Heat-Shock Protein 70 and Dough-Quality Changes Resulting from Heat Stress During Grain Filling in Wheat

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ABSTRACT

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In an attempt to further elucidate the molecular mechanisms that determine the loss of dough strength associated with heat stress of growing wheat, the roles of heat-shock proteins (HSP) and heat-shock elements upstream of glutenin genes were investigated. A range of genotypes differed in the extent of synthesis of high molecular weight glutenin subunits (HMW-GS) and HSP during heat stress. The concentration of HSP 70 remaining in mature grain increased as a result of a few days' heat stress of wheat plants. The amount of HSP 70 in mature grain samples from heat-stressed plants of 45 genotypes was not strongly correlated with loss of dough strength. There was much less evidence for this mechanism than for other molecular hypotheses from the literature, particularly, changes in glutenin-to-gliadin ratio, size distribution of the glutenin polymer, and the involvement of HSP and chaperones during grain-protein synthesis. HSP 70 was purified from heat-stressed grain, and was added to (or incorporated into) dough in the direct-drive mixograph. The HSP behaved similarly to several other hydrophilic proteins when added at a level of 2 mg/2 g of flour. It showed no dramatic effects on dough properties that could constitute a major explanation for the dough-weakening effects of heat stress, even though the level of addition was well above the maximum levels that might be encountered in field-grown, mature grain. Furthermore, sequencing of the genes (upstream of the coding region) for HMW-GS failed to show the presence of heat-shock promoters, even for genotypes that differed considerably in their reactions to heat stress. The findings simplify the range of possibilities that cause heat-related loss of dough strength, focusing attention on the degree of polymerization of the glutenin chains, and on the roles of HSP and chaperones in the developing grain.

High temperatures (>32°C) for as few as one or two days during grain filling have the capacity to drastically alter the dough properties of wheat (Randall and Moss 1990; Blumenthal et al, 1991a,b, 1993, 1995). Despite an increase in the proportion of grain protein (relative to starch content), the protein composition changes in such a way as to cause general decreases in dough strength for many wheat genotypes (Ciaffi et al 1995, Borghi et al 1995, Graybosch et al 1995). Nevertheless, exceptions to this generalization have been reported for some wheats that are apparently tolerant to the dough-weakening effects of heat stress (Stone et al 1994, Blumenthal et al 1995).

It is important for us to better understand the molecular reasons for these environmental and genetic phenomena, given the relevance of dough properties to the processing quality and market value of wheat for virtually all end uses. Elucidation of the molecular mechanisms involved should, in turn, lead to the development of more efficient procedures to: 1) test grain at harvest for the likelihood of quality change due to heat stress; 2) devise strategies to produce genotypes that are tolerant to the effects of heat stress on grain quality; 3) develop methodologies to screen for heat-tolerant genotypes as potential parent or progeny lines in breeding programs.

Several hypotheses have been advanced to account for the observed changes in dough strength due to heat stress during grain filling. Evidence for these was considered in planning the studies described in this article.

Hypothesis 1. Blumenthal et al (1990b,c; 1994) demonstrated that the ratio of glutenin to gliadin decreased as a result of heat stress because gliadin synthesis continued during heat stress while there was a greatly decreased synthesis of glutenin protein. In these studies, glutenin (>100 kDa) and gliadin (<100 kDa) proteins were quantified using size-exclusion (SE) HPLC of an unreduced extract of grain protein (Blumenthal et al 1993, 1994). This explanation was backed by the observation of heat-stress elements (HSE) in the upstream regions of some gliadin genes, but such HSE have not been found in published sequences of glutenin genes (Blumenthal et al 1990b,c).

Hypothesis 2. Heat stress reduces the size of glutenin polymers in the mature grain, thereby weakening the resulting dough. This conclusion was drawn following analysis of the size distribution of the glutenin proteins from heat-stressed and control grain by multistacking SDS-gel electrophoresis (Blumenthal et al 1995) and RP-HPLC (Ciaffi et al 1995). Greater dough strength was statistically associated with a greater proportion of very large glutenin polymers, or of the more insoluble glutenin, and a heat-stress history for grain was associated with a lower proportion of the larger sizes of glutenin polymers. As glutenin polymer size is largely a function of disulfide bonding between glutenin peptides, this phenomenon could result from the heat-sensitivity of the enzymes involved in the formation of SS bonds (e.g., protein disulfide isomerase).

Hypothesis 3. The reports of Bernardin (1994) and Bernardin et al (1994) have suggested that the heat-shock proteins (HSP) are implicated in the heat-related loss of dough strength, due to their presence in the mature grain. The synthesis of HSP has been demonstrated for many parts of the wheat plant (Necchi et al 1987, Vierling 1991, Hendershot et al 1992), including the developing grain (Giorini and Galili 1991). Their synthesis is a part of the ubiquitous reaction of all organisms to heat and other stress conditions (Ashburner 1982, Pelham 1986, Blumenthal et al 1990a, Howarth 1991). Although it has been demonstrated that there is also considerable breakdown of these proteins in wheat endosperm following the stress event, their presence in the mature grain has been reported (Blumenthal et al 1990a, Bernardin et al 1994). If the heat-stress proteins are capable of modifying dough structure, then their presence in the mature grain could make a significant contribution to the heat-related loss of dough quality. Bernardin et al (1994) pointed especially to a 70-kDa heat-shock protein (HSP 70) as having such a role. These authors have also suggested that the HSP would be valuable markers of a history of

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heat stress in mature grain, thereby providing industry with an assay to indicate the possibility of unexpectedly weak dough properties. Furthermore, a world patent application (Anonymous 1995) has claimed that quantification of a "heat stress peptide in the endosperm" would indicate whether the wheat had been exposed to elevated temperatures during the grain-filling period.

