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Thiols in Flour and Baking Quality

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ABSTRACT

Baking quality (LV) was defined as loaf volume per gram of loaf protein. Further attempts to find negative correlation between LV and small thiols bound by disulphide bonds (SS) to gluten failed, but this was almost certainly because the method was flawed. A new approach was devised to measure on the same sample: free sulphydryl groups (SH) in glutenin, diffusible thiols in flour, and thiols bound to protein by SS.

Both methods showed the existence of bound thiols; corrected values in the old method had a mean of 1·5 μmol per gram of gluten; in the new they ranged from ~0·9 to 2·3 μmol per gram of flour protein with a mean of 1·6.

Altogether three ways were tried of measuring diffusible thiols in flour (possibly the rheologically active ones). The most specific reagent measured 14–16% of accessible thiol as diffusible. A second method gave slightly lower values but this could be explained by side reactions. High values, with a third reagent, were almost certainly due to incomplete reaction.

Probably for the first time, it was shown that untreated glutenin molecules in flour carry free SH. The use of phenyl mercuric acetate suggested ~1/8SH a glutenin chain (of average mol. wt 50 000). Higher results with iodoacetamide ranged from 0·17 to 1·1, but these probably included extra SH, from reaction of glutenin with active thiols before they could be blocked, and/or from attack on labile SS in glutenin. The amount of free SH on glutenin was negatively correlated with LV in the Chorleywood Bread Process (CBP), but not significantly in the 3 h long fermentation process (LFP).

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The results suggest that an active thiol attacks a glutenin SS when flour is wetted to add both SH and bound thiol; it can also form an SS with an SH on glutenin by oxidation.

The hypothesis that the mol.wt of glutenin controls about half the variation in baking quality is compatible with recent findings that baking quality depends on the type of high molecular weight subunit in glutenin. Payne found one-third to two-thirds the variation in baking quality was linked with variation of subunit composition, so both the mol.wt of glutenin, and its high mol.wt subunits, affect baking quality. Further studies of the effect of thiols on the mol.wt of glutenin (and therefore on baking quality) could well be done on wheats of constant subunit composition.

With a commercial flour the CBP was less tolerant of added thiols than the LFP. Cysteine is only about a tenth as active as the others in the LFP. Its effect (when large amounts were used) did not seem to involve an enzyme since the D- *and* L-*forms were equally active. In a trial with 24 flours, protease activity was uncorrelated with LV. A further measurement was made of SH in three stored flours.*

INTRODUCTION

Despite the valuable findings of Payne and co-workers (1979, 1984) that baking quality is linked with certain glutenin subunits of high mol.wt, it has not been easy to explain this in the past. Baking quality may at least partly depend on the sulphydryl groups (SH) in flour, because they can lower dough strength and loaf volume. A long-known action of improvers seems to be to remove SH so preventing their harmful effect of breaking glutenin molecules.

Ewart (1988*b*) could find no other reactions, besides oxidation of SH, that explained how a few 10s of ppm of improvers could be effectual: improvement of flour in the first few months of storage may also be due to loss of SH by aerial oxidation. It was concluded that only part of the accessible thiols were effectual. Jones *et al.* (1974) used rheological tests to show that only 25–35% of SH in flour were active. (Ewart (1988*a*) unfortunately omitted this paper from a brief review.)

The disulphide interchange reaction is fast, and when flour is wetted it is possible that free thiols naturally present, especially those of low mol.wt, would attack glutenin disulphide bonds (SS) and (if the SS is interchain) break the molecule. Each thiol would add itself to a glutenin molecule. A thiol could also block an SH on glutenin when oxidised to form an SS with it. Ends of glutenin molecules may have residual SH that failed to form SS with SH on other molecules during biosynthesis. Free SH on glutenin, either original or from reaction with thiols, should thus be related to the number of

ends, or breaks, and so be inversely related to the number-average mol.wt. The number-average mol.wt (or degree of polymerisation, since the mol.wts of individual chains should average out in a large number of molecules) may be related to baking quality (Ewart, 1968, 1980, 1985).

The aim of this work was to try to measure thiols that are diffusible, SH on glutenin, and thiols that are bound to glutenin by SS to form mixed disulphides.

MATERIALS AND METHODS

Gluten preparation

Two doughs, made by mixing 28 g of flour and the volume of 0·1% NaCl equal to that of the water absorption in a Minorpin mixer for 1 min, were combined and washed out to gluten in 2 litres of 0·1% NaCl. The gluten was stirred overnight in 350 ml of 0·1M acetic acid and freeze-dried.

Glutenin preparation

Gluten (3 g) was stirred for 1·5 h with 200 ml of 70% (v/v) ethanol. After being centrifuged for 30 min at 1000*g*, the pellet was extracted as before for 1 h, stirred overnight with 0·1M acetic acid and freeze-dried.

Dithizone solution

Dithizone (diphenylthiocarbazone; Sigma) was dissolved in carbon tetrachloride (15 ml for each mg) in a flask wrapped with foil. After ~30 min stirring the solution was filtered into a flask wrapped in foil and stored at ~5°C. For use it was diluted tenfold in dim light to give an A_{625} of ~0·850.

