Determination of Protein–Glutathione Mixed Disulfides in Wheat Flour

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An HPLC method was adapted for measurement of protein–glutathione mixed disulfides (PSSG) in flour and flour protein fractions. It involves extraction of unbound glutathione with perchloric acid (PCA), release of glutathione from PSSG by dithiothreitol (DTT) reduction, and separation and quantification of the carboxymethylated and dinitrophenylated peptide by HPLC on amino-bonded phase silica. PSSG levels ranged from 70 to 150 nmol/g in flours from different wheat cultivars. PSSG also varied among flour protein fractions obtained by Osborne fractionation. Glutenin had the highest value (897 nmol/g of protein) and albumin a somewhat lower value (796 nmol/g of protein). Levels in globulin and gliadin were considerably lower (239 and 76 nmol/g of protein, respectively). SDS-unextractable glutenin macropolymer preparations also had high levels (1062 nmol/g of protein, cv. Glenlea; 1538 nmol/g of protein, cv. Bussard). Availability of this methodology for GSH, GSSG, and PSSG analysis provides a powerful tool for elucidating the importance of glutathione in flour and dough systems.

Keywords: *Wheat; glutathione; protein; mixed disulfides*

INTRODUCTION

The reduction–oxidation (redox) reactions that occur in wheat flour and dough have long been recognized as being important in relation to dough rheology and bread quality, but the (bio)chemistry of these reactions is still poorly understood. Such redox reactions, including those involving exogenous oxidizing bread improvers, are thought to affect, either directly or indirectly, the disulfide-mediated polymeric structure of the wheat glutenin protein fraction (Fitchett and Frazier, 1986; Grosch, 1986).

Particular attention has been focused on the possible role of the tripeptide, glutathione (γ-glutamylcysteinylglycine), which occurs endogenously in flour in both reduced (GSH) and oxidized (GSSG) unbound forms (Schofield and Chen, 1994, 1995). Protein–glutathione mixed disulfides (PSSG) are also present in flour proteins (Ewart, 1985; Sarwin et al., 1992). PSSG may also be formed during dough mixing either through reaction of GSH with protein disulfide (SS) bonds (Kuninori and Sullivan, 1968) or through reaction of GSSG with flour protein sulfhydryl (SH) groups (Jones and Carnegie, 1971), both via SH/SS interchange reactions. Although the latter reaction does not result directly in the scission of SS bonds, GSH is released, and it can take part in further SH/SS interchange reactions with flour protein SS bonds. Thus, both reactions may result in glutenin being depolymerized, thus weakening the dough (Sullivan, 1936; Ziegler, 1940; Villegas et al., 1963; Jones and Carnegie, 1969b).

Nevertheless, the real technological significance of glutathione and its reactions remains unclear. A major problem is uncertainty about the true levels of the peptide in flour; values reported in the literature vary by orders of magnitude (Grosch, 1986; Schofield and Chen, 1994, 1995). Furthermore, a convenient and reliable method for measuring GSH, GSSG, and PSSG individually has also not been available, making it difficult to obtain a complete picture of the reactions that glutathione undergoes in dough.

Recently, we established a relatively rapid and straightforward HPLC method for measuring GSH and GSSG in flour (Schofield and Chen, 1994, 1995); this is an adaptation to flour of the method described by Reed et al. (1980). In the present paper, we have established an extension to that method, by which PSSG in the protein fraction of flour can be determined. This method is also an adaptation to flour of a procedure developed by Meredith (1982); it now enables us to measure GSH, GSSG, and PSSG in flour individually.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Merck-BDH Ltd., Poole, Dorsetshire, U.K., except reduced and oxidized glutathione, which were obtained from Sigma Chemical Co., Poole, Dorsetshire, U.K.

Wheat and Flour Samples. Grain samples of eleven U.K. wheat cultivars (1989 harvest; information on growing locations not available) were purchased from Plant Breeding International (Cambridge) Ltd., Cambridge, U.K. Grain was milled into "straight-run" flours using a Brabender Quadrumat Junior Mill (Brabender, Duisberg, Germany) fitted with a 200 μm aperture size sieve. Flour extraction rates (100 × weight of flour produced ÷ weight of grain milled) were within a narrow range (64–68%). The moisture contents of these flours were between 10.5% and 11%, and the results are expressed on an "as is" basis. Two other straight-run flours were milled on different Buhler experimental mills (Model MLU 202) from different lots of cv. Mercia grain (both 1990 harvest but from different locations). These flours, termed Mercia A and Mercia B, were used in experiments to determine the optimum level of dithiothreitol (DTT) in the reduction step of the procedure (Mercia A) and in experiments to determine recoveries and to determine the precision of the measurements (Mercia B). The Mercia A and B flours were provided by RHM Technology Ltd,

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High Wycombe, Bucks, U.K., and by Dr. B. A. Brockway, The University of Reading, Department of Food Science and Technology, Reading, U.K., respectively. Extraction rate data were not available, but extraction rates are generally in the range ~70–73% for wheats such as cv. Mercia.

