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Comments on Recent Hypotheses for Glutenin

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ABSTRACT

Some recent hypotheses of glutenin structure are criticised. A linear molecule whose subunits were held together by secondary forces would probably not have the necessary strength. Branched models do not satisfactorily explain the toughness of dough, its work-hardening and development, fibril formation, the observed molecular weight distribution of glutenin, or results from viscosity studies. A recent branched hypothesis is doubtful because, among other drawbacks, there may not be enough room for both branches and recognition sites.

At present a linear molecule built up from subunits joined by SS seems likelier than branched models. Viscosity of dough depends on cooperating secondary forces between protein molecules. The elastic restoring force arises because the folded native conformations of subunits are incomparably more probable than a stretched state. The linear model explains work-hardening of dough, its development by mixing, its ability to control the expansion of gas cells, and the formation of fibrils.

Suggestions that Osborne's nomenclature should be changed are misguided and should be rejected.

INTRODUCTION

Ewart (1972*b*) discussed some of the current hypotheses for the structure of glutenin, the protein in wheat flour that gives dough its viscoelastic nature (Grosskreutz, 1960, 1961*a*, *b*; Meredith, 1964; Tracey, 1964; Jones & Carnegie, 1971). This paper comments on recenter work.

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AN AGGREGATED STRUCTURE FOR GLUTENIN

Bernardin and Kasarda's hypothesis

This is essentially a linear structure, the subunits being joined to each other by specific secondary forces (Bernardin & Kasarda, 1973*b*; Bernardin, 1975). The ingenious hypothesis is well explained and illustrated in a review by Kasarda *et al.* (1976). Agents that break SS could alter the configuration of a subunit so that it no longer fitted its neighbour and the strand would divide. Since many high polymers naturally form helices, aggregates of strands could take helical or wavelike shapes. The crests of these could associate with those of other aggregates to give continuity throughout dough and form a springlike structure. Stress on the whole structure would not disturb the conformation of any particular subunit enough to interfere with the strong binding site, but these small changes in conformation of the subunits would accumulate and flatten the waves in the aggregates, so letting the structure extend. The tendency to revert to lowest free energy would provide the elastic restoring force, while viscosity is easily explained by the resistance of secondary forces as groups of strands move past each other.

The junctions between individual subunits and the sides of the long strands are, however, very vulnerable to stress. A strand could be broken if the secondary forces at any junction were successively, but rapidly, parted by even weak tension (analogously to the action of a zip-fastener). Similarly, strands could be peeled apart longitudinally.

A polypeptide chain naturally folds into its stablest state and SS can strengthen that state J. D. Schofield (1989, pers. comm.) has asked whether the breaking of SS by thiols would normally disturb the stable conformation; therefore thiols may not break such strands.

The hypothesis also does not agree (Ewart, 1979) with work of Beckwith and Wall (1966) on reoxidation of reduced glutenin at different concentrations. Nevertheless, it is an intelligent attempt to explain the facts and, even if eventually proved wrong, will have played a valuable part by stimulating research.

BRANCHED HYPOTHESES

Bietz, Huebner and Wall

Bietz and Huebner (1980) and Bietz and Wall (1980) have postulated branching among some fractions of glutenin. As it is extremely difficult with present techniques to prove how the SS are distributed in glutenin, circumstantial evidence has to be invoked.

A branched glutenin could not be work-hardened in the way that a linear glutenin could. Indeed, a highly branched glutenin should not need development by mixing; since it could not be oriented or overlapped, it would only need thorough wetting to reach maximum development. It is clear from electron micrographs of Bernardin and Kasarda (1973*a*) that glutenin forms fibres spontaneously when flour is wetted. Fibre formation would not be expected of a branched structure. Further, in branched polymers the largest fraction, by weight or number, is monomeric (Flory, 1946, 1953); there is no evidence that this is so in glutenin.

Branched glutenin should show a point of inflection in the fall of viscosity with time when gluten solutions are reduced, but attempts to find one failed (Ewart, 1979). Further viscosity studies were compatible with a linear model (Ewart, 1988).

Branched molecules would not intermesh well with other molecules to form a cohesive dough because only polypeptide chains protruding from the main aggregate would be best fitted for this. In heat-damaged wheat there may be branches, owing to SS interchange among denatured proteins (Schofield *et al.*, 1983): the glutenin becomes insoluble and the dough is very weak. This further erodes the belief that there is any significant branching in normal gluten.

