Atomic Force Microscopy (AFM) Study of Interactions of HMW Subunits of Wheat Glutenin

Andrew D. L. Humphris,[1] Terence J. McMaster,[1] Mervyn J. Miles,[1] Simon M. Gilbert,[2] Peter R. Shewry,[2] and Arthur S. Tatham[2,3]

ABSTRACT

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Atomic force microscopy (AFM) has been used to study the noncovalent interactions of alkylated HMW subunit 1Dx5 and a M_r 58,000 peptide derived from the central repetitive domain. Both protein and peptide align side-by-side to form fibrils, the HMW subunit forming a branched network, and the peptide forming linear rods. The N- and C-terminal domains of the subunit would, therefore, appear to contain regions that interact through noncovalent interactions in the absence of disulfide bond formation. These regions may be of importance in facilitating disulfide bond formation during protein body development.

The gluten proteins of wheat are crucial for breadmaking and other food uses as they confer unique rheological properties, a combination of elasticity and viscous flow, to doughs. Wheat gluten is a complex mixture of proteins broadly divided into two groups, the monomeric gliadins and the glutenins that form polymers linked by interchain disulfide bonds. Both of these groups of proteins contribute to the rheological properties of gluten and dough, but one subgroup of glutenins is thought to be of particular importance, the high molecular weight (HMW) subunits of glutenin. Early studies showed that the differences in the HMW subunit composition of bread wheat cultivars were associated with variation in breadmaking quality (Payne 1987) while more recent studies have shown direct effects of HMW subunit composition on the proportion of high M_r glutenin polymers (Popineau et al 1994, Gupta et al 1995) and the level of gluten viscoelasticity (Popineau et al 1994). The structures and interactions of the HMW subunits are, therefore, of considerable interest in relation to understanding and manipulating gluten functionality.

A number of HMW subunit genes have been cloned and sequenced, providing complete sequences of the encoded proteins (Shewry and Tatham 1997). These show that the proteins typically comprise between 630 and 830 amino acids, with M_r ranging from 67,500 to 88,000. Their sequences can be divided into three domains, with an extensive central domain consisting of repeated sequences flanked by shorter nonrepetitive domains of 81–104 residues at the N-terminus and 42 residues at the C-terminus. The nonrepetitive domains contain most or all of the cysteine residues, some of which form interchain disulfide bonds (see Shewry and Tatham 1997) to stabilize the high M_r glutenin polymers. In contrast, the repetitive domains contain either a single cysteine or no cysteine residues, and consist of tandem and interspersed repeats based on hexapeptide (consensus PGQGQQ), nonapeptide (consensus GYYPTSP/LQQ) and, in some subunits only, tripeptide (consensus GQQ) motifs. Analyses of the data from spectroscopic (Shewry et al 1993, Belton et al 1995), viscometric (Field et al 1987) and small angle X-ray scattering (Matsushima et al 1992, Thomson et al 1999) analyses have indicated that the repetitive sequences adopt an unusual supersecondary structure, which is a loose spiral comprised of β-reverse turns. Direct images of this structure have been provided by scanning tunneling microscopy (Miles et al 1991), showing good agreement with the molecular dimensions determined by viscometric analysis (Field et al 1987) and predicted by molecular modeling (*unpublished data*).

Although interchain disulfide bonds are clearly crucial for stabilizing the high M_r glutenin polymers, recent NMR studies indicate that hydrogen bonding between adjacent subunits, and between HMW subunits and other proteins, may be important in stabilizing the structure of gluten (Belton et al 1995). The formation of extensive hydrogen bonds between HMW subunits would be facilitated by the high proportions and regular spacing of glutamine residues. Belton (1994) has proposed a loop and train model for the contribution of such hydrogen bonding to gluten elasticity.

