Mixing Properties as a Measure of Reversible Reduction and Oxidation of Doughs

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ABSTRACT

The effects of reductants and oxidants on the functionality of gluten proteins during dough mixing were studied for seven mixing parameters using a prototype 2-g mixograph. Structural changes in the dough proteins were monitored by size-exclusion high-performance liquid chromatography and by multistacking sodium dodecyl sulfate polyacrylamide gel electrophoresis. Using dithiothreitol as the reducing agent, experimental conditions were established that resulted in partial reduction of the glutenin polymers and radical changes in mixing parameters. Studies of the effects of iodate, bromate, permanganate, and hydrogen peroxide on reduced doughs showed that careful selection of oxidant concentration and oxidation conditions allowed an essentially complete recovery of the original dough mixing properties. Size-exclusion high-performance liquid chromatography and electrophoresis studies showed that samples reoxidized under optimum conditions had protein size distributions almost identical to those of control samples. This test system provides a basis for directly evaluating hypotheses on the functional roles of specific glutenin subunits by incorporating them into reduced doughs by oxidation.

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The mixing characteristics of flours and the rheological properties of the resulting doughs are mainly determined by the proteins of the flours. In particular, the glutenin fractions of the doughs may play a major role in determining the functional properties of the doughs. It is now generally accepted that these glutenin fractions are built up from smaller subunit proteins, covalently linked through disulfide bonds into hydrophobically stabilized polymers of very high molecular weight. The molecular weight range of these subunits is 30,000–100,000. There is strong evidence that the specific subunit composition plays an important role in determining the rheological properties of doughs (Payne 1987).

There is also a well-documented relation between the functional properties of doughs (mixing time and tolerance to overmixing) and the average molecular weight of the gluten protein (MacRitchie 1992). The effects of reducing agents on dough properties can be attributed to the rupture of disulfide bonds within the dough structure. Thus, a reduced average molecular weight accompanies a reduction of the mixing requirement and a decreased tolerance to overmixing. The effects of oxidizing agents presumably are the result of the cross-linking of sulfhydryl groups into disulfide linkages, which increases the average molecular weights of the proteins in the dough.

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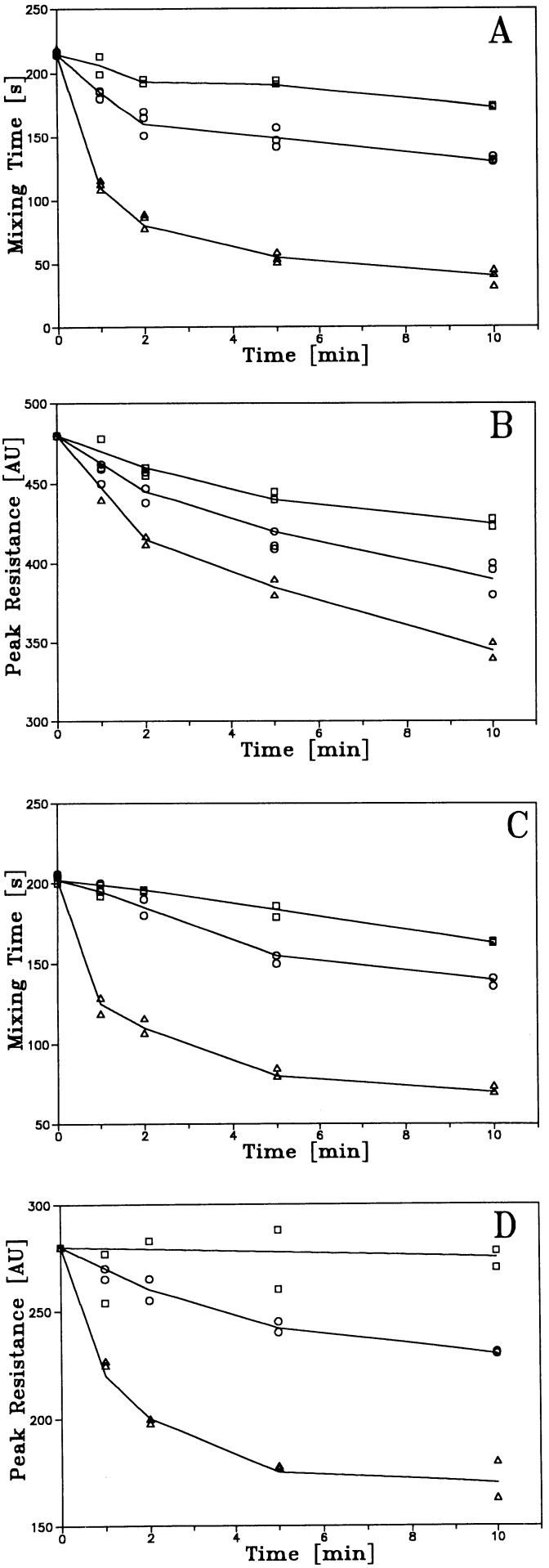
Reconstitution studies, which might give more direct evidence on the effects of individual proteins on the functional properties of doughs, are hindered by the difficulty of isolating undenatured gene-products. By isolating the genes for the desired proteins and inserting them into foreign genomes such as *Escherichia coli*, yeasts, or insect cell lines, it may be possible to have the gene-product expressed in a system where separation is much simplified (Shewry et al 1992).