Determination of Protein–Glutathione Mixed Disulfides. The method we have established for analysis of PSSG is based on that of Meredith (1982) with some modifications. Unbound GSH and GSSG were first removed by perchloric acid (PCA) extraction as described previously (Schofield and Chen, 1995). Flour (*ca.* 0.2 g) was weighed accurately into a 10 mL centrifuge tube. To remove oxygen from the flour sample, the tube was placed in a vacuum desiccator, which was then evacuated for 10 min using a water pump. O_2-free N_2 was then allowed into the desiccator. This procedure was repeated three times. The flour sample was then suspended in ice-cold 5% (w/v) PCA (2 mL). Oxygen was removed from the PCA solution prior to extraction by bubbling O_2-free N_2 through the bulk extractant solution using a 1.0 mm i.d. syringe needle at a bubble rate of 5 per second for 30 min. O_2-free N_2 was then passed through the flour suspension in PCA to exclude O_2 from the extraction tube before it was sealed with a plastic stopper. Extraction was carried out at 4 °C for 1 h. The tubes were shaken about 20 times manually every 15 min. After centrifugation at $29000g_{max}$ at 4 °C for 15 min, the supernatant was removed and used for determination of GSH and GSSG. The pellet was washed twice with ice-cold 5% (v/v) PCA, after which it was resuspended in 0.025 M dithiothreitol (DTT)/0.05 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, pH 8.5 (1.0 mL). After incubation for 1 h at 40 °C with gentle magnetic stirring, the solution was cooled to 4 °C in an ice bath and 70% (w/v) PCA (0.05 mL) was added to reprecipitate the protein. After centrifugation at $29000g_{max}$ at 4 °C for 15 min, the supernatant was decanted and an aliquot (1.0 mL) was taken for derivatization of the released glutathione by alkylation with iodoacetic acid (IAA) and dinitrophenylation with 1-fluoro-2,4-dinitrobenzene (FDNB). The derivatization procedure and HPLC separation and quantification were as described previously (Schofield and Chen, 1995).

Flour Protein Fractionation. Flour protein was fractionated according to the Osborne procedure using flour from the cv. Mercia essentially as described by Sarwin et al. (1992). For extraction of the albumin and globulin fractions, flour (*ca.* 20 g, weighed accurately) was extracted sequentially with 0.01 M *N*-ethylmaleimide (NEMI; 200 mL) at 20 °C and with 0.5 M NaCl (2 × 200 mL) and water (1 × 150 mL) at 4 °C. In each case, extraction was carried out for 1 h with magnetic stirring. After centrifugation at $28000g_{max}$ for 30 min, the combined extracts were dialyzed overnight twice against distilled water at 4 °C. The precipitated globulin fraction was recovered by centrifugation at $28000g_{max}$ for 15 min. Both the soluble albumin fraction and the precipitated globulin fraction were frozen and freeze-dried. Gliadin was then extracted from the flour residue, from which the albumin and globulin had been removed, using 70% (v/v) ethanol (2 × 200 mL) at 20 °C for 1 h with magnetic stirring. After centrifugation at $28000g_{max}$ for 30 min, the combined extracts were concentrated by rotary evaporation at 40 °C and then frozen and freeze-dried. The residue, containing mainly starch and glutenin protein, was also frozen and freeze-dried.

Glutenin Macropolymer Preparation. Two glutenin macropolymer (Weegels et al., 1994) or "gel protein" samples from the cultivars Bussard and Glenlea were provided by Dr. A. Graveland, Unilever Research Laboratorium Vlaardingen, The Netherlands. They were prepared by extraction of flour with 1.5% (w/v) sodium dodecyl sulfate (SDS) as described previously (Graveland et al., 1982).

Protein Determination. Protein was determined according to a micro-Kjeldahl procedure (AACC method 46-08, 1984). A Kjeldahl nitrogen to protein conversion factor of 5.7 was used.

