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Thiols in Flour and Breadmaking Quality

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ABSTRACT

A differential pulse polarograph was used to measure accessible thiols in wheat flour suspensions at pH 4·2 with phenyl mercuric acetate as the reagent. Loaf volume per gram of loaf protein (LV) showed some inverse correlation with accessible thiol content of flour in a trial on 53 English wheats from 39 varieties ($r = -0{\cdot}523$; $P < 0{\cdot}001$). At least four measurements were done on each flour. The mean level of accessible thiol for the 53 flours was 0·85 μmoles g^{-1}. From trials in which bread was baked from doughs with added thiols it was concluded that bread quality began to fall off in the Chorleywood Bread Process when 0·2–0·3 μmol thiol per gram of flour were added. Evidence from elsewhere as well as this paper suggests that not more than about quarter the endogenous thiols are rheologically active. This would explain why better correlation between thiol content and LV was not found.

Twenty-three of the 53 results were done on nine varieties, and these showed that accessible thiol varied significantly within a variety.

Three more attempts to find significant amounts of thiol in glutenin failed. Excess L-*cysteine or glutathione acting on glutenin at dough pH in the absence of denaturing agents reduced ~90% and ~35%, respectively, of its disulphide bonds.*

When five flours were analysed for free cyst(e)ine a mean value of 76 nmol Cys/2 per gram of flour was found (range 59 to 86).

INTRODUCTION

The thiol and disulphide contents of wheat flour have been of interest for many decades, but surprisingly few quantitative studies have been made.

Goldstein (1957) was the first to suggest that thiol groups catalysed disulphide interchange reactions in dough: he rejected the then current idea that thiols were oxidised to form new disulphide bonds (SS). Water-soluble thiols outnumbered those of the acetic acid-soluble proteins by two or three times (Matsumoto & Hlynka, 1959). Bromate and iodate caused some loss of thiols in dough (Matsumoto & Hlynka, 1959; Lee & Samuels, 1962). Thiolated gelatin (McDermott & Pace, 1961) or radioactive cysteine became bound to gluten during mixing: the binding seemed to be by disulphide interchange rather than oxidation (Mauritzen & Stewart, 1963, 1966; Stewart & Mauritzen, 1966) though about quarter the thiol groups were lost by oxidation during mixing. Low-molecular-weight thiols were more effective (Villegas *et al.*, 1963), or became bound to dough proteins more readily (Lee & Lai, 1968), than protein-bound thiols. Mixing increased the reaction of glutathione with flour proteins (Kuninori & Sullivan, 1968).

Sullivan (1954) stated that the lower the grade of flour, the higher its reducing value and the greater its response to oxidising agents. Reducing values did not correlate with the amount of oxidant needed, however, nor with the resulting improvement in properties. From a study on seven flours Tsen & Bushuk (1969) reported that total and reactive thiols were inversely related to mixing strength. Lee & Lai (1967) found a high correlation between the less accessible thiols and load volume, which is hard to explain: if, however, one bases both their variables on unit protein the correlation vanishes. Archer (1972) noticed, in ten flours, a strong negative correlation between free glutathione and mixing tolerance, resistance, Pelshenke, or Zeleny readings. Though he did not normalise the glutathione to unit protein, when this is done the correlations are not seriously altered. Strictly speaking, the dough properties should also be normalised to unit protein but there is a lack of evidence that dough properties are linearly related to protein content of dough. Archer (1979), in further tests on twenty-two flours, found no significant correlation of rheologically important thiol content with resistance, extensibility or dough breakdown. If his thiol figures are normalised to unit protein, correlation reaches significance ($P < 0{\cdot}05$) for dough breakdown ($r = 0{\cdot}48$) and extensibility ($r = -0{\cdot}49$). Coventry *et al.* (1972) got highly significant negative correlation between total glutathione or half-cystine residues and resistance; the correlation is still significant $P < 0{\cdot}001$ when GS- and Cys- levels are combined and normalised to unit protein. The thiol content per gram of protein was less in a strong flour than in a weak one according to Stevens (1966).

Therefore, six references support the view that endogenous thiols could lower dough strength. And although Bloksma (1972) stated in his summary that there was no unequivocal relation between total thiol content and various rheological properties, he found a positive correlation between

reactive or total thiol and dough compliance in his Figure 22, giving further support to a link between thiol content and protein quality.

The aim of the present work is to test further the relation between accessible thiol content of flour and baking quality, when measurements are done within a month of the baking test.