Flours and baking tests

The flours were English, covering a wide range of baking quality, together with Canada Western Red Spring (CWRS). The baking tests were by the 3 h long fermentation process (LFP) (Draper & Stewart, 1980) and the Chorleywood Bread Process (CBP) (Axford *et al.*, 1978). If any thiols were added they were dissolved in tap water, degassed with N_2, shortly before mixing.

SH measurement in flour

This was done by sieving flour (~1 g) into excess phenyl mercuric acetate (PMA) solution at pH 4·2, with or without 8M urea, and measuring the

unreacted PMA by pulse polarography as before (Ewart, 1988*a*). The mean of at least two measurements was taken.

Phosphate buffer

M/3 phosphate buffer, pH 8·5, was made by dissolving 10·9 g of $Na_2HPO_42H_2O$ and 0·2 g of $NaH_2PO_42H_2O$ in 300 ml of water.

Diffusible thiols by AEDANS method

The same ten flours, varying widely in LV, were used for the AEDANS and PMA methods.

Flour containing 2·5 μmol of accessible thiol was sieved into a rapidly stirred mixture of 20 ml of phosphate buffer and 1·0 ml of freshly made 2·5 mM AEDANS (*N*-iodoacetyl-*N'*-(5-sulphonyl-1-naphthyl) ethylene diamine) in a centrifuge tube. After 5 min stirring the mixture was centrifuged at 3500*g* for 15 min and stood overnight in the dark at ~5°C. Next day the weighed supernatant was poured into a double-knotted dialysis sac (Visking 18/32 in), put in a weighed boiling tube with 20 ml of water and shaken for 24 h at ~5°C. From the final weight of the tube the liquid contents were known after subtracting 0·5 g for the dry sac. The diffusate was read at 337 nm, and the concentration of the AEDANS derivatives of thiols calculated by assuming an ε of 6000 litres mol^{-1} cm^{-1} (Hudson & Weber, 1973). The amount of original diffusible thiol was calculated with the factor 21/(volume of supernatant used for dialysis). The mean of three measurements was taken.

Diffusible thiols by PMA method

Flour containing 5·0 μmol of accessible thiol was sieved into 5 ml of 1 mM PMA (i.e. 5 μmol) and 19 ml of 0·063M acetic acid with rapid stirring. After 5 min the suspension was centrifuged for 15 min at 3500*g* and the supernatant was dialysed as above for ~26 h. Diffusate (4 ml) was mixed with 6 ml of 10M HCl and hydrolysed at 105°C for 24 h in screw-cap tubes fitted with Teflon washers. When the tubes were cold their contents were rinsed into stoppered 100 ml measuring cylinders with 70 ml of water, final strength 0·75M HCl. In dim light 5 ml of diluted dithizone solution were put in each cylinder, which was kept in a cupboard. Two at a time were given 30 vigorous shakes then stood. This was repeated three times after settling so that each cylinder had a total of 120 up and down shakes. In daylight, but with the CCl_4 layer shielded from light by hand where possible, the first cylinder was tilted and the upper layer removed. The CCl_4 layer was filtered through a Whatman

No. 54 12·5 cm paper into the spectrophotometer cell and read immediately at 625 nm. A calibration curve gave the amount of Hg (corresponding to diffusible thiol). This was corrected by 24/(weight in g of supernatant dialysed). The mean of six measurements was taken.

SH on glutenin with PMA

Flour (2 g) was sieved into 100 ml of fast-stirred 8M urea, 0·1M acetic acid, 0·05 mM PMA; then stirred more slowly for 1 h. The suspension was dialysed against 1 litre of 8M urea acidified with ~1 ml of acetic acid for 3 days with four changes, and then for 40 h with three changes against water acidified with a few drops of acetic acid.

The solid matter was recovered by centrifuging at 3500*g* for 15 min in a weighed tube. The tube was filled with alcohol to give ~65% v/v ethanol, total volume 170 ml, and stirred for 0·5 h. After being centrifuged as before, the pellet was resuspended in 70% (v/v) ethanol with a Silverson homogeniser, stirred for 1 h, and centrifuged. The pellet was broken up by homogenising in 0·5M NaCl, stirred for 0·5 h and centrifuged. Enough 10M HCl, 's.g. 1·16', was added to the pellet to make the mixture 6M. It was hydrolysed in a screw-capped tube at 105°C for 24 h. The cool solution was diluted to 0·75M HCl and analysed as above for Hg.

Flours with blocked thiols

These were given the treatment in the last section, as controls. Free thiols in flour were blocked in two ways.

(1) N-*Ethyl maleimide (NEMI)*
Flour (5 g) was sieved into 100 ml of fast-stirred 0·1M phosphate buffer, pH 7·0, 8M urea, 5 mM EDTA, 0·6 mM NEMI. (The NEMI, in ~10 × molar excess over thiol, was dissolved just before the flour was added.) The suspension was stirred at a lower rate for 30 min, acidified with acetic acid, dialysed against water at ~5°C for 3·5 days with five changes, and freeze-dried.

