The cd measurements were made with a Jasco J40 CS dicograph. Spectra were obtained at various temperatures using a heated cell holder and a thermocouple probe of a Comark Thermometer inserted directly into the solution. Far and near-UV spectra were measured in 0.2 mm and 1 cm pathlength cells, respectively; for variable temperature work, once solutions had reached the required temperature they were equilibrated for approximately 5 min. The results were calculated using an average monomer molecular weight of 110, calculated from published amino acid compositions<sup>9</sup>; the units are degrees cm<sup>2</sup>/mol. All measurements were duplicated, and an average spectrum calculated. Absorption spectra of the same solutions were recorded with a Cary 210 spectrophotometer. Solvents were of the highest purity available, protein solutions were filtered before use (Millex GV filters, 0.22  $\mu$ m, Millipore Corp.).

Protein samples were extensively dialysed against distilled water and freeze-dried. Samples were dissolved in the appropriate buffer and in 6 M urea and protein concentration was determined by comparison with previously reported values in the latter solvent.<sup>10</sup> Although care was taken in

determining the extinctions, and the concentrations from these, errors in these determinations could result in errors in the calculated dichroic absorptions. However, we are confident that these could not be sufficiently great to affect the conclusions of the study.

### *Amino acid analyses*

Amino acid compositions of the two  $\omega$ -gliadin fractions were determined using an LKB 4400 amino acid analyser. These were typical of the  $\omega$ -gliadins<sup>6</sup> with approximately 42–43 mol% glutamate + glutamine, 26–27 mol% proline, 8.5–8.7 mol% phenylalanine and no cysteine.

### *Cysteine modification*

Disulphide bonds in the  $\gamma$ -gliadins were reduced with 2-mercaptoethanol and alkylated with acrylonitrile ( $\text{CH}_2=\text{CHCN}$ ), a UV-transparent reagent<sup>11</sup>. Protein (5 mg) was dissolved in 2.5 ml of alkylation buffer (0.3 M Tris, 1 mM KCl, 1% 2-mercaptoethanol, pH 7.5 with  $\text{HNO}_3$ ) and shaken overnight; 1.5% acrylonitrile was added, shaken for 2 h, then acidified with 5% (v/v) acetic acid to pH 5.0, dialysed against distilled water and freeze-dried.

## **Results and Discussion**

### *Purification and characterisation of the gliadin fractions*

Fractions containing  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins were prepared by a combination of gel filtration and ion exchange chromatography. Electrophoretic analyses are shown in Fig. 1. Lactate-PAGE (Fig. 1(a)) showed slight contamination of the  $\beta$ -gliadins with  $\gamma$ -gliadins, but little or no contamination of the  $\alpha$ - and  $\gamma$ -gliadins. The SDS-PAGE showed that most of the  $\alpha$ -gliadins had lower relative molecular masses ( $M_r$ s) and the  $\gamma$ -gliadins higher, although the  $M_r$ s of the three groups overlapped.

Two  $\omega$ -gliadin fractions were prepared by ion-exchange chromatography. The first gave a major slow doublet on lactate-PAGE (Fig. 1(a), track c) and a single major band on SDS-PAGE (Fig. 1(b), track b). The second gave two major faster bands on lactate-PAGE (Fig. 1(a), track d) and several lower  $M_r$  bands on SDS-PAGE (Fig. 1(b), track c).

### *The cd spectra of $\alpha$ -, $\beta$ -, $\gamma$ - and $\omega$ -gliadins*

The far-UV (below 250 nm) cd spectrum is indicative of the conformation of a protein molecule, the spectrum arising from the absorption of the peptide bond. The far-UV cd spectra of the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins in 70% (v/v) aqueous ethanol show typical  $\alpha$ -helix-rich conformations<sup>12</sup>, with minima around 208 and 222 nm, and maxima in the range 190–200 nm (Fig. 2(a)). The two  $\omega$ -gliadin fractions had spectra that were almost identical to each other, but distinctly different from those of the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins, with none of the characteristics associated with the  $\alpha$ -helical or  $\beta$ -sheet conformation<sup>12</sup>. The spectrum of  $\omega$ -gliadin fraction 2 is shown in Fig. 2(a) and in subsequent figures. Although the spectra resembled that of the random coil conformation<sup>13</sup> certain differences were apparent, notably a minimum at 204 nm rather than between 196 and 200 nm, and a shoulder around 230 nm. A similar spectrum has recently been reported