Graveland's hypothesis

Graveland *et al.* (1985) put forward a branched structure for glutenin. The repeating unit, glutenin IIa (Fig. 1), has a mol. wt of $\sim 8 \times 10^5$ and a main chain of high mol. wt (HMW) subunits 1 (or 3), 10 and 5 joined by SS. Subunit 10 has four branches, each of three low mol. wt (LMW) subunits joined by SS. The glutenin I polymer of ~ 12 or more repeating units has a mol. wt of $> 10^7$ and is insoluble. The hypothesis was put forward to explain the various fractions of glutenin found by gel filtration, their subunit compositions, numbers of SS and SH and the effects of partial reduction.

Difficulties raised by Graveland model

Unfortunately the authors do not say how such a large specific structure is formed nor what stops indefinite growth of the branches to form a giant molecule with other units. It is too big for any known enzyme to hold the parts together while the interchain SS are formed. It was suggested to the author that the subunits assemble because specific sites recognise one another (possibly having opposite electric charges), and the SS form later. If so, six pairs of complementary sites are needed, as shown in Fig. 1, to explain how the branches have exactly three subunits. (The ends of the

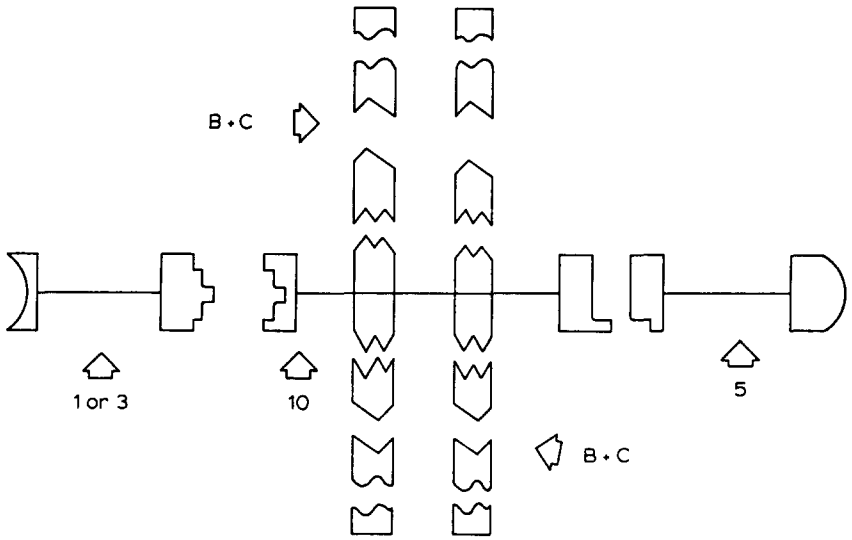

Fig. 1. Sketch of model for glutenin proposed by Graveland *et al.* (1985) showing that six pairs of complementry sites are needed for self assembly. Numbers refer to HMW subunits. B + C are LMW subunits.

branches have only site (or one SH) to stop unlimited growth.) Though recognition sites are common among proteins, they seem only to be found where they are essential to some process or structure, having arisen through natural selection. It is far from clear why there should be any in a protein that is only a food supply. So, with no obvious explanation of their presence, these twelve sites are an exacting need, an *ad hoc* assumption that Occam's razor would shave off. But it may be possible to cut the needs to two pairs (i.e. four sites) without seriously altering the model (Fig. 2).

Suppose the model is an average picture and that the three HMW subunits may be arranged in all six ways, then the HMW units only need two sites. Also if it is assumed that three is the *average* branch length, the side chains also only need two sites, some subunits, of course, having only one. Four times as many LMW subunits as HMW, would ensure an average of three a branch. Even this model demands *ad hoc* assumptions including the serious one, for which there is no evidence, that only certain junctions between glutenin subunits are possible. If, as seems likely, any subunit could combine with any other, the presence of a hexafunctional chain would yield giant molecules.

Sequence data and the Graveland model

Thompson *et al.* (1985) and Forde *et al.* (1985) have sequence data on ID and 1A genes for HMW subunits respectively (the latter may not be expressed).

