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Cereal science and technology for feeding ten billion people: genomics era and beyond

Zaragoza : CIHEAM / IRTA

Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 81

2008

pages 119-122

Article available on line / Article disponible en ligne à l'adresse :

<http://om.ciheam.org/article.php?IDPDF=800818>

To cite this article / Pour citer cet article

Ferrante P., Gianibelli M.C., Larroque O., Lafiandra D., Masci S. **Considerations about the effect of incorporation of two rare LMW-GS in durum wheat in comparison to bread wheat doughs.** In : Molina-Cano J.L. (ed.), Christou P. (ed.), Graner A. (ed.), Hammer K. (ed.), Jouve N. (ed.), Keller B. (ed.), Lasa J.M. (ed.), Powell W. (ed.), Royo C. (ed.), Shewry P. (ed.), Stanca A.M. (ed.). *Cereal science and technology for feeding ten billion people: genomics era and beyond.* Zaragoza : CIHEAM / IRTA, 2008. p. 119-122 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 81)



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# Considerations about the effect of incorporation of two rare LMW-GS in durum wheat in comparison to bread wheat doughs

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## Introduction

Gluten is a protein complex composed of monomeric gliadins and polymeric glutenins. After chemical reduction, glutenins release high and low molecular weight subunits (HMW-GS and LMW-GS, respectively). LMW-GS have been classified into three groups, B, C and D, on the basis of their electrophoretic mobility and isoelectric point. In particular, the sequences of B subunits can be subdivided into LMW-s, LMW-m and LMW-i types, whose first N-terminal amino acids in the mature polypeptides are Ser, Met and Ile, respectively (rev. in D'Ovidio and Masci, 2004). Whereas LMW-m and LMW-s are the most common, only recently it was demonstrated that LMW-i are actually expressed in wheat endosperm, being part of the glutenin fraction (Ferrante *et al.*, 2004; Ikeda *et al.*, 2004). So far no information is available about their functionality, i.e. if they behave as chain extenders or chain terminators of the growing polymers. Regarding the C and D groups, they are mainly composed of  $\alpha/\beta$ -,  $\gamma$ - and  $\omega$ -gliadins mutated in the number of cysteine residues. It is supposed that they are incorporated into the gluten polymeric network by virtue of unpaired cysteines, likely acting as chain terminators of glutenin polymers.

In the present work we investigated the functional properties of two unusual LMW-GS, an i-type LMW-GS and a modified  $\gamma$ -gliadin with an additional cysteine located at the beginning of the repetitive domain, corresponding to a C type LMW-GS (Ferrante *et al.*, 2006). Each of the polypeptides was purified from bacterial cells in which they were heterologously expressed, incorporated using a 2-g Mixograph into durum and bread wheat doughs, and their effects on the mixing behaviour of the reconstituted doughs evaluated.

## Materials and methods

### Production of heterologous proteins

The recombinant LMW-i and the modified  $\gamma$ -gliadin were produced in *E. coli* cells according to the method described by Ferrante *et al.* (2004, 2006). Inclusion bodies were extracted after three hours induction with 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside according to the protocol of Patacchini *et al.* (2003).

### Incorporation protocols

To set up a reversible reduction/oxidation procedure for incorporating the recombinant polypeptides, different amounts of the reducing agent DTT and of the oxidant KIO<sub>3</sub> were added to flour and semolina at constant water absorption of 60%. The range of DTT and KIO<sub>3</sub> additions was established by choosing values close to those used previously for incorporation in bread wheat doughs (Békés *et al.*, 1994; Verbruggen *et al.*, 2001). To carry out incorporation tests we used the amounts of reducing and oxidant agents that caused the lower change in MT with respect to the control dough (in which only water was added). All the mixing tests were performed in duplicate and, wherever possible, in triplicate, with a prototype 2-g Mixograph, using a modification of the standard method for 35 g of flour scaled down to the 2-g size (Rath *et al.*, 1990).