Hypothesis 4. Irrespective of the possible role of heat-shock proteins in the mature grain on dough properties, the general family of HSP and chaperones has a clear role in monitoring the formation, folding, and polymerization of polypeptides in the developing grain. The HSP may have the additional role of disaggregating and hydrolyzing proteins that are malformed as a result of stress conditions (Pelham 1986, Parcell et al 1994, Hartl 1996, Hendershot et al 1996, Hesterkamp et al 1996). These mechanisms, operating at the site and time of the synthesis and polymerization of gluten proteins, are of primary importance in the determination of dough quality in general. These mechanisms are thus also likely to be involved in the modification of dough-protein function due to the activities of the HSP and chaperones during heat stress.

Although all four of these factors (and possibly more) may be involved to some extent in determining the effects of heat stress on dough properties, we selected two of them (Hypotheses 1 and 3) as being amenable to further study: the possible occurrence of heat-shock elements in the promoter regions of glutenin genes (thus explaining the tolerance of certain genotypes to heat stress) and the possible contribution of HSP 70 in mature grain to weakness during dough mixing.

It was, therefore, the aim of the project reported here to determine the extent to which the presence of heat-inducible proteins (particularly the HSP 70 family and the high molecular weight glutenin subunits HMW-GS) account for the alteration of dough properties caused by heat stress during grain filling. The approach taken was to analyze for HSP 70 in immature grain and in mature grain that had been characterized for dough strength, and to purify the HSP 70 family to test its effect when added directly to dough. We were thus able to examine the direct effects of HSP 70 on dough strength (Hypothesis 3) compared to other factors that might explain the effects of heat stress on dough quality. Attempts to explain variations in the responses of certain HMW-GS to heat stress were made by sequencing the promoter region of these genes, seeking to identify the presence of heat-shock elements in the glutenin genes examined.

MATERIALS AND METHODS

Determining Rate of Synthesis of Proteins Induced Under Heat Shock

Plants of the 10 genotypes studied by Blumenthal et al (1994) (Pinnacle, Sonalika, Lyallpur, Trigo 1 and 3, Kalyansona, Hartog, Sunco, Banks, and ME 71) were grown under a continuous temperature regime of 18°C (day) and 13°C (night). Heads were harvested at 31 days after anthesis (duplicates of each cultivar) and were placed in a head culture of a 0.1*M* potassium phosphate buffer (pH 6.2) (80 µCi L-[^{35}S]-methionine (>800 µCi/mmol for 24 hr at 21 or 38°C). A small volume (200 µL) was used initially to ensure total uptake of the label. Heads were then frozen in liquid nitrogen. The frozen endosperm was ground in a cold mortar and pestle, and 40 mg of the powder was placed in 170 µL of 1.8% (w/v) SDS in 0.5*M* Tris-HCl, pH 7.5. After sonicating for 10 sec, 10% 2-mercaptoethanol was added and the sample was left at 50°C overnight. The samples were then centrifuged at 12,000 × *g* for 30 min. Aliquots (5 µL) of the supernatant were placed on filter paper disks and incorporation of label into protein was determined by scintillation counting after fixing in 10% trichloroacetic acid (TCA). Proteins synthesized during the heat shock were analyzed by SDS-PAGE. Equal counts (35,000 cpm) of TCA-precipitable material for control and heat-shocked samples were loaded onto a 12% polyacrylamide gel. Gels were stained with 0.2% Coomassie blue R250. They were destained in 40% methanol and 10% acetic acid. After destaining, gels were incubated (Amplify, Amersham) for 30 min before drying. Autoradiography was performed on dried gels using preflashed Kodak X-ray film. Fluorography was performed at –70°C. The extent of ^{35}S incorporation into the individual electrophoretic components was determined by image analysis of the X-ray film, with particular attention to the HSP 70 family and the subunits of glutenin.

Gel Electrophoresis and Probing Immunoblots

Flour sample (50 mg from either control or heat-shocked plants) was mixed by vortexing with 250 µL of 0.5% phosphate buffer (pH 6.9) containing SDS and 2-mercaptoethanol; the mixture was sonicated for 15 sec and centrifuged for 10 min, and the supernatant was used for SDS-PAGE as described by Blumenthal et al (1990a). For immunoblotting, the gel strip (before staining) was placed onto a nitrocellulose sheet and protein zones were transferred by electroblotting. The immuno-visualization procedure was similar to that described for dot-blotting. For immuno-analysis of grain extracts, 1-µL duplicate spots of the supernatant from SDS-phosphate extraction were spotted onto a nitrocellulose sheet and allowed to dry. The sheet was treated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) overnight at room temperature. The strip was washed three times for 5 min with PBS containing Tween (0.05%). The strip was then incubated, with rocking, for 2 hr at room temperature (20°C) with a 1/2500 dilution of monoclonal anti-human HSP 72 kDa (Amersham Cat. No. RPN 1197). The strip was then washed four times for 5 min each time with PBS/Tween. The second antibody (anti-mouse IGG [H+L] alkaline phosphatase conjugate, from Promega Cat. No. S3721) was diluted 1/2500 in 1% BSA-PBS and incubated with rocking for 1 hr at room temperature. The strip was washed five times for 5 min each time in Tris-buffered saline-Tween (0.05%) (TBST). The nitrocellulose sheet was incubated at room temperature in substrate solution (NBT-BCIP in alkaline phosphate buffer) until background started to show. The reaction was stopped with the addition of 10 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA. The blots were scanned to quantify staining intensity.