Statistical Methods. Statistical analyses were carried out using the Minitab statistical computer software package. For the data for flour samples from the different wheat cultivars, analysis of variance (ANOVA) was used to establish that there was significant variation among the means of duplicate

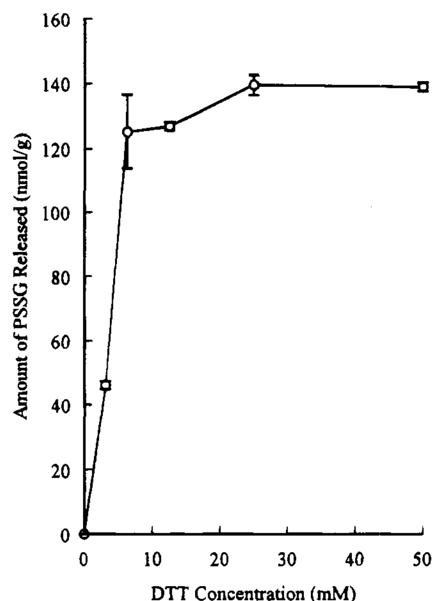

Figure 1. Effect of DTT concentration on the release of glutathione from the flour protein of cv. Mercia flour (Mercia flour sample A). The data points represent the means of duplicate determinations, and the bars indicate the ranges of the duplicate values.

determinations. The differences between individual pairs of means were then investigated using Student's *t* test on each pair. Differences were considered significant at $P < 0.05$. These procedures were also used to obtain estimates for the standard deviation (SD) for a single determination and for the least significant difference (lsd) between the mean values.

RESULTS

Effect of DTT Concentration on the Measured Levels of PSSG. In the procedure that we established recently for the analysis of GSH and GSSG in wheat flour (Schofield and Chen, 1994, 1995), GSH and GSSG are removed from the flour by extraction with ice-cold 5% (w/v) PCA. In the method described here, PSSG was then released from PCA-insoluble protein by reduction with DTT. The released glutathione was then separated from the protein by reprecipitation of the latter with PCA, and the glutathione in the PCA supernatant was analyzed as described before (Schofield and Chen, 1995).

A critical step in this procedure is the release of glutathione from flour PSSG by the reductant DTT. To ensure maximal reduction of PSSG, the effect of DTT concentration was examined. Aliquots of the same cv. Mercia flour (Mercia A) were incubated with increasing concentrations of DTT, and the glutathione released was determined. As shown in Figure 1, the amount of glutathione released was dependent on DTT concentration up to about 6.25 mM. Above this DTT concentration, the amount of glutathione released appeared to reach a plateau. The average amount of glutathione released from this particular cv. Mercia flour sample in this plateau region (6.25–50 mM DTT) was 133 nmol/g of flour (SE = 3.5; SD = 9.5). A similar concentration-dependent release of glutathione from model bovine serum albumin–glutathione mixed disulfides by DTT was reported by Meredith (1982). Although 6.25 mM DTT was enough to release glutathione from flour protein PSSG, a DTT concentration of 0.025 M was used routinely throughout this work to ensure that complete reduction of PSSG occurred with an adequate margin of safety. In the absence of DTT, no glutathione was detected, showing that GSH and