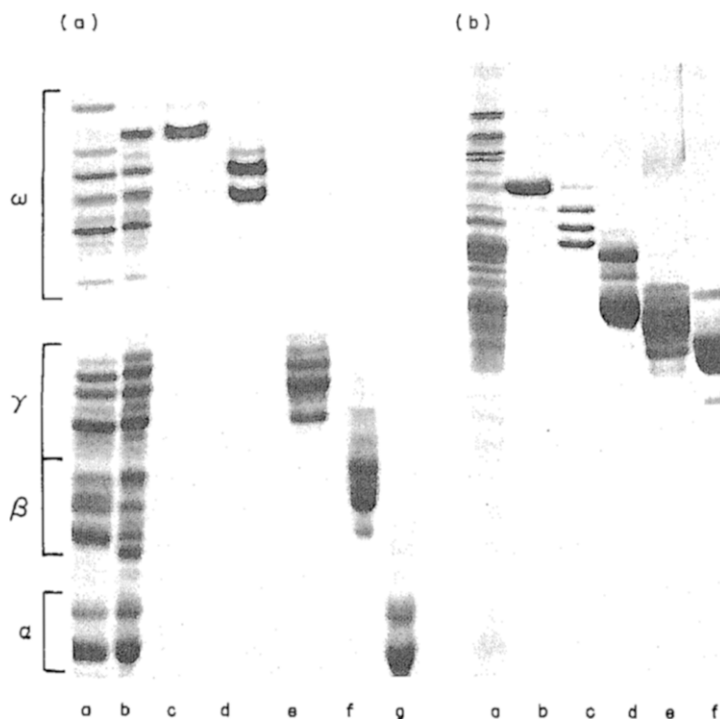


FIGURE 1. Electrophoretic analyses of gliadin fractions. (a) By lactate-PAGE at pH 3.1. Maris Butler is a mixture of seed types (biotypes) differing in their gliadin patterns. Two of these are shown in tracks a and b. Track c is  $\omega$ -gliadin fraction 1, d is  $\omega$ -gliadin fraction 2, e is  $\gamma$ -gliadins, f is  $\beta$ -gliadins and g is  $\alpha$ -gliadins. (b) By SDS-PAGE. Track a is total seed proteins, b is  $\omega$ -gliadin fraction 1, c is  $\omega$ -gliadin fraction 2, d is  $\gamma$ -gliadins, e is  $\beta$ -gliadins and f is  $\alpha$ -gliadins.

for C-hordein, the homologous S-poor prolamins of barley, where it was shown to be associated with a  $\beta$ -turn rich conformation<sup>14</sup>.

Absorption in the near-UV (250–300 nm) region arises principally from the aromatic and disulphide chromophores. The spectra of the gliadins show characteristic phenylalanine band structure (Fig. 2(b), (c)), with two vibronic components centred at 262 and 268 nm<sup>15</sup>. The phenylalanine contribution in this region is relatively strong; this would not be expected for a freely rotating side chain and indicates that in all the gliadins the phenylalanine side chain adopts a fixed conformation. The band at 275–276 nm arises probably from the tyrosyl side chain<sup>15</sup> as discussed previously for the aromatic spectrum of an  $\alpha$ -gliadin by Kasarda *et al.*<sup>16</sup> No contribution from the tryptophan side chain could be detected, though Kasarda *et al.*<sup>16</sup> reported a shoulder at 294 nm for  $\alpha$ -gliadin in aqueous solution. The fine phenylalanine band structure was not reported for A- ( $\alpha$ -) gliadin in dilute HCl<sup>16</sup>; however, in aqueous alcohol such structures become apparent (Fig. 2(b)). Such a change in the aromatic region could arise from the reorientation of the aromatic side chains in solvents of different polarities, with little effect on the overall

