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**Development of Assay Systems for Bioengineering
Proteins that Affect Dough Quality and Wheat
Utilization**

O.D. Anderson, G. Galili

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Investigators:
(Principal first)

Olin D. Anderson

Gad Galili

Affiliated Institution:

Agric. Res. Service, US Dept. Agric.

Weizmann Institute, Israel

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Olin D. Anderson
 Signature:
 Principal Investigator

WJH

E. L. Civerdo
 Signature:
 Authorizing Official, Affiliated Institution

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ABSTRACT

The quality and utilization of wheat is largely dependent upon the exact physical/chemical properties of the doughs made from flour/water mixtures. Among the wheat seed components most correlated with dough viscoelastic parameters are the high-molecular-weight (HMW) glutenin subunits whose disulfide cross-linked macropolymer is critical for dough functionality. We have used the tools of molecular biology, wheat transformation, heterologous expression of HMW-glutenin subunits in bacteria, and dough micro-mixing experiments to examine some of the molecular basis of HMW-glutenin functionality. In addition, we have developed sets of modified and synthetic gene constructs and transgenic wheat lines that will allow further examination of the role of the HMW-glutenins. Among the results from this work is evidence⁽¹⁾ that the HMW-glutenin repeat domain is directly related to dough properties, the demonstration⁽²⁾ that interaction between subunits is dependent upon domain presence but not order, a novel⁽³⁾ understanding of the restrictions on intra- vs inter-chain disulfide bonds, the demonstration that⁽⁴⁾ HMW-glutenin genes can be transformed into wheat for simultaneously high expression of the transgene and suppression the endogenous genes, and the construction of a set of modified⁽⁵⁾ HMW-glutenins capable of being cpitope tagged for studying polypeptide subcellular processing and the fate of HMW-glutenins in dough mixing experiments.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

General objectives: 1) Develop lines of wheat with defined transgenic HMW-glutenin genes in a background of specific glutenin expression. 2) Analyze the assembly of transgenic native and modified HMW-glutenins into the glutenin matrix. 3) Continued development of heterologous expression systems as an integral part of wheat protein bioengineering programs.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

Objective #1: Develop lines of wheat with defined transgenic HMW-glutenin genes in a background of specific glutenin expression.

It was decided by the Principle Investigators to carry out transformation experiments in the highly regenerable hexaploid wheat cultivar Bobwhite, and then use these transformants to cross transgenic HMW-glutenin loci into whatever genetic background was appropriate for specific experiments. Additional copies of the native Dx5 and Dy10 HMW-glutenin genes have been transformed into wheat (not shown) and are available for further work under this objective. These lines are now being crossed into additional genetic backgrounds to test the effects of overexpressing specific HMW-glutenin subunits. As an example of the types of experiments and questions these lines can address is the question of the effect of overexpressing a single subunit. This can be addressed both by direct study of existing lines and by attempting to pyramid multiple transgenic loci for a specific gene in order to test a series of increasing dosage and the effect on formation and organization of the gluten matrix.

In addition, an unexpected result from several transgenic lines present some unique opportunities. The basis of this original objective was to produce wheat lines expressing defined numbers of specific HMW-glutenin genes. The example of producing a wheat line synthesizing only the transgenic subunits would require either developing transformation for a wheat line null in all HMW-glutenin expression, or crossing the transgenic loci into a null line. Figure 1A shows a Dy10 subunit transgenic wheat line where the Dy10 are expressing at a high level while the endogenous HMW-glutenin genes are evidently suppressed. Another line (Fig. 1B) likewise expresses transgenic Dx5 + Dy10 genes but not the endogenous genes. We have shown (A. Blechl, unpublished) that this cosuppression effect in such lines is dominant and is thus ideal for the original Objective #1: the transgene locus, expressing a specific HMW-glutenin (s) can be crossed into any genetic background and evidence the specific HMW-glutenin expression pattern in the first generation. The above mentioned lines are being multiplied and the transgenic loci crossed into other genetic backgrounds.

Objective #2: Analyze the assembly of transgenic native and modified HMW-glutenins into the glutenin matrix.

