Isolation Treatments and Effects of Gliadin and Glutenin Fractions on Dough Mixing Properties

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ABSTRACT

The effects of a range of solvents and buffers commonly used in the isolation of gliadin and glutenin polypeptides were evaluated on the functionality of gliadins and glutenins in a small-scale dough mixer. Fractions from two wheat cultivars that differed in mixing behavior and high molecular weight glutenin subunit composition were studied. Dialysis or addition of dilute acetic acid rather than water before drying was critical for mixing behavior to remain unaltered. The mixing behavior of gliadin and glutenin fractions treated with a range of buffers and solutions, including sodium dodecyl sulfate, urea, aqueous alcohols and acetonitrile, and low pH buffers was unaltered after dialysis against acetic acid. In contrast, exposure of both gliadins and glutenins to reducing agents altered the mixing behavior of these fractions. The results will guide research on the isolation of single polypeptides for functionality studies and development of a simple dough for structure and function studies.

A major focus for our current research is the direct investigation of the effects of individual flour proteins on the functional properties of doughs, using small-scale testing equipment (Bekes and Gras 1992). In these experiments, 2–10 mg of the purified protein is added or incorporated into a base flour, and the changes in rheological properties are assessed. One of the main challenges in experimental design is to devise purification conditions for the wheat protein that do not introduce changes to functionality. This is especially relevant for wheat storage proteins because they are typically of low solubility in aqueous buffers. Gluten fractions often contain a large number of polypeptide components with similar physicochemical characteristics, so in many cases a combination of fractionation strategies will have to be utilized.

Several methods have been described for gluten protein fractionation, including: 1) fractional precipitation from aqueous propanol, using acetone (Melas et al 1994); and 2) cation exchange chromatography on carboxymethyl- or sulfopropyl-derivatized ion exchange gels (Oh and Gehrke 1965, Kasarda et al 1983, Tatham and Shewry 1985). These often use acidic buffers, such as acetate or glycine-HCl, together with urea to aid in solubilization. In a third method, size-exclusion chromatography, eluents such as aqueous solutions of urea, acetic acid, or a mixture of urea and acetic acid solutions (Nielsen et al 1968, Huebner et al 1974, Khan and Bushuk 1979, Payne and Corfield 1979, Huebner and Bietz 1993) have been used. More recently, aqueous acetonitrile and trifluoroacetic (TFA) acid, acetic acid, acetic acid and ethanol and TFA (Huebner and Bietz 1993), or sodium dodecyl sulfate (SDS) (Singh et al 1990) have also been used. SDS is used for high-performance liquid chromatography (HPLC).

However, the ability of these treatments to leave protein functionality unaltered has not been systematically studied. As part of a research plan to isolate individual gliadin and glutenin polypeptides to study the effects on dough processing behavior and the structure of the protein polymer within doughs, it is important to ensure that the solvents and buffers used for protein isolation do not irreversibly change the properties of the target polypeptides. Assaying for such effects is complicated by the absence of a readily assayable parameter, such as enzyme activity, for wheat storage proteins. In the present study, we treated gliadin and glutenin-rich fractions with common solvents, and after solvent removal by dialysis, we analyzed the effects on mixing properties. In addition, we compared the effects of several methods of solvent removal on functionality after exposure to aqueous ethanol.

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MATERIALS AND METHODS

Flour Samples and Preparation of Fractions

Gliadin- and glutenin-rich fractions from flour from two wheat cultivars, Rosella (high molecular weight glutenin subunit (HMW-GS) composition (2*, 7+8, 2+12) and Suneca (HMW-GS 1, 17+18, 5+10) that differed markedly in mixing behavior (Skerritt et al 1994) were prepared using a fractionation method based on extraction (20 ml of solvent per gram of flour) using dilute hydrochloric acid (adapted from MacRitchie 1985). This method had earlier been reported (using large-scale fractionation and reconstitution) to not alter functionality. The gliadin-rich fraction was defined as that fraction solubilized after titration to pH 5.3 (after prior removal of water-solubles). The glutenin-rich fraction was defined as that solubilized after titration to pH 3.9 (after sequential extraction at pH 5.3, 4.9, and 4.1) and neutralized and lyophilized after preparation. Control fractions were not treated further. The gliadin and glutenin subunit composition of the fractions was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), both before and after reduction with 2% (v/v) 2-mercaptoethanol (Skerritt and Underwood 1986).