Grain Used for Isolating HSP 70

Triticum aestivum L. cv Hartog was grown (eight plants per pot, three tillers per plant) in a 1:1 mixture of Perlite and Vermiculite under an 18/13°C, 16/8 hr day-night temperature regime in the Canberra Phytotron growth chambers (Morse and Evans 1962). At one week after anthesis, half the pots were transferred to a growth cabinet where they were subjected to a 40/15°C temperature regime (16/8 hr day-night cycle) for the duration of grain filling. The plants were watered frequently to ensure that they were not water stressed.

HSP Purification by ATP-Affinity Column Chromatography

Grain from the severely heat-stressed Hartog was milled to white flour in a Quadrumat Jr. mill (Brabender, Germany). HSP 70 was purified using the procedures of Welsh and Feramisco (1985) and Giorini and Galili (1991). Aliquots (10 g) of flour were vortexed in 40 mL of 0.1*M* Tris-HCl (pH 8.0), containing 100 m*M* potassium chloride, 50 m*M* magnesium acetate, 1 m*M* dithiothreitol (DTT), and 1 m*M* phenymethyl-sulphonyl fluoride (PMSF). This mixture was sonicated for 5 min, centrifuged for 10 min at 50,000 × *g*, and filtered through a 0.45-µm membrane. The supernatant was applied to an ATP-agarose column that had been pre-equilibrated with 0.02*M* Tris-acetate (pH 7.5), containing 0.02*M* sodium chloride, 0.1 m*M* EDTA, 15 m*M* β-mercaptoethanol, and 3 m*M* magnesium chloride. The column was then washed extensively with high- and low-salt buffers (Welsh and Feramisco 1985, Giorini and Galili 1991). The column was developed with 3 m*M* ATP resulting in the coelution of several ATP-binding proteins. A major protein band eluted with an apparent average

molecular mass of ≈68 kDa. There were also two proteins with apparent molecular masses of 35 and 38 kDa, and two more proteins with apparent molecular masses of 16.5 and 18.5 kDa. Eluates from both control and heat-shocked flour were analyzed on a 10% SDS-polyacrylamide gel. The gels were fixed and stained with 0.05% (v/v) Coomassie Brilliant blue R250 in 40% (v/v) isopropanol, 10% (v/v) acetic acid for 2 hr. The relevant spots were excised from the gel and eluted at 37°C in 0.1% (w/v) SDS in 100 m*M* sodium acetate, pH 8.8. The eluate was then placed on Pro-Spin cartridges for sequencing (Walsh et al 1995).

To obtain a further purified sample of HSP 70 for use in dough-addition experiments, attempts were made to remove the contaminating proteins. The column was first developed with 1 m*M* GTP resulting in the elution of the proteins with apparent molecular mass of 16.5 and 18.5 kDa. To remove the 35- and 38-kDa contaminating proteins, to remove salts, and to concentrate the sample, the eluate was loaded onto a 50-K Macrosep centrifugal concentrator (Filtron), centrifuged at $1,000 \times g$ in a fixed-angle rotor, and concentrated to a volume of 1.5 mL. Concentrates were pooled, freeze-dried, and used for dough-quality studies.

Dough-Mixing Studies

Dough properties were determined as mixing time, peak resistance, and resistance breakdown, using a direct-drive mixograph, with a modification of the standard method for 35 g of flour scaled to the 2-g size (Rath et al 1990). The computer program was modified to enable portions of the recording to be automatically excised when the mixing was halted briefly. A reversible reduction-oxidation procedure for incorporation of added polypeptides into glutenin was used (Bekes et al 1994). A base flour, Janz (obtained from BRI Australia, Ltd.) was chosen on the basis of its mixing time of 174 sec and its protein content of 11%. All mixing experiments were performed in duplicate. Statistical analysis was performed using a one-way analysis of variance and comparisons of means were conducted by the Student's *t* test.

DNA Extraction, PCR reaction, Cloning, and Sequencing

Genomic DNA was extracted from leaf tissue of the genotypes Banks, ME71, Wyuna, and 6386, using 3% sarcosyl buffer, including 10 m*M* EDTA, by grinding in a mortar and pestle in liquid nitrogen. DNA was further purified by phenol-chloroform extraction, precipitation with cold ethanol, and resuspension in 10 m*M* Tris–1 m*M* EDTA, pH 8.0 (TE buffer), containing 10 µg/mL of RNAase for incubation at 37°C for 30 min. After repeated phenol-chloroform extraction, the DNA was resuspended in TE buffer.