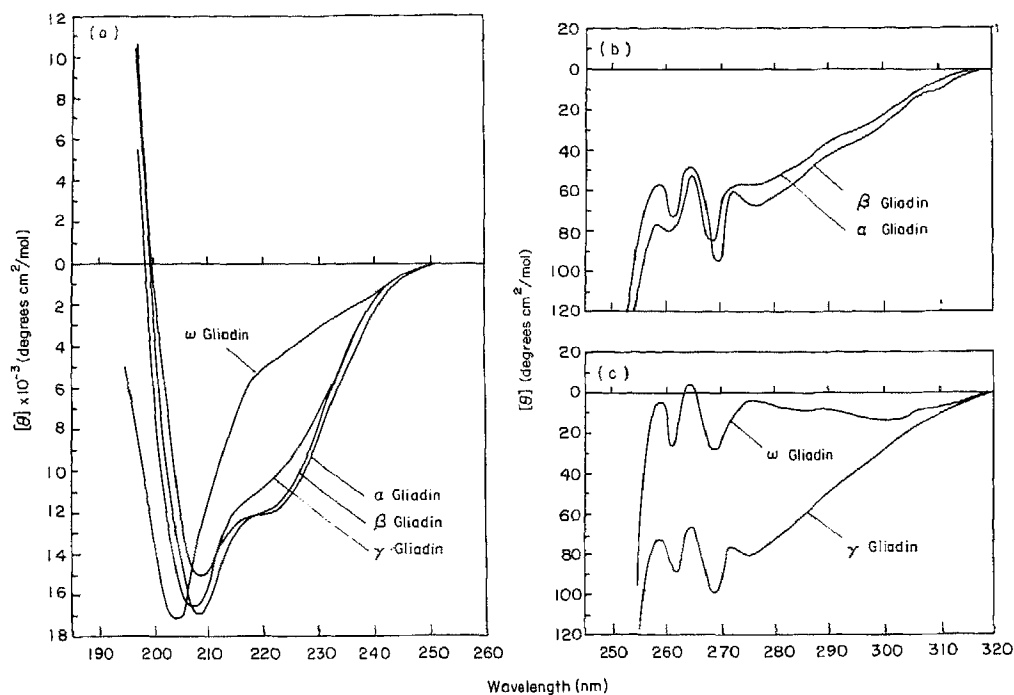


FIGURE 2. The cd spectra of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins in 70% (v/v) aqueous ethanol. (a) Far-UV spectra of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins at 20 °C. (b) Near-UV spectra of  $\alpha$ - and  $\beta$ -gliadins. (c) Near-UV spectra of  $\gamma$ - and  $\omega$ -gliadins. The protein concentration was 1 mg/ml.

conformation of the peptide backbone. One marked difference between the near-UV spectra of the  $\omega$ -gliadin fractions and of the other gliadin fractions is the broad absorbance associated with the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins in the region 260–320 nm (Fig. 2(b)). The  $\omega$ -gliadin fractions contained no cysteine residues, whereas the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins contain about 2 mol% cysteine<sup>9</sup>. The broad absorbance between 260 and 320 nm is probably associated with the rigid cystine disulphide chromophore<sup>17</sup>.

It is generally accepted that the cd spectrum of a protein is a direct reflection of its secondary structure content. Different approaches have been made to correlate the two, with varying degrees of success. One method involves the use of cd spectra of polypeptides of known conformations as the reference spectra for calculation<sup>18, 19</sup>. The second involves calculations based on the cd spectra of globular proteins of known secondary structure, usually determined by X-ray crystallography<sup>20, 21</sup>. A number of assumptions are inherent in both approaches, for example, that the reference spectra are appropriate, that there may be more structures than allowed for, and a number of mathematical and statistical constraints<sup>22</sup>. Using a method of the first type<sup>18</sup>, the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins were found to have similar  $\alpha$ -helical contents of 33–34%, 34–35% and 30–31%, respectively. The value reported for A- ( $\alpha$ -) gliadin in aqueous solution using

the same method was 33–34%<sup>18</sup>, suggesting a similar conformation in aqueous and ethanolic solution. A method of the second type<sup>20</sup> gave  $\alpha$ -helical contents of 36–37%, 36–37% and 33–34% for the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins, respectively.

Analysis<sup>20</sup> of the  $\alpha$ -gliadin spectrum estimated 36–37%  $\alpha$ -helix, 11–12%  $\beta$ -sheet and 52–53% aperiodic structure which would include the random coil and the  $\beta$ -turn conformation. Similar values of 34–35%  $\alpha$ -helix, 8–9%  $\beta$ -sheet, 33–34%  $\beta$ -turn and some 22% random coil were predicted<sup>23</sup> from the amino acid sequences reported for A- and  $\alpha$ -gliadin<sup>24, 25</sup> (authors' unreported results).