Direct addition of expressed proteins to a base flour could allow measurement of the roles of individual protein components in the functional behavior of dough. The effects of simple addition of monomeric glutenin subunits to a base flour would not indicate the real effect of the subunits on dough properties because the subunits would not form part of the glutenin structure. Instead, the expressed protein would have to be chemically incorporated into the glutenin network of the base flour. The molecular structure of glutenin proteins implies that the effects of any particular subunit on dough properties are not exerted by the monomeric subunit, but rather as a contribution to the structure of the glutenin polymer. Meaningful estimates of the effects of added glutenin subunits on dough properties could be made only if they could be chemically incorporated into the glutenin polymer. Techniques that allow the partial reduction and reoxidation of glutenin without significant changes to its functionality are a prerequisite for such studies.

By careful manipulation of doughs with reducing agents, the average molecular weight of the gluten proteins can be decreased by disruption of intermolecular disulfide bonds. There is also



evidence that oxidation of reduced doughs can at least partially restore the mixing properties of the dough (Bushuk 1961, Belderok 1967). Therefore, it should be possible to incorporate glutenin subunits into the dough structure by partial reduction of a mixture of a specific subunit and a base flour, followed by reoxidation. In practice, such a procedure would only be of value if the reduction-oxidation steps were effectively reversible when applied to the base flour alone. That is, doughs manipulated by the chosen process should have effectively the same mixing properties as doughs that had been mixed using the same regimen but without the addition of reducing or oxidizing agents.

The production of highly purified glutenin subunits, either by expression or by conventional purification procedures, is lengthy and expensive. To date, mixing studies of such proteins have been impractical because of the large amounts of protein required by conventional mixing tests. The development of the 2-g mixograph, and the associated automated interpretation of the resulting mixograms, provides results for mixing parameters that are effectively identical to those obtained from larger instruments, while using much smaller flour samples (Gras et al 1990, Rath et al 1990). This has allowed mixing studies to be applied in situations where samples sizes are limited, such as measurement of the heritability of mixing properties of flours from early generation breeding lines (Gras and O'Brien 1992) or determination of the effects of adding very small amounts of isolated flour fractions (Bekes and Gras 1992). The 2-g mixograph, therefore, provides a practical method for determining the contributions of particular glutenin subunits to mixing behavior on a scale never before possible.

This article describes an investigation of the extent to which the partial reduction of dough can be reversed by various oxidants as measured by the mixing properties of doughs, by size-exclusion high-performance liquid chromatography (SE-HPLC), and by multistacking sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Medium-protein (MPF) and high-protein (HPF) commercial bakers' flours (10.3 and 13.0% protein [N × 5.7], respectively), used as base flours in this study, were obtained from George Weston Foods Ltd. (St. Leonards, NSW). Mixing tests were conducted with a prototype 2-g mixograph (National Manufacturing Division, TMCO, Lincoln, NE), using a modification of the standard method for 35 g of flour scaled down to the 2-g size (AACC 1983). All mixing tests were performed in triplicate. The coefficients of variation varied from parameter to parameter, but they were typically about 3%. The mixing properties of reduced doughs were determined using a mixture of 2 g of base flour, 1.10 ml of water, and 0.10 ml of water containing 0 (control), 5, 10, 25, 50, 75, 100, 125, 150, or 200 μg/ml of dithiothreitol (DTT) or cysteine solution in the bowl of the 2-g mixograph. The concoction was mixed for 30 (or 60) sec; allowed to react without mixing for 2, 4, 6, 8, or 10 min; and then mixed for a further 10 min as in a conventional mixograph.

The mixing properties of reduced-oxidized doughs were determined using reduced doughs prepared from 2 g of flour, 1 ml of water, and 0.10 ml of water containing DTT solution (50 μg/ml). The concoction was mixed for 30 sec and allowed to react for 4 min. The reduced doughs were then treated with 0.10 ml of oxidant solution containing either 0, 50, 100, 150, or 200 μg/ml of potassium iodate solution or the stoichiometric equivalents of potassium permanganate, potassium bromate, or hydrogen peroxide. Mixing was continued for 30 sec; the dough was allowed to react for 1, 2, 5, or 10 min; then the dough was mixed for

Fig. 1. The effects of reducing conditions (dithiothreitol [DTT] concentration and reaction time) on mixing times (**A** and **C**) and peak resistance (**B** and **D**) for medium-protein (**A** and **B**) and high-protein (**B** and **D**) commercial bakers' flours. □ = Control experiment (no DTT); ○ = 0.10 ml of water containing DTT solution (50 μg/ml); △ = 0.10 ml of water containing 100 μg of DTT solution.

a further 10 min as in a conventional mixograph determination.