PCR experiments were performed in a volume of 10 µL using 60-ng genomic DNA, 1 unit of Amplitaq DNA polymerase (Perkin Elmer), Taq PCR buffer (Biotech), 80 ng each of the two primers, 1.5 m*M* magnesium chloride, and 10 µ*M* of each deoxyribonucleotide. Three of the primers used for the 5′ flanking region of the *Glu-D1* genes were those reported by Mackie et al (1996):

P1:- 5′ TCTCGCTGGGGTAGAAGGATCCGGCCTT 3′
P2:- 5′ CACACTTCTGCAGACAATACACCAGAAC 3′
P3:- 5′ GAACACTTGGAGATCCATAGAA 3′

A fourth primer was synthesised:
5′ GGTCTGCTAAAGCCACGTAATGG 3′

based on the gene sequence reported by Mackie et al (1996). The location of three of these primers in the gene sequence is shown in Mackie et al (1996). Amplified products were analyzed on a 1.5% agarose gel. The amplified products, corresponding to a region of -800 bp to +312 bp of the *Glu-D1-2* gene (nomenclature of Mackie et al 1996), were cloned into the pGEM-T vector (Promega) or were subjected to direct nucleotide sequence analysis. This analysis was made using a dye terminator cycle sequencing kit (Applied Biosystems).

RESULTS AND DISCUSSION

Synthesis of Heat-Inducible Proteins in Developing Grain

Most studies of heat-inducible proteins have concentrated on their synthesis in the vegetative, growing plant (Krishnan et al 1989, Vierling and Nguyen 1992, Nguyen et al 1994), with surprisingly little reference to the residual amounts in the mature, reproductive organs, such as the economically important grains (Bernardin et al 1994). For the growing grain, the synthesis of heat-inducible proteins can most readily be demonstrated by the incorporation of ^{35}S-amino acids into grain proteins, followed by autoradiography. The results of such an experiment are shown in Fig. 1 for wheat grain from plants that have been subjected to heat stress at 38°C for 24 hr, after the provision of ^{35}S methionine. For the immature grain from heat-shocked plants, there was the sudden appearance of new bands due to the synthesis of the heat-shock proteins. Most evident was a pair of bands, whose migration corresponds to molecular weight values of 68,000 and 72,000, which we shall refer to interchangeably as the "HSP 70 family" and "HSP 70". Image analysis of fluorographs of SDS gels, for the full set of 10 wheats examined, showed that the increase in this region of the pattern ranged from 35 to 140% (Table I).

In addition, there were increases in the density of the fluorograph for the region corresponding to the mobilities of the HMW-GS (Fig. 1) in the case of immature grain from the line ME 71 and the cultivar Hartog (Table I). However, grain from most genotypes showed a marked decrease in ^{35}S uptake for the HMW-GS region of mobility, providing further evidence for Hypothesis 1. Because we were seeking information about genotypes that might be tolerant to the loss of dough strength due to heat stress, the genotypes ME 71 and Banks were selected for further study as being par-

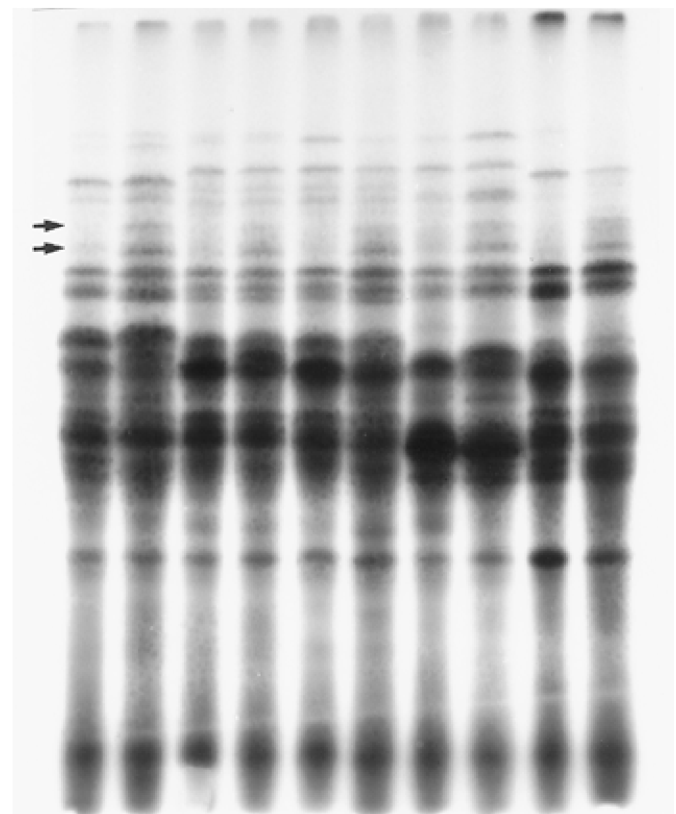

Fig. 1. Fluorograph of an SDS electrophoresis gel showing the uptake of ^{35}S following a brief heat stress of developing grain. In each pair of patterns, the control is on the left, the pattern for the heat-stressed sample is on the right. Lanes: Hartog (1,2), Sunco (3,4), Banks (5,6), ME 71 (7,8), Pinnacle (9,10). Arrows indicate the positions of the proteins with apparent molecular weights of 68,000 and 72,000 that appear after heat stress.

ticularly tolerant and susceptible to heat stress, respectively, on the basis of the criterion of synthesis of HMW-GS polypeptides during heat stress (Table I).

Nucleotide Sequencing for HMW-GS Genes

For gene-sequencing studies, these two genotypes (ME 71 and Banks) were used, together with the two (line 6386 and cultivar Wyuna) that had been the most tolerant and most susceptible to heat stress, respectively, in an earlier survey of the modification of dough properties by heat stress for 45 genotypes (Blumenthal et al 1995). The specific aim of this sequencing experiment was to determine whether the wheats with contrasting tolerance to heat might also differ with respect to the presence of heat-stress promoters upstream of the coding regions for the glutenin genes, a finding that could be consistent with the observation of continuing glutenin synthesis (or lack of it) during heat (for ME 71 and Banks), or the contrasting reactions to heat of 6386 and Wyuna.

