J. Plant Biotechnology *Vol. 1. pp.13~19*

Journal of Plant Biotechnology

Changing Wheat Quality with the Modification of Storage Protein Structure

László Tamás[1,3]*, Ferenc Békés[2], Matthew K. Morrell[3], Rudi Appels[3]

*[1]Eötvös Loránd University, Budapest, H-1445 PO Box 330, Hungary, tamasl@ludens.elte.hu; [2]CSIRO Plant Industry, Sydney, North Ryde, NSW 2113, Australia; [3]CSIRO Plant Industry, Canberra, ACT 2601, Australia (*author for correspondence)*

Key words: dough testing, gene manipulation, protein expression, storage proteins, structure/function relationships, wheat quality,

Abstract

The visco-elastic properties of gluten are major determinants of the processing properties of doughs. These visco-elastic properties are strongly influenced by the ratio of monomeric and polymeric proteins and the size distribution of the polymeric proteins, which make up the gluten fraction of the dough. Recent studies have revealed that other features, such as the number of the cysteine residues of the HMW-GS, also play an important role in determining the functional characteristics. To modify the processing properties at molecular level, the relationship between the structure of molecules and dough properties has to be understood. In order to explore the relationships between individual proteins and dough properties, we have developed procedures for incorporating bacterially expressed proteins into doughs, and measuring their functional properties in small-scale equipment.

A major problem in investigating the structure/function relationships of individual seed storage proteins is to obtain sufficient amounts of pure polypeptides from the complex families of proteins expressed in the endosperm. Therefore, we have established a simplified model system in which we produce specific protein genes through bacterial expression and test their functional properties in small-scale apparatus after incorporation into base flour. An S poor protein gene has been chosen as a template gene. This template gene has been modified using standard recombinant DNA techniques in order to test the effects of varying the number and position of cysteine residues, and the size of the protein. Doughs have been mixed in small-scale apparatus and characterised with respect to their polymeric composition and their functional properties, including dough mixing, extensibility and small scale baking. We conclude that dough characteristics can be manipulated in a predictable manner by altering the cysteine residues and the size of high molecular weight glutenins.

Introduction

Wheat prolamins can be divided into two groups. The gliadins are soluble in aqueous alcohols and are present as monomeric proteins that either lack disulphide bonds (omega-gliadins) or have only intr-chain disulphide bonds. The other group, glutenins, are insoluble in aqueous alcohols and consist of protein subunits present in polymers stabilised by inter-chain disulphide bonds. Reduction of these bonds results in subunits that are soluble in ethanol, or i-propanol/water mixtures and are classified into high molecular weight (HMW) and low molecular weight (LMW). The gliadin/glutenin classification is based on the disulphide-bonding behaviour of the proteins, rather than their primary amino acid sequences. Polypeptides of the prolamin fraction are structurally related. Their amino acid sequences consist of three separate domains: N-terminal non-repetitive domain, central repetitive domain and non-repetitive C-terminal domain. The cysteine residues are mainly located in the terminal domains. The repetitive domain consists of tandem or interspersed repeats based on one, two or three peptide

motifs, rich in proline and glutamine. Structural studies indicate that the highly conserved repetitive primary structure results in a similarly conserved supersecondary structure. This is a spiral based on beta-turns in HMW prolamins and beta-turns interspersed with poly-L-proline II structure in S-poor prolamins, giving a less compact structure. The central repetitive domain can undergo deformation/reformation under stress/relaxation. Deforming this domain results in disruption of a stable state, the reformation of which contributes to elasticity [7,8,13].

Wheat storage proteins form the gluten fraction, whose properties are largely responsible for the ability to use wheat flour to make bread, other baked goods and pasta. The glutenin sub-fraction of wheat gluten is generally accepted as being crucial to bread-making quality. Particular attention has been focused on that fraction of gluten that is unextractable in sodium dodecyl sulphate (SDS) solution, referred to as the glutenin macropolymer (GMP) [16]. Strong relationships between the GMP in flour and quality parameters, such as loaf volume and physical dough properties have been observed [4]. The structure and properties of gluten are determined by molecular interaction within the GMP. It is important that these should be understood if the functional properties of gluten are to be manipulated.