#### *Thermal denaturation study of $\gamma$ -gliadin*

The thermal denaturation of native and cysteine-modified  $\gamma$ -gliadin was studied to determine the influence of intramolecular disulphide cross-linking and the inherent conformational stability of the  $\gamma$ -gliadins, as a model for the sulphur-rich monomeric gliadins. Kasarda *et al.*<sup>16</sup> showed a small but reversible loss of the  $\alpha$ -helical content of A- ( $\alpha$ -)gliadin on heating.

The effects of heating on the backbone conformations of native and modified  $\gamma$ -gliadin in 70% (v/v) aqueous ethanol are shown in Fig. 3(a), (b). In both cases increases in temperature resulted in monotonic decreases in regular structure content. The effects of temperature were found to be reversible for both native and modified  $\gamma$ -gliadin. Both sets of curves exhibit isocircular dichroic points at 199 nm, indicating that there is a transition between two defined conformations. The position of the isocircular dichroic point indicates that the denaturation involves a helix-coil transition<sup>26</sup>. The near-UV spectra of the native and modified  $\gamma$ -gliadin show marked differences (Fig. 3(c), (d)), the broad absorbance associated with the disulphide chromophore being absent from the spectrum of the modified gliadin (Fig. 3(d)). The intensity of the phenylalanine absorbance, however, was not affected by reduction of the cystine residues, and this together with the near-identical backbone spectra would suggest that the conformation of the modified gliadin is similar to that of the native protein at 20 °C. There was loss of detail attributed to the tyrosine residues in the modified protein, which indicates that they were probably exposed to the solvent and, hence, in a different environment to that in the native protein. These results are consistent with the recent study of Cole *et al.*<sup>27</sup>, who showed that reduction and alkylation of A-gliadin had little effect on the conformation as determined by intrinsic viscosity. On heating, similar changes were found in both the native and modified material. A loss of aromatic detail in the near-UV region suggests a 'loosening' of the overall conformation, with phenylalanine residues being exposed to the solvent with increasing temperature. The far-UV spectra indicate a small loss of  $\alpha$ -helical content. The loss over the temperature range 20–80 °C, calculated by the method of Greenfield and Fasman<sup>18</sup>, was 3–4% and 6–7% for the native and modified protein, respectively; and by the method of Chen *et al.*<sup>20</sup> as 6–7% and 10–11%, respectively. The native  $\alpha$ - and  $\beta$ -gliadins were as stable to heating as the native  $\gamma$ -gliadins (not shown), and in all cases the changes were completely reversible on cooling.

It would appear that although the disulphide bonds confer some stability to the secondary structure of the  $\gamma$ -gliadins, the helices are inherently quite stable. The marked loss of phenylalanine band structure on heating indicates that the conformation is not