(2) Iodoacetamide (IAM)
As in (1) except that the buffer was 0·33M Tris-HCl, pH 8·6, 8M urea, 2·7 mM EDTA, 0·5 mM IAM.

Calibration curve

1 mM PMA was made by stirring 0·337 g of PMA in a stoppered 1 litre volumetric flask at room temperature for a few hours till dissolved.

Solutions (0·1 mM), i.e. 100 nmol per ml, of the PMA derivative of L-cysteine or glutathione (GSH) were made by mixing the stoichiometric volume of PMA with nine times its volume of water containing enough acetic acid to make the final solution 0·05M, then adding to the right weight of L-cysteine hydrochloride or GSH. (Care should be taken with >0·2 mM solutions owing to the low solubility of these compounds: 0·5 mM solutions are supersaturated and soon get cloudy.) Various amounts of these compounds, up to 1200 nmol, were loaded into dialysis sacs with enough 0·05M acetic acid to make 20 ml. These were dialysed with shaking at ~5°C for ~26 h, then 4 ml samples of the diffusates were hydrolysed and analysed as above. The A_{625} readings were corrected by adding 0·850 − (A_{625} of the dithizone solution used). The corrected readings were plotted against nmol Hg originally in the sac.

From 0·02 mM solutions, known quantities of these compounds were also put in hydrolysis tubes with 1·5 × their volume of 10M HCl, hydrolysed and analysed as above. This related A_{625} to the Hg actually present.

Diffusible thiols by iodoacetamide method: 'triple attack'

In a third test to measure diffusible thiols, flour (2 g) was sieved into 15 ml of fast-stirred 0·25M ammonium bicarbonate buffer, pH 8·6, 5 mM EDTA and 1 ml of fresh 10 mM IAM (in water). After half an hour's stirring under Al foil, the mixture was centrifuged at 3500*g* for 15 min. The supernatant was put in a dialysis sac (18/32 in) and dialysed at ~5°C for ~27 h against 15 ml of water in a test-tube. The tubes were shaken briefly three times during dialysis. The diffusate was freeze-dried after addition of a drop of acetic acid to decompose ammonium carbamate. The freeze-dried diffusate was rinsed into a test-tube with 2 × 0·5 ml of constant-boiling HCl, sealed in a vacuum (~0·05 mm Hg), held at 105°C for 1 day and rotary-evaporated at room temperature. The residue was taken up in 1 ml of LKB loading buffer, spin-filtered (0·2 μm) and loaded (100 μl) on the amino acid analyser. Diffusible thiol was measured as carboxymethyl cysteine (CMC).

Measuring bound thiols with vinyl pyridine

The pellet from the last section was broken up with a rod in ~50 ml of 70% ethanol, magnetically stirred for 30 min, and centrifuged as above. The supernatant was rejected. The pellet was stirred with 10 ml of 0·35M ammonium acetate (pH 7·5) and 0·10 ml of 2-mercaptoethanol (ME) for 1·5 h in the dark under N_2. 4-Vinyl pyridine (155 μl) was added and stirring continued for 1·5 h. The supernatant after spinning was dialysed as above against 10 ml of water for 24 h. The diffusate was freeze-dried,

dissolved in ~1 ml of water, freeze-dried again, then analysed as in the last section. Bound thiols appeared as pyridylethyl cysteine (PEC). Tests were also done without the alcoholic extraction.

The last pellet from centrifugation was slurried in ~10 ml of water, dialysed at ~5°C versus 1 litre of water for 24 h with one change, and a sample of it freeze-dried. About 30 mg of this were hydrolysed and analysed as above. Both PEC and CMC were measured and related to flour protein via the amino acid totals for the glutenin, assuming this was 45% of the flour protein.

In earlier trials on another set of flours, bound thiols were measured as cysteic acid, by performate oxidation followed by dialysis, hydrolysis and amino acid analysis (Ewart, 1985), with minor changes.

Special blanks free from bound thiols (for the performate method)

To free gluten from bound thiols, 2 g were stirred with 40 ml of 0·35M Tris-HCl, pH 8·6, 8M urea, 5 mM EDTA, 0·68M ME, for 4 h in a stoppered flask. The sample was dialysed against 400 ml of 8M urea, 5 mM EDTA, with four changes in 3 days, then against water with four changes in 4 days, and freeze-dried.

Glutenin (1 g) was treated similarly except that the ME was 1·3M and dialysis was against 1 litre of 8M urea, 5 mM EDTA, for 2 days with five changes before changing to water.

Removing free amino acids from gluten

Gluten (1 g) was stirred in 0·5M acetic acid, 8M urea, for 4 h, then dialysed against the same solvent for 3 days with four changes. Dialysis was continued against water for 4 days with four changes before freeze-drying.

Protease activity

This was done in duplicate at pH 4·7 by the American Association of Cereal Chemists Approved Method 22-60: soluble nitrogen was measured after digestion of haemoglobin for 5 h at 40°C.