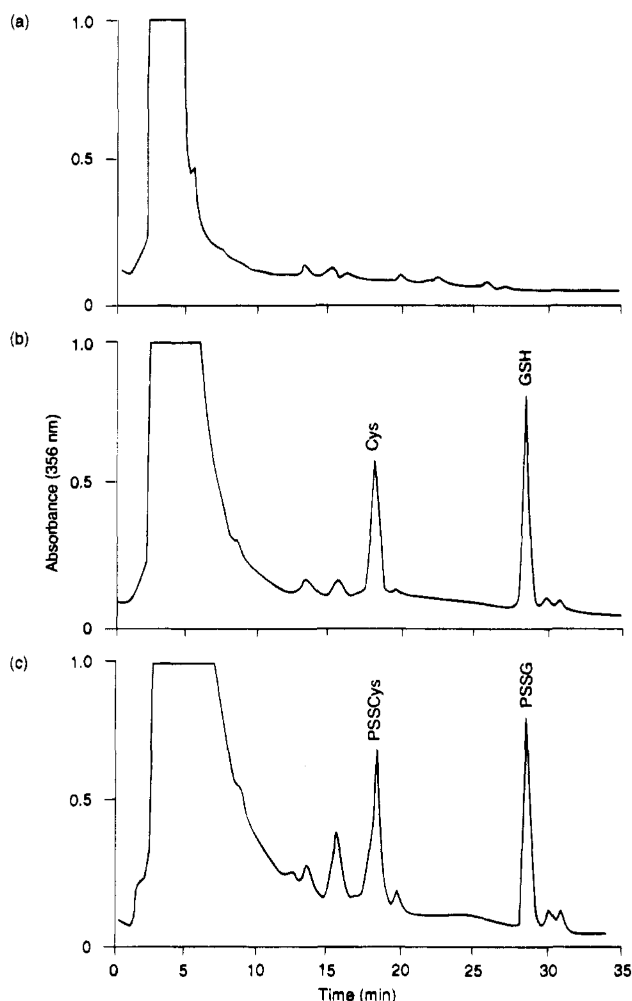

Figure 2. Chromatograms showing the separation by HPLC of (a) compounds extracted from the PCA residues of cv. Mercia flour by extraction with MOPS buffer in the absence of DTT, (b) cysteine and glutathione standards, and (c) compounds released by reduction with DTT from the protein in the residue after PCA extraction of cv. Mercia flour. The flour sample was Mercia A.

GSSG were removed efficiently from flour by the PCA extractant.

HPLC Separation of the Compounds Released by Reduction with DTT. An HPLC chromatogram of GSH (reduced from a GSSG standard) and cysteine (reduced from a cystine standard) is shown in Figure 2, together with a chromatogram obtained for the compounds released by DTT reduction of the cv. Mercia flour (Mercia A sample) protein residue after GSH and GSSG extraction with PCA. The elution time of the released glutathione in these experiments was 28.9 min. Cochromatography of a GSH standard confirmed the identity of glutathione in the flour chromatograms. The elution time for the released glutathione was slightly different from that reported previously (Schofield and Chen, 1995). As noted before, variation in GSH and GSSG elution times occurs from one column to another and with the age of the column.

It is clear from Figure 2 that compounds other than glutathione were present in the chromatograms. Since they were also released by reduction with DTT and were labeled with FDNB, they were presumably sulfhydryl peptides and/or sulfhydryl amino acids also present in the form of mixed disulfides with the flour protein. The elution time of one of these peaks corresponded to that of cysteine. The other peaks were not characterized in this work.

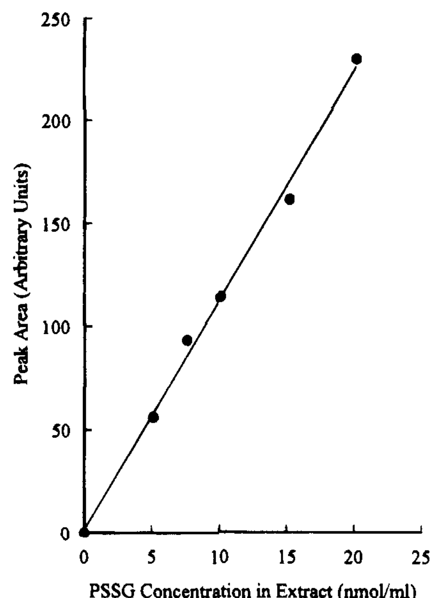

Figure 3. Calibration curve showing the linearity of the detector response for reduced glutathione released from an oxidized glutathione standard during the DTT reduction step. The data points represent the means of duplicate determinations. In all cases the duplicate values were within 1.6% of the mean value or less.

Calibration Curve. Protein standards with known protein–glutathione mixed disulfide contents were not available. To construct a calibration curve, a GSSG standard was reduced with DTT under the conditions used for the PSSG determination, and the peak area for the resultant GSH was measured. The results (Figure 3) showed that the relationship was linear up to at least 40 nmol of released GSH/mL of the reaction solution after addition of PCA. This concentration range was chosen since our experiments showed that the glutathione concentrations of extracts of flour samples containing released PSSG fell within this range (see below). The correlation coefficient of the regression line was 0.99.