As result of the preliminary experiments, the following incorporation conditions were chosen: 25 mg of LMW-i or modified  $\gamma$ -gliadin were incorporated into doughs of both durum and bread wheat. In case of durum, dough was partially reduced with 50  $\mu$ l DTT (2 mg/ml) and finally re-oxidised with 16  $\mu$ l  $\text{KIO}_3$  (5 mg/ml), whereas bread wheat dough was first partially reduced with 40  $\mu$ l DTT (2 mg/ml) and then re-oxidised with 24  $\mu$ l  $\text{KIO}_3$  (5 mg/ml). As a control, the same doughs from durum and bread wheat were used, in which only DTT and  $\text{KIO}_3$  were added.

## Results and discussion

### Incorporation in bread wheat

Incorporation tests performed on bread wheat doughs have shown that the modified  $\gamma$ -gliadin decreased the overall dough strength reducing MT by 18.5%, PR by 20.8% while having a slight positive effect on the dough tolerance to over-mixing, given the decrease in RBD by 11.1%. Conversely, the LMW-i type did not change mixing requirements significantly, as evidenced by the slight increase in MT (by 0.7%), the weak decrease in PR (by 1.5%) and null effect on RBD. The effects exerted by the chemically incorporated LMW-i type and the modified  $\gamma$ -gliadin are also visually evident by comparing the shape of the mixing curves relative to the control (Fig.1, panel A) and reconstituted doughs (panel B and C). It is clear that the incorporation of the recombinant LMW-i (panel B) did not consistently modify the shape of the mixing curve with respect to the control dough. On the other hand, the incorporation of the modified  $\gamma$ -gliadin (panel C) led to a general flattening of the mixing curve and reduced the time required to reach optimum mixing. The data obtained support the hypothesis of the modified  $\gamma$ -gliadin acting as a chain terminator, because of the presence of an extra cysteine residue in the N-terminal position that would be available in forming only one inter-molecular disulphide bond. The role of chain extender of the LMW-i here considered can be inferred from the observation that the rheological parameters do not change significantly after its incorporation, likely because the bread wheat flour here used is particularly strong and it seems possible that it cannot be improved further. It is likely that incorporation of this subunit in a weaker bread wheat flour than Kukri might exert a detectable effect of this subunit on dough mixing properties, because the weaker is the flour and the easier is to detect a strengthening effect.

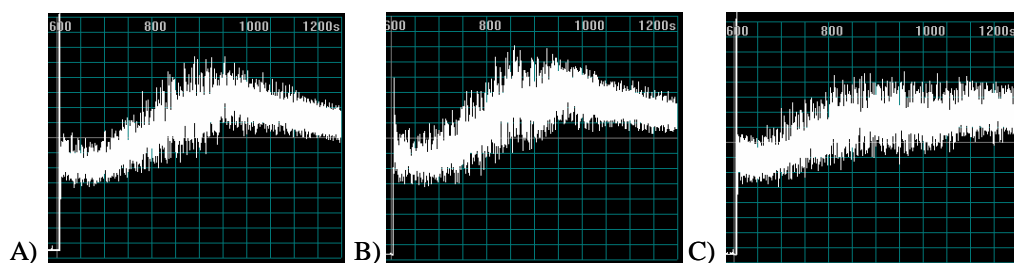


Fig. 1. Mixing curves of control and reconstituted doughs obtained from the bread wheat cv. Kukri. A) Mixing curve relative to the control dough simply reduced and re-oxidised; B and C, mixing curves of bread wheat doughs obtained by incorporating the recombinant LMW-i (B) and the modified  $\gamma$ -gliadin (C). For clearness, the mixing curves refer only to the 10 min mixing after that the reduction and the oxidation have taken place.