Thiols in dough

A dough was mixed with 28 g of flour, 0·56 g of NaCl, 0·56 g of yeast, 16·8 ml of tap water and a known weight of thiol (1–3 μmol). The yeast was slurried with the minor ingredients before these were added to the flour and mixed for 1 min in a Minorpin mixer.

Before mixing the dough, 50 ml of 0·4M sodium acetate buffer, pH 4·2, and

2 ml of 1 mM PMA had been degassed and the PMA peak read on a pulse polarograph. Newly mixed dough, ~1 g accurately weighed, was added to the cup. The Silverson homogeniser probe was dipped into the cup and run at the lowest speed for 30 s. (With care splashing could be avoided: the stirrer magnet could be held clear at the side of the cup with an outside magnet.) After 10 min stirring under N_2 and 10 min more degassing, the PMA peak was read.

The rest of the dough was kept covered. A sample was taken from inside the piece ~3 h after mixing and treated as above.

Fluorescent labelling of flour glutenin SH

(a) Lynx flour (2 g) was sieved into 100 ml 0·2M Na phosphate buffer, pH 7·0, 6M urea, 5 mM EDTA, 0·4 mM NEMI, stirred for 30 min, acidified, dialysed versus 0·01M acetic acid, and freeze-dried.
(b) Another sample was treated as in (a) but no NEMI was added.
(c) Untreated Lynx flour (2 g).

Flour samples (a), (b) and (c) were each halved.

One set was reacted with 25 ml of M/3 Tris-HCl, pH 8·0, 6M urea, 2·7 mM EDTA containing 3 μmol of AEDANS for 1 h, then another 25 ml were added and reacted for 1 h, the flask being covered in foil throughout. The other set was given the same treatment except that for the first hour AEDANS was replaced by 3 μmol of IAM.

All six samples were treated with 5 ml of 1M trimethylamine acetate, pH 9·6 (to form a tetraalkylammonium salt with AEDANS or IAM), exhaustively dialysed under foil for 5 days versus water, and freeze-dried. They were run on ME SDS PAGE and looked at under UV light before staining.

Definition of baking quality

In this work loaf volume divided by weight of loaf protein (LV) was used to correct for the fact that higher-protein flours tend to make bigger loaves. Past workers have sometimes used a more scientific measure: the slope of the volume versus loaf protein graph. It was decided to see which way was better.

A good approximation of the slope results from subtracting 363 ml (a mean volume for the ingredients of the loaf made with 280 g of flour) from the loaf volume, before dividing by the weight of loaf protein. (Varying water absorption and loss of water during fermentation would not cause errors bigger than the variation in volume between replicate bakes on a given flour.)

TABLE 1
Correlation Coefficients (r) of LV or 'Slope' versus Variable in Left-Hand Column

	LV	*'Slope'*	*Degrees of freedom*
Intrinsic viscosity of glutenin	0·738 0	0·774 8	34
Intrinsic viscosity of gluten	0·749 5	0·711 4	34
Accessible SH (μmol g^{-1} flour)	−0·522 2[a]	−0·455 7	51

[a] This differs slightly from −0·523 because rounded-off mean values were put in the computer instead of raw data as before (Ewart, 1988*a*).

For LV versus 'slope', $r = 0{\cdot}975\,8$ (viscosity data) and 0·985 8 (SH data).

All eight values of r were significant at $P < 0{\cdot}001$.

The slope of volume versus loaf protein ('slope'), and LV, were each correlated with three variables (Ewart, 1980, 1988*a*). Table 1 shows the correlation coefficients.

These figures show that LV need not be corrected for the volume of ingredients because doing so changes r insignificantly.

Abbreviations for wheat proteins

As the three commonest names for wheat proteins all confusingly start with gl, others may find helpful the symbols used here for many years: UT, glutenin; IA, gliadin; EN, gluten.

RESULTS AND DISCUSSION

(1) Added thiols and loaf volume

Further tests were done to see how added thiols affected the loaf volume of a commercial flour in the LFP. The slope of the best straight line gave the change of loaf volume (in ml) for 1 μmol of added thiol for each gram of flour.

The hydrochlorides of L-cysteine, DL-cysteine and D-cysteine had respective slopes of −38, −47 and −46, which did not differ significantly and agreed with −62 found earlier for L-cysteine HCl (Ewart, 1988*a*). (One control had 8·7 μmol of DL-serine HCl per gram of flour, but there was no significant fall in volume compared with the other control.) No enzyme seems involved in the action of cysteine since the L- and D-forms are equally active.

Cysteamine hydrochloride ($CyNH_2$), thioglycollic acid (TGA) and ME

had respective slopes of −260, −500 and −670. At 0·5 μmol of ME per gram of flour there was a slight rise in volume (*cf.* Ewart, 1988*a*), at 1·0 μmol there was a slight fall, but at 1·5 μmol the fall was catastrophic. TGA and $CyNH_2$ also did not begin their harmful action till ~0·5 or ~1 μmol per gram of flour was added, respectively.