For each reductant-oxidant combination described above, dough samples were taken for characterization by SE-HPLC and multistacking SDS-PAGE. Samples were frozen in liquid nitrogen and held at −80°C until characterization. Dough proteins were extracted by SDS-sonication (Singh et al 1990). The molecular weight distributions of the extracted proteins were determined by SE-HPLC (Batey et al 1991) and by multistacking SDS-PAGE (Khan and Huckle 1992).

Mixing parameters were determined using a modification of an existing computer program (Gras et al 1990). This modification automatically excised portions of the recording when mixing was halted. Parameters determined were: the time to peak dough resistance (mixing time), peak dough resistance, bandwidth at peak dough resistance, breakdown in resistance, breakdown in bandwidth, time to maximum bandwidth, and maximum bandwidth. All mixing parameters were calculated using the moving mean of the recorded data (center line of recorded data). Breakdowns were calculated as the change in the value of the resistance or bandwidth at peak resistance and at peak resistance plus 3 min. Breakdowns were expressed as a percentage of the value at peak resistance. The extent of restoration of dough properties of any particular mixing parameter was defined as a percentage of the difference between untreated and reduced doughs:

$$R = 100 \times [(x - r)/(c - r)] \quad (1)$$

where: R is the percentage of restoration of the dough property; x is the value of the measured mixing parameter; r is the value for the measured mixing parameter after reduction; and c is the value for the measured mixing parameter with no reduction or oxidation.

The overall restoration of the mixing properties ($\bar{R}$) was expressed as the mean of the values for each mixing parameter for each combination of oxidant and its concentration.

RESULTS AND DISCUSSION

Partial Reduction of Doughs

The rates of reaction of reductants such as DTT and cysteine with doughs are such that the reaction is only close to completion in times comparable to normal mixing times as determined on the mixograph. For meaningful measures of the effect of reducing agents on the mixing behavior of doughs, the reducing agent must be quickly dispersed into a dough and then allowed to react with the protein before measurement of the mixing parameters can be made. The dispersion step should be as short as possible but sufficient to allow intimate contact between the heterogeneous reaction components. Preliminary experiments with aqueous dye solutions had shown that mixing for 30 sec was sufficient for effective dispersion of the reductant.

Mixing parameters from a trial where the dough is mixed for 30 sec and allowed to react with reductant for a variable reaction period have to be compared with experiments where the preliminary mix, water addition, and reaction times exactly duplicate the conditions of reduction experiments, but without the reducing agent. Measurement of the mixing properties of control samples where the reaction time was varied showed small but progressive changes in each of the measured parameters for each of the base flours as the reaction times increased from 0 to 10 min (Fig. 1A–D, upper lines). These changes were observed previously and attributed to continuing enzyme activity in the dough (Tipples and Kilborn 1977).

The mixing properties of reduced doughs showed dramatic changes. For additions of 5 and 10 μg of DTT solution (0.65 and 1.3 μequiv, respectively), the changes in dough mixing time and peak resistance were well advanced after 2 min of reaction time. Despite the significant changes observed, the flour-water-reductant mixtures still behaved as doughs, albeit very weak doughs. This is consistent with the previous observations that these levels of DTT cause only a partial reduction of the glutenin structure (Ng et al 1991, Werner et al 1992). After 4 min of reaction time, changes in most of the mixing parameters were no greater than the changes in the same parameters obtained from the control samples (Fig. 1A,C,D). Peak dough resistance was the only exception to this generalization; in one of the base flours, changes