The *Glu-1* alleles, thought to be derived from *T. tauschii*, were characterized using primers as described by Mackie et al (1996). Two PCR products were obtained using these primers. Sequence analysis of the large and small PCR products revealed conservation between these sequences in the four genotypes that we examined, as well as identity to the Bx17 and Bx7 sequences previously described (Reddy and Appels 1993).

The smaller PCR fragment lacked the "-300 bp" sequence, that was reported by Kreis et al (1985) to be located at approximately -450 bp in the expressed HMW-GS genes, and which is believed to be involved in tissue-specific expression. In addition, the smaller PCR product had an 11-bp deletion from - 689 to -700.

The larger PCR fragment contained a 53-bp duplication that is absent from the smaller PCR product. This 53-bp sequence has been absent in the silent Ay gene of Cheyenne (Anderson et al 1989).

These two sequences, from the four genotypes examined, showed no differences (such as heat-stress elements) that could be construed as explaining the observed differences in the reactions of these wheats to heat stress. It thus seemed more likely that heat-inducible proteins such as the HSP 70 chaperone family and other HSP may play a role in the changes seen as a result of heat stress.

TABLE I
Changes in the Synthesis of Wheat Endosperm Proteins Due to Heat Stress[a]

Genotype[b]	Region of Mobility for HSP 70 Family	Region of Mobility for High Molecular Weight Glutenin Subunits
Sunco	+35%	–20%
Hartog	+40%	+5%
Sonalika	+50%	–25%
Trigo 3	+60%	–5%
Banks	+65%	–30%
Pinnacle	+75%	–35%
Kalyansona	+80%	0
Trigo 1	+85%	0
ME 71	+90%	+15%
Lyallpur	+140%	–10%

[a] Expressed as % of ^{35}S uptake for the mobility region in the control samples.
[b] Arranged according to the increase in heat-shock protein 70 (HSP 70).

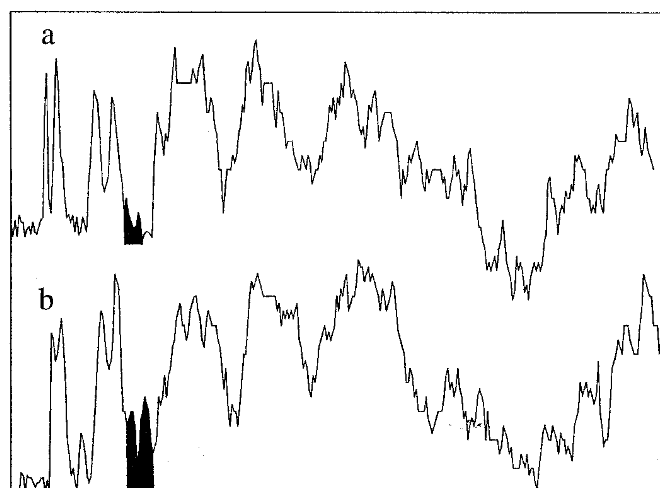

Fig. 2. Scans of a Coomassie-blue-stained gel following SDS-gel electrophoresis of grain proteins (extracted with SDS buffer [pH 6.9]) containing mercaptoethanol) from control plants (a) and from plants of mature Hartog wheat that had been heat-stressed (b) throughout grain filling. Family of heat-shock 70 proteins is indicated in solid black.

Synthesis of the HSP 70 Family and Tolerance to Heat Stress

The appearance of this group of HSP bands has been reported in several studies of the effects of heat stress, especially in tissues other than the endosperm (Hendershot et al 1992, Weng and Nguyen 1992, Joshi et al 1996). Due to their prominence in the profile of the developing wheat endosperm, attention has focused on them as a possible cause of the quality changes associated with heat stress (Bernardin et al 1994). One approach to testing this possibility was to determine the rate of their synthesis during a heat shock, and to see if this index related to the extent of heat-related change in the dough properties of the mature grain. Accordingly, the proportion of this pair of HSP was determined for a set of 10 genotypes (studied by Blumenthal et al 1994) by image analysis of X-ray films from autoradiography following the SDS-PAGE of a reduced extract of the developing grain, comparing heat-stressed with control grain samples. The fluorograph for five of these is shown in Fig. 1. The change in film opacity was analyzed with respect to the total opacity of the full lane.

Compared to the control (unstressed samples, C), heat stress (HS) produced considerable increases in the proportion of radioactivity incorporated in the region of the film corresponding to the HSP 70 family for all these genotypes (ranging from 35 to 140% over the control samples) (Table I). This increase was greatest for Lyallpur, and least for Sunco. The increase (HS – C) in the amount of the HSP 70 family correlated significantly with changes in the protein content of the mature grain ($r = 0.78$, $P < 0.05$). However, the rates of HSP synthesis thus determined were not significantly correlated with any other of the heat-associated changes in the mature grain or flour samples, including the range of dough properties reported by Blumenthal et al (1994).