There is a hint from these results that a positive charge on thiols reduces activity. The LFP also tolerates up to ~1 μmol SH per gram of flour fairly well, the level depending on the thiol. In the LFP with a commercial flour, though added thiols may shorten glutenin molecules, the consequent improved orienting and overlapping (Ewart, 1989) could compensate for this; better SS interchange may also reduce mechanical degradation. The LFP tolerates added thiol better than the CBP, where more than ~0·2 μmol SH per gram of flour reduces volume (Ewart, 1988*a*). Orientation is probably near optimal in the CBP so extra thiol tends only to be harmful by shortening glutenin molecules.

(2) Thiol loss in dough

Measurements of the total SH in dough when extra GSH, cysteine, or ME had been added were not accurate, possibly because dough is not easily dispersed. Despite this, means values showed that there was a fall in accessible thiol on mixing but no significant change after 3 h fermentation. Air gets beaten into dough during mixing and presumably oxidises thiols. Unfortunately the work threw no light on why cysteine is so inactive in the LFP. The possibility that yeast uses it does not seem likely since the D-form was also ineffectual. Fresh yeast samples varied from 17 to 19 μmol SH per gram.

(3) Bound thiols and baking quality (by the performate method)

Ewart (1985) suggested that glutenin-bound thiols (EB) were inversely related to mol. wt because they marked the ends of glutenin molecules: hence the name end-blockers, and this abbreviation has been kept for continuity.

No correlation appeared between EB and LV in a trial on 38 English glutens with the performate method.

Since glutenin had been used previously (Ewart, 1985), glutenins were prepared from the gluten samples, but there was no correlation for these either. A third trial was done with glutens, in quadruplicate instead of duplicate, with dialysis at ~5°C, norleucine as an internal standard, and constant protein. The constant protein should have kept the degradation of protein fairly constant, so no blanks were done in this trial. No correlation resulted.

In a search for the cause of these puzzling results, high correlation

($r = 0{\cdot}84$, 0·76 (glutens), 0·83 (glutenins); $P \ll 0{\cdot}001$) of EB with other amino acids was found. Clearly, EB levels were linked with the amount of peptide that got through the sac.

The idea that endogenous flour proteases caused this was tested by measuring protease activity per gram in 24 of the flours; there was no correlation between it and EB, LV or other amino acids. (A negative correlation between protease activity per gram of flour protein and flour protein content ($r = -0{\cdot}552$; $P < 0{\cdot}001$) merely implied that as storage protein increases, enzymes form a smaller part of it, as was long known.)

Efficiency of dialysis of EB

Mean recovery of cysteic acid added to a gluten with its EB removed (special blank) was 86% when put through the treatment given to blanks.

If the performate treatment given to samples was used, recovery was 96%. These dialyses, done at $\sim 5°C$, confirmed a previous finding (Ewart, 1985) when, however, no protein was used, that dialysis of cysteic acid was nearly complete in 1 day in performated samples. (When using norleucine, recovery was too variable, even with similar samples, to be of any use for checking the recovery of dialysis. The reason is unknown.) The coefficient of variation for EB was 13·5% among repeats in the third trial, and most of this must be due to variable dialysis, despite its apparent efficiency in tests with added cysteic acid.

Degradation by formic acid

Separate tests showed that as dialysis time increased so did the total EB and especially other amino acids. Increasing the temperature of dialysis from 5 to 37°C also showed that formic and performic acids were degrading the protein, because the amino acid total increased.

Blanks were not good controls

In blanks, up to four peptide bonds need hydrolysing to free a peptide containing cystine. In the oxidised samples a cysteic acid residue could be released by breaking two bonds at most. Therefore the blanks were not good controls for the release of cysteic acid residues by hydrolysis as claimed by Ewart (1985).

Eliminating effect of amino acids by extrapolation

When the results of the third test series were plotted against extraneous amino acids and extrapolated to zero, values of EB at zero amino acids were still not significantly correlated with LV ($r = -0{\cdot}26$), but the errors of extrapolation were very large. When the combined results were extrapolated, the EB value at zero was 1·5 μmol per gram of protein. Since the

mean value for EB was 3·0 μmol per gram of protein, about half this was due to degradation of the protein, causing a very large error in the measurement of EB. The method was flawed.

Since the extraneous amino acids per gram of protein were not significantly correlated with LV in the glutenin trial and the second gluten trial, the extra cysteic acid brought with them would probably not have been correlated either and so may have masked a correlation of cysteic acid with EB. The previous results (Ewart, 1985), where two kinds of blank correction were tried, may be valid because correlations of LV and intrinsic viscosity of glutenin with EB per gram of protein were little changed even when blanks were ignored (both $r = -0{\cdot}92$; $P \sim 0{\cdot}01$).