Treatment of the Fractions

The lyophilized fractions were dissolved or suspended at 1 mg/ml in 70% (v/v) ethanol. After 16 hr of end-over-end mixing at room temperature, the solvent was removed. In the first experiment, different replicate fractions were treated: a) ethanol removed by dialysis (eight changes, 72 hr) against 200 vol of purified water; b) dialysis against 0.1 m*M* acetic acid; c) ethanol removed by rotary evaporation at 35°C; d) ethanol removed by evaporation at 35°C after addition of 0.1 m*M* acetic acid. In the second experiment, treatments involving isopropanol, urea, acid, and SDS were evaluated. In each case, the solvent was removed by dialysis against 0.1 m*M* acetic acid. The treatments (at room temperature unless shown) were: a) 50% (v/v) isopropanol; b) 50% (v/v) isopropanol at 60°C (4 hr only); c) 50% (v/v) isopropanol + 0.1% (w/v) dithiothreitol (DTT); d) 50% (v/v) isopropanol for 16 hr, followed by addition of acetone (to 80%, v/v) and incubation of

this mixture for 2 hr at room temperature (Melas et al 1994); e) 2*M* urea (Batey 1984); f) 2*M* urea + 0.1% (w/v) DTT; g) 8*M* urea; h) 8*M* urea + 0.1% (w/v) DTT; i) 0.1*M* acetic acid; j) 5 m*M* sodium acetate, pH 3.5 (Oh and Gehrke 1965); k) 3*M* urea and 10 m*M* glycine and acetate, pH 4.6 (Tatham and Shewry 1985); l) 0.1*M* acetic acid and 12% (v/v) ethanol and 0.03% TFA (Huebner and Bietz 1993); m) 50% (v/v) acetonitrile and 0.1% (v/v) TFA in water (Batey et al 1991); n) 0.1% (w/v) SDS and 50 m*M* sodium phosphate, pH 6.9 (Singh et al 1990).

Analysis of the Fractions

Mixing tests were conducted with a prototype 2-g mixograph using a modification of the standard method for 35 g of flour scaled to 2-g size (Gras and O'Brien 1992). The base flour was a commercial medium protein (10.3% protein by near infrared reflectance [NIR]) bakers' flour. Five milligrams of either the glutenin or gliadin fractions were directly added to the flour as the lyophilized powder. The ability of the mixing protocol we used to distribute the added fraction evenly through the flour was confirmed in preliminary experiments using dye (Bekes et al 1994). Addition of 5 mg of the gliadin-rich fraction increased the total gliadin content of the flour by 4.8%, and addition of 5 mg of the gluten-rich fraction increased the total glutenin content by 5.4%. Water absorption of the flours was determined using the approved method (AACC 1995), with water additions increased in samples that contained the additional protein (Gras et al 1990). All experiments were performed in duplicate; data shown are means of replicates that differed by <5%. Although several parameters were measured, for brevity only effects on mixing time (MT) and resistance breakdown (RB) are reported. Statistical analyses using MSUSTAT (developed by R. E. Lund, Montana State University, Bozeman, MT) used multiple linear analyses of variance, and compared the effects of addition of untreated (control) fractions with the treated fractions.