EXPERIMENTAL

Flours

The 53 flours used were samples from 39 English varieties embracing the range from the best breadmaking to the weak biscuit types and including feed wheats. Winter and spring varieties, including hard and soft milling wheats, were included.

Thiol measurement

0·4M sodium acetate buffer, pH 4·2 (50 ml) and 2 mM phenyl mercuric acetate (PMA; 1·0 ml) were pipetted into the glass cell of an EDT Differential Pulse Polarograph, model ECP100. The solution was stirred and degassed with O_2-free N_2 for 10 min then two peaks were read on an x,y recorder (J. J. Lloyd Instruments) scanning from 0·15 to −0·85 V at 2 mVs^{-1} at 200 nA full scale sensitivity. The drop time of the mercury electrode was 1 s^{-1}. The auxiliary and reference electrodes were platinum and calomel, respectively. The gain was 1 and pulse amplitude usually 75 mV. A quantity of flour containing 100 mg of protein was added slowly with stirring through a small sieve (7 mesh cm^{-1}) to prevent clotting. The mixture was stirred, with N_2 playing over the surface for 10 min then bubbling through the mixture for a further 10 min. Two peaks were read. The areas of the peaks were calculated by multiplying the height of the peak, perpendicular to the sloping baseline, by the width at half height parallel to this baseline. The fraction of original PMA peak area lost gave the fraction used. Protein was taken as 5·7 × N, which was measured on a Kjeltec Auto 1030 Analyser.

Baking test

The baking test by the long fermentation method (Draper & Stewart, 1980) included bromate in the recipe (except when testing the added thiols). When the Chorleywood Bread Process was used to test the effects of added thiols the method was as before (Axford *et al.*, 1978) except that the oxidant level was 30 mg kg^{-1} on the flour for ascorbic acid and 45 mg kg^{-1} for potassium bromate.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used in 10% gels as described by Laemmli & Favre (1973).

N-Ethyl maleimide (NEMI)-treated glutenin

Glutenin (1·6 g) prepared as before (Ewart, 1985), was stirred with a fresh solution of 2 g litre^{-1} NEMI in 0·1 M imidazole HCl, pH 6·9, 8 M urea (50 ml) for 15 min, acidified, dialysed against dilute acetic acid, and freeze-dried.

Free thiols in glutenin

Glutenin ($\sim$ 50 mg protein) was stirred under N_2 for 1·5 h in a 6 ml plastic ultracentrifuge tube with 0·1 M Tris-HCl, pH 8, 50 g litre^{-1} SDS, 8 M urea, 2 mM Ellman's reagent. The tube was again flushed with N_2 after the magnet had been removed, then recapped and centrifuged at 107 000 *g* for 15 min. The clear supernatant layer was read at 412 nm against a control done on an equal weight of NEMI-treated glutenin.

Phenyl mercuric acetate treatment of glutenin

Glutenin ($\sim$ 100 mg protein) was stirred for 1 h with a mixture of 20 g litre^{-1} PMA in dimethylformamide (0·1 ml) and 8 M urea (5 ml); it was put in a 22/32 in dialysis sac, using 8 M urea to rinse the tube, and dialysed against at least a fivefold volume of 8 M urea, changing once a day for 7 days. The urea was then removed by dialysis against water and the contents of the sac freeze-dried. The samples were hydrolysed at 105°C with constant-boiling HCl for 24 h and rotary-evaporated at room temperature after adding EDTA (1 mg).

In other tests three-quarters or half as much protein was taken, the PMA solution was made 0·5 M in acetic acid, the reaction time increased to 4 h, and the protein was not hydrolysed.

Mercury was measured spectrophotometrically at 490 nm after complexing with dithizone by a modification of the method of Tyuma *et al.* (1966), the organic material having been oxidised with acid potassium permanganate at 70°C. Parallel tests were done on NEMI-treated glutenin.

Blocking thiols with a fluorescent reagent

Glutenin, NEMI-treated glutenin, bovine serum albumin and barley β-amylase were each treated with 1 mM solution of *N*-iodoacetyl-*N'*-(5-sulpho-

1-naphthyl) ethylene diamine (1,5-I-AEDANS) in M/3 Tris HCl, pH 8·0, 8 M urea, 8 mM EDTA (5 ml) and stirred in the dark for 2 h. 1·2 M Trimethylamine acetate, pH 9·6 (5 ml) was added to each, and the samples were dialysed in the dark against water for 3 days and freeze-dried. The samples were treated with ME and run on SDS PAGE. They were examined under ultra-violet light before staining.