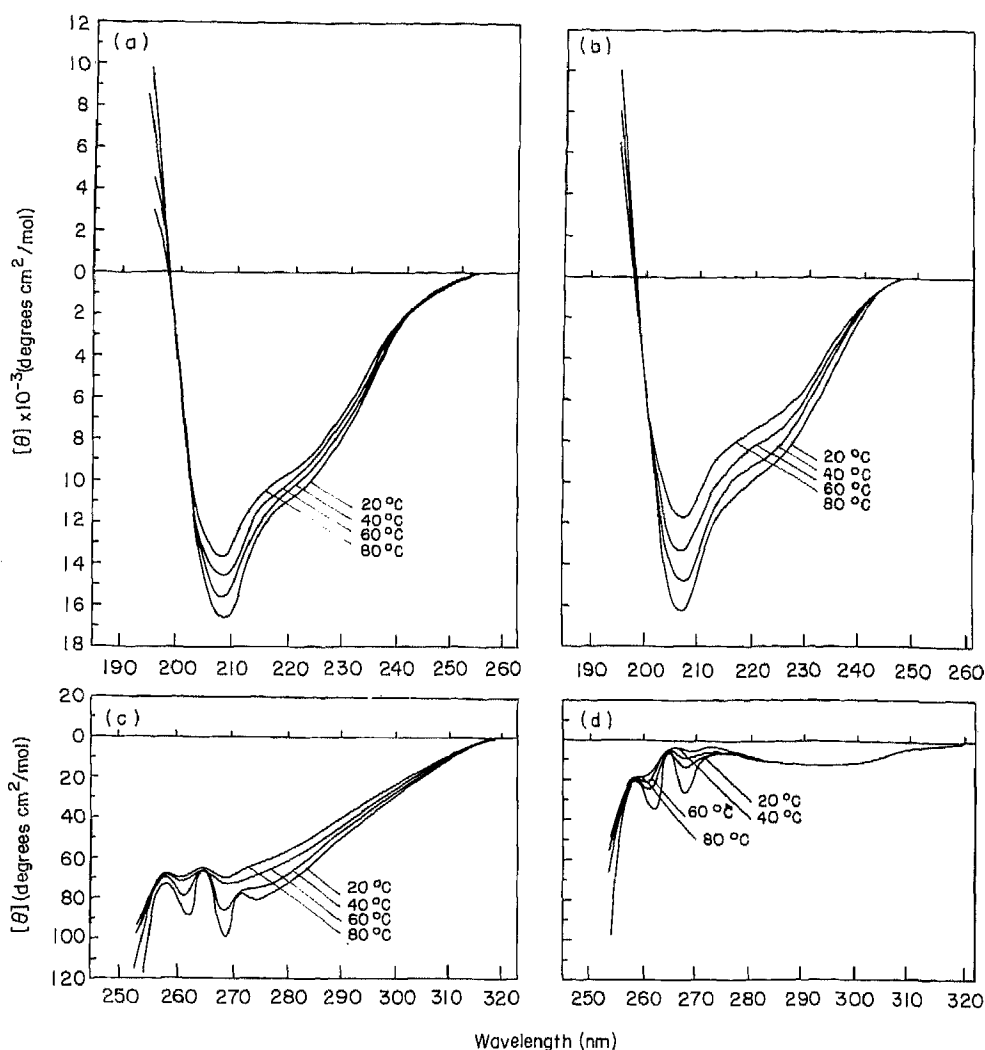


FIGURE 3. Effect of temperature on the cd spectra of native and cysteine-modified  $\gamma$ -gliadin in 70% (v/v) aqueous ethanol. (a) Far-UV spectra of native  $\gamma$ -gliadin, (b) far-UV spectra of modified  $\gamma$ -gliadin, (c) near-UV spectra of native  $\gamma$ -gliadin, (d) near-UV spectra of modified  $\gamma$ -gliadin.

stabilised by hydrophobic interactions involving these residues. Therefore, although increasing the temperature would be expected to decrease hydrogen bond and electrostatic contributions to stability, it must be concluded that one or both of these are important in stabilising the helices. Hydrogen bonding may be more extensive than in most proteins, and would be facilitated by the high content of glutamine, which could form hydrogen bonds with other glutamine residues and possibly into the peptide backbone. Because of this extensive hydrogen bonding the protein would be more stable to heating.

*The effect of heat on  $\omega$ -gliadin*

The effect of temperature on the conformation of  $\omega$ -gliadin in 70% (v/v) aqueous ethanol was also studied. Increasing temperature resulted in an increase in absorption of the 204 nm minimum, with a simultaneous decrease in the intensity of a shoulder centred at 228–230 nm (Fig. 4). The differential absorption was not monotonic, as for the native and modified  $\gamma$ -gliadins; indicating that the conformational change that was occurring was reaching completion near 60 °C.  $\omega$ -Gliadin dissolved in 1 mM acetic acid showed similar changes on heating (spectra not shown). A plot of  $[\theta]/[\theta]_0$  (ellipticity divided by ellipticity at 20 °C at 228 nm) vs. temperature for  $\omega$ -gliadin in 70% aqueous ethanol and