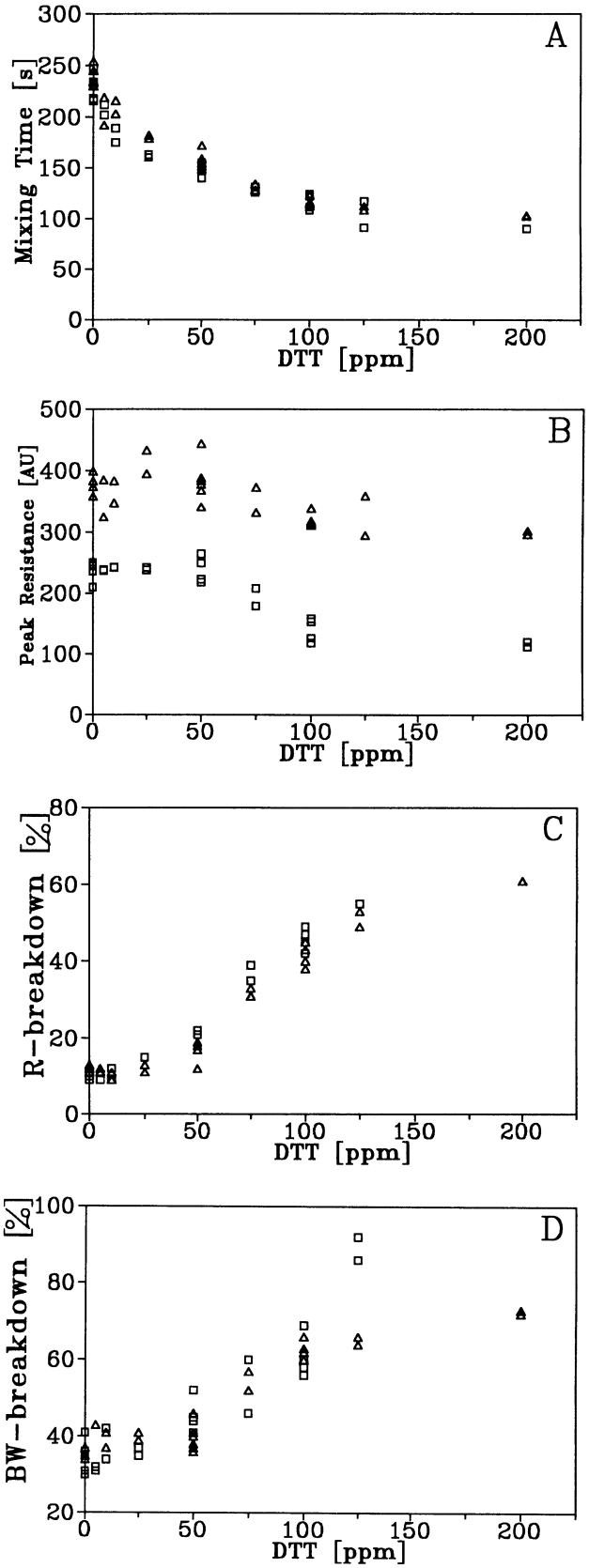


Fig. 2. Relationships of dithiothreitol (DTT) concentration with: mixing time (**A**), peak resistance (**B**), resistance (R) breakdown (**C**), and bandwidth (BW) breakdown (**D**) of high-protein (△) and medium-protein (□) commercial bakers' flours. Reaction time = 4 min.

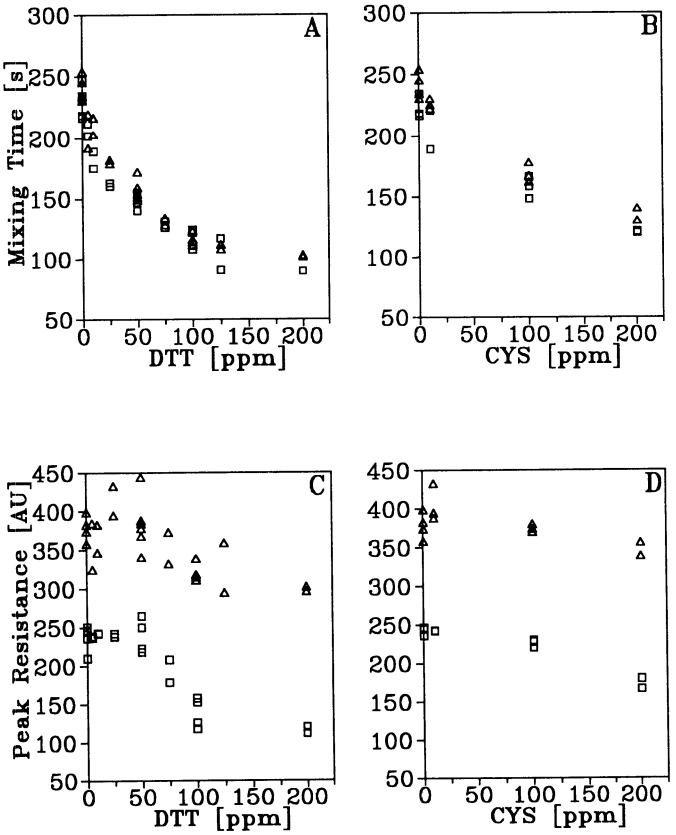


Fig. 3. Comparison of the effects of dithiothreitol (DTT) (**A** and **C**) and the effects of cysteine (**B** and **D**) on the mixing time (**A** and **B**) and peak resistance (**C** and **D**) of high-protein (△) and medium-protein (□) commercial bakers' flours.

were still continuing after 4 min (Fig. 1B).

The changes in mixing properties were dependent upon the amount of reductant, as expected (Fig. 2A–D). Higher amounts of DTT (>0.65 μequiv) did not have much further impact on the already measured, poor dough mixing characteristics. The resemblance of the mixture to dough decreased.