Recovery of Glutathione. Since protein standards of known PSSG content were unavailable, recoveries of glutathione were determined by addition of GSSG standards to a cv. Mercia flour sample (Mercia B) during the DTT reduction step. GSSG standard solutions (5.1, 10.1, and 20.2 nmol/mL) were prepared in 0.05 M MOPS buffer, pH 8.5, and added to the pellet from 5% PCA extraction of flour. DTT was also included to a final concentration of 0.025 M. After incubation for 1 h at 40 °C with gentle magnetic stirring, the solution was cooled to 4 °C in an ice bath and 70% PCA (0.05 mL) was added to reprecipitate the protein. An aliquot (1 mL) of the supernatant after centrifugation was taken for derivatization of the released glutathione as described above.

Recoveries of GSH (derived by reduction of GSSG) in these experiments were very similar at each level of GSSG included at the reduction step, averaging 93% (Table 1). Although recovery experiments were not conducted here with flour samples that gave high and low values for PSSG contents in the analysis of flours from different cultivars (see below), previous experiments (Schofield and Chen, 1995) showed that recoveries of GSH and GSSG did not vary among high- and

Table 1. Recoveries of Free Oxidized Glutathione (GSSG) as Free Reduced Glutathione (GSH) When Different Amounts of Free Oxidized Glutathione Were Included at the Reduction Step during the Analysis of a Sample of Cv. Mercia Flour (Mercia Flour Sample B)

amt of GSSG standard added[a] (nmol/g of flour)	amt of GSH recovered (nmol/g of flour)	recovery (%)
202	182	91
101	94	93
51	48	94
av recovery		93

[a] GSSG values given as GSH equivalents.

low-value samples. In those experiments, recovery values were high for both GSH and GSSG (98% and 86%, respectively). These results suggest that there are unlikely to be any differences in the efficiency of extraction of GSH derived by reduction of PSSG among flour samples from different cultivars, *i.e.* no differences in "matrix effects" among flour samples from different cultivars. The different PSSG values reported below for different flour samples are unlikely, therefore, to be due to different matrix effects that affect the recoveries of PSSG.

Precision of Protein–Glutathione Mixed Disulfide Determinations. The precision of the PSSG determinations was determined by carrying out five replicate analyses on one sample of a cv. Mercia flour (Mercia B). The PSSG analysis showed very good precision, the individual values for this particular cv. Mercia flour sample being 73.7, 74.2, 79.7, 76.1, and 78.3 nmol of PSSG/g of flour, with a mean of 76.4 (SE = 1.2; SD = 2.6; coefficient of variation = 3.4%).

PSSG Levels in Flours Milled from Different Wheat Cultivars. Once established and optimized, the analytical procedure was applied to determine the PSSG contents of straight-run Brabender Quadrumat Junior Milled white flours from a set of single samples of grain of U.K.-grown wheat cultivars. These cultivars represented a range of baking qualities, and the GSH and GSSG contents of flours milled from those samples had been determined (Schofield and Chen, 1994, 1995). The flours used in these experiments were analyzed immediately after milling. The results, which are presented in Table 2, are the means of duplicate determinations and are expressed on the basis of nanomoles per gram of flour ("as is" moisture basis and uncorrected for recoveries). For purposes of comparison, the GSH and GSSG values determined previously (Schofield and Chen, 1995) are also shown. As was found for GSH and GSSG values (Schofield and Chen, 1995), examination of the duplicate values for PSSG contents indicated no dependence of the standard deviation (SD) on the magnitude of the mean values, and ANOVA gave an estimated SD of single determinations of 3.1. The least significant difference ($P < 0.05$) for PSSG was estimated to be 6.6.

Considerable variation was noted in the PSSG contents, with values ranging from 70 to 150 nmol/g of flour for the 11 cultivars examined. The PSSG values were generally of an order of magnitude similar to that for total free glutathione (GSH plus GSSG). The cv. Mercia flour sample (termed Mercia C), the results for which are presented in Table 2, was milled from a different grain sample from those used in the optimization, recovery, and precision experiments described above. The value obtained for the Mercia C sample was different from those obtained for the Mercia A and B samples. The reasons for these differences are not known, but they may indicate that intracultivar variation in glutathione levels exists, *i.e.* between different grain samples of the same cultivar, as well as intercultivar variation.