Scanning probe microscopy provides a unique opportunity to study the interactions of biomolecules under conditions similar to those in biological systems. We have therefore previously used scanning tunneling microscopy (STM) to image single gluten molecules and protein arrays in the hydrated solid-state (Miles et al 1991, Thomson et al 1992, Shewry et al 1997, Wannerberger 1997), which corresponds to the form of the proteins in the wheat grain and in gluten. In the present study, we used a different type of scanning probe microscopy, atomic force microscopy (AFM), to show that a purified HMW subunit can form extensive networks in the absence of disulfide bond formation. This behavior is compared with that of a recombinant peptide derived from the repetitive domain of the HMW subunit.

MATERIALS AND METHODS

Protein and Peptide Purification

Reduced and pyridethylated HMW subunit 1Dx5 was prepared from a wheat near-isogenic line (Olympic × Gabo) as described by Marchylo et al (1989), followed by ion-exchange chromatography on CM cellulose in 3*M* urea, 10 m*M* glycine-acetate buffer, pH 4.6, and eluted with a linear gradient of NaCl (0–250 m*M*). An M_r 58,000 peptide corresponding to residues 102–645 of subunit 1Dx5 was expressed in *Escherichia coli* and purified as described by Buonocore et al (1998).

Atomic Force Microscopy (AFM)

The protein and peptide were dissolved in 0.05*M* acetic acid to give solutions with concentrations of 1 mg/mL to 1 μg/mL. A 10-μL drop of solution was deposited on either freshly cleaved mica (Agar Scientific Ltd., Essex, UK) or highly oriented pyrolytic graphite (HOPG) (Advanced Ceramics Corp., Cleveland OH) substrate. The substrate was placed in a controlled environment and the solvent was allowed to evaporate. By changing the environmental conditions (atmospheric concentration of solvent and temperature), it was possible to control the evaporation rate and

[1] H.H. Wills Physics Laboratory, University of Bristol, Tyndall Avenue, Bristol BS8, 1TL, UK.
[2] IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK.
[3] Corresponding author. Phone (44) 1275 549330. Fax (44) 1275 394281 E-mail: arthur.tatham@bbsrc.ac.uk

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thus deposition rate of protein to the substrate. Samples were left for >18 hr under the selected deposition conditions to fully dry before imaging.

A Nanoscope III controller with a multimode AFM head and extender box (Digital Instruments, Santa Barbara, CA) was used for all imaging. The microscope was operated in tapping mode under air using standard tapping mode silicon cantilevers, spring constant 33–64 Nm^{-1}. A range of scan tubes was used to obtain large area and high-resolution images. Data were recorded using at least two channels during scanning, one for height and one for amplitude. The height image displays topographic data by maintaining near-constant oscillation amplitude of the probe using feedback to alter the tip-sample separation (defined as the z-axis). Oscillating the tip and tapping the sample surface applies less shear force to the sample and reduces potential damage. By recording the amplitude of the oscillation, an error image is produced that can enhance the imaging of high-frequency spatial features and so produce images of higher resolution.

RESULTS

Deposition of the HMW subunit 1Dx5 on a graphite substrate by evaporation of a 0.01 µg/mL solution in 0.05*M* acetic acid revealed a structure composed of interlinked filaments (Fig. 1A). The diameter of the filaments was ≈20 nm, which is larger than that observed and calculated for the repetitive domains of the HMW subunits (≈2 nm) (Field et al 1987, Miles et al 1991). The observed filaments must therefore consist of multiple HMW subunits. Small-angle X-ray scattering from dilute solutions of HMW subunits also indicated side-by-side aggregation of the protein rods (Thomson et al 1999). The complex nature of the filament is also apparent at higher magnification, which shows some loops (Fig. 1B). When deposited from a higher concentration solution (0.1 µg/mL), the HMW subunit completely coated the substrate (Fig. 2A) but with crack-like gaps that could have arisen from shrinkage during drying (Fig. 2B). High-resolution imaging of the edge region (Fig. 2C) showed the presence of a network of subunit protein that may have been pulled out from the retracting edge during drying. This network shows branch points at ≈40–50 nm intervals, corresponding to the approximate length calculated for a single HMW subunit (Field et al 1987).