Changes in mixing parameters also depended on the reducing agent. Thus, chemically equivalent amounts of cysteine and DTT had qualitatively similar effects on mixing parameters, although the effects were quantitatively different (Fig. 3). The differences resulted from different rates of reaction. Cysteine is slower acting than DTT (data not shown). These observations, together with the rapid cessation of change in mixing properties of DTT mixtures, imply virtual completion of the reduction reaction within 4 min, as previously reported (Jones et al 1972).

The aim of this experiment was to produce a small but reversible reduction of the dough without total destruction of its structure. Therefore, a reaction time of 4 min with 0.10 ml of DTT solution (50 μg/ml, 0.65 μequiv) as the reductant was chosen as the most practical method of partial reduction. This choice allowed significant change in dough properties while retaining some doughlike properties. It also minimized the contribution of the slow changes that occur without the addition of reductant and maximized the effect of reduction within a reasonable time.

Multistacking SDS-PAGE and SE-HPLC confirmed that these conditions provided only a partial reduction, as desired (Fig. 4A and B). A comparison of the multistacking SDS-PAGE patterns from untreated and reduced doughs (Fig. 4Aa and Ab) showed that reduced doughs had decreased amounts of the highest molecular weight material.

Oxidation of Reduced Doughs

Oxidation studies require that an oxidant be adequately dispersed into the reduced dough. As with reducing agents, the reactions take a finite time and must be allowed to proceed close to completion before mixing to obtain useful results. Preliminary

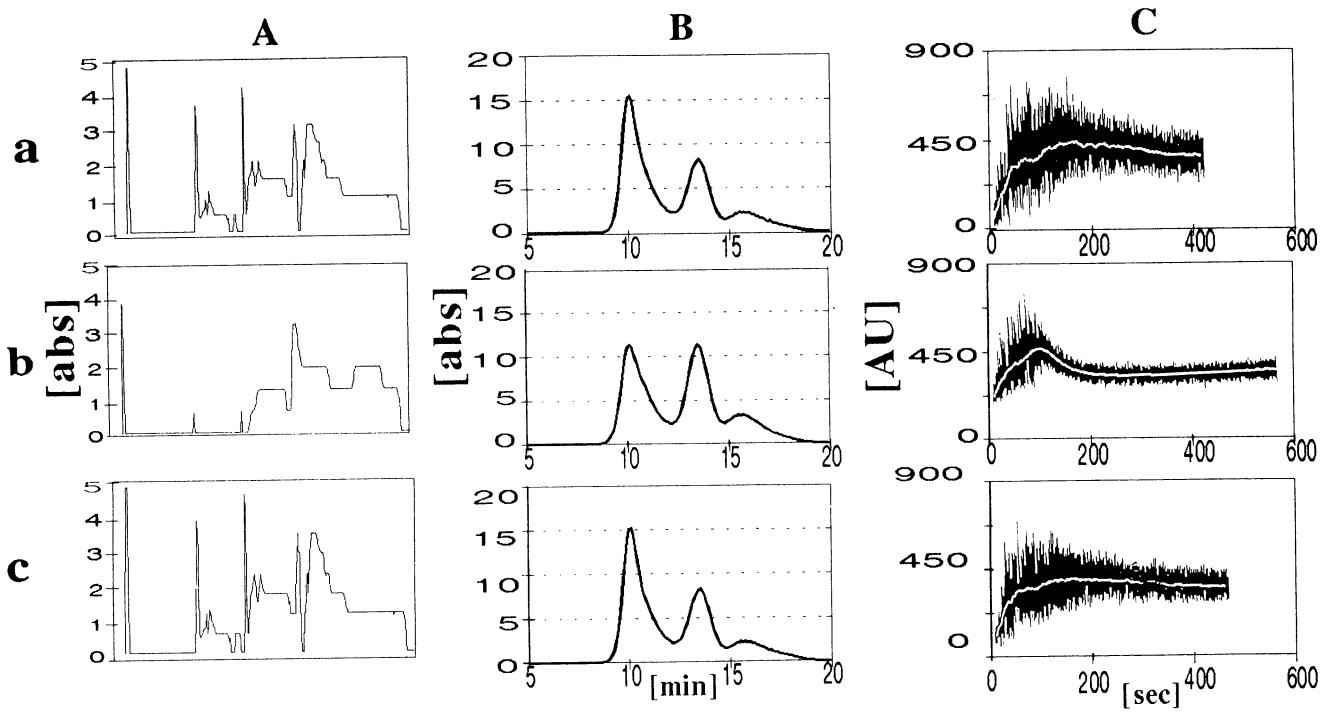


Fig. 4. The effects of partial reduction and subsequent oxidation on the molecular weight distribution of proteins extracted from doughs by sodium dodecyl sulfate sonication. Densitograms of the glutenin regions of multistacking sodium dodecyl sulfate polyacrylamide gel electrophoresis (**A**); size-exclusion high-performance liquid chromatography (**B**); and the mixing curves (**C**). **a,** Control—untreated high-protein flour; **b,** partially reduced dough from high-protein flour using 0.5 μg of dithiothreitol, and **c,** reduced and oxidized dough using 0.5 μg of dithiothreitol and 0.54 μequiv potassium iodate.