RESULTS AND DISCUSSION

Analysis of Gliadin- and Glutenin-Rich Fractions

The gliadin and glutenin-rich fractions were analyzed by size-exclusion HPLC following dissolution using sonication. The method described by Batey et al (1991) was followed, except that a column with a greater exclusion limit (BioSep S-4000, Phenomenex, Torrance, CA) was used. These studies showed that 75–80% of the fractions described herein as "glutenin" was polymeric (M_r > 200,000), while a similar proportion of the "gliadin-rich" fraction eluted with monomers. The composition of the fractions was further checked using SDS-PAGE (Fig. 1). In the fractions extracted using the higher pH, polypeptides corresponding to gliadin mobility were detected in the unreduced samples (lanes a,b), while the fraction extracted at the lower pH ran mainly as a "streak", except for some polypeptides with mobilities corresponding to the D-LMW-GS (lanes c,d). After reduction, most of the higher pH extract exhibited a typical gliadin profile (lanes e,f), while in the low pH extracted material (lanes g,h), most polypeptides corresponded to HMW- and LMW-GS. There were some polypeptides that were apparently common to both fractions, probably because of a slight contamination of the glutenin fractions (lower pH extracts) with gliadin.

Comparative Effects of Gliadin- and Glutenin-Rich Fractions

Rosella and Suneca were chosen as a source of gliadin and glutenin fractions as they differed in allelic composition at most gliadin and glutenin loci; Suneca also had somewhat stronger mixing properties (Fig. 2A and D). In keeping with this behavior, addition of the glutenin fractions from both flours increased MT (Fig. 2B) and decreased RB (Fig. 2D), with greater effects on both parameters noted for Suneca. Addition of the gliadin fractions had weakening effects on dough properties (Fig. 2A and C), with decreases in MT and increased RB being observed. Interestingly, Suneca gliadin had greater weakening effects than did the corresponding Rosella fraction.

Method of Drying

The presence or absence of acetic acid during drying had greater effects on functionality than did the method used for ethanol removal and drying (rotary evaporation vs. dialysis and freeze-drying). Glutenin fractions from both cultivars dried in the presence of acetic acid (HOAc) exhibited similar RB and MT behavior to the control glutenin fractions (Fig. 2B and D). The MT and RB values for the 70% ethanol-treated and dried fractions were compared with the control fractions using multiple linear analysis of variance. In no cases were the MT and RB values for the acetic acid dialyzed or evaporated fractions significantly different from the corresponding values for the control fractions. In contrast, in 14 of 16 cases the water-dialyzed or evaporated fractions had significantly different ($P < 0.05$) MT and RB values from the controls. Exceptions were for the water-evaporated Suneca gluten fraction (MT) and Rosella gliadin fraction (RB).

Drying from water did not abolish the increase in MT elicited by glutenin but actually increased it; effects on RB were less pronounced. The increased activity of this glutenin fraction could be due to exposure of additional sulfhydryls, leading to the ability of the "denatured" glutenin to form additional bonds upon addition to doughs. Similar trends were seen with gliadins (Fig. 2A and C). Drying in the presence of dilute acetic acid maintained effects on MT observed with starting material, while they were almost abolished after drying from water.