One of the major limitations to evaluate the contributions of various groups of gluten proteins to dough functionality has been the lack of appropriate test systems that allow specific proteins to be incorporated and tested within doughs. The other one has been the hardship to purify individual proteins from the mixture of components that exhibit polymorphism. However, the situation has recently changed due to two advances. The first is the development of small-scale testers and methods (2g Mixograph, Extensograph, micro baking) together with procedures for incorporating exogenous proteins into GMP of dough [1]. Advantages of these systems are the requirement for only small amounts of proteins and the ability of rapid multiple test of individual polypeptides, produced by, for example, protein engineering. The second progress was the establishment of large-scale expression method of soluble, correctly folded storage proteins in heterologous system [9]. It is evident from previous papers [2] that small scale testers and genetically engineered proteins provide a useful experimental system to identify genes that alter flour processing properties, and study stucture/function relation to design molecules for different needs, to use in wheat transformation.

This paper studies the effects of length of the repetitive domain and number of cysteine residues within a model protein, on rheological properties of wheat dough. C hordein, a barley homologue of the omega-gliadins of wheat [14], was used as a model storage protein to alter systematically, using genetic engineering methods. The mutant proteins (called Analogue Glutenins, ANG) were expressed in Eschericia coli and purified in sufficient quantity to use in small scale mixing, extensibility and micro-baking experiments.

Materials and Methods

Cloning of ANG genes for expression

A region of the C hordein genomic fragment (lambda) hor1-17 (accession X60037) was used as a template to create Analogue Glutenin (ANG) protein molecules with new characteristics, replacing amino acid residues with cysteine, and reducing the size of the central repetitive domain. Manipulation and cloning of four molecules (wild type, Cys7, Cys236 and Cys7Cys236) into pET-11d expression vector has already been reported [12]. For this study three extra molecules, with shorter central domain, have been cloned using PCR method. Sequences of two oligonucleotides, used this work, correspond to the N-terminal region of the gene, have been published earlier [9].

Both oligonucleotides 1 and 3 correspond to the 5' end of the gene and are very similar to each other. Oligonucleotide 3 is longer and has an A to T substitution at position 25. Two new oligonucleotides (called 2* and 4*) were designed and synthesised to delete part of the gene and substitute threonine to cysteine in oligonucleotide 4*. PCR reaction was performed in an FTS-4000 Thermal Sequencer (Corbet Research, Australia) with 1 cycle of 5 min at 94℃, 20 sec at 55℃, 1 min at 72℃; 36 cycles of 30 sec at 94℃, 45 sec at 72℃. The reaction was carried out in 50 μl, containing 45 μl of Supermix (GIBCO BRL) and 1 ng of template DNA and 50 pmol of each of two appropriate oligonucleotides in 5 μl altogether. Amplified DNA was purified following the QIAquick protocol (QIAGEN) and cloned, using the pGEM-T Vector System I (Promega) as was recommended by the manufacturer.

oligonucleotide 2*:

*** Val Met Thr Gln Gln Pro

5' GGATCC CTA GAC CAT ACT CCA GAT GGT TTG TTG GGG GAC TGG TTG AGG 3'
BamH I

oligonucleotide 4*:

*** Val Met Cys Gln Gln Pro

5' GGATCC CTA GAC CAT ACT CCA GAT GCA TTG TTG GGG GAC TGG TTG AGG 3'
BamH I

White transformant colonies selected for growth in LB medium supplemented with ampicillin (100 mg/l) were screened for insert bearing plasmid DNS by PCR. Plasmid DNA was purified from positive clones using standard method and the insert was sequenced in both direction, using the Prism dye terminator cycle sequencing protocol (Perkin-Elmer). One plasmid isolate, containing the short ANG gene was designated and used for cloning into the expression vector.

Genes were excised with BspHI and BamHI enzymes and cloned into pET-11d vector as reported earlier [11].