experiments with aqueous dye solutions showed that the addition of a reagent in 0.10 ml of water to the reduced dough, followed by mixing for 30 sec, was sufficient for effective dispersion. As with the reduction experiments, control samples must be prepared exactly as the test samples were. For oxidation experiments, this dictated: 1) the use of 1 ml of water, 0.10 ml of reductant solution, and 2 g of flour to prepare the reduced dough; 2) 4 min of reduction reaction time; 3) the addition of 0.10 ml of oxidant solution; and 4) 30 sec of mixing to disperse the oxidant. Smaller volumes would have been more desirable, but they were too small to accommodate the desired levels of some oxidants.

Four oxidants (potassium bromate, potassium iodate, potassium permanganate, and hydrogen peroxide) were tested for their ability to reconstitute the original dough using a range of concentrations and reaction times.

Each oxidant caused changes in the mixing parameters that were consistent with the reversal of the changes induced by reduction. However, the rate and extent of the reversal of reduction or restoration of dough properties were dependent on both the reaction time (Fig. 5) and the quantity of oxidant. At chemically equivalent quantities, it was apparent that potassium permanganate and potassium iodate were significantly more effective than potassium bromate or hydrogen peroxide (Fig. 5A–D). As with the reduction step, this appeared to be a kinetic effect. A reaction time of 5 min for the oxidation step was selected as a practical compromise between allowing the oxidation to proceed to completion and performing the mixing step within a reasonable time.

Using the selected conditions for oxidation of the reduced doughs, some restoration of dough properties was observed for every mixing parameter. For example, adding 20 μg of potassium iodate (0.71 μequiv) effectively restored the mixing time to that observed when the dough was mixed with water only (Fig. 6). Peak resistance was slightly less than that of the control sample. The breakdowns in resistance and bandwidth over the 3 min after peak dough development were slightly higher than those in the control. Despite these small changes, the overall behavior of the oxidized dough was remarkably similar to that of the control dough. Similar changes were observed in all the other measured mixing parameters, although the extent of recovery varied from parameter to parameter and from one oxidant to another. In every case, the reproducibility of restoration of the dough property was satisfactory. The standard deviation of each mean restoration value (determined from triplicates) was less than 5% (Tables I and II.)

The mean values of the extent of restoration were close to 100% at the highest levels of oxidation tested for all oxidants used, except for hydrogen peroxide. However, when the restorations of individual parameters were examined, it was clear that the restoration was better in some parameters than in others, depending on the oxidant. The best overall restoration was observed with 0.71-μequiv potassium iodate as the oxidant; the restoration was close to 100% for all the tested parameters. This was not true for the other oxidants tested. Optimum restoration is determined as 100%; restorations of higher or lower values are less successful.

While all of the seven mixing parameters give specific information about the properties of the doughs during mixing, the most important parameters, from a commercial point of view, are the mixing time and peak resistance. If the measure of restoration is taken to be the mean of the restorations for these two parameters, then the overall measures of restoration for iodate and permanganate are between 100 and 103% for both flours.

SE-HPLC and multistacking SDS-PAGE testing of samples taken at various stages of the oxidation process showed the expected shift to higher molecular weight in the molecular weight