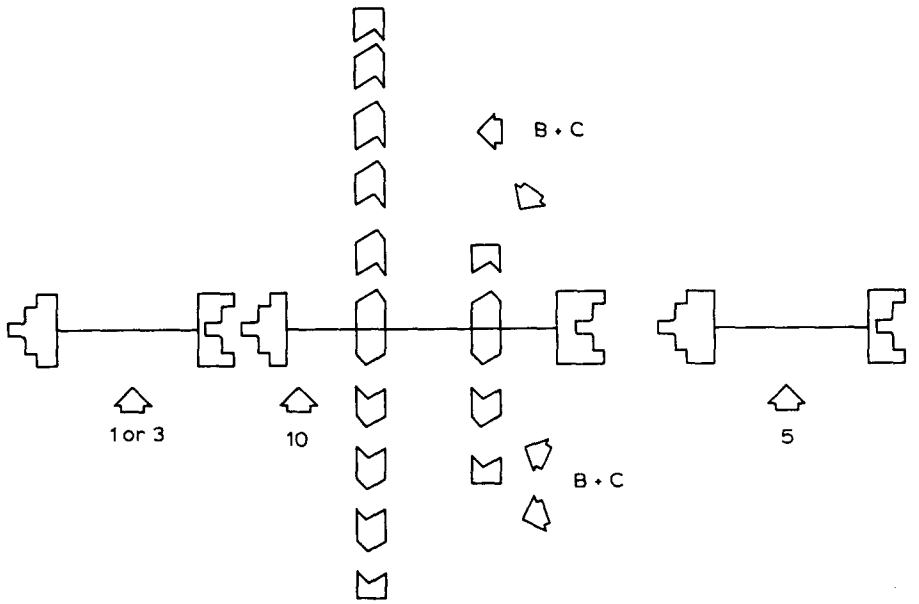

Fig. 2. Minor changes reduce the number of pairs of complementary sites to 2.

Studying their diagrams shows that on the N-terminal side of the molecule the distances from one half-cystine to the next are 12, 22, 1 and 10 residues. (Calling the half-cystine residues of proteins with no SH groups 'cysteines' is confusing because it implies the presence of SH.) If the polypeptide chain were in the extended form, as in β-keratin, there would be ~ 16 nm between the extreme half-cystine residues, 45 residues apart; the side-chains would fit easily, but five recognition sites would be difficult to construct on a short stretch of *extended* polypeptide chain. Kabat (1966) suggests from work with antibodies that a binding site needs at least five residues, and these are not normally adjacent in the polypeptide chain. If, as is much likelier, the polypeptide chain is folded or coiled, at least two subunits could find it a tight fit on, say, an α-helix 6·6 nm long when they are at least 4·5 nm in diameter. It is asking a lot to fit five different recognition sites, or at least four similar ones and one different one, on this compact section of chain. Again, there is no obvious evolutionary pressure for such a complex and precise structure.

Owing to the close relation between HMW subunits it is unlikely that the distances between half-cystine residues will vary much among them. Sugiyama *et al.* (1985) have given the sequence of a different HMW subunit. Unfortunately they have not mentioned the half-cystine residues in the text nor given the totals of amino acids (which would be helpful in checking amino acid analyses). Study of the sequence shows that there are only three half-cystine residues at the N-terminus, 15 residues apart, and one at the C-terminus.

Graveland *et al.* (1985) briefly mention the possibility of subunit 10 having only one side-branch instead of four, which would resolve these difficulties. If the branch were linear it would need twelve pairs of recognition sites for self-assembly, though this could be reduced to one by taking twelve for an average number, as mentioned above. But the assumption that subunits cannot combine unless they have recognition sites may well be false, in which case even a single branch should lead to giant molecules.

The author does not share Graveland's belief (1988) that his transmission electron micrographs of glutenin suspended in water show the branched structure. The pictures are easily explained by association and folding over of molecules. Attempts have been made for many years in these laboratories to cooperate with electron microscopists in getting molecular resolution of glutenin but without success.

Alternative explanation of results of Graveland *et al.*

A simpler explanation of the results of Graveland *et al.* (1985) could be that, since 1·5% SDS is not a perfect solvent for glutenin molecules, the bigger ones will tend to associate to colloidal particles that could form the HMW peak in their gel filtration curves. This is the likelier because neither linear nor branched polymerisations should have the bimodal distributions they found, chapters 8 and 9 (Flory, 1953).

It is also possible that SDS could so extend the conformations of glutenin molecules that they would diffuse very slowly into sepharose particles and most would come off the column early, with an apparently HMW. If so their IIb fraction may be the main fraction of soluble glutenin, while the III fraction is soluble in 70% ethanol because it lacks HMW subunits. Dimers and trimers containing an HMW subunit may precipitate with the ethanol-insoluble II fraction, because HMW subunits were insoluble in 70% ethanol (Bietz & Wall, 1973; Graveland *et al.*, 1985).

Several objections to Graveland's hypothesis also apply to the branched concept outlined by Shewry *et al.* (1984).

LINEAR GLUTENIN

Khan and Bushuk (1978, 1979), Lasztity *et al.* (1970) and Ewart (1968*b*, 1972*a*, 1989) have put forward hypotheses for linear molecules in glutenin. The subunits are joined to neighbours by an SS. Ewart (1972*a*) thought there were two SS at each junction between subunits, but later concluded that there was probably only one (1979, 1987, 1988).

There are at least twelve different subunits in glutenin and they vary in

mol wt. In the linear hypothesis the subunits are arranged at random along glutenin molecules. The average mol. wt of a subunit is 50 000 but varies from about 30 000 to 70 000. (Mol. wt, being a mass ratio, has no units. Many workers still express mol. wts in daltons (Da), often using the wrong symbol. The longer term 'relative molecular mass' seems not to have eliminated these errors).