Expression and purification of proteins

Ten pg DNA of each ANG-pET clones were freshly transformed into E.coli strain AD494(DE) and one colony from ampicillin containing plate was used for expression. Small-scale expression was carried out in 5 ml 2YT medium, supplemented with ampicillin (100 mg/l). After, about 5 hours of inoculation (OD600=0.4) expression was induced by addition of 0.4 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG). Culture was further incubated for 4 hours at 30℃. Expression was monitored by SDS-PAGE in 12 % gel.

Large-scale expression was performed in shaking flask. One litre of 2YT medium with 100 mg/l of ampicillin was inoculated with 1 ml of overnight culture and incubated at 37℃. Expression was started with the addition of 0.4 mM of IPTG, in final concentration, when cell density reached 0.5 at 600 nm wavelength. Cells were harvested after overnight incubation at 30℃.
Purification was carried out as described elsewhere [9] with slight modification. After extraction with 70 % (v/v) ethanol protein solution was dialysed against 10 mM of acetic acid. The pellet after centrifugation (10,000 g 10 min at 4℃) was dissolved in 0.1M acetic acid and freeze-dried.

HMW glutenin subunit 1Bx7 was purified from flour of wheat cv. Galahad 7, which contains only this subunit, as published earlier [10].

Small scale tests

Mixing tests were conducted with a prototype 2g Mixograph using a modification of the standard method for 35 g of flour, scaled down to the 2g size [5]. Mixing parameters were determined using a modification of a previously reported computer program [3]. Parameters were determined were mixing time (MT) and break down in resistance (BDR). A reversible reduction/oxidation procedure for incorporation of 5 mg of added, purified protein into glutenin, was used [1]. Base flour and its HMW-GS composition was the same as in earlier experiments [12].

A small-scale extension tester was used, providing results for maximum resistance and extensibility, which are closely related with those from the Brabender Extensograph. Sample preparation and handling method was the same as published earlier [6].

Test baking was carried out using a recently developed procedure, employing 2.4 g dough prepared in the 2g Mixograph [15].
All tests were performed three times. The least significant differences calculated by Student test.

Results and Discussion

Design, construction and cloning of ANG molecules

The gene selected for this work is coding for C hordein, a storage protein from barley, homologue to omega-

gliadins of wheat. For expression of the gene in E.coli, the signal peptide was removed and replaced with a methionine (ATG) initiation codon. All genes have been flanked with restriction endonuclease cleavage sites to clone them into pET-11d expression vector. Oligonucleotides 1 and 3 have BspHI, while oligonucleotide 2, 4, 2* and 4* have BamHI cloning sites. Four oligos (1, 2, 3 and 4) were used to amplify up and modify the gene for the whole size ANG molecules, for details see [9]. Genes for mature proteins (molecular weight 28 kDa) have 723 nucleotides, including a 669 bp long fragment for a central repetitive domain. Codon (TGC) for cysteine residue was introduced into the non-repetitive N- and/or C-terminal domains, replacing the 7th serin (AGC) and/or 236th threonine (ACC). Three engineered genes, two with single cysteine residue (ANGCys7 and ANGCys236) and one with codons for two cysteine residues (ANGCys7Cys236) were amplified and cloned into the expression vector as published earlier [12].

Genes for short ANG molecules were also cloned in this study. Oligonucleotide 2* and 4* were designed to reduce the size of the central repetitive domain, by approximately one third of its original length. Sequence of the 3' end of both oligos correspond to the sequence between 427 and 447 nucleotides, which is rather unique within the strongly repetitive region and codes for an amino acid sequence of PQPVPQQ. These oligos contain a sequence to restore the whole C-terminal unique region (6 amino acids), and add a restriction site for BamHI enzyme, immediately after the stop codon. Differences between oligonucleotide 2* and 4* are, that the second one has a C at position 26 and an A at position 27 to substitute G and T, respectively. Using oligo 4* enables us to introduce a codon for cysteine residue to change the threonine. This is the 6th residue from the end of the protein, which corresponds to position 236 of the full size molecule.

These new genes for analogue glutenin molecules code for proteins with 155 amino acid residues, which have a molecular weight of 18.4 kDa. ANGΔ (short analogue glutenin) proteins consist of unique N- and C-terminal domains of only 6 and 12 amino acid residues, as the full size ANG molecules, separated by 137 amino acids of repeats. This repetitive domain is 86 residues shorter than in the full size protein (233 a.a.), so Δ is equal 86 amino acids. Structure of ANG and ANGΔ genes are shown in Figure 1.