Further evidence for EB

When glutenin was treated with ME in a denaturing solvent and dialysed against 8M urea, EB must have been lost because their level fell to about that in the blanks (Ewart, 1985). Similar experiments on a second sample and two on gluten have confirmed this. When gluten was freed from amino acids and peptides by dialysis in a swelling solvent the EB level fell only insignificantly. This was further proof that most EB did not come from free amino acids and peptides but were bound to gluten by SS.

The mean free cyst(e)ine found in five flours (Ewart, 1988*a*) was 0·74 μmol (measured as $\frac{1}{2}$ cystine) per gram of protein (range 0·6 to 0·9). When the diffusate was hydrolysed, this rose to 1·3 μmol per gram of protein (range 1·1 to 1·5). A mean value for EB of 3·0 μmol per gram of protein could not, therefore, all be supplied by free amino acids and peptides in flour, which is further support for EB being covalently bound.

(4) Low molecular weight thiols in flour

Dithizone method for mercury

The method used for measuring mercury with dithizone was a modification of Fridovich and Handler (1957) in which the loss of A_{625} measures the reagent that has complexed with Hg. (This avoids extraction of excess reagent with NH_3, after which the absorption of the complex with Hg is measured.)

Their paper is misleading in implying that when a mercurial reagent has reacted with a thiol it cannot complex with dithizone. Possibly it cannot if the thiol is protein-bound, but here compounds of PMA with low mol.wt thiols complexed with dithizone. Increasing concentration of acid in the aqueous layer reduced A_{625} markedly, therefore tests and calibration curves must have the same concentration of HCl. Here it was finally fixed at 0·75M HCl. Hydrolysis destroyed surface-active material that prevented separation

of the CCl_4 layer in some flours. Hydrolysis may at least partly have converted phenylHgSR to $HgCl_2$ because it increased dithizone binding, though less than twofold.

Obviously this simpler method is useless if unreacted PMA is present.

'Active' thiols

Low mol.wt thiols in flour may be more active than those on proteins: thiols on enzymes sometimes lie in a protected pocket. Data of Tipples and Tkachuk (1965) and Tkachuk and Tipples (1966) suggest that the major enzyme, β-amylase, does not add more than $\sim$0·04 μmol SH per gram to flour.

Three methods were tried for measuring diffusible (presumed active) thiols: they were based on a fluorescent agent (AEDANS), PMA or IAM. No method showed significant correlation between LV and 'active' thiol. The correlation coefficients of diffusible thiol (PMA method) with LV were $-0{\cdot}32$ (LFP) and $-0{\cdot}57$ (CBP).

Visking tubing has $\sim$0·1% of sulphur, left by the viscose process, which could interfere with mercury compounds. The recommended method of purification was not used; instead calibration curves were set up by giving known quantities of the PMA derivatives of cysteine and GSH the treatment given to flour samples. This took care of troubles caused by sulphur and by increasingly slow dialysis at low concentration. The modified method for Hg described above could be used because PMA was added equivalent to accessible thiol, found by the polarograph. With PMA, only 14–16% of the accessible thiols were diffusible. This is about the same as the amount of EB: $\sim 1{\cdot}5$ μmol per gram of protein. At least some active thiols may finish up as EB.

The AEDANS method found 32–39% of accessible thiols were active, but AEDANS probably reacted incompletely, and it or its side-products with amino acids or water diffused through to give too high a result. The third try at measuring diffusible thiols (with IAM) is discussed in Section (6).

(5) Thiols attached to glutenin

Test of Graveland's method for thiols

There seem to have been very few attempts to see whether glutenin molecules have SH. Kawamura *et al.* (1985) and Ewart (1985, 1988*a*) failed to find any, but Graveland *et al.* (1985) did.

The author applied Graveland's method to one of the glutenin samples. After the solution had been centrifuged it was still hazy. The A_{412} value (0·31) was higher than the one they presumably got (0·17) for 2 moles of SH in a mole of protein of mol.wt 8×10^5. But this A_{412} was almost certainly due to haze, since a control done with reduced and blocked glutenin of the same variety gave a similar value. It is therefore possible that slight haze could

have caused their positive result, because they did not say they took steps to clear the solution.

SH on glutenin by the PMA method

The PMA method applied to flour, not prepared glutenin, showed that a weak English wheat (Apollo) and a strong wheat (CWRS) had about 1/8 SH per glutenin chain. This leads to ~16 chains per molecule (if there was an SH at each end), and to a number-average mol.wt of $\sim 8 \times 10^5$, numerically the same as the mol.wt Graveland *et al.* (1985) found for glutenin IIa; this had, however, been through a fractionation scheme where SH might have become oxidised. As far as the author knows, there has been no other direct measurement on untreated glutenin. The SH amounts to ~1/8 the accessible thiol in flour. If, during biosynthesis, subunits polymerise to glutenin by oxidation of their SH, a number-average mol.wt of 8×10^5 corresponds to a *b* value (Ewart, 1987) of 1/16, leading to a weight-average mol.wt of $1{\cdot}5 \times 10^6$, fairly close to a literature value (Jones *et al.*, 1961).