Free amino acids in flour

Flour (10 g) was suspended in sterile water (40 ml) by magnetic stirring then dialysed at 4°C for 24 h against sterile water (150 ml). Drops of toluene were added to both sac and diffusate. After the volume of diffusate had been measured, an aliquot was rotary-evaporated, taken up in 'LKB loading buffer' (0·2 M sodium citrate, pH 2·2; 8 ml), spin-filtered (0·2 μm) and analysed for amino acids.

Reaction of glutenin with cysteine or glutathione at dough pH

Glutenin (0·1 g) was stirred overnight under N_2 with L-cysteine (1 g) in 0·12 M Na_2HPO_4, 0·039 M citrate, pH 5·8 (10 ml) or glutathione (0·1 g) in the same buffer (20 ml) after adjusting the pH back to 5·8. The calculated quantity of iodoacetamide in M Tris HCl, 27 mM EDTA, pH 8·6 (20 ml) was added. After the pH had been continually adjusted to 8·5 with NaOH for 15 min, the solution was acidified with acetic acid, dialysed in the dark for at least 4 days and freeze-dried.

Blanks were treated with the pH 8·6 buffer (20 ml), acidified and freeze-dried. The reduction was also done on glutenin that had already been reduced with mercaptoethanol (ME) and blocked with iodoacetamide in 7 M guanidine hydrochloride. This corrected for the amount of cystine, i.e. unreacted SS, that came from oxidised reagent not removed by dialysis.

Samples were hydrolysed *in vacuo* (0·05 mm Hg) for 24 h with constant boiling HCl and analysed for cystine.

RESULTS AND DISCUSSION

Reaction time

The acid buffer for polarography was chosen to reduce the chance of breaking labile disulphide bonds at alkaline pH in the presence of a mercurial reagent. When the flour was allowed to react for 1 to 2 h instead of 10 min no increase in thiol level was found. Therefore PMA appeared to react with all the accessible thiols within 10 min.

Standard solutions of glutathione were analysed; the coefficient of variation was 7·4%, similar to that found with the flours.

Flour properties

Protein contents ranged from 7·8 to 12·3%: mean, 9·4%, sd, 0·972. Loaf volumes ranged from 955 to 1595 ml: mean, 1350 ml, sd, 144.

Accessible thiol levels

The SH groups of 53 English wheat flours (kept at room temperature) were measured within a month of milling and in the absence of denaturing agents. These accessible SH groups are likely to include the ones that are rheologically active in dough (Frater *et al.*, 1960). At least four measurements were done on each flour and the means were used. The mean value of μmoles of accessible SH per gram of flour for the 53 flours was 0·85 with a range of 0·66 to 1·07; the standard deviation (sd) of a single measurement was 0·062. The mean μmoles of accessible SH per gram of protein was 9·1 with a range of 7·1 to 11·0; sd of a single measurement was 0·63.

The use of bromate in baking tests

The fact that bromate, varying in level according to the flour characteristics, was added in the baking test (Draper & Stewart, 1980) will seem anomalous. Bromate is known to destroy the very SH groups whose influence was being studied. Bloksma (1972) said that bromate seems to react more specifically with reactive, i.e. accessible, SH (which probably include those that lower the molecular weight of glutenin and also the baking quality). Over half a century ago, before bromate was regularly used in baking tests, Larmour (1930, 1931) showed that loaf volume was correlated with protein content in baking tests with and without bromate: his samples were mostly from three varieties, but from hundreds of different sites. Bromate greatly improved the correlation. Aitken & Geddes (1934) tested ten composite flours, each a blend of hundreds of samples such that all variables except protein content were considered to be averaged out. Their Fig. 1 shows that bromate gives a steeper regression line of volume versus protein content. The action of bromate is slow, however, which may be why correlation was found between intrinsic viscosity of glutenin from *untreated* flours and LV from a baking test *with bromate* (Ewart, 1980). It seems that the presence of bromate by no means completely vitiated the expected correlation between baking quality measured with bromate and a property that baking quality depended on

measured without bromate. Therefore although a baking test without added oxidant would have been preferable, since it was not available for routine testing the work was put in hand without it.