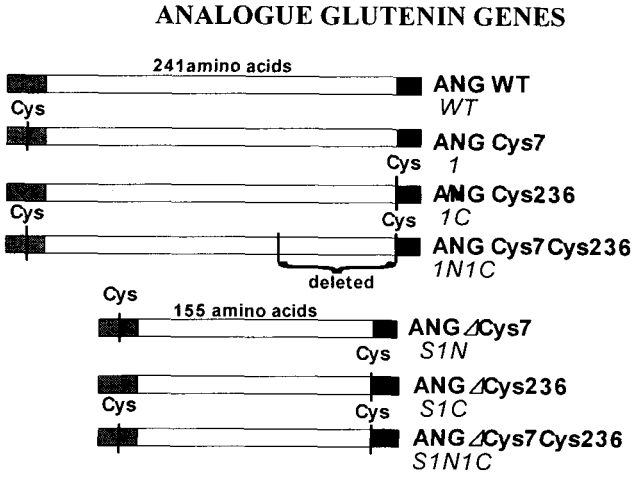

Figure. 1. Structure of Analogue Glutenin proteins used in this study. N-and C-terminal regions are marked with grey box. The deleted region from full size molecule to create the short version is also marked. Abbreviation in italic means the short name of each construct.

Expression and purification of ANG proteins

The level of expression appeared to be around 8% of the total extracted protein, using qualitative gel scanning technic. Both ANG and ANGΔ proteins were readily extracted from lysed cells with 70% (v/v) ethanol with a yield in excess of 20 mg/litre of culture. The resulting preparations, in the presence of reducing agent (0.1M DTT), gave a single homogenous band by SDS-PAGE. Under non-reducing conditions SDS-PAGE showed a mixture of monomeric and dimeric forms for both size of ANGs with single cysteine residue, and a ladder of bands for proteins with two cysteine residues.

Small scale studies of dough characteristics

Mixing

Data of mixing time (MT) and breakdown in resistance (BDR) of dough samples using full and short length of ANG protein molecules are shown in Figure 2 and 3, respectively. In incorporation type of experiments, ANG proteins with two cysteine residues (*1N1C* and *S1N1C*), increased MT and decreased BDR. These effects are similar to the incorporation of HMW glutenin subunit (HMW-GS) 1Bx7, although of lower magnitude. Differences can

also be observed between full and short size ANG proteins. The longer molecule made the dough stronger (longer MT) and more stable (smaller BDR).

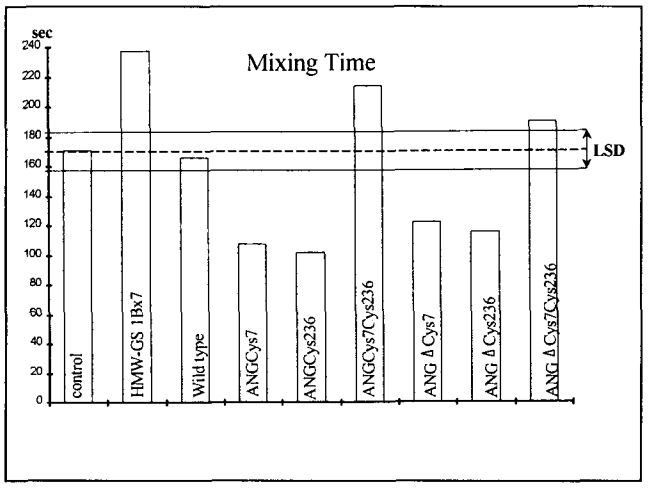

Figure. 2. Comparison of mixing time result, measured by 2g mixograph, of samples with different modified chemical composition. Bars represent mean values of three replicate analyses. Two parallel lines represent LSD values for data.