Quantitation of HSP in Mature Grain (Maximum Limits)

Although it appeared to be a reasonable proposition to attempt to relate changes in quality attributes to the rate of HSP synthesis, it is most obviously the amount of HSP remaining in the mature grain that is likely to contribute directly to quality changes (according to Bernardin's hypothesis), especially since the rate of breakdown of HSP will be as important as their rate of synthesis.

An assessment of the maximum amount of HSP 70 that might be expected in mature grain was sought by subjecting wheat (Hartog) to daily maxima of 40°C for 8 hr during every day after the first week of grain filling. Cool (15°C) night temperatures prevailed, and plants were given an adequate supply of water. The HSP 70 pair of bands could be seen clearly in a Coomassie blue-stained SDS-PAGE of a reduced extract of the mature grain from the heat-stressed plants. This region of the electrophoretic pattern is indicated in solid black in the scans of the gel patterns shown in Fig. 2. There was some protein at this position for the control grain extract (also marked in Fig. 2), but much less than for the heat-stressed sample. This material in the control pattern is probably a combination of other (background) proteins with similar mobility, plus some HSP 70, since it is reportedly a constitutive protein (Bednarek and Raikhel 1992, Buchner 1996).

Quantification of the amount of protein at the position of the HSP 70 family of bands indicated that this material accumulates

to the extent of 0.06% of the grain protein in the Hartog wheat under the extreme conditions of heat stress used in this experiment, corresponding to 0.01% HSP 70 on a grain-weight basis. This figure could be reduced slightly to allow for the possibility that part of the small amount of protein in this part of the pattern for the control sample was not HSP 70. In addition, of course, the extreme conditions of heat stress for almost every day of grain filling are unrepresentative of field conditions.

Quantitation of HSP in Mature Grain (Variation Among Genotypes)

Specific detection and quantification of the HSP 70 family in many grain samples was rendered more efficient by using a monoclonal antibody to human HSP 70. This antibody reacted exclusively with the smaller protein (HSP 68) of the pair of HSP 70 bands but not with any other of the wheat-grain proteins in the SDS gel pattern, as reported by Bernardin (1994), and as demonstrated by us using the antibody in an immunoblotting experiment, after SDS-PAGE of a reduced extract of mature grain from heat-stressed plants.

This immunological method was lent greater convenience by developing it into a dot-blot method, which was used for more extensive screening for the presence of HSP 68 in mature grain. Figure 3 shows that there was more of the HSP in the heat-stressed (HS) grain (40°C for three successive days, applied at about mid-grain filling) than in controls for most of 45 genotypes that were characterized by Blumenthal et al (1995) for heat tolerance.