### Incorporation in durum wheat

The two heterologous polypeptides were also incorporated into semolina doughs obtained from two durum wheat genotypes, Lira biotype 42 and Lira biotype 45, showing poor and good technological properties, respectively. The incorporation tests showed that both polypeptides decreased the overall dough strength and had a detrimental effect on dough tolerance to over-mixing, especially in Lira 45 biotype doughs. In particular, in reconstituted doughs of Lira biotype 42, the modified  $\gamma$ -gliadin decreased MT and PR by 16.2% and 9.1%, respectively, and it increased RBD by 10.5%. The LMW-i had nearly the same weakening effect, reducing MT and PR by 17.5% and 18.6%

respectively and increasing RBD by 10.5%. In incorporation tests performed using Lira biotype 45 semolina, both proteins had a more evident detrimental effect on dough strength and stability than in Lira biotype 42 doughs. This might be explained by considering the stronger nature of Lira biotype 45 doughs compared to Lira 42 doughs that makes the detection of negative effects more evident. In particular the modified  $\gamma$ -gliadin and the LMW-i respectively reduced MT by 35% and 36.4%, PR by 18.1% and 11.6%, whereas they increased RBD by 36.4% and 72.7%.

Since it is known that monomeric gliadins weaken the dough in which they are added or incorporated (Khatkar *et al.*, 2002), we first checked if the two heterologous subunits were present in the semolina doughs as monomers rather than as glutenins. The incorporation of both the modified  $\gamma$ -gliadin and the LMW-i was clearly demonstrated by 2D-gels, RP-HPLC and SE-HPLC analyses performed on reconstituted and control doughs. In Fig. 2 a two-dimensional map of glutenins extracted from the dough of Lira biotype 42 reconstituted with the modified  $\gamma$ -gliadin and collected at MT is shown. The spot indicated by the arrow most likely corresponds to the chemically incorporated  $\gamma$ -gliadin, given that it showed the same electrophoretic mobility as the recombinant protein, besides being present in the control dough, although in a lesser amount.

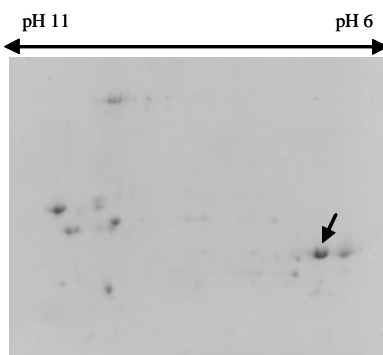


Fig. 2. Two-dimensional gel of the glutenin fraction extracted from the dough of Lira biotype 42 reconstituted with the recombinant  $\gamma$ -gliadin and collected at MT. The spot indicated by the arrow most likely corresponds to the  $\gamma$ -gliadin chemically incorporated into the dough, given that it showed the same electrophoretic mobility as the recombinant protein.

Moreover SE-HPLC, RP-HPLC analyses clearly confirmed the presence of the recombinant LMW-i and modified  $\gamma$ -gliadin in the glutenin polymeric fraction. RP-HPLC analyses have shown that the HMW-GS/LMW-GS ratio is lower in reconstituted doughs than in the control doughs of both durum biotype as expected if the recombinant proteins had been incorporated. Finally SE-HPLC analyses of reconstituted and control doughs showed that the incorporation of the modified  $\gamma$ -gliadin and the LMW-i increased the percent value of unextractable polymeric proteins (UPP%) of reconstituted doughs as compared to control doughs, both at mixing time and time of break down, giving further evidence of their incorporation into the glutenin matrix (data not shown). The results obtained by incorporating the modified  $\gamma$ -gliadin and the LMW-i in durum wheat doughs are clearly in contrast with those for bread wheat doughs, leading to the suggestion that the reduction-oxidation conditions used to chemically incorporate the recombinant proteins in durum wheat doughs might not be completely appropriate and effective. Whereas incorporation tests are well documented for bread wheat, the tests here described are the first report of chemical incorporation into durum wheat doughs, thus it is possible that the reduction and oxidation conditions here used were not completely appropriate and effective for durum wheat doughs. Some factors as different semolina/ flour particle size, different degree of starch damage and a possible different glutenin polymers organization might affect the reduction-oxidation protocols employed to chemically incorporate the recombinant proteins into semolina doughs. If is this the case, the reduction-oxidation condition in durum wheat doughs will need further adjustment.

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