The purpose of this objective was to test the utility of studying the effect of transgenic native and modified HMW-glutenins on the formation and structure of the disulfide cross-linked gluten matrix. The basic rationale was that by perturbing existing matrix patterns novel insights into glutenin structure/function relationships to dough visco-elastic properties would be obtained. Two major published accomplishments resulted thus far (reprint Attachment A). Number one is the first demonstration that transgenic HMW-glutenin genes

will express glutenin subunits that incorporate into the disulfide cross-linked matrix in a similar pattern with endogenously encoded subunits. Until this was demonstrated it was unknown if the transgenically encoded subunits would synthesize and undergo appropriate post-translational processing. The second result was the unexpected finding that the N- and C-terminal non-repetitive domains of a single HMW-glutenin subunit can form an intramolecular disulfide bond. Such a bond has not been previously observed and commonly believed to be not allowed because the repetitive central domain of the HMW-glutenin subunit formed a rigid enough structure to prevent such a disulfide bond from forming. The finding that such a bond can form has resulted to two novel suggestions on the formation of the glutenin macropolymer forms: 1) The normal lack of N- to C-terminal intramolecular disulfide bonds may be due to selection against the affinity of available cysteine residues to form such bonds and not physical constraints. 2) The central repetitive domain cannot be strictly a rigid rod, but must either be flexible or at least have one or more "hinge" structures to allow the two termini to come into proximity.

A second line of experiments under this objective was to modify one or more HMW-glutenin genes to allow post-translational monitoring of a specific glutenin during subcellular transport, protein body deposition, and glutenin macropolymer formation. The proposed strategy was divided into three stages, the engineering of unique restriction sites bracketing the stop codon in HMW-glutenin genes, the use of these new sites to replace the existing gene terminus with a terminus encoding an added 11-17 aa epitope as the specific polypeptide marker, and finally, the transformation and analysis of transgenic wheat lines. After numerous technical problems (likely related to PCR replication errors in using genes with

repetitive motifs) the first stage is now complete. Figure 2 shows two HMW-glutenin genes with added restriction sites: based on the hybrid gene described by Blechl and Anderson (1996) (Fig. 2A) and the native Dy10 gene (Fig. 2B). In both cases the gene has been mutated at 3 bases to produced unique rare 8-bp restriction recognition sites for the enzymes *FseI* and *SseI* near the stop codon. Experiments are continuing to insert the reporter peptide for use in studying subcellular processing. Besides efforts in the Principle Investigators laboratories, other laboratories have queried for the availability of these constructs for a number of similar and unrelated studies, and will be made available as soon as feasible.

Objective #3: Continued development of heterologous expression systems as an integral part of wheat protein bioengineering programs.

The purpose of this objective was to continue the development of an *E. coli* heterologous expression system to produce HMW-glutenin polypeptides of native and modified amino acid sequence for use in structure/function studies. The collaborative work was begun under a previous BARD grant and has been continued between grants and with a continuing collaboration with scientists at CSIRO, Sydney in testing bacterially produced HMW-glutenins in dough micro-mixing protocols (Dr. Frank Bekes and Dr. Peter Gras). The rationale has been to test effects of modifications of the HMW-glutenin polypeptide on dough properties. As an example of the utility of this approach, we have shown that the Dx5 and Dy10 HMW-glutenin subunits interact in a synergistic manner to effect dough property. The genes for these two subunits are highly linked and highly correlated with dough properties. However, traditional research approaches have not been able to study the effects of the

subunits individually since the two genes have never been genetically separated. Results from other laboratories had previously suggested a specific interaction of these two subunits, but could not address the effect of this interaction on functionality. The results of this objective have not only allowed us to separate the effects of the subunits, but allows novel gene/subunit manipulations. Previously we had shown (in collaboration with Dr. Frank Bekes, CSIRO) that the Dx5 subunit had a greater positive effect on dough mixing time than the Dy10 subunit. Figure 3 (and reprint Attachment B) shows that not only do the Dx5 and Dy10 have more of an effect on mixing time when together, but the synergistic effect can even be seen when domains of the two subunits are exchanged; i.e., as long as all the domains of the two polypeptides are present, the synergistic effect occurs. This opens the door to future experiments to determine exactly which are the critical domains and specific amino acid residues.