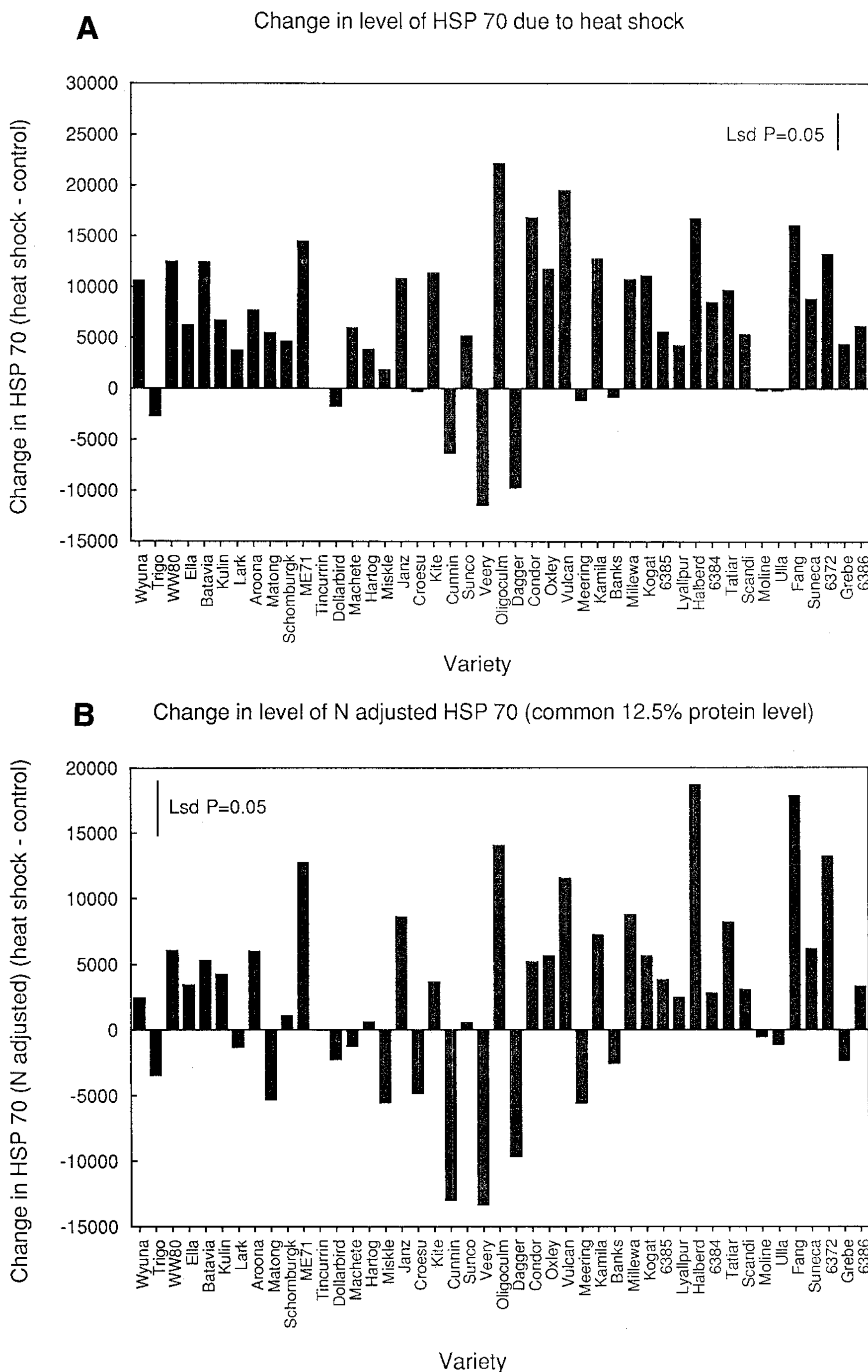

Fig. 3. Changes in the amounts of heat-shock proteins (HSP 70) family in mature grain of 45 wheats examined (Blumenthal et al 1995), expressed as the difference heat-shock (HS) grain minus control (C), based on dot blots with a monoclonal antibody to human HSP 70. **A,** Differences (HS – C) in HSP based on flour dry weight. **B,** Adjusted to a constant flour-protein content (12.5%). Wheats are arranged from most tolerant (6386) to most susceptible (Wyuna) to the effects of heat stress on mixograph mix time (Blumenthal et al 1995, 1996).

Differences (HS – C) in the amount of the antibody-quantified HSP were calculated both on the basis of % in flour (Fig. 3A) and normalized (to 12.5% flour protein) for variations in protein content (Fig. 3B). The wheats are arranged across the two histograms (Fig. 3) in order of their tolerance to the effects of heat stress on dough strength (as change in time to peak in the mixograph), but there was no clear relationship to the relative presence of HSP at maturity. Furthermore, the wheats showing a decrease in HSP 68 were not those that were outstandingly tolerant or susceptible to heat stress in the previously reported assessment of their reactions to heat (Blumenthal et al 1995). There was no correlation between the amount of HSP 68 (adjusted to equivalent protein level) and any of the quality attributes reported by Blumenthal et al (1995). For HSP 68 on a flour-weight basis, only dough-mixing time showed any significant correlation ($r = -0.37$, $P < 0.05$) to the amount of HSP. Nevertheless, this modest correlation is in the direction predicted by the hypothesis: an increase in HSP makes the resulting dough weaker (shorter time to peak development).

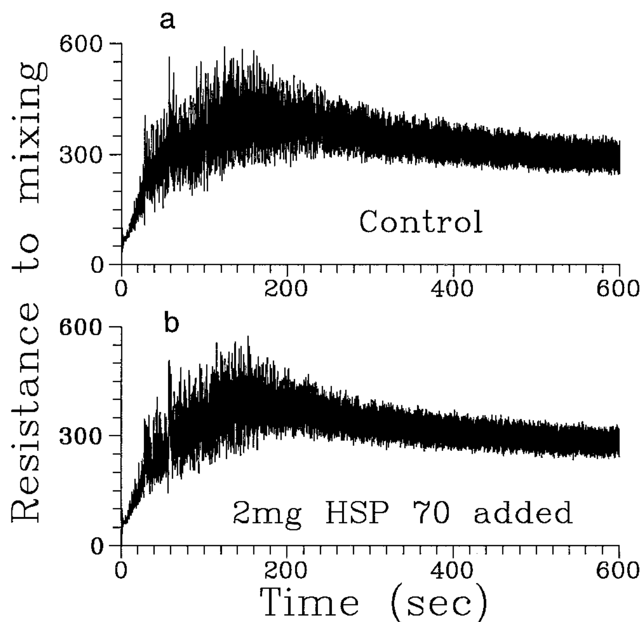

Fig. 4. Mixing traces for the base flour (a) and for the base flour with the addition of 2 mg of purified heat-shock proteins (HSP 70) (b).

TABLE II
Dough Quality After Addition of Several Pure Proteins (as measured in 2-g mixograph)

Protein (mg)	Mix Time (sec)	Peak Resistance (arbitrary units)	Resistance Breakdown (%)
HSP 70[a]			
0	179b[b]	344ab	17.5ab
2	169b	340ab	20.0c
6	138a	353ab	50.0d
Hemoglobin			
0	186b	345ab	15.5a
2	180b	361b	20.0c
6	182b	352ab	15.5a
Ovalbumin			
0	187b	345ab	15.5a
2	188b	332a	18.0bc
6	186b	346ab	18.0bc
Water-soluble flour protein			
0	187b	345ab	15.5a
2	166b	342ab	19.0bc
6	176b	338ab	19.5bc
LSD[c]	24.1	27.8	2.3

[a] Heat-shock protein 70.
[b] Values followed by different letters are significantly different ($P < 0.05$).
[c] Least significant difference ($P < 0.05$).

Purification of HSP 70

In a more direct approach to elucidating the possible role of heat-shock proteins in dough, an ATP affinity column was used to purify HSP 70 from the mature grain of Hartog wheat that had been heat-stressed daily during grain filling. Three protein components were obtained, as in the results of Giorini and Galili (1991), whose method we followed. These had apparent molecular weights of 35,000, 38,000, and 68,000, based on their migration rates on SDS-PAGE compared to standard proteins. N-terminal amino-acid sequencing of the three ATP-binding proteins revealed the following sequences:

1. The 35,000 protein had the N-terminal amino-acid sequence (N/S/G/R)KIKIGINGFGRIGR (using the single-letter designation for amino acids). A homology search revealed this sequence was identical to the first 15 residues of cytosolic glyceraldehyde-3-phosphate dehydrogenase in maize (*Zea mays* L.), GKIKIGINGFGRIGR.