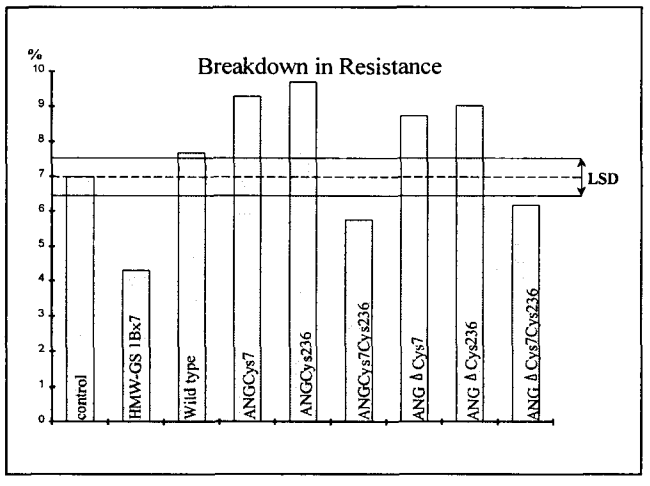

Figure. 3. Comparison of breakdown in resistance results, measured by 2g Mixograph, of samples with different modified chemical composition. Bars represent mean values of three replicate analyses. Two parallel lines represent LSD values for data.

When single cysteine residue containing analogue glutenin proteins (*1N, 1C, S1N* and *S1C*, see Figure 1) were studied in incorporation, MT of dough was significantly reduced, compared to control dough sample. Dough with shorter macropolymer requires shorter mixing time for dough development. It seems when protein with single cysteine residue is incorporated into the gluten macropolymer; the added molecule stops the extension of the polymer. We think that ANG molecules with single cysteine act as chain terminator. It is remarkable that reducing the strength of dough is not followed by strong reduction in stability, as the effect on BDR was less marked than on MT. Length of the molecule has also got effect on dough handling properties. The shorter proteins have decreased MT and increased BDR in less extent.
Differences in MT and in BDR between ANGCys7 and ANGCys236 proteins are not significant but consistent for both size of ANG molecules. Protein with cysteine residue in the C-terminal region has higher effect on MT and on BDR, compares to the control sample, in mixing experiments.

Extensibility

Having incorporated of expressed and purified proteins into dough by reduction/oxidation procedure, dough was mixed until resistance reached maximum level (optimal mixing time). Extensibility was measured in a small scale Extensograph on, approximately, 1.3g of dough samples. Extensibility and Rmax values are shown in Figure 4 and 5, respectively.

Incorporation of polypeptides (HMW-GS 1Bx7, *1N 1C* and *S1N1C*) can enlarge the size of polymeric protein fraction and have significantly decreased the extensibility. The extent of this decrease seems to be related with the size of the polypeptides. HMW glutenin subunit has the strongest effect on extensibility, while *S1N1C* has the lowest. On the other hand, ANGs containing 1 cysteine residue have increased the extensibility. There are small differences in the extent of this increase. Full size ANGs have slightly stronger effect than the short analogue glutenins. ANGCys7 caused larger increase on extensibility, although not significantly, than ANGCys236 protein. It seems that both the size of the polypeptide and the environment of the single cysteine residue alter strength of the chain termination effect of the molecule, which seems to be directly related to extensibility.
The most remarkable effect on extensibility was found using the molecule without cysteine residue (*WT*). The strong effect can be explained with the reduced ratio of polymeric to monomeric proteins in the dough.

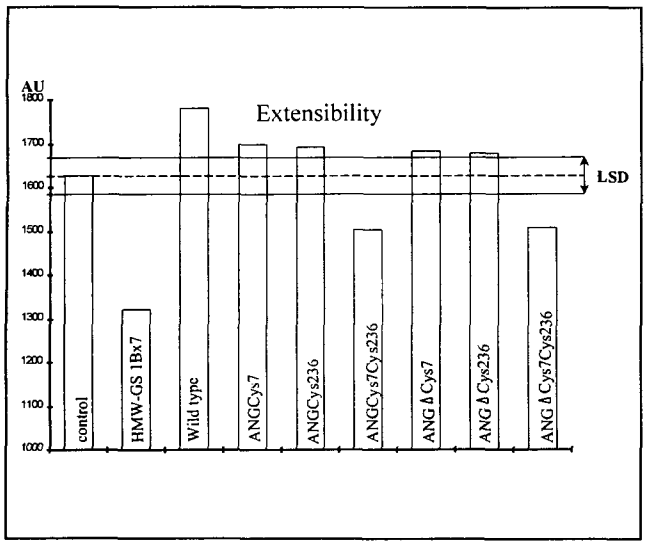

Figure. 4. Comparison of extensibility results, measured by small scale Extensograph, of samples with different modified chemical composition. Bars represent mean values of three replicate analyses. Two parallel lines represent LSD values for data.