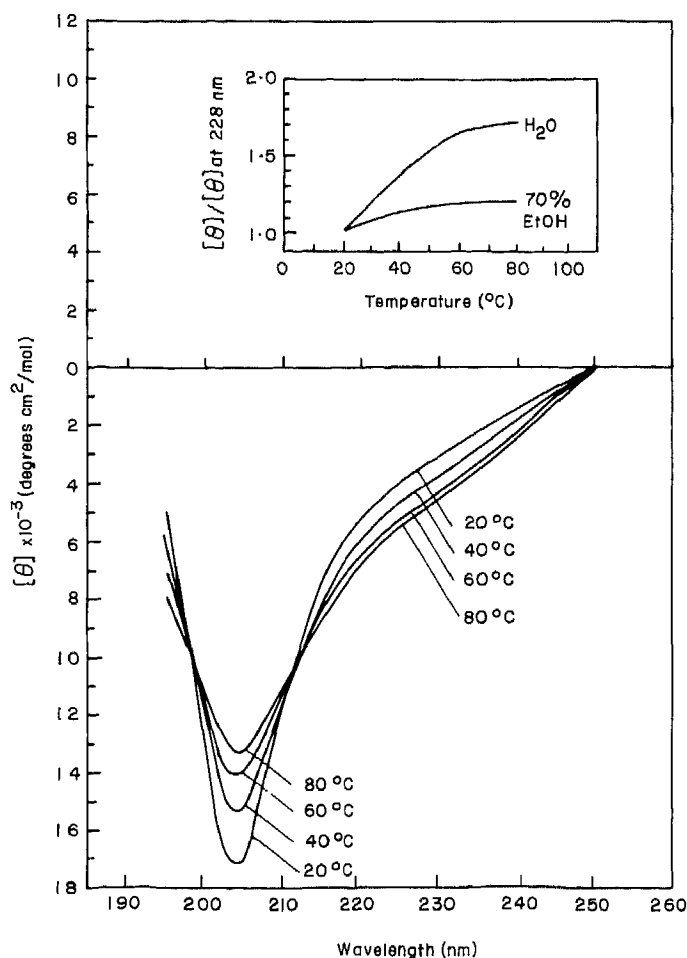


FIGURE 4. Effect of temperature on the cd spectra of  $\omega$ -gliadin in 70% (v/v) aqueous ethanol. Insert:  $[\theta]/[\theta]_0$  at 228 nm, for  $\omega$ -gliadin in 70% (v/v) aqueous ethanol and 1 mM acetic acid.

1 mM acetic acid shows that the conformational changes in both solvents reach completion at about 60–70 °C (Fig. 4 insert). The set of curves exhibited two isocircular dichroic points at 211–212 nm and 198 nm, suggesting two defined conformations, one at high and one at low temperature with a negligible concentration of intermediates<sup>28</sup>. The near-UV spectra, however, showed little change over the same temperature range (spectra not shown, see Fig. 2(c)), indicating an ordered protein structure at all temperatures. This can only be explained if the secondary structure is stabilised, at least partially, by hydrophobic interactions, which are favoured at higher temperatures<sup>29</sup>. The phenylalanine residues would remain in a fixed conformation on heating and not be exposed to the solvent. The effects of temperature on the  $\omega$ -gliadin spectra were completely reversible.

A far-UV difference spectrum was obtained by subtracting the 20 °C spectrum from the 80 °C spectrum (Fig. 5). The spectrum corresponds to a class B,  $\beta$ -turn spectrum, according to the classification scheme of Woody<sup>30</sup>, with minima in the region 220–230 nm and maxima in the region 200–210 nm. This class of  $\beta$ -turn spectrum is the most common associated with any  $\beta$ -turn conformation<sup>31</sup>. There is, however, no unique cd spectrum associated with the  $\beta$ -turn conformation; Woody<sup>30</sup> calculated four different classes of cd spectra from the theoretical  $\beta$ -turn backbone conformations described by Venkatachalam<sup>32</sup>. Proteins rich in  $\beta$ -turns may, therefore, be expected to contain