The bread-making classifications for the cultivars analyzed here are also given in Table 2. These classifications are derived on the basis of tests on multiple grain samples produced in official National List Trials [National Institute of Agricultural Botany (NIAB), Cambridge, U.K.] over several seasons. The classification gives an indication of the bread-making quality to be expected from a given cultivar. Baking tests were not carried out on the particular flour samples analyzed here. Flours from cultivars classified as being of good bread-making quality (such cultivars are given bread-making classifications of A or B in this classification system) generally had higher PSSG contents than those from cultivars classified as being of poorer bread-making quality (bread-making classifications of C and D). The cultivar Fresco is grouped with the wheats with good bread-making classifications in Table 2 because its classification of C is due to its having very strong gluten characteristics rather than weak gluten characteristics as for most of the poorer wheats in Table 2. The total amount of glutathione in disulfide-bonded form (PSSG plus GSSG) likewise tended to be higher for the better bread-making cultivars, but this was mainly a reflection of their higher PSSG values.

The poor bread-making cultivar Riband was a clear exception, however, the PSSG value for this cultivar being the same as that for the very strong gluten cultivar Fresco. Doughs from cv. Riband, in fact, have rheological properties that are intermediate between those of other poor bread-making wheats and those of good bread-making wheats. The poor bread-making characteristics of this cultivar may be related, at least in part, to factors other than the quality of its gluten (G. D. Oliver and C. J. Brock, Campden-Chorleywood Food Research Association, U.K., unpublished results).

The ratios of total oxidized glutathione (PSSG plus GSSG) to GSH for better and poorer bread-making flours covered similar ranges of values. Although no clear-cut relationship emerged between bread-making quality and either the GSH, GSSG, or PSSG contents, the relationship between the PSSG contents of flours and their bread-making performances merits further examination.

PSSG Levels in Osborne Flour Protein Fractions. Considerable differences were observed between the PSSG contents of the different protein fractions extracted from cv. Mercia flour (Mercia B) by the Osborne fractionation procedure (Table 3). PSSG values (expressed on a nanomoles per gram of protein basis) were highest in the residue (comprising mainly glutenin protein) and albumin fractions (897 and 796 nmol/g of protein, respectively) and lowest in the gliadin fraction (76 nmol/g of protein). The globulin fraction had an intermediate value (239 nmol/g of protein).

These results, in particular those for the globulin and gliadin fractions, are similar to those reported by Sarwin et al. (1992) for protein fractions. They obtained rather lower proportions of the total flour protein in their albumin and residue fractions, however (11% and 36%, respectively), than observed here (22% and 49%, respectively). Determination of the PSSG value for their albumin fraction was confounded by the presence of GSH and presumably GSSG. From the results pre-

Table 2. Free Reduced Glutathione (GSH), Free Oxidized Glutathione (GSSG), and Protein–Glutathione Mixed Disulfide Contents of Freshly Milled White Flours from U.K.-Grown Wheats[a]

cv.	PSSG[b]	GSH[c]	GSSG[c]	total free glutathione[d]	total flour glutathione[d]	moisture content	bread-making classification[e]
Fresco[f]	131a	31	24	79	210	10.7	C
Hereward	150b	62	22	106	256	10.8	B
Mercia C	102de	74	27	128	230	10.7	B
Pastiche	100e	45	18	81	181	10.6	B
Avalon	108d	75	13	101	209	10.8	B
Norman	73f	74	15	104	177	10.9	C
Galahad	70f	81	17	115	185	10.6	D
Tara	73f	47	12	71	144	10.7	D
Riband	131a	64	19	102	233	10.8	D
Beaver	72f	56	19	94	166	11.0	D
Haven	89g	18	20	58	147	10.8	D
SD[g]	3.1	1.7	1.0				
lsd[h]	6.6	3.6	2.1				

[a] Values were rounded to whole integers and are in nmol/g of flour "as is" basis. Results are uncorrected for recoveries. [b] Values within columns that are followed by the same letter are not significantly different ($P < 0.05$). [c] GSH and GSSG data are from Schofield and Chen (1995); they are included to facilitate comparison with PSSG data. [d] Total free glutathione (GSH + GSSG) and total flour glutathione (GSH + GSSG + PSSG) are given as GSH equivalents. [e] A and B, bread wheats; C and D, biscuit/feed wheats. The best bread wheats are given a classification of A and the poorest D. [f] Fresco is a cultivar with very strong gluten characteristics. [g] SD, estimated standard deviation of a single determination. [h] LSD, least significant difference between the two means of two determinations.