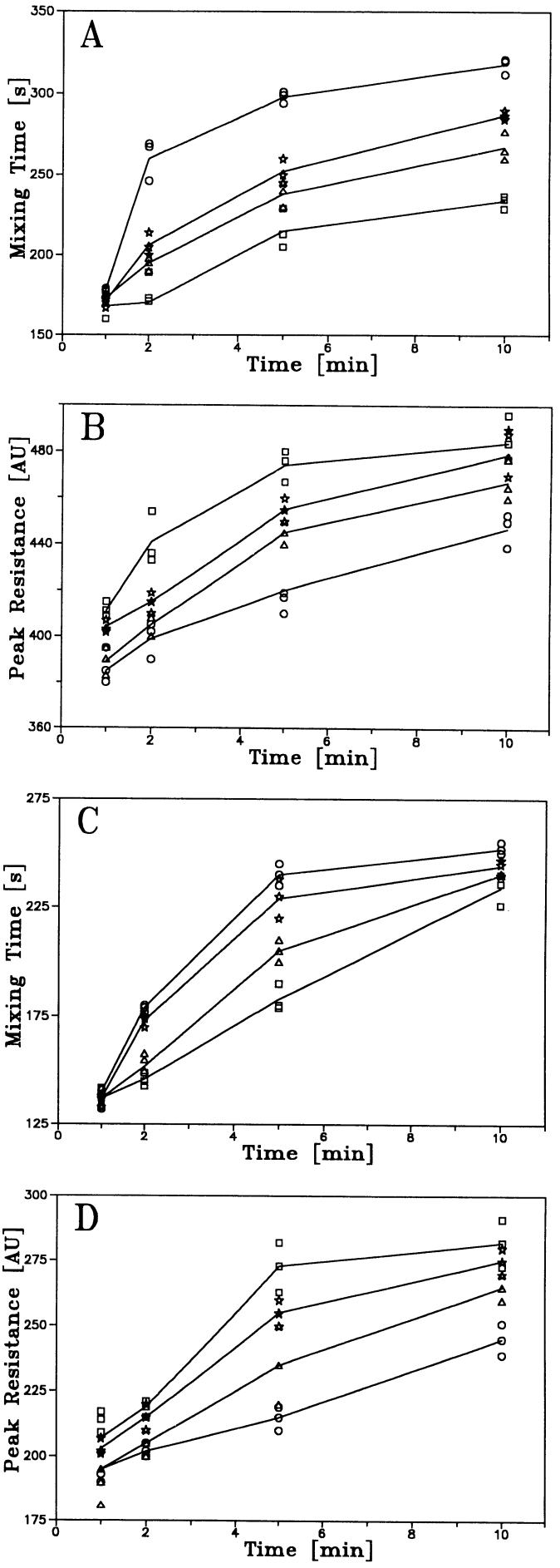


Fig. 5. The effects of oxidation on the mixing time (**A** and **C**) and peak resistance (**B** and **D**) on reduced doughs of high-protein flour (**A** and **B**) and medium-protein flour (**C** and **D**). △ = Potassium bromate, ○ = potassium iodate, ★ = potassium permanganate, □ = hydrogen peroxide.

distribution and the electrophoretic patterns (Fig. 4A and B). The extent of these changes was a function of the reaction time and the oxidant concentration. They paralleled the changes in dough mixing properties. In fact, for the conditions that afforded the best recovery of flour properties, the multistacking SDS-PAGE patterns of proteins from the reoxidized doughs were identical with the corresponding patterns from the untreated samples (Fig. 4Aa and Ac), within the experimental limits of the techniques used. Similarly, the apparent molecular weight distributions of the glutenin from the control and reoxidized doughs were identical (Fig. 4Ba and Bc). A comparison of the mixograms of the control and reoxidized flours (Fig. 4Ca and Cc) show the very close similarity between the two resulting doughs, with effectively identical mix times, peak resistances, and breakdowns. There are some differences in the curves, but these are most obvious in the first minute, where the oxidation-reduction steps are conducted. Subsequent behavior is much more comparable, particularly up to the 3 min after peak dough development.

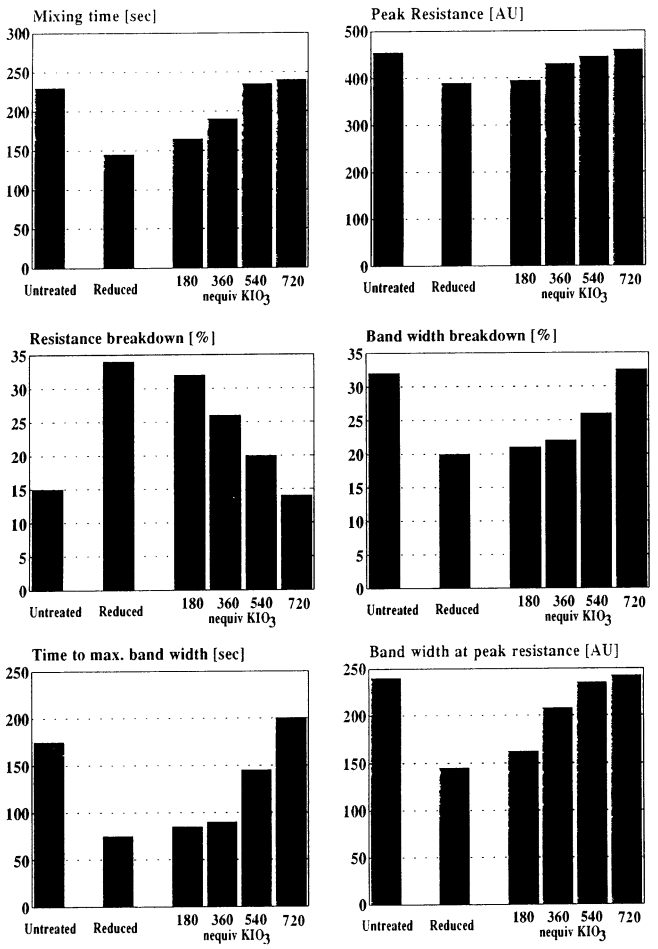


Fig. 6. Effect by the amount of potassium iodate on mixing properties of high-protein flour in reduction-oxidation experiments.

Further study is required to determine whether the baking performance of the reduced-oxidized doughs matches that of the control flours, as implied by the close matching of the mixing curves of the control and the reduced-oxidized doughs.