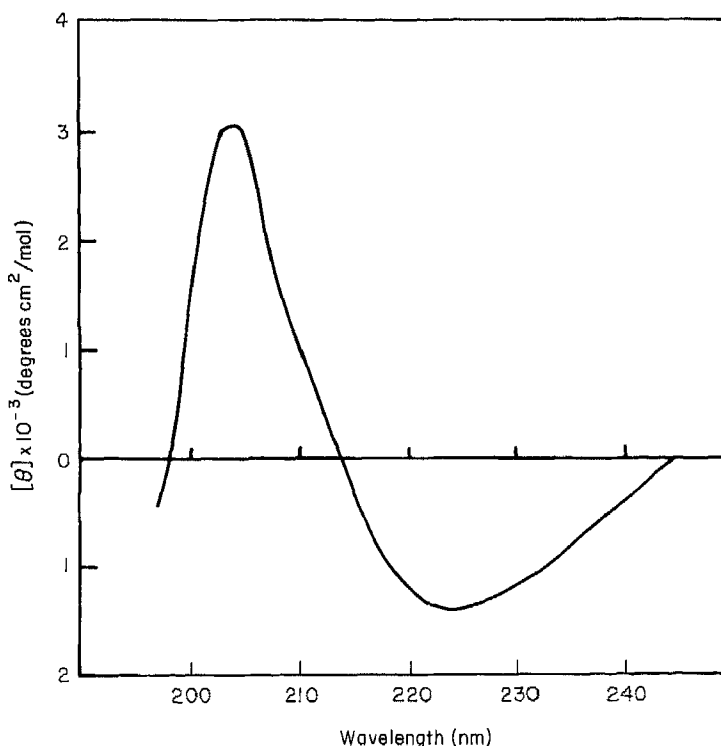


FIGURE 5. Far-UV difference spectrum of  $\omega$ -gliadin (see text for details).

mixtures of turn types, which would be reflected in their cd spectra. The  $\omega$ -gliadin cd spectrum resembles that of other  $\beta$ -turn-rich proteins with minima around 202–204 nm and shoulders between 220–230 nm<sup>33–35</sup>.  $\omega$ -Gliadins contain few or no cysteine residues and we suggest that the  $\beta$ -turn-rich conformation is stabilised by hydrophobic interactions between aromatic residues, and by hydrogen bonding between glutamine residues and other glutamine residues and possibly the peptide backbone. The conformational changes on heating would be expected to be due to increases in the strength and contributions of hydrophobic interactions and decreases in the strength and contributions of hydrogen bonding to the structure. The resulting increase in class B  $\beta$ -turns could be due to the introduction of additional  $\beta$ -turns of this class, or changes in the conformations of the  $\beta$ -turns already present.

### General discussion

The present study indicates that the S-rich ( $\alpha$ -,  $\beta$ - and  $\gamma$ -) gliadins and S-poor ( $\omega$ -) gliadins have different secondary structures, with different degrees of stabilisation by covalent disulphide bonds and non-covalent hydrogen bonds and hydrophobic interactions. The  $\omega$ -gliadins are rich in  $\beta$ -turns, with no detectable  $\alpha$ -helix or  $\beta$ -sheet; these  $\beta$ -turns are probably interspersed with random coil. This is similar to the conformation proposed for the S-poor C-hordein of barley on the basis of cd studies and computer prediction from amino acid sequences<sup>14</sup>. Disulphide bonds are absent and hydrophobic interactions are very important, especially at high temperatures, where an increase in their intensity leads to a conformational change that results in an increase in the content of class B  $\beta$ -turns.

In contrast the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins contain 30–35%  $\alpha$ -helix and 10–20%  $\beta$ -sheet. Heating results in a partial loss of the  $\alpha$ -helical content. The high stability of the helices is probably due partly to extensive hydrogen bonding, although disulphide bonds are clearly also important. Because the cd spectra of  $\beta$ -turns are masked by those associated with  $\alpha$ -helix and  $\beta$ -sheet, it is not possible to assess the importance of  $\beta$ -turns in the  $\alpha$ -,  $\beta$ - and  $\gamma$ -gliadins by cd spectroscopy. An alternative approach is to determine secondary structure by computer prediction from amino acid sequences. Available amino acid sequences of S-rich prolamins show the presence of structural domains, including a repetitive proline-rich domain near the N-terminus<sup>24, 25, 36</sup>. The repetitive domain is also rich in phenylalanine with a repeat motif related to that in C-hordein<sup>36</sup> (and probably also  $\omega$ -gliadins). Computer predictions using the methods of Chou and Fasman<sup>28</sup> and Garnier *et al.*<sup>37</sup> indicate that the  $\alpha$ -helix is confined to the non-repetitive domains, while the repetitive domain is rich in  $\beta$ -turns (authors' unpublished results). However, the rapid loss of phenylalanine band structure in the near-UV indicates that, unlike the  $\omega$ -gliadins, the  $\beta$ -turn conformation in this domain is not strongly stabilised by hydrophobic interactions.

The results reported here indicate that the individual gliadin monomers have conformations stabilised by strong non-covalent forces. The presence of similar interactions between gliadins and between gliadins and glutenins, could provide much of the viscosity of whole gluten.

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