Table 3. Protein–Glutathione Mixed Disulfide (PSSG) Contents of Protein Fractions from Cv. Mercia Flour (Mercia Flour Sample B) Obtained by a Modified Osborne Fractionation Procedure

		PSSG content		
protein fraction	protein found in fraction (mg/g of flour)	protein basis (nmol/g of protein)	flour basis (nmol/g of flour)	proportion of total flour PSSG (%)
albumin	22.4	796	17.9	27
globulin	4.6	239	1.1	2
gliadin	25.5	76	2.0	3
residue	50.2	897	45.1	68

sented, however, the albumin PSSG value was evidently lower than the value obtained in the present research, but the PSSG value obtained by Sarwin et al. (1992) for the residue fraction was rather higher than that obtained here (1441 nmol/g of protein compared with 897 nmol/g of protein).

When the results were expressed on the basis of flour weight (Table 3), it was clear that the residue fraction contained by far the greatest amount of PSSG (45.1 nmol/g of flour), accounting for 68% of the total flour PSSG. This reflected the much higher proportion of the total flour protein found in this fraction than in the other protein fractions. The albumin fraction contained a smaller, although still substantial, amount of PSSG (17.9 nmol/g of flour), accounting for 27% of the total flour PSSG. The amounts present in the globulin and gliadin fractions were very small (1.1 and 2.0 nmol/g of flour, respectively), however, accounting for only 2% and 3%, respectively, of the total flour PSSG. Therefore, the bulk of the PSSG in flour was present in the residue fraction comprising mainly glutenin. These findings are similar to those reported by Sarwin et al. (1992, 1993).

PSSG Levels in Glutenin Macropolymer Preparations. Two SDS-unextractable glutenin macropolymer (gel protein) preparations were also examined. The PSSG value for the glutenin macropolymer from cv. Glenlea, a very strong gluten Canadian cultivar, was 1062 nmol/g of protein, whereas that from cv. Bussard, a weak gluten French cultivar, was 1538 nmol/g of protein. Control experiments, in which DTT was omitted at the reduction stage of the procedure, showed that GSH and GSSG were absent from these glutenin macropolymer preparations. This information is too limited to draw any conclusions about relationships between bread-making quality and PSSG contents of glutenin preparations. The experiments do demonstrate once again, however, the utility of the methodology established here for determining the PSSG contents of glutenin preparations.

DISCUSSION

This is the first time that a method has been established for the analysis of PSSG in wheat flour. A procedure was described recently for measuring GSH and total glutathione (GSH plus GSSG plus PSSG) in wheat flour (Sarwin et al., 1992, 1993). That procedure cannot be used to measure PSSG specifically except in isolated protein fractions. Neither can it be used to measure GSSG specifically. Our method is also much less complex and requires, in addition to the simple derivatization steps, only one HPLC chromatographic step. In contrast, the methodology of Sarwin et al. (1992, 1993), while not only giving less information, requires, in addition to the derivatization step, one conventional gel filtration step, two HPLC steps, and a number of intermediate freeze-drying steps. It also necessitates access to radiochemical handling and liquid scintillation counting facilities. For these reasons, we believe that our procedure has significant advantages for studies of glutathione and its reactions in flour and dough systems.

The method we have established exploits a number of advantages conferred by using PCA as the extractant. PCA is a strong chaotrope (Harris and Angal, 1990). It therefore provides efficient extraction of unbound GSH and GSSG, as shown by the fact that GSH and GSSG were not detected when DTT was omitted during the first step of the PSSG analysis procedure (Figure 2). The pH during PCA extraction is also low (pH <1.0), which prevents dissociation of SH groups, thus preventing SH/SS interchange reactions from occurring during the extraction procedure. Finally, PCA is an efficient protein precipitant. This results in an effective separation of GSH and GSSG (which are extracted with PCA) from PSSG (which are present in the PCA-unextractable protein fraction), thus facilitating measurement of glutathione in all three pools.

Although glutathione was the major component present in the chromatograms of the components released by reduction with DTT, substantial amounts of

other components were also found to be present (Figure 2). Since these components contained one or more amino groups that could be blocked with FDNB, they are presumably cysteine-containing peptides and cysteine itself, which are also present as protein mixed disulfides. One of these components did, in fact, cochromatograph with cysteine; the others have not yet been identified.

Ewart (1985) also detected glutathione and cysteine in the form of mixed disulfides in glutenin. He referred to glutathione and cysteine in such a form as "end blockers" to indicate their putative role in blocking sites for further polymerization of glutenin through disulfide bond formation, which, in turn, might affect glutenin functional properties. Ewart's methodology could not differentiate quantitatively PSSG from other cysteine-containing protein mixed disulfides, although a number of cysteine-containing peptides are known to occur in flour (Jones and Carnegie, 1969a).