2. N-terminal amino-acid sequencing of the protein with apparent molecular weight of 38,000 provided the sequence (A/G)PIKIGINGFGRIGR, identical to the first 15 residues of glyceraldehyde-3-phosphate dehydrogenase in garden snapdragon (*Antirrhinum majus*), APIKIGINGFGRIGR.

3. Sequencing of the 68,000 protein extracted from the heat-shocked flour revealed the sequence AKGEGPAIGIDLGTT-YSXV, which is almost identical to the N-terminal amino-acid sequence of HSP 70 of maize. The unidentified amino acid ("X") in this sequence is probably cysteine, as it is conserved in all the available sequences of HSP 70.

Given that the sequence of this last component indicates that it is an HSP, it was taken to be a suitable preparation for use in the proposed dough studies. It was further purified, and prepared in quantity for experiments involving additions to dough, taking care in its preparation to avoid possible denaturation (MacRitchie 1980).

Dough-Mixing Experiments

This HSP preparation was evaluated in dough-mixing experiments using the direct-drive 2-gram mixograph (Rath et al 1990). In designing this set of experiments, we chose 0.10% as the level of HSP addition to flour to test the upper limit of HSP levels possible in mature grain. This is 10× the level (0.01%, grain-weight basis) that we measured in severely heat-stressed Hartog. In deciding on this level, we were acknowledging the finding that Hartog was one of the more conservative genotypes with respect to the synthesis of HSP 70 in the developing grain compared to nine others (Table I). Accordingly, 2 mg of purified HSP was used with 2 g of base flour. In addition, to ensure maximum opportunity for a result for the HSP, a few experiments were also performed with 6 mg of HSP 68 in 2 g of flour (30× the level indicated in the Hartog grain). Figure 4 shows the mixing traces obtained for the base flour with and without the addition of 2 mg of purified HSP. Similar mixing properties were obtained whether HSP was included in the dough by simple addition or by incorporation into the gluten matrix (by sequential partial reduction of SS bonds and subsequent re-oxidation).

Table II shows the results for one of these approaches, the addition of the HSP preparation to the dough. The level of 0.1% (2 mg of HSP to 2 g of flour) caused no statistically significant changes in mixing time or in peak resistance, using comparison of means by Student's *t* test. Changes in breakdown after the peak were significant. All these changes were much greater for a 6-mg addition of HSP, but this treatment was acknowledged to be well beyond physiological conditions. However, these changes in the mixing profile were similar to those that resulted from the addition of other water-soluble proteins, such as hemoglobin, ovalbumin, and water-soluble flour proteins at the same levels. We, therefore, concluded that there was no HSP 70-specific effect on dough properties, but that the inclusion of excess quantities of hydrophilic protein appears to alter dough behaviour slightly, as reported by Wrigley et al (*in press*).

CONCLUSIONS

Genotypes differed in the synthesis of HMW-GS and HSP during heat stress. The extent of the increase in HSP 70 varied among 10 genotypes from 35 to 140% (for heat-stressed developing grain as compared to the control samples).

The concentration of HSP 70 remaining in mature grain increased as a result of a few days' heat stress of wheat plants (for most of a large number of genotypes examined), due to synthesis at the time of stress, and to incomplete breakdown thereafter.

The amount of HSP 70 in mature grain samples from heat-stressed plants of 45 genotypes was not significantly correlated with any of several indicators of dough strength, apart from a correlation at $P < 0.05$ between the time to peak mixing and the amount of HSP 70, when HSP content was expressed on the basis of flour dry weight (not if based on % in flour protein). This relationship was much weaker than that for the other molecular hypotheses: glutenin-to-gliadin ratio, size distribution of the glutenin polymer, and the involvement of HSP and chaperones during grain filling.

HSP 70 added to (or incorporated into) dough behaved similarly to several other hydrophilic proteins added at a level of 2 mg to 2 g of flour. HSP 70 showed no dramatic effects on dough properties that could constitute a major explanation for the dough-weakening effects of heat stress, even though this level of addition (2 mg) was well above the maximum levels that might be encountered in field-grown mature grain.

Sequencing of the first 800 base-pairs upstream of the genes for HMW-GS failed to show the presence of heat-shock promoters, even for genotypes that differed considerably in their reactions to heat stress.

Failure to identify heat-stress elements upstream of the coding regions of the HMW-GS genes does not necessarily exclude the possibility of incorporating such promoters into these genes as a means of alleviating the heat-associated decrease in glutenin level (with respect to the level of gliadin). The findings simplify the range of possibilities that cause heat-related loss of dough strength, focusing attention on the degree of polymerization of the glutenin chains, and on the roles of HSP and chaperones in the developing grain. It is likely that the HSP modify the folding and aggregation of gluten proteins in situ during grain filling, especially during stress situations, thereby altering their dough-forming potential, especially since HSP have been implicated in these functions in other organisms (Pelham 1986, Howarth 1991, Parsell et al 1994, Hendershot et al 1996, Hesterkamp et al 1996).

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