A cross-section of a network similar to that in Fig. 2C is shown in Fig. 3. The strands of the protein vary in height and, by studying cross-sections, the strands were observed to have a characteristic minimum width of ≈10 nm and a minimum height of ≈1.8 nm. This height is consistent with the diameter of an individual subunit (1.5–2.0 nm) (Field et al 1987, Miles et al 1991). In contrast, the minimum width is consistent with the strands comprising a

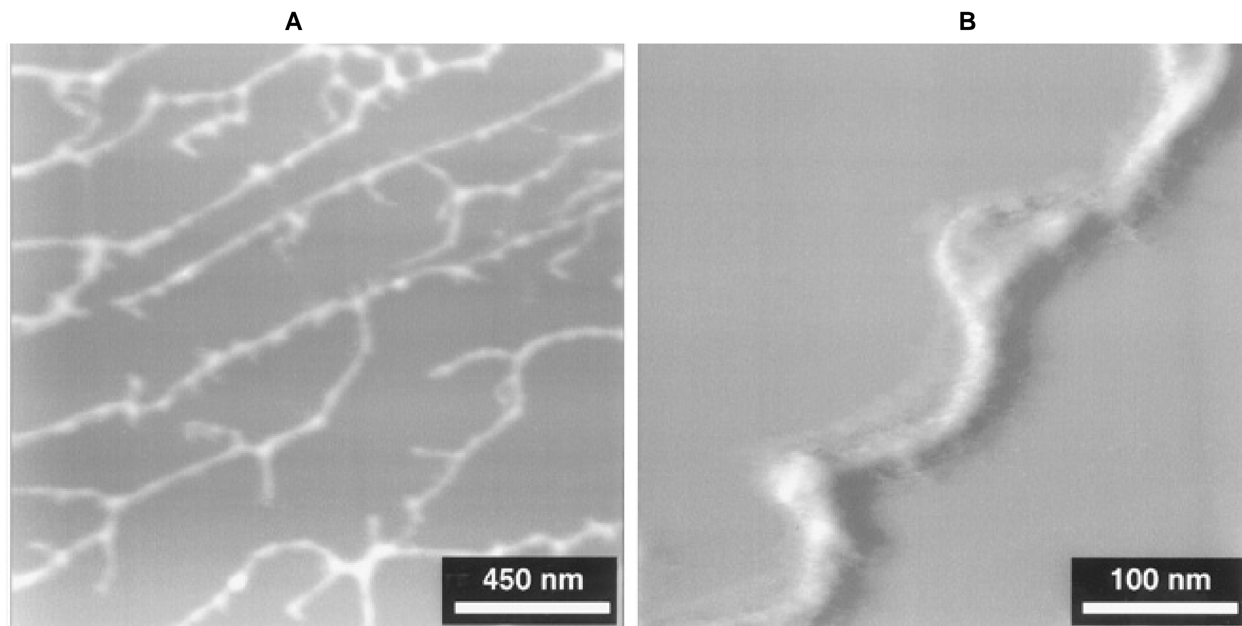

Fig. 1. HMW subunit deposited from 0.05*M* acetic acid at a concentration of 0.01 mg/mL to highly oriented pyrolytic graphite (HOPG) substrate. Images taken in tapping mode in air.