Another major accomplishment under this objective was the completion of a completely synthetic HMW-glutenin gene designed for easy modification and high levels of bacterial expression (reprint Attachment C). Features of the synthetic gene include: preferred codon usage consistent with high levels of protein synthesis in *E. coli*; incorporation of regular unique restriction sites to allow easy future modifications; modular assembly of unique, terminal domains, and the central, repetitive domain; and a protocol for creating repeat domains of any specified length. In addition to the initial gene, other constructs derived by the same procedures are shown in Figure 4, and encode a series of HMW-glutenin polypeptides with the terminal domains and repetitive domains composed of 0, 16, 32, and 48 copies of the single motif PGQGQPPYYPTSPQQ. In these initial constructs, a single

repetitive motif was used to construct the central, repetitive domain of the synthetic HMW-glutenin, in contrast to the natural pattern of interspersed variants of 2-3 motifs. This series demonstrates the advantage of using this approach: the terminal domains and the composition of the repetitive domains are all held constant while varying only the length of the repetitive domain. After expressing these genes in *E. coli* and isolating the HMW-glutenin subunits, a set of micro-dough-mixing experiments were carried out to test the effect of repeat length on dough "mixing time," a common parameter used to test dough strength. Figure 5 shows that there is a clear relationship between dough strength and length of the repetitive domain, the first direct demonstration of such a relationship between a dough macro-property and a specific polypeptide feature. The termini of these synthetic genes were based on the amino acid sequence of the natural Dx5 HMW-glutenin. A second series of repeat length variants were constructed by Dr. Renato D'Ovidio (University of Tuscia, Italy) in a collaboration also interested in HMW-glutenin domain functionality. This second series also used the natural Dx5 gene and constructed several repeat length variants using the natural repeat motif structure. When the two sets of Dx5 repeat variants (uniform, synthetic single repeat, or interspersed variants of three natural repeat motifs) are compared in micro-mixing experiments, the results are plotted in Figure 6. Both sets of constructs show the near linear relationship between mixing time and repeat length of the added HMW-glutenin subunits synthesized in bacteria and is the basis for the Patent Application shown in Attachment D ("Confidential"). More surprising was that there was no apparent difference of the effect using either repeat structure. Previously it had been theorized that the different motifs in the repetitive domain each served functions in the higher order structure of the glutenin

macropolymer. This must now be re-examined in light of our findings and suggests other purposes of more than one motif; i.e., the DNA encoding a single motif might be unstable during DNA replication while 2-3 interspersed repeat motifs are less likely to undergo rapid unequal cross-overs or slip-mismatching of the DNA.

Similar to as described under Objective 2, a unique restriction site (*FseI*) has been engineering just upstream of the stop codon for two repeat length variants of the Dx5 bacterial expression construct (Figs. 7A and 7B). When combined with the unique *StuI* restriction site found in all HMW-glutenin genes near the signal peptide encoding DNA, these *FseI* sites will allow swapping of intact HMW-glutenin coding regions between bacterial and plant expression constructs. Thus, a new coding region can be quickly checked for integrity in the bacterial system, and if appropriate, be easily moved into a plant expression context.

DESCRIPTION OF THE COOPERATION

The transformation experiments for producing wheat lines expressing specific HMW-glutenin subunits were carried out in the US laboratory. Crosses with lines into other genetic backgrounds for further study have been, and are ongoing, in both the US and Israeli laboratory. Analysis of assembly of transgenic glutenin subunits into the glutenin macropolymer were performed in Israel. Planning for the construction of tagged glutenins was a collaboration between the two laboratories, and the plasmid constructs were prepared in the US. Both laboratories are further modifying these constructs to tag specific glutenins for sub-cellular monitoring experiments to be carried out in Israel. The synthetic HMW-glutenin constructs were

made in the US and are starting to be distributed to other laboratories (University of Bristol, United Kingdom; University of Groningen, Netherlands).