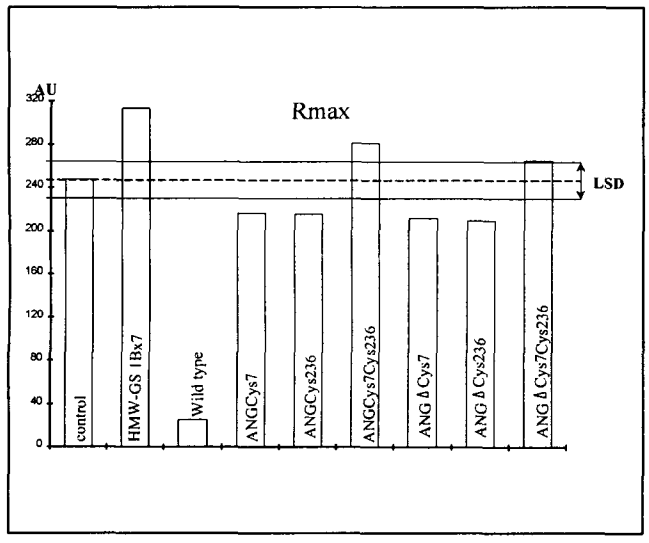

Figure. 5. Comparison of resistance in maximum results, measured by small scale Extensograph, of samples with different modified chemical composition. Bars represent mean values of three replicate analyses. Two parallel lines represent LSD values for data.

Resistance in maximum (Rmax) has changed in the opposite way, compared to extensibility. When proteins with two free cysteine residues (HMW-GS, *1N1C* and *S 1N1C*) were incorporated into the dough, the Rmax was increased, due to the increased glutenin/gliadin ratio and size distribution of polymeric fraction. In case of ANGs with one cysteine, Rmax was reduced because of the decreased size distribution. The size of the molecule and the position of cysteine residue have altered the extent of these effects, the larger polypeptide has the larger effect.

Baking

Doughs with incorporated, engineered proteins were baked using a recently developed method. Loaf height (LH) of the small breads was measured and results can be seen on Figure 6. It is clear from our results that height of small loafs are in connection with the value of Rmax. In case of the incorporation of chain extender proteins, both protein content of dough and size distribution of the polymeric proteins in gluten macropolymer were increased, which increased the loaf height. Chain terminator proteins increased protein content of dough, but decreased size distribution, and the result was decreased LH. The size of the incorporated protein has also altered the extent of the effect, probably, through the shift on molecular size of macropolymer. It seems that using WT protein, the negative effect of decreased glutenin/gliadin ratio was compensated by the increased protein content and the LH did not change. From our data we propose that the most important factor related to the baking properties is the size distribution of the polymeric proteins in the gluten polymer.

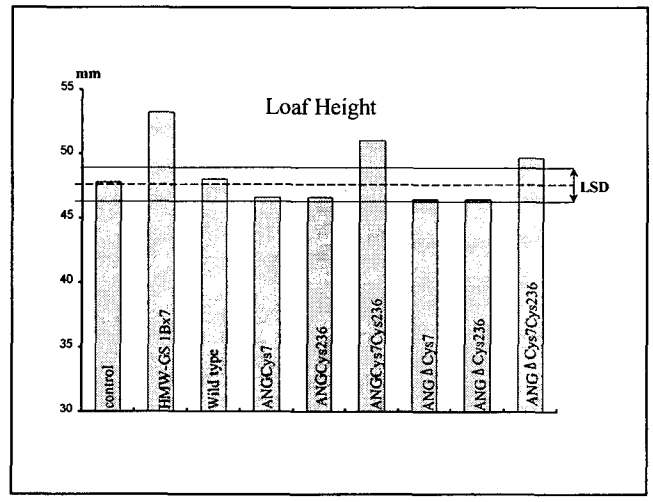

Figure. 6. Comparison of loaf height results of samples with different modified chemical composition. Bars represent mean values of three replicate analyses. Two parallel lines represent LSD values.