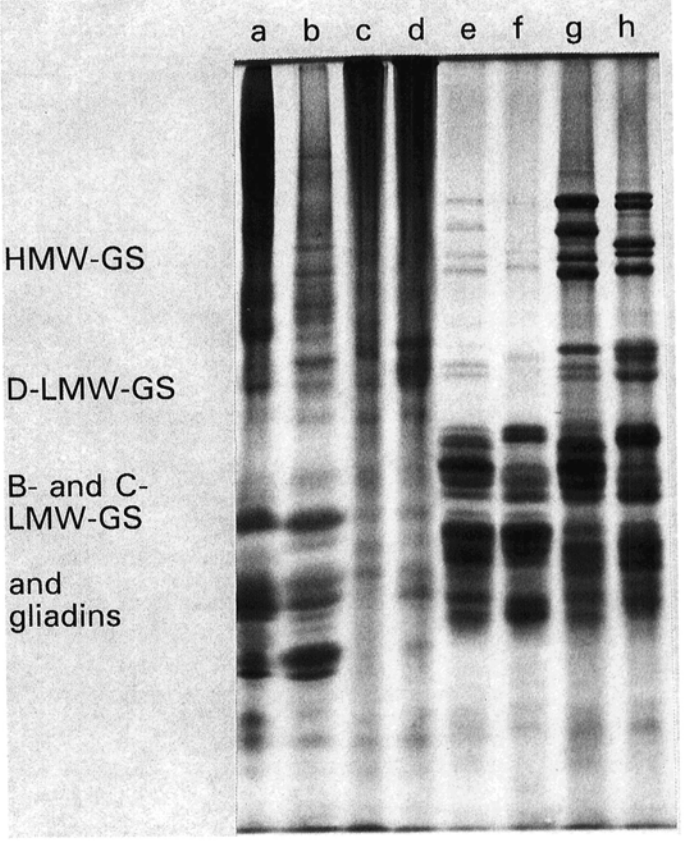

Fig. 1. Analysis of gliadin-rich and glutenin-rich fractions from Rosella and Suneca flour by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lanes a–d: unreduced extracts. Lanes e–h: reduced extracts. Rosella gliadin-rich fraction (lanes a and e); Suneca gliadin-rich fraction (lanes b and f); Rosella glutenin-rich fraction (lanes c and g); Suneca glutenin-rich fraction (lanes d and h). HMW-GS = high molecular weight glutenin subunits. LMW-GS = low molecular weight glutenin subunits.

In subsequent experiments, fractions were analyzed after dialysis against dilute acetic acid. While the data also showed that low temperature evaporation of ethanol was satisfactory, this approach was not pursued further because it is not applicable to other extractants or eluents such as urea or buffers and it is harder to use routinely for large numbers of samples.

Effects of Solvents and Buffers on Gliadin and Glutenin Behavior

Both the MT (Fig. 3) and RB data (Fig. 4) indicated that glutenins can be exposed to a wide range of solvents (including those based on isopropanol, urea or acid) in the absence of reducing agents, with full retention of functionality. Treatment with more concentrated acetic acid also caused activity loss for Rosella but not Suneca. However, in many cases reducing agents are required to dissolve glutenin and fractionate it at the subunit level.

In contrast to glutenin, the gliadin fraction dissolved in each of the solvent systems tested. None of the treatments blocked the weakening effect of both the control Rosella or Suneca gliadin preparations (Fig. 3A and B and 4A and B), although the DTT-containing recipes tended to partially reduce the decrease in MT for the gliadin preparations compared with the control. In contrast, the DTT treatments reduced the increases in MT for the Suneca and Rosella glutenin fractions. These effects were statistically significant for all of the 50% isopropanol and DTT treatments and all of the 2*M* urea and DTT treatments (except for Suneca glutenin). Surprisingly, the effects of 8*M* urea and DTT were less marked, with only the effect on the MT of Rosella glutenin fraction reaching statistical significance. The only other treatment to have a statistically significant effect on MT was 0.1*M* acetic acid, with a (just) significant reduction in the lengthening of MT caused by Suneca glutenin.

The major treatments that significantly decreased the effects of gliadin and glutenin fractions on RB were also those that employed a reducing agent, in this case 8*M* urea and DTT. Effects of these treatments on RB were not significant for the Rosella gliadin fraction, probably because the control fraction caused only a modest change in mixing properties. Some other treatments also displayed significantly different RB values when compared to the control treatments: acetonitrile and TFA and water (increased RB for Rosella gliadin, decreased for Suneca gliadin); sodium acetate (slight decrease in RB for Suneca glutenin); and 8*M* urea