EVALUATION OF THE RESEARCH ACHIEVEMENT

This project proposed to develop knowledge, tools, and resources to form a broad approach to the eventual creation of novel and improved wheat cultivars engineered for enhanced dough processing characteristics. Progress and degrees of success have been achieved with all three objectives thus far, and further individual and collaborative work between the two laboratories will continue based on this progress. Approach #1 to Objective 1 was dropped in favor of stated Approach #2 when the Principle Investigators decided the effort to develop transformation protocols for lines null in some or all HMW-glutenin loci was relatively inefficient. The alternative strategy that was adopted is to simply cross transgenic loci into appropriate genetic backgrounds and back-cross. Such experiments on ongoing by the two laboratories and recently additional such experiments in other, collaborating, laboratories has begun. In addition, the fortuitous discovery that some of the transgenic lines are suppressing endogenous loci in trans while overexpressing the transgenic locus will provide a faster approach than previously conceived. These initial transgenic lines are still being multiplied in preparation to begin physical/chemical analysis of dough properties, and crosses to other genetic backgrounds are underway. Work on Objective Two will be ongoing. Not all of the original proposed experiments have been completed, but the initial analyses published in Shimoni et al. (Attachment A) has already changed our views of HMW-glutenin higher order structure and disulfide bond formation. In addition, a collaborator (Dr. Dr. Kasarda, ARS, Albany, CA) has

used the finding of HMW-glutenin monomers to begin an analysis on the intramolecular disulfide bond formation of HMW-glutenins, an analysis previously not possible because all subunits normally are part of the complex glutenin macropolymer structure. Under Objective Three the synthetic HMW-glutenin gene was successfully completed (Attachment C) and a further set of genes constructed (Fig. 4). Previous and new modified natural gene sequences are still being tested for effects on mixing parameters by the collaborators at CSIRO, but the results have already shown direct correlations between HMW-glutenin polypeptide structure and dough properties (Fig. 6).

CONCLUSIONS

Progress under this collaborative grant has shown the utility of a multi-faceted approach to understanding wheat gluten structure and function. Both planned experiments and the use of novel results has provided new insights into HMW-glutenin structure and the relationship to dough macro-parameters. The collaboration between the U.S. and Israeli laboratories has allowed them to synergize their efforts in this important applied research area. Although the official term of the project has ended, it is anticipated that not only will the two laboratories will be able to continue collaborating in this area, but other laboratories, worldwide, will benefit from the resources and information developed. Among the research results are new insights into HMW-glutenin structure, disulfide bond formation, contributions of specific polypeptide motifs to dough properties, and the generation of novel transgenic wheat lines expected to yield more information of the glutenin macropolymer. In addition, constructs for tagging the HMW-glutenin polypeptide are now ready to proceed to follow, for the first time, a specific wheat

gluten polypeptide in post-translational processing and macropolymer formation. Although additional resources are always desired, the Principle Investigators believe there are now on hand sufficient resources and protocols to make substantial progress in understanding the molecular basis of HMW-glutenin polypeptides' contributions to dough visco-elastic parameters.

LIST OF PUBLICATIONS

- Shimoni, Y., Blechl, A.E., Anderson, O.D., and Galili, G. (1997) A recombinant protein of two high molecular weight glutenins alters gluten polymer formation in transgenic wheat. *J. Biol. Chem.* 272:15488-15495.
- Anderson, O.D., Kuhl, J.C., and Tam, A. (1996). Construction and expression of a synthetic wheat storage protein gene. *Gene* 174:51-58.
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- Blechl, A.E., Le, H.Q., Bekes, F., Gras, P.W., Shimoni, Y., Galili, G., and Anderson, O.D. Applications of molecular biology in understanding and improving wheat quality. Proceedings of the International Wheat Quality Conference. Manhattan, KS. 1997. (accepted May, 1997).
- Blechl, A.E., Le, H.Q., Anderson, O.D., McCue, K., Gras, P.W., Bekes, F. Flour improvement by genetic engineering of wheat. *Proceedings of 7th International Symposium on Seed Proteins of Plants. J. Plant Physiology.* (accepted June, 1997).

Blechl, A.E., Anderson, O.D., and Bekes, F. Wheat quality improvement via biotechnology.

Proceedings of the International Wheat Quality Conference. Manhattan, KS. (accepted May, 1997).

PATENTS

Anderson, O.D. Patent Application Serial No. 08/706,391, "Altering dough viscoelasticity with modified glutenins." (filed with PTO August 30, 1996, action pending - Confidential).