The extent of restoration observed for the mixing parameters, coupled with the compelling evidence of restoration from the SE-HPLC and SDS-PAGE results, appears to justify the use of this technique as a model system for testing the effects of incorporating specific glutenin subunits on the properties of doughs. The test system requires at least three separate parts: 1) mixing without reduction or oxidation, 2) mixing with reduction and oxidation, and 3) mixing of the flour plus the added subunit preparation with reduction and oxidation. The results presented here show that a particular set of conditions affords optimum restoration of dough properties with both base flours used. Nevertheless, the use of other base flours may require slightly different conditions to obtain optimum restoration. This is not a simple test system, but the technique offers promise of directly measuring some of the effects of particular subunits on dough properties.

CONCLUSIONS

The use of a carefully controlled reduction-oxidation procedure provides a method for direct testing of the contributions of individual polypeptides (glutenin subunits) to the functional properties of doughs. The results also highlight the wide range of aspects of dough functionality that can be studied with the 2-g mixograph.

TABLE I
Extent of Restoration of Mixing Parameters[a] of a High-Protein Flour (%) Using Different Oxidizing Conditions

Oxidant[b]	Amount (μequiv)	Mixing Parameters[c] for High-Protein Flour								
		MT	PR	BWPR	RBD	BWBD	TMBW	MBW	MR7	MR2
I	0.18	74	80	70	223	91	74	172	107	77
I	0.36	79	82	75	200	94	79	170	107	81
I	0.54	100	84	77	161	134	108	120	110	92
I	0.71	109	93	90	114	110	117	100	110	101
M	0.18	68	96	81	61	60	52	169	81	82
M	0.36	80	98	88	69	80	58	175	90	89
M	0.54	90	100	94	100	85	75	112	101	95
M	0.71	106	101	98	107	97	89	115	109	103
H	0.18	69	99	80	61	62	69	157	83	84
H	0.36	95	101	88	69	94	80	160	97	98
H	0.54	95	106	120	130	117	80	157	112	101
H	0.71	106	109	133	138	145	100	110	124	107
B	0.18	73	101	100	107	74	51	172	93	87
B	0.36	77	102	101	123	94	70	170	101	90
B	0.54	86	101	97	130	108	75	120	100	94
B	0.71	87	100	107	146	131	82	100	104	94

[a] Percentage of the difference between untreated and reduced doughs.
[b] Oxidants used were potassium iodate (I) potassium permanganate (M), hydrogen peroxide (H) and potassium bromate (B). All measures of extent of restoration are the means of triplicate determinations, with standard deviation < 5 in all cases.
[c] MT = time to peak dough resistance (mixing time); PR = peak dough resistance; BWPR = bandwidth at peak dough resistance; RBD = breakdownin resistance; BWBD = breakdown in bandwidth; TMBW = time to maximum bandwidth; MBW = maximum bandwidth; MR7 = mean restoration of the seven mixing parameters; MR2 = mean restoration of MT and PR.

TABLE II
Extent of Restoration of Mixing Parameters[a] of a Medium-Protein Flour (%) Using Different Oxidizing Conditions

Oxidant[b]	Amount (μequiv)	Mixing Parameters[c] for Medium-Protein Flour								
		MT	PR	BWPR	RBD	BWBD	TMBW	MBW	MR7	MR2
I	0.18	94	99	69	84	94	83	96	89	97
I	0.36	98	93	85	100	102	94	89	94	96
I	0.54	95	98	101	89	105	121	107	101	97
I	0.71	100	100	105	84	116	114	92	103	100
M	0.18	75	94	71	52	59	87	86	74	85
M	0.36	83	88	96	52	64	98	96	82	86
M	0.54	95	90	86	57	94	127	86	91	93
M	0.71	108	98	105	68	91	116	96	103	101
H	0.18	78	88	77	42	72	127	89	81	83
H	0.36	71	89	100	63	97	82	93	85	80
H	0.54	104	114	71	52	62	94	85	81	109
H	0.71	76	113	96	47	91	81	87	83	95
B	0.18	71	98	66	68	70	89	96	78	85
B	0.36	76	96	73	78	81	97	89	83	86
B	0.54	75	96	85	84	97	94	107	89	86
B	0.71	69	95	99	89	132	93	101	93	82

[a] Percentage of the difference between untreated and reduced doughs.
[b] Oxidants used were potassium iodate (I) potassium permanganate (M), hydrogen peroxide (H) and potassium bromate (B). All measures of extent of restoration are the means of triplicate determinations, with standard deviation < 5 in all cases.
[c] MT = time to peak dough resistance (mixing time); PR = peak dough resistance; BWPR = bandwidth at peak dough resistance; RBD = breakdownin resistance; BWBD = breakdown in bandwidth; TMBW = time to maximum bandwidth; MBW = maximum bandwidth; MR7 = mean restoration of the seven mixing parameters; MR2 = mean restoration of MT and PR.

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