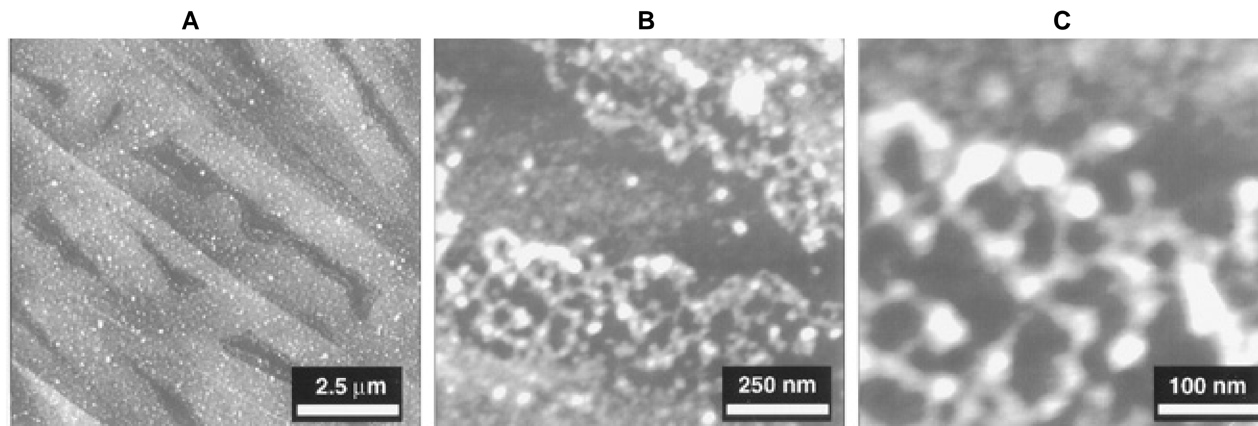

Fig. 2. HMW subunit deposited from 0.05*M* acetic acid at a concentration of 0.1 mg/mL to highly oriented pyrolytic graphite (HOPG) substrate. Images taken in tapping mode in air.

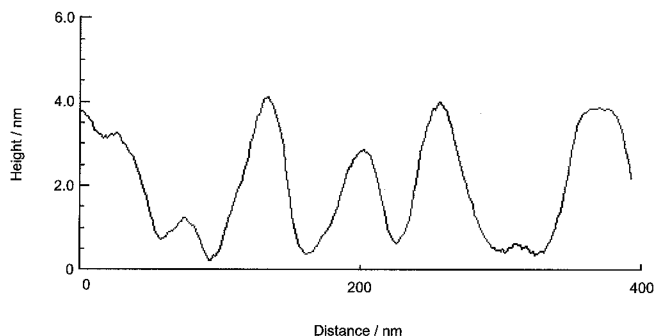

Fig. 3. Cross-section of a network pulled from the edge of HMW subunit under the conditions in Fig. 2. Range of branch cross-sections was observed with a characteristic minimum height and width of ≈1.8 and ≈10 nm, respectively.

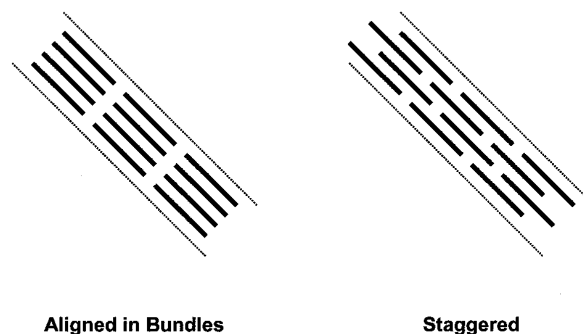

Fig. 5. Possible arrangements of peptide molecule in fibrils. Structure showing alignment in bundles as seen in Fig. 4.