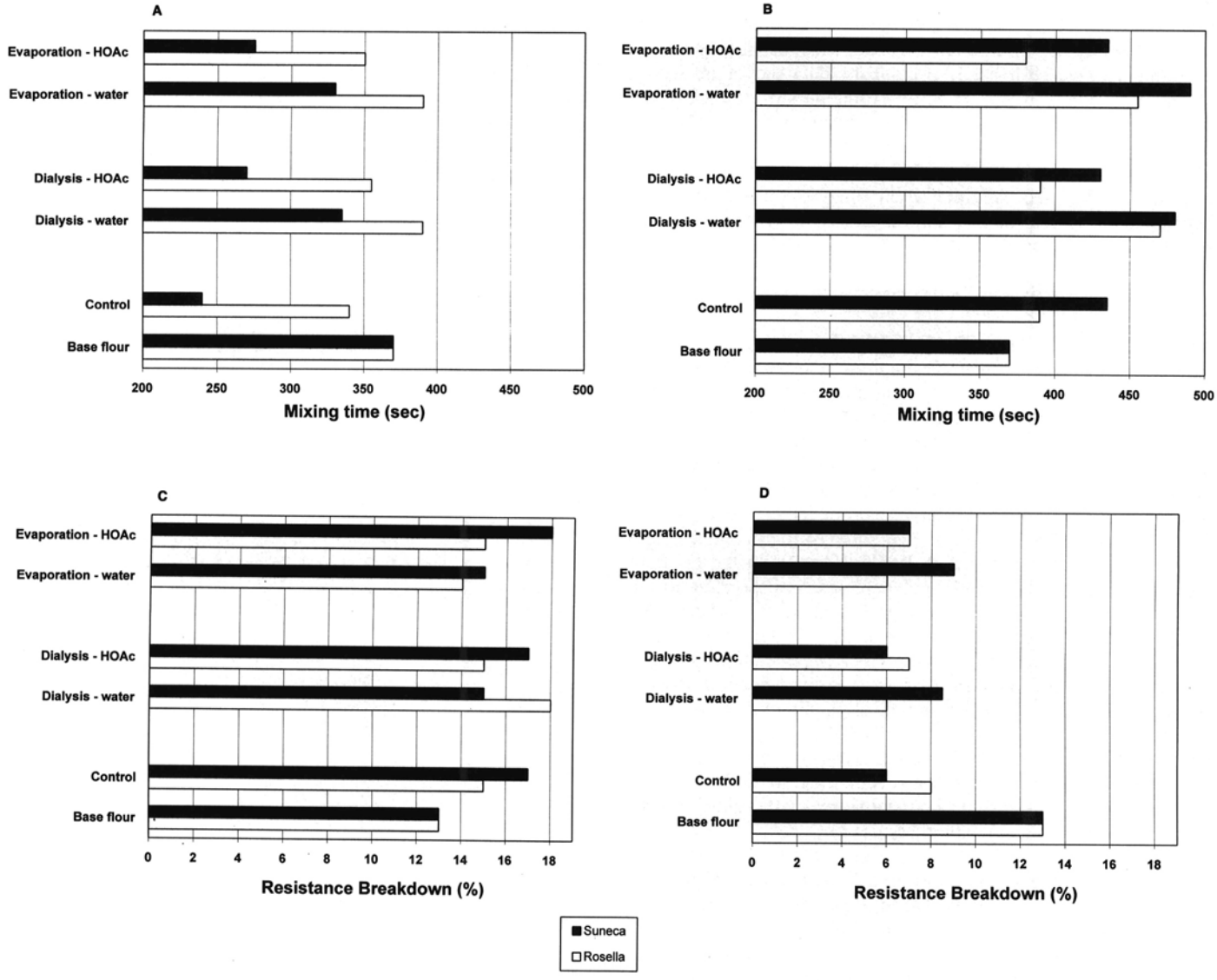

Fig. 2. Effects of solvent removal method on fractions isolated from Suneca and Rosella flours. **A,** gliadins and mixing time (LSD: 34 sec for Rosella, 26 sec for Suneca); **B,** glutenin and mixing time (LSD: 39 sec for Rosella, 41 sec for Suneca); **C,** gliadins and resistance breakdown (LSD: 1.6% Rosella, 1.0% for Suneca); **D,** glutenin and resistance breakdown (LSD: 1.0% for Rosella, 1.4% for Suneca). LSD = least significant difference ($P < 0.05$) Student's *t*-test; HOAc = acetic acid (0.1 m*M*).

(decreased RB for Suneca gliadin, slightly increased RB for Rosella glutenin).

Therefore, in some cases these effects were quantitatively minor and in no case were the effects of the same type observed for the fractions from both cultivars. The ability of reducing agents to affect the behavior of gliadin in mixing studies suggests that the status of the intramolecular disulfide bonds can affect dough behavior. The reduction in the strengthening effects of glutenins on dough behavior upon DTT treatment probably arises from the inability of the added treated material to incorporate into the gluten network, since a subsequent oxidizing step was not used for this purpose. The treated glutenins thus become gliadin-like in their behavior. A reversible reduction-oxidation strategy has been developed to enable the incorporation of glutenin subunits into doughs for assessment of their behavior (Bekes et al 1994). The effects of DTT treatment were less marked in the high-urea preparations. Possibly, these subunits were able to more readily reoxidize.