The present results clearly raise questions as to the possible importance of these other protein–peptide/amino acid mixed disulfides in relation to bread-making quality. Although Ewart (1985) observed an inverse relationship between the level of total end blockers in glutenin and bread-making quality, further research is needed to determine quality relationships of PSSG in comparison with other protein mixed disulfides. It is interesting to note in this context, however, that, of a number of disulfide peptides present in flour, only GSSG was effective in affecting the rheology of doughs to which it was added (Jones and Carnegie, 1969b). This implies some specificity in the reactivity of GSSG with flour proteins during dough mixing. We have found that the reaction of GSSG with flour proteins is one of the major reactions involving glutathione during dough mixing (X. Chen and J. D. Schofield, unpublished results; see below).

Considerable variation in PSSG values was observed for white flours milled from U.K.-grown wheat cultivars (Table 2). The source of this variation has not been explored in detail. Flours from cultivars with better bread-making quality tended to have higher PSSG contents, but the results are too preliminary at this stage to allow definite conclusions to be drawn about relationships of PSSG with bread-making quality. Only a limited set of cultivars was examined, and, furthermore, the bread-making performances of the grain lots analyzed here were not determined directly in this work.

The PSSG analyses carried out here with different cultivars were all performed with freshly milled flours. We have observed that both the GSH and GSSG contents of flours fall during flour storage (Schofield and Chen, 1995). Similarly, the PSSG contents of flours also fall during flour storage (X. Chen and J. D. Schofield, unpublished results). As with GSH and GSSG analysis, therefore, it is important to standardize the times between milling grain and analysis of the flour to obtain meaningful comparative results.

The highest PSSG content in the different flour protein fractions obtained by a modified Osborne fractionation procedure was observed for the residue fraction containing mainly glutenin. PSSG were also confirmed as being present in glutenin, the major protein component of the Osborne residue fraction, through analysis of two samples of SDS-unextractable glutenin macropolymer (gel protein) isolated from the cultivars Glenlea and Bussard. This is of interest and potential importance since variation in bread-making quality among wheat cultivars is known to be due to differences in the properties of this fraction [for a review see Schofield (1994)]. In contrast, much lower values were observed for the gliadin fraction. It is well-known that the Osborne fractionation procedure does not achieve a complete separation of the different protein classes in wheat flour and, in particular, that 70% (v/v) ethanol is capable of extracting from wheat flour substantial amounts of small glutenin polymers enriched in low M_r subunits of glutenin (Payne and Corfield, 1979; Bietz and Wall, 1980; Bottomley et al., 1982). Therefore, even the low gliadin PSSG content observed here could be an overestimate if, as seems likely, some of the PSSG detected in the gliadin fraction is associated with small glutenin polymers.

Assuming an average glutenin subunit M_r of 50 000 (Ewart, 1985), the glutenin macropolymer and Osborne glutenin PSSG values can be used to calculate the average frequency of occurrence of PSSG on glutenin polymers. The calculated values ranged from 1 PSSG in 13 glutenin subunits in the cv. Glenlea glutenin macropolymer to 1 PSSG in 22 glutenin subunits for cv. Mercia Osborne glutenin.

Our methodology for measuring PSSG, as well as that for measuring GSH and GSSG, has also been applied to determine the dynamics of glutathione in these three pools during dough mixing and to study the effects of oxidizing improvers (X. Chen and J. D. Schofield, unpublished results). That work has confirmed a rapid loss of GSH and an increase in GSSG levels during the initial stages of dough mixing, and the accentuation of these changes by oxidizing improvers, as observed also by Sarwin et al. (1993). However, we have also observed a substantial increase in PSSG levels during the early stages of dough mixing, which was also accentuated by inclusion of oxidants. These observations suggest that the importance of the formation of PSSG during dough mixing may have been overlooked.

The establishment of the present procedure for measuring GSH, GSSG, and PSSG individually should allow more definitive and precise information to be obtained on the fate of glutathione during flour processing and should afford a better understanding of the significance of glutathione in flour/baking technology.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; GSH, free reduced glutathione; GSSG, free oxidized glutathione; PSSG, protein–glutathione mixed disulfides; SH, sulfhydryl; SS, disulfide; PCA, perchloric acid; IAA, iodoacetic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; NEMI, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; cv., cultivar; ANOVA, analysis of variance; M_r, relative molecular mass.

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