Viscosity is of course explained, as it is in any liquid, by intermolecular forces. Those on long molecules with a covalent backbone cooperate to form strong resistance.

Elasticity

The normal conformation of most proteins is a globular shape in which the polypeptide chain folds in a complex way with many sharp turns of $\sim 180°$ (hairpin bends), as has been known since the pioneering work of Kendrew and Perutz (Kendrew *et al.*, 1958; Cullis *et al.*, 1961). (These have lately been dignified by a rather uninformative but presumably more impressive name, β-turn). If, as seems so with glutenin subunits, there are long repetitive sequences, which Kasarda (1980) first brilliantly predicted, these could unfold into extended structures by frictionless rotation about bonds, when long strands of subunits underwent stress. The enormous number of possible folded conformations of similar energy means that folded ones are incomparably likelier than extended ones. When stress is released, Brownian motion ensures rapid refolding. Hence dough is elastic. (Ewart (1989) forgot that most bonds are in their vibrational ground states at 25°C. This fact also shows why bond stretching cannot cause elasticity in gluten.)

In describing the forces that help chains refold, the ofen used term 'hydrophobic force' or 'bond' is now thought inaccurate, the author being as guilty as anyone of misusing it. Burley and Petsko (1988) point out that the right term is *hydrophobic effect*: the actual forces involved, for non-aromatic hydrocarbons at least, are very weak (London forces).

Gas cells and fibrils

Shear stress in any kind of dough mixing causes flow in all the pores between starch granules. This flow orients the linear molecules and overlaps them to give satisfactory cohesion despite the water, gliadins and lipids that are trapped by the strands of oriented glutenin molecules. As there are many pores pointing in random directions between any two gas cells in dough, these can cope with tangential forces that the surfaces of expanding bubbles exert in all directions (Ewart, 1989).

The linear hypothesis accounts for the streamers of protein that shoot out from flour particles on wetting (fibrils) (Ewart, 1989).

Insoluble glutenin

In some literature reports, e.g. (Miflin *et al.*, 1983), the linear glutenin hypothesis is wrongly described as 'linear... with only a small amount of branching'. Only a few cross links, however, are needed to change the properties of a polymer. (1% by number of bisacrylamide molecules present in the monomer makes the resulting polyacrylamide insoluble.) Even if only one glutenin subunit were tri- or (more probably) tetra-functional it would have a major effect on the structure and properties of the glutenin, and would destroy the hypothesis.

Small amounts of crosslinking by other bonds than SS could come from the action of free radicals (e.g. from lipid peroxides), aldehydes or mechanical splitting of SS, but provided that these crosslinks are so few that they do not materially affect dough rheology they need not invalidate the hypothesis (Ewart, 1968*a*, *b*).

OSBORNE'S NOMENCLATURE

Miflin and coworkers (Miflin *et al.*, 1983; Shewry & Miflin, 1985; Kreis *et al.*, 1985; Shewry *et al.*, 1986) have decided to change Osborne's nomenclature by classing glutelins and prolamins as prolamins, because sequence work suggests a descent from a single ancestral protein. This is, however, a confusing and retrograde step that should be rejected. They claim that Osborne would have supported their suggestion. This is unlikely.

Osborne was a great American chemist and pioneer in protein research. To a chemist, the presence of interchain SS, i.e. a structural difference that gives glutelins different properties from prolamins, is the overriding reason for classing them separately. The fact that some glutelins are alcohol-soluble when their SS are reduced does not entitle them to be called prolamins, because the treatment has altered their chemical structure. Similar logic would class practically all proteins as water-soluble because that is what they become after prolonged extraction with HCl.

Chymotrypsins A and B, trypsin and elastase have nearly quarter their sequences in common, but this close evolutionary relation is eclipsed by differences in chemical behaviour (mainly due to a few mutations at the substrate-binding sites) (Shotton & Hartley, 1970). Because of these small structural *differences* the enzymes get different names; the evolutionary kinship and the fact that all are proteases are quite secondary to their chemical properties.

Osborne's glutelins include nearly all the storage proteins, high in Gln and Pro, which have formed, or (in the case of the monomers) are able to form,

multichain structures linked by SS. By this definition the glutenin soluble in 70% ethanol (Beckwith *et al.*, 1966; Elton & Ewart, 1966) is LMW glutenin, not HMW gliadin. One does not expect solvent extraction to give sharp separations of polymers because solute–solute forces are so strong, but his four groups are as useful today as when he discovered them early in the century. If protein sequences and genetics throw light on relations that were not even imagined in Osborne's day, it is easy enough to invent new names for the categories. To devalue such a well established name as prolamins is against good practice.

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