CONCLUSIONS

A major finding of the study is the importance of using dilute acetic acid, rather than water for drying gluten protein fractions, if their functionality in mixing studies is to be maintained. The amount of acetic acid used for dialysis and drying is rather low; a

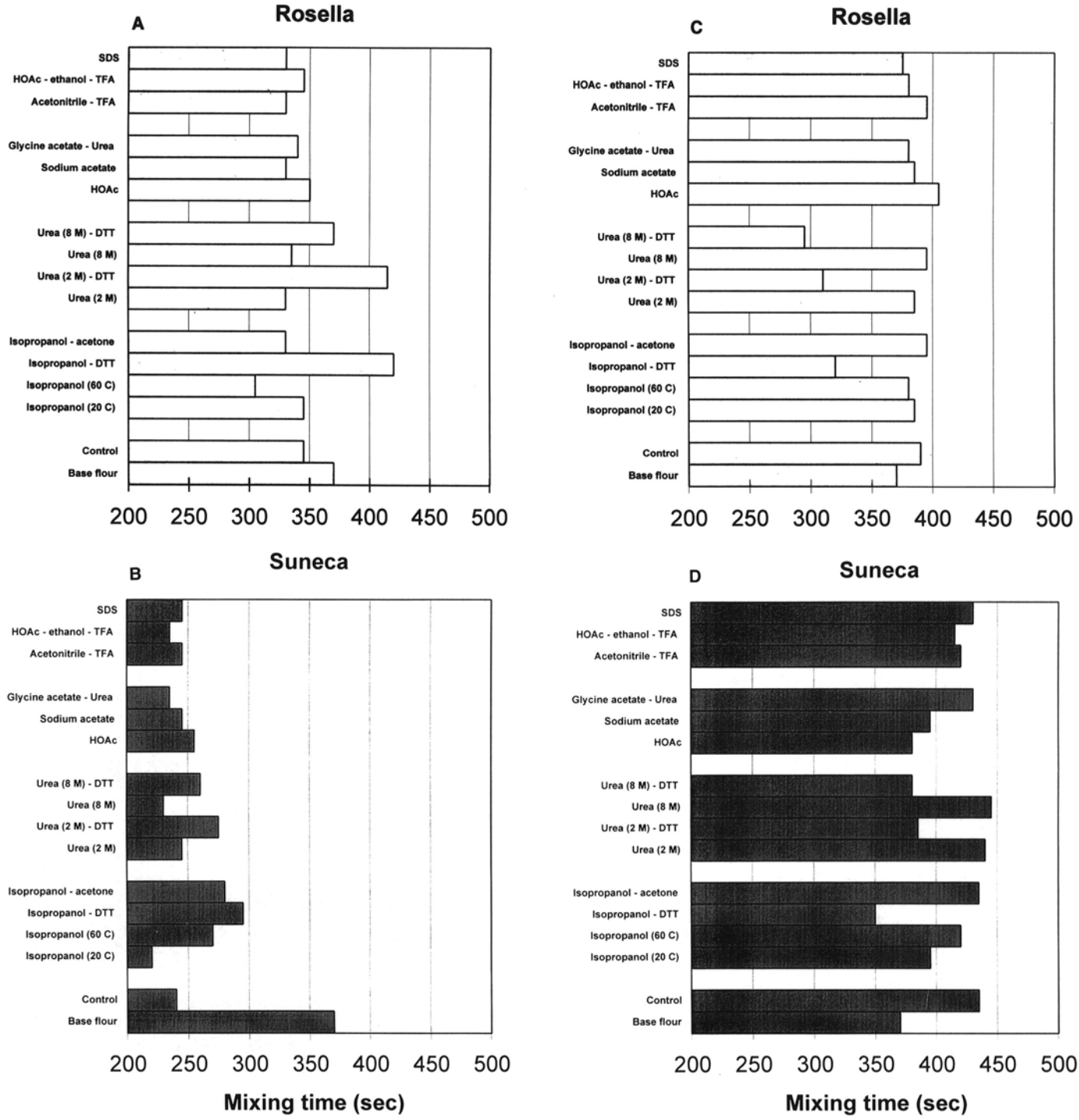

Fig. 3. Effects of treatment of fractions from Suneca and Rosella flours with various solvents on mixing time. **A** and **B:** gliadins (LSD: 27 sec for Rosella, 19 sec for Suneca). **C** and **D**: glutenin (LSD: 29 sec for Rosella, 31 sec for Suneca). LSD = least significant difference ($P < 0.05$) Student's *t*-test; SDS = sodium dodecyl sulfate (0.1%, w/v); TFA = trifluoroacetic acid (0.1%, v/v); DTT = dithiothreitol (0.1%, w/v).

0.1 m*M* solution of acetic acid has a pH close to that of dough (≈pH 6) (Hoseney and Brown 1983). The fractions in this study had also already been exposed to acid in the initial preparation. This was the result for water removal by either dialysis or evaporation. Use of water instead of acetic acid increased the MT for both gliadin fractions (decreasing the reduction in MT of the control fractions) and glutenin fractions (increasing the potentiated MT of the control fractions).

An encouraging outcome is that, provided samples are dialyzed against dilute acetic acid, a range of extractants can be used without apparent alteration to the mixing properties of the bulk fractions. Weegels et al (1994) also found that a higher concentration of acetic acid (1%, v/v) can be used for dialysis of gliadin with retention of functionality. In their view, an important role of the acid is the avoidance of aggregation during dialysis. Several other studies with gluten and glutenin have suggested loss of functionality after exposure of the proteins to aqueous alcohols (Hoseney et al 1969, MacRitchie 1985, Chakraborty and Khan 1988). In these studies, the protein extract was not subsequently dialyzed against acetic acid.

The weakening effect during subsequent addition to doughs of glutenin fractions that had been exposed to a reducing agent was expected, given that intermolecular disulfide bonds had been broken and that only some of these may reform in the usual configu-