Figure 1. HMW-glutenin subunit expression accompanied by suppression of endogenous genes. SDS-PAGE gels of seed extracts from plants transformed with plasmids bearing the natural Dx5 and Dy10 HMW-glutenin subunit genes show that occasionally transgenic lines are obtained where the endogenous HMW-glutenin genes are suppressed. Positions of the transgenic Dx5 and Dy10 subunit bands are shown on the outside of each figure. A) Regenerated wheat lines transformed with the Dy10 gene. Dots to the right of the control (BW) lane indicate the positions of the endogenous HMW-glutenin subunit bands on a protein gel. Diamonds indicate the highly synthesized Dy10 subunit. Lanes 1 and 2 show lines expressing both the transgene and the endogenous HMW-glutenin genes. Lanes 3a and 3b show lines expressing the transgene but suppressing the endogenous genes. B) Regenerated wheat lines co-transformed with the Dx5 and Dy10 genes. Pyramids indicate the transgenic bands. Lane a is the control. Lanes b and e are transformed lines not expressing additional genes. Lanes c and d show two lines overexpressing Dy10 and Dx5 genes but not the other endogenous HMW-glutenin genes.

Figure 2A. Restriction site engineering in HMW-glutenin genes. Restriction map is shown for the GluY(H)X hybrid gene of Blechl and Anderson (1996). Unique *Fse*I and *Sse*I restriction sites were engineered to bracket the stop codon.

Figure 2B. Restriction site engineering in HMW-glutenin genes. Restriction map is shown for the natural Dy10 gene. Unique *FseI* and *SseI* restriction sites were engineered to bracket the stop codon.

Figure 3. Synergistic effect of HMW-glutenin subunits Dx5 and Dy10. The effects of dough mixing time were tested by supplementing 2g of dough in a Mixograph test. Equal amounts of bacterially produced HMW-glutenin subunits were used in each test. Results showed that using Dx5 and Dy10 subunits together caused more dough strengthening than either subunit alone. Similar results were obtained using hybrid subunits where the N-terminal domains of the Dx5 and Dy10 subunits are exchanged *via* DNA manipulation of their genes.

Synthetic HMW Glutenin Gene Constructs

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Figure 4. Synthetic HMW-glutenin gene construct encoded polypeptides. A) Four constructs are based on the Dx5 subunit. The encoded termini are identical to the Dx5 subunit and the repeat domain is composed of 0, 16, 32, and 48 copies of a 15 amino acid consensus motif (PGQGQQGYPTSPQQ). B) The 32 and 48 copy repeat domains were inserted into an expression vector designed to express the repeat domains with or without two adjacent cysteine residues on each termini.

Figure 5. Dough micro-mixing experiments using HMW-glutenin subunits encoded by synthetic genes. The 2 g Mixograph was used to measure dough resistance after supplementation with HMW-glutenin subunits isolated from the bacterial expression system. The synthetic gene series all included the identical terminal domains of the natural Dx5 subunit, but contain 0, 16, 32, or 48 copies of a single repeat motif. The same experiment with a flour extracted Dx5 subunit preparation is shown for comparison. Results are shown as percent increase in mixing time relative to the Dx5 subunit.

Figure 6. HMW-glutenin repeat length increases dough mixing time. The results of HMW-glutenin supplementation of 2g of dough is plotted versus the repeat size for all HMW-glutenin gene constructs containing the Dx5 termini. This includes the natural Dx5 sequence (6), three variants of the natural sequence with different sized repeat domains (3,5,8), and the four different synthetic gene constructs (1,2,4,7). Since the experiments were carried out using different base flours, the raw scores were not comparable. However, the natural Dx5 subunit was included in all experiments and all scores were normalized to Dx5 and represented as a percent of the Dx5 subunit control for each experiment.

Figure 7A. Restriction site engineering in HMW-glutenin bacterial expression constructs. A unique *FseI* restriction site was engineered into the bacterial expression vector pET3a containing a variants of the Dx5 gene encoding a 441 amino acid repetitive domain.

Figure 7B. Restriction site engineering in HMW-glutenin bacterial expression constructs. A unique *FseI* restriction site was engineered into the bacterial expression vector pET3a containing a variant of the Dx5 gene encoding a 853 amino acid repetitive domain.