(If the cysteine residue found in the repetitive part of glutenin subunit 12 by Thompson *et al.* (1985) was the cause of this SH content, most of the high mol.wt subunits of both wheats would have to be subunit 12.)

Fluorescent labelling of flour

Of the samples reacted with AEDANS, only untreated flour gave a strong fluorescent pattern on ME SDS PAGE: there was weak fluorescence of the background tailing and at the origins of large- and small-pore gels. IAM and NEMI must have effectually blocked flour SH. Even the exposure of flour to pH 7 buffer only, followed by dialysis and freeze-drying, lost all the SH, presumably by oxidation and dialysis. Non-specific labelling of protein by AEDANS (e.g. with NH_2 or SS) was negligible compared with its reaction with SH. These results could imply that most glutenin subunits carry SH. This would occur if at least some ends of glutenin molecules had SH and the order of subunits in molecules was random. The valuable work by Singh and Shepherd (1985) also supports a random arrangement of subunits in glutenin. The likeliest explanation, however, is that SS interchange was so fast at pH 8 that there was significant transfer of SH from low mol.wt thiols to glutenin, which were labelled together with those already there. So, unfortunately, no conclusions can be drawn about the arrangement of subunits. If similar results followed with a large excess of AEDANS over SH instead of only 3:1, they would be more convincing.

(6) Measuring bound and free thiols on the same sample

'Triple attack' on thiols in flour

The previous work suggested that it would be worth trying to measure, on

the same flour sample, all the SH (on glutenin, bound to glutenin by SS, or diffusible). The idea was to block accessible thiols quickly by sieving flour into iodoacetamide solution, dialyse, then remove gliadins from the solid residue, reduce and block the residual glutenin with vinyl pyridine, and dialyse again. Diffusible thiols would be measured as CMC in the first diffusate, EB as pyridylethyl cysteine in the second diffusate, and the SH originally on glutenin would appear as CMC when the residue was hydrolysed.

Owing to the author's retirement there was only time for a short trial on 22 flours with few duplicates. Five of these had no CBP LV, and data on another were incomplete.

Diffusible thiols

No significant correlation emerged between diffusible thiols in flour and LV whether by the LFP ($r = -0{\cdot}38$) or the CBP ($r = -0{\cdot}36$), though the negative correlation noted in the Hg method was confirmed. Diffusible thiols were only ~5–9% of the total accessible thiol in flour but tests showed that this could be raised by ~25% to correct for incomplete dialysis. (The higher figure of 10–14% given by the Hg method may be because PMA reacts virtually instantly with SH, so preventing the loss of thiols by reaction with glutenin or oxidation that may occur when IAM is used.)

EB on glutenin in the 'triple attack'

Surprisingly, the correlation was positive between EB (removed by ME) and LV. For the CBP LV, $r = 0{\cdot}63$, $P < 0{\cdot}01$ for EB per gram of flour protein and $0{\cdot}61$, $P < 0{\cdot}01$ for EB per gram of flour. For the LFP LV, $r = 0{\cdot}44$, $P < 0{\cdot}05$ for EB per gram of protein and 0·27 NS for EB per gram of flour.

The mean value, 1·55 μmol per gram of flour protein, agrees well with that of the performate method, 1·5 μmol per gram of gluten protein, and this work again confirms that low mol.wt thiols are bound to glutenin by SS. The amounts ranged from 0·9 to 2·3 μmol per gram of flour protein. When the 70% ethanol extraction was included there was a 42% drop in the mean EB level ($P \ll 0{\cdot}001$). This drop may be due to the loss of low mol.wt glutenin, which would be disproportionately rich in EB if these are linked with the ends of molecules. It could, however, also be due to loss of some gliadins bearing EB. (If, say, each γ-gliadin carried an EB, these alone would make up more than the original EB total.)

The correlation of LV with EB on higher mol.wt glutenin (i.e. after alcoholic extraction) was no longer positive but nil, so it would be wise not to try to explain a possibly spurious positive correlation till it is confirmed.

SH on glutenin in the 'triple attack'

SH on glutenin showed some significant correlation when expressed as μmol

per gram of flour protein ($r = -0{\cdot}60$, $P \sim 0{\cdot}01$: CBP LV, but $r = -0{\cdot}20$, NS: LFP LV). When expressed as μmol per gram of flour, $r = -0{\cdot}49$, $P < 0{\cdot}05$: CBP, but $r = -0{\cdot}28$: LFP. The fact that the negative correlation was only significant in the CBP ties in with its lower tolerance of added thiols.

This IAM method gave values in the range of 0·17 to 1·1 SH per average chain of mol.wt 50000, as against 0·12 by the PMA method for only two flours. It is conceivable that IAM during the 30 min reaction at pH 8·6 could attack SS bonds as well but probably fast SS interchange at pH 8·6 of thiols with glutenin could also give it extra SH. The PMA reaction was done at weakly acid pH to try to avoid this.