Results of our experiments indicate that specific and predictable modification can be achieved in gluten struc-

ture and dough quality parameters via protein engineering.

Acknowledgement

The authors are grateful to Dr. P.Gras for his valuable help in micro testing and to Mrs. C.Tam s and D.Murray for their excellent technical support. We thank to Goodman Fielder Ltd and Group Limmagrain for financial support.

References

1. Békés F, Gras PW, Gupta RB (1994) Mixing properties as a measure of reversible reduction/oxidation of doughs. Cereal Chemistry 1: 44-50.
2. Békés F, Anderson OD, Gras PW, Gupta RB, Tam A, Wrigley CW, Appels R (1994) The contribution to mixing properties of 1D glutenin subunits expressed in a bacterial system. In Henry R and Ronalds J (eds), Improvement of Cereal Quality by Genetic Engineering, pp.97-104.
3. Gras PW, Hibberd GE, Walker CE (1990) Electronic sensing and interpretation of dough properties using a 35 gram Mixograph. Cereal Foods World 35: 568-571.
4. Gupta RB, Batey IL, MacRitchie F (1992) Relationships between protein composition and functional properties of wheat flours. Cereal Chemistry 69: 125-131.
5. Rath CR, Gras PW, Wrigley CW, Walker CE (1990) Evaluation of dough properties from two grams of flour using the Mixograph principle. Cereal Foods World 35: 572-574.
6. Rath CR, Gras PW, Zhen Z, Appels R, Békés F, Wrigley CW (1994) In Panozzo JF and Downie PG (eds) Proceedings of the 44th ACC Conference, Ballarat, Australia pp. 122-126.
7. Shewry PR, Tatham AS (1990) The prolamin storage proteins of cereal seeds: Structure and evolution (Review Article). Biochem J 267: 1-12.
8. Shewry PR, Tatham AS, Barro F, Barcelo P, Lazzeri P (1995) Biotechnology of breadmaking: Unraveling and manipulating the multi-protein gluten complex. Bio/Technology 13: 1185-1190.
9. Tamás L, Greenfield J, Halford NG, Tatham AS, Shewry PR (1994) A beta-turn rich barley seed protein is correctly folded in *Escherichia coli*. Protein Exp and Purification 5: 357-363.
10. Tamás L, Békés F, Greenfield J, Tatham AS, Gras PW, Shewry PR, Appels R (1995) Heterologous expression and mixing studies on genetically modified C hordeins. In Williams YA and Wrigley CW (eds) Proceedings of the 45th Australian Cereal Chemistry Conference, Adelaide pp.482-488.
11. Tamás L, Békés F, Gras PW, Morrell MK, Appels R (1996) Studying the structure function relation of storage protein in mixing experiments. In Wrigley CW (ed) Proceedings of the 6th International Gluten Workshop, Sydney, Australia pp184-189.
12. Tamás L, Békés F, Greenfield J, Tatham AS, Gras PW, Shewry PR, Appels R (1998) Heterologous expression and dough mixing studies of wild-type and mutant C hordeins. J of Cereal Science 27: 15-22.
13. Tatham AS, Drake AF, Shewry PR (1989) Conformational studies of a synthetic peptide corresponding to the repeat motif of C hordein. Biochem J 259: 471-476.
14. Tatham AS, Shewry PR (1995) The S-poor prolamins of wheat, barley and rye. J Cereal Science 22: 1-16.
15. Uthayakumaran S, Békés F, Gras PW (1997) Effect of varying protein content and glutenin to gliadin ratio on the functional properties of wheat dough. In Tarr AW and Ross AS (eds) Proceedings of the 47th ACC Conference, Perth, Australia pp. 212-216.
16. Weegels PL, Hamer RJ, Schofield JD (1996) Functional properties of wheat glutenin. J Cereal Science 23: 1-17.