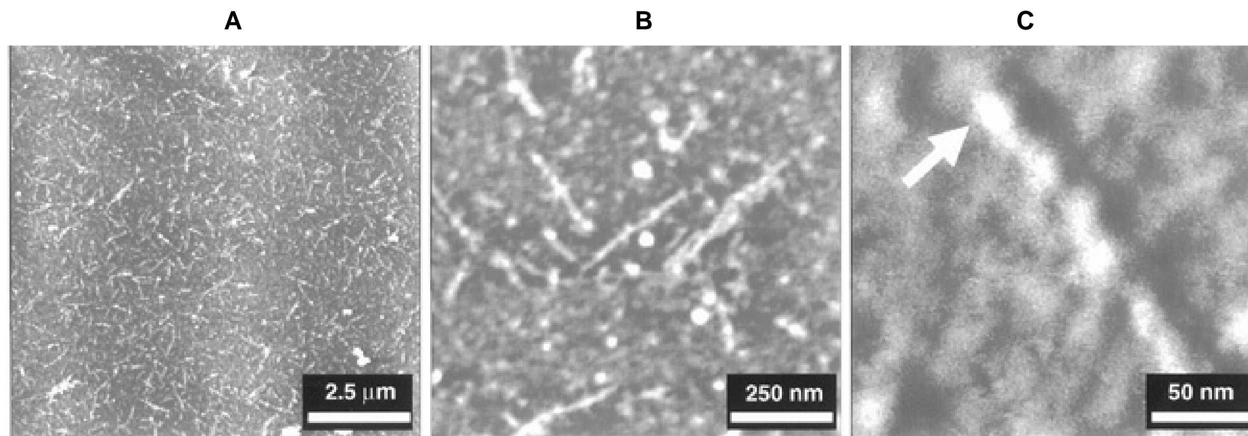

Fig. 4. M_r 58,000 peptide deposited from 0.05*M* acetic acid at a concentration of 0.1 mg/mL to a mica substrate. Images taken in taping mode in air. Arrow indicates aligned bundle of peptide ≈40 × 20 nm.

number of subunits aligned side-to-side. The observed width will also be increased due to the geometrical properties of the tip.

An M_r 58,000 peptide derived from the repetitive domain of subunit 1Dx5 was also deposited under similar conditions to the subunit to determine whether it was also able to form networks (Fig. 4). In contrast to the subunit, the peptide formed linear rod-shaped structures with only one connection at each branch point, rather than a branched network (Fig. 4A). However, it did resemble the HMW subunit in that the connection points were present at 45–50 nm intervals (Fig. 4B), and the diameter of the rods was >2 nm (Fig. 4C), indicating the presence of several peptide molecules aligned side-to-side. In addition, the images suggested that the ends of the peptide molecules were aligned within the bundles rather than staggered (Fig. 5).

DISCUSSION

The images reported here demonstrate that both the HMW subunit and the M_r 58,000 peptide molecules can align side-by-side to form fibrils with diameters of ≈20 nm. This side-to-side alignment probably arises from intermolecular hydrogen bonding between glutamine side chains and the amide groups in the peptide backbones of adjacent polypeptide chains. Fourier transform infrared studies of HMW subunits have shown that the content of β-sheet structure increases with increasing protein concentration (Belton et al 1995), indicating that intermolecular hydrogen bonding can stabilize the fibrilar structures formed.

Only the whole subunit formed a branched network; the repetitive peptide formed linear rods. Also, the individual peptide molecules seemed to be precisely aligned, in contrast to whole proteins, which were staggered. The latter may be due to the presence of nonrepetitive N- and C-terminal domains preventing the formation of the precise alignment observed for the wholly repetitive peptide. This indicates that branching sites must be present in the nonrepetitive N- and C-terminal domains. This is also the case in glutenin polymers where interchain disulfide bonds are thought to be formed between cysteine residues present in these domains. However, in the present study, the stabilizing forces must be noncovalent, the subunit being reduced and S-pyridethylated to prevent disulfide bond formation.

The ability of the HMW subunits to form a branched network in the absence of disulfide bond formation suggests that the ability to form disulfide bonds, in part, is directed by noncovalent interactions. Certainly, charged amino acid residues are present in the N- and C-terminal domains, and these could interact by charge repulsion or attraction to bring cysteine residues into close proximity. In contrast, the only charges present in the peptide are associated with the amino and carboxyl groups present at the N- and C-termini, respectively.

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