Proteins may act as EB

Kasarda (1989) has studied published sequences of γ-gliadins (Okita, 1984; Sugiyama *et al.*, 1986; Scheets & Hedgcoth, 1988) and shown that they have odd numbers of half-cystine residues and so presumably at least one SH. (It is a pity that so few of those who go to the trouble of getting a sequence publish totals of amino acids and bases. The author counted the half-cystine residues as 7, 7 and 9, respectively. Their work, incidentally, means that Ewart's claim (1977), on the thinnest evidence, that γ-gliadins had no SH was foolish.)

So it seems that some gliadins may have to be considered, either as EB themselves or as binding EB, in tackling the problem of protein quality. Also Rowsell and Goad's work (1962) implied that β-amylase was bound to glutenin, and so could act as an EB, though the possibility of entrapment may not have been quite excluded. It should not be difficult to prove whether or not gliadin (freed from low mol.wt glutenin) contains SH, either free or blocked by EB.

Coefficient of variation

In the 'triple attack' CMC and PEC peaks were measured by triangulation to get a set of results to compare with those of the integrator. Coefficients of variation were 37% (integrator) and 27·5% (triangulation). Triangulation not only avoided one or two wild results but was responsible for such correlations as appeared. Further improvements in accuracy could well bring to light more relations.

(7) Conclusing remarks

Though the results are inconclusive (which is not unknown in cereal chemistry) there are several positive features.

Glutenin almost certainly binds low mol.wt compounds (EB) by SS, and two methods agree on their mean amount, $\sim 1{\cdot}5$ μmol per gram of protein.

Credible mol.wts can be calculated from the formula 50 000(2C/E), where C and E are the moles of chain and EB, respectively (Ewart, 1985).

Glutenin also seems to carry SH, which were significantly correlated with LV in the CBP, despite the analytical errors. These SH may be inversely related to mol.wt, if they mark the ends of molecules, predicting a similar value to that from EB. (There may be SH on glutenin that are buried in the core (cf. Thompson *et al.*, 1985). If these took no part in polymerisation during biosynthesis, they would not be markers for the ends of molecules.) The problem that both SH and EB are marking ends of molecules is resolved by using the factor (4C/E), which was found to agree better with viscosity data than (2C/E) (Ewart, 1988*c*). This formula depends on active thiols being as likely to attack labile intrachain SS as interchain, so for each molecule there should be one intrachain SS attacked on average. Attack on an SS puts an SH on one half and an EB on the other.

Though the diffusible SH in flour did not correlate significantly with LV, two methods gave negative values of r which is in line with significant negative correlations for all the accessible flour SH with LV, found by several workers (Ewart, 1988*a*). All this suggests that active SH control the mol.wt of glutenin and are the target of improvers.

Ziegler (1985) stated that, while protein mixed disulphides undoubtedly exist, current methods could not measure their low concentrations accurately. This work confirms his statement but holds out hope that the problems will soon be overcome now that there is a strategy for measuring more than one variable on the same sample: radioactive counting would greatly improve accuracy and gel electrophoresis could replace dialysis. Methods for measuring individual low mol.wt thiols in flour would be valuable.

No conflict with Payne's work

Since the mol.wt of glutenin of UK wheats (as measured by the intrinsic viscosity) only accounts for about half the variation in baking quality (see the correlation coefficients in Table 1), there is no conflict between the importance of SH (bound or free) and the work of Payne *et al.* (1979, 1984). One-third to two-thirds the variation in baking quality (depending on the country of growth of the wheats) is linked with types of high mol.wt subunits (Payne, 1986; Lukow *et al.*, 1989). Kasarda (1989) has pointed out that low mol.wt subunits have nothing like the capacity to unfold between their interchain SS that high mol.wt subunits have (cf. Ewart, 1978), hence these are essential for elasticity. The possibility that their quantity is more important than their type, raised by Lawrence *et al.* (1988), is well worth pursuing. They made the important discovery that a wheat without high mol.wt subunits had very poor baking quality.

Future studies on the effect of free and bound thiols on the baking quality of glutenin could well be done on groups of wheats having a constant composition of high mol.wt subunits. The dependence of baking quality of glutenin roughly equally on its subunit composition and on its mol.wt (which thiols affect) is probably why workers have seldom found high correlations between baking quality and free or bound thiols.

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APPENDIX

Extra result on loss of thiol with time

In the study of thiol loss in flour on aging (Ewart, 1988*b*) further points were added at about 20 months (Table 2). When these points were combined with the earlier ones, the thiol contents were all negatively correlated with storage time at room temperature ($P < 0{\cdot}05$) (last column of Table 2).

TABLE 2
Thiol Content of Flour Stored at Room Temperature

Wheat	*Days after milling*	*Mean accessible thiol (μmol/g of flour)*	*Mean total thiol (μmol/g of flour)*	*Correlation coefficient (r)*
CWRS	598	—	0·96 (8)	−0·940
	605	0·95 (6)	—	−0·816
Avalon	625	—	0·82 (6)	−0·832
	632	0·77 (6)	—	−0·866
Galahad	610	—	0·71 (6)	−0·842
	614	0·69 (6)	—	−0·816

Number of determinations in parentheses.