Loaf volume and protein content

Loaf volume was not significantly correlated with protein content ($r = 0{\cdot}140$; NS) which is further evidence that proteins of wheats vary in quality. Baking quality is here defined as loaf volume per gram of loaf protein (LV), the yeast protein (amounting to $\sim 0{\cdot}25\%$ of the flour) being ignored. LV was inversely correlated with protein content ($r = -0{\cdot}624$; $P < 0{\cdot}001$) but this is merely because loaf volumes of these wheats did not correlate with protein contents, and so volume divided by protein content would amost certainly be inversely correlated with protein content.

Fourteen of the 53 observations were later found to be repeats of nine varieties. When the relations of the twenty-three observations were studied in this subset, LV was correlated with accessible thiols per gram ($r = -0{\cdot}716$; $P < 0{\cdot}001$); in general, plots of the two to four points for a given variety agreed qualitatively with this correlation. As before, there was no significant correlation with thiol per gram of protein.

Accessible thiol per gram of flour did not vary significantly ($P > 0{\cdot}1$) more among varieties than within varieties, and so did not seem to be a constant for a variety. Accessible thiol per gram of protein varied significantly ($P < 0{\cdot}01$) more among varieties than within varieties, but again the intravarietal variation was significantly greater than experimental error: this real variation within a variety was at least partly a reflection of the tendency found in the 53 samples for accessible thiol per gram of protein to fall as protein content rose ($r = -0{\cdot}403$; $P < 0{\cdot}01$).

Baking quality and thiols

Apart from the artifact mentioned earlier, the highest correlation was between LV and accessible SH per gram of flour ($r = -0{\cdot}523$; $P < 0{\cdot}001$). This result agrees with the balance of evidence cited in the Introduction that endogenous SH groups in flours have a negative effect on dough strength and baking quality.

Loaf volume alone was not significantly correlated with accessible SH per gram of flour ($r = -0{\cdot}109$; NS), which shows the importance of normalising loaf volume to unit protein. The absence of correlation meant that when both variables were normalised to unit protein, they also were not correlated ($r = 0{\cdot}092$; NS).

Level of active thiol

Although the deleterious effect of thiols on the baking quality of flour has long been known, data on the amounts needed are remarkably hard to find. The addition of 0·66 μmol of cysteine per gram of flour increased loaf volume (Swanson & Andrews, 1944), which contrasted with only 0·03 μmol g^{-1} flour of the improver, iodoacetic acid, being needed for optimum loaf volume (Tkachuk, 1972).

Tests here, however, showed that when the Chorleywood Bread Process was used, additions of L-cysteine, 2-mercaptoethanol (ME), or glutathione (GSH) all began to lower bread quality at 0·2 to 0·3 μmol g^{-1} flour (Fig. 1). In the 3 h bulk fermentation test (Fig. 2), when bromate was omitted, GSH was similarly effective; this agrees with a statement of Baker *et al.* (1944) that 100 mg (i.e. 0·33 mmol) kg^{-1} of GSH is enough to produce marked effects in breadmaking. About 0·5 μmol g^{-1} flour of ME was needed, however, and cysteine, even at 1 μmol g^{-1} flour, had no significant deleterious effect (Fig. 2): the effect was so slight that the scale of the abscissa in Fig. 2 for cysteine is increased tenfold to get the points on the Figure. At first sight this was surprising because tests with L-cysteine or GSH in large excess on glutenin showed that cysteine can attack ~90% of SS in the absence of urea at pH 5·8, near that of dough, whereas GSH only attacked about a third. Thiols

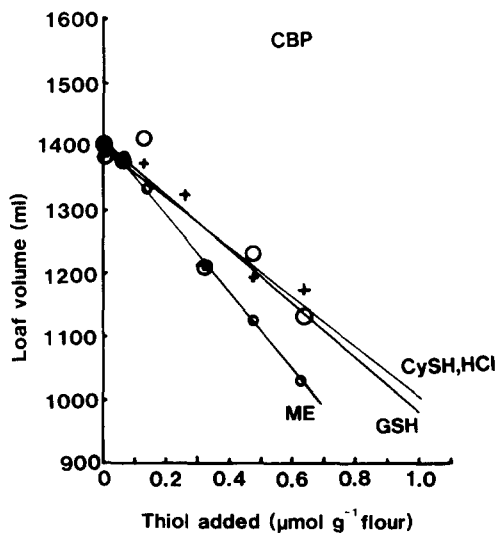

Fig. 1. Plot of loaf volume versus added thiols in the Chorleywood Bread Process (CBP). ○, GSH; +, L-cysteine, HCl; ○, ME. Slopes of the least squares regression lines

$$\left(-\frac{\text{d(ml)}}{\text{d}(\mu\text{mol})}\right) = \text{GSH, 430; CySH, HCl, 390; ME, 600.}$$