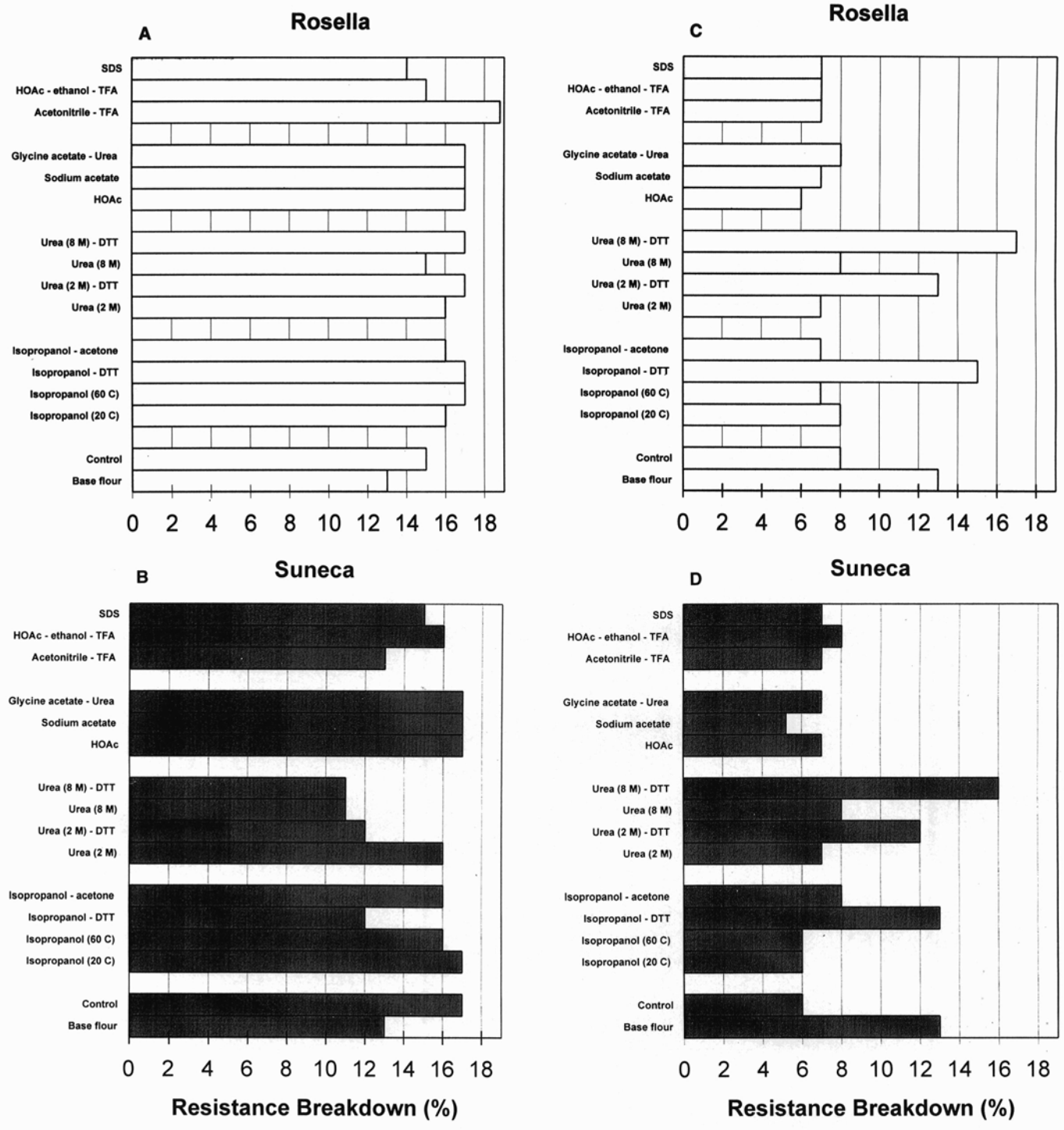

Fig. 4. Effects of treatment of fractions from Suneca and Rosella flours with various solvents on resistance breakdown. **A** and **B:** gliadins (LSD: 2.0% for Rosella, 1.3% for Suneca); **C** and **D:** glutenin (LSD: 1.3% for Rosella, Suneca for 1.4%). LSD = least significant difference ($P < 0.05$) Student's *t*-test; SDS = sodium dodecyl sulfate (0.1%, w/v); TFA = trifluoroacetic acid (0.1%, v/v); DTT = dithiothreitol (0.1%, w/v).

ration during the dialysis step. Because the use of reducing agents is critical for fractionation of glutenin at the subunit level, it will be important to assess the effects of solvent treatments on glutenin subunit functionality after subunit incorporation into doughs. However, the marked effects of reducing agent on the subsequent behavior of the gliadin fractions was unexpected. In a few other cases, a particular solvent had an effect on the gliadin or glutenin fraction from one cultivar but not the other. Treatment with SDS detergent at 0.1% in a neutral buffer did not significantly affect functionality of any of the fractions studied. Since preparative electrophoresis involving SDS-containing buffers has been shown to be a useful means of purifying glutenin subunits (Curioni et al 1989, 1995), it is possible that these subunits may retain functionality after exposure to SDS.

We have already been able to produce functional glutenin subunits and subfractions following reversed-phase HPLC fractionation and propanol solubilization and precipitation. It will be important to also check treatments systematically on extensibility and microbaking behavior. It also cannot be ruled out that compensating effects on different components within the fraction may have been induced by the solvents. There was some evidence for differences in effects of agents on gliadins and glutenin in this study. Thus it will be necessary to recheck effects of selected solvents to be used for purification on individual subunits. Because gluten proteins lack a readily assayable biological activity (such as enzymatic activity), the only practical way to do this will be to compare the same subunits after purification using two or three different methods. Even so, in such work it would be important to at least compare results obtained within a set of related material (alleles, homologous chromosome arms) using fractions made the same way.

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