The significance level of all the slopes was $P < 0{\cdot}001$ except for GSH which was $P < 0{\cdot}01$.

such as cysteine and ME, however, are more easily oxidised than GSH, probably because the negatively charged GSH molecules repel each other and so, in a bulk fermentation test, higher amounts of ME and, especially, cysteine (Fig. 2) have to be added so that enough will survive the 3 h fermentation. Therefore, thiols added at about 0·2 μmol g^{-1} flour begin to affect bread quality adversely.

This figure is confirmed by an average level of ~0·2 μmol of gluten-bound thiol per gram of English flour found here when measured by methods similar to those described (Ewart, 1985). Since only rheologically active thiols could bind to gluten, by reacting with SS bonds, 0·2 μmol g^{-1} flour is a reasonable estimate of the mean level in English flours. Tkachuk's (1972) showing that only 0·03 μmol of iodoacetate was necessary for the best loaf volume of a flour he was studying is in accord with a low value for the level of rheologically active thiols. All this confirms Bloksma's (1972) statement that rheologically active thiols form only a small fraction of the total. Since the mean value of accessible thiols was 0·85 μmol g^{-1} flour it seems, therefore, that rheologically active thiols make up less than quarter the total (0·2 ÷ 0·85). This would explain why the inverse correlation of LV with accessible thiol content of flour only accounts for a small part of the total variation, as measured by the square of the correlation coefficient (r^2):

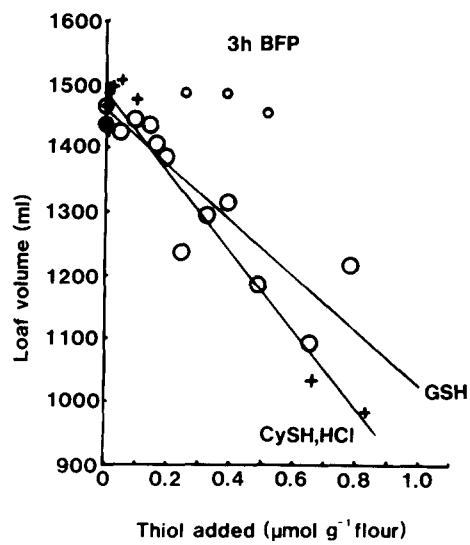

Fig. 2. Plot of loaf volume versus added thiols in the 3 h bulk fermentation process (3 h BFP). ○, GSH; +, L-cysteine HCl; ○, ME. Slopes of the least squares regression lines

$$\left(-\frac{\text{d(ml)}}{\text{d}(\mu\text{mol})}\right) = \text{GSH, 430; CySH, HCl, 62; ME, —.}$$

The significance level of all the slopes was $P < 0{\cdot}001$. The figures on the abscissa for the plot of CySH, HCl must be multiplied by 10.

random variation in the large excess of inactive thiols probably accounts for the rest and also swamps any possible correlation between LV and accessible thiols per gram of protein. It should be noted that reactive thiols are not necessarily rheologically active (Sokol *et al.*, 1960; Sullivan *et al.*, 1963; Mauritzen & Stewart, 1966; Tanaka & Bushuk, 1973).

Free cyst(e)ine in flour

The mean value for cyst(e)ine (measured as cystine) on five flours was 9·1 μg g^{-1} flour, range 7·1 to 10·3 (or a mean of 0·076 μmol of Cys (i.e. half-cystine), range 0·059 to 0·086). This shows that less than 10% of accessible thiols could be present as cysteine.

Thiol groups absent from glutenin

Some of these inactive thiols may be in protected sites on metabolic proteins, but none seems to be attached to glutenin because in three further tests (with Ellman's reagent, phenyl mercuric acetate, and a fluorescent reagent) significant amounts of SH groups on glutenin were not found, in agreement with three other negative tests (Ewart, 1985), and the finding of Kawamura *et al.* (1985). Graveland *et al.* (1985) found appreciable quantities of thiol in glutenin using Ellman's reagent. At FMBRA, Ellman's reagent yielded weak positive results, but since these also occurred with NEMI-treated glutenin it is unlikely that they were caused by thiol groups in glutenin.

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