# Role of enzymes in improving the functionality of proteins in non-wheat dough systems

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## Abstract

Gluten free systems lack the viscoelastic network required to resist gas production and expansion during baking. Enzymatic treatments of the GF flours have been proposed initially for creating protein aggregates that mimic gluten functionality but then also for modifying proteins changing their functionality in GF systems. To better exploit the technological function and the potentials of enzymatic processing for improving GF bread quality, it is important to understand the key elements that define the microstructure and baking functionality of GF batters as compared to wheat dough. In this review, some keys are pointed out to explain the different mechanisms that are available for understanding the action of enzymes to effectively design GF viscoelastic matrixes. Focus will be on protein modifying enzymes, because they play a decisive

role in the formation of the fine network responsible for improving the expansion of rice batters.

**Key words**: enzymes; gluten free batters; transglutaminase; protease

## 1. Introduction

In the Western world, bread is one of the most important staple foods. Bread quality largely relies on the unique viscoelastic properties of gluten. In fact, once the flour is hydrated, the gluten confers extensibility and good gas holding ability to the dough. However, pathologies associated with gluten consumption prompt food technologist and the food industry to find suitable replacements for breadmaking purposes.

When hydrated, flours from gluten free (GF) cereals result in a batter rather than dough as their proteins do not possess the viscoelastic properties typically found in gluten. Furthermore, gas holding is more difficult (Cauvain, 1998). For such reasons, the replacement of gluten in GF products requires the supplementation of existing functional ingredients in the bread formula but also the development of new functional ingredients and advanced processing techniques (Zannini et al., 2012). Enzymatic processing offers a sustainable, specific bio-processing tool able to deliver products which are natural, contain a reduced amount of chemicals and possess appealing sensorial properties. Enzymes can be applied in the processing of cereals to obtain: (i) modified fibrous structures alternative to commercially available hydrocolloids and gums (ii) protein and/or polysaccharide based functional ingredients and (iii) natural pre-biotics. Enzymes are commonly applied in the baking industry in order to improve the characteristics and quality of wheat flour based products (Rosell and Collar, 2008). Comprehensive reviews are available, which describe in detail the mechanism of action

of enzymes commonly used in the baking industry, the implication at molecular level on the main flour constituents and their influence on baking properties, textural and sensorial quality, and nutritional aspects (Gerits et al., 2014; Joye et al., 2009; Poutanen, 1997; Goesaert et al., 2009). However, the focus of such reviews is on wheat based products, which implies that the technological function described for each enzyme might not be directly translated to application in GF bread products. In fact, the microstructure and rheological properties of wheat dough and GF batters are inherently different and enzymatic treatments are pursuing the improvement of dough viscoelastic properties (Rosell, 2009). To better exploit the technological function and the potentials of enzymatic processing for improving GF bread quality, it is important to understand the key elements that define the microstructure and baking functionality of GF batters as compared to wheat dough. After a short review of such differences, the application of enzymatic processing in GF breads is discussed with focus on protein modifying enzymes.

## 2. GF batter microstructure as compared to wheat dough

Immediately after mixing, wheat dough consists of a dispersion of discrete gas cells that are embedded in a continuous starch-protein matrix (Figure 1A). The gluten–starch matrix is the primary stabilizing factor for expanding gas cells against disproportionation and coalescence as controlled by the strain hardening behaviour of gluten (Sroan et al, 2009; Gan et al., 1995; Bloksma, 1990; Hoseney, 1992). Strain hardening is considered a key factor, controlling the breadmaking properties of the dough (Dobraszczyk et al., 2003) owing to the entanglement of large glutenin molecules in the gluten network (MacRitchie and Lafiandra, 1997; Singh and MacRitchie, 2001). During baking, the gluten matrix provides the dough with both the extensibility to respond to the increasing gas pressure as well as the strength to resist collapse (Sroan et

al, 2009). Later in the baking process, the increase in the elastic-like behaviour of the gluten–starch matrix as result of starch gelatinization and gluten polymerization, results in the rupture of the matrix and the formation of a permanent network. Consequently, the bread dough undergoes a structural transformation from foam to an open sponge (Figure 1A) (Gan et al., 1995), which is associated with a sharp increase in the release of gas from the dough. It is reported by some authors (Sroan et al, 2009; Turbin-Orger et al., 2012; Gan et al., 1995) that during rupture of the gluten-starch matrix, a secondary stabilizing mechanism involving thin liquid lamellae at the gas–liquid interface prevents the coalescence and disproportionation of gas cells coming in close contact with each other. The liquid film contains surface active proteins and (polar) lipids which stabilizes the gas cells.

These mechanisms are crucial to provide the soft sponge structure typical of a wheat bread crumb, which can be macroscopically described as a high volume fraction of air ($\geq$0.8) dispersed in a solid matrix of mostly open cell walls (Lagrain et al., 2012). At microstructural level, the solid matrix of the crumbs consists of a continuous phase of gelatinised starch (Pomeranz et al., 1984; Durrenberger et al., 2001; Zannini et al., 2012) and a continuous gluten network which encloses the starch granules and fibre fragments (Figure 1B).

In GF batters, a continuous protein-starch matrix is missing as compared to wheat dough (Figure 2A). Starch becomes the primary structural element due to the lack of gluten, but only during the baking stage, when the batter temperature reaches those of starch gelatinization. During mixing, the stabilizing mechanism for the dispersed gas cells primarily relies on the viscosity of the medium, which also prevents starch and yeast from settling. For such reasons, hydrocolloids and gums are typically used in starch-containing products, such as GF batters (Rosell et al., 2001) as they can partially

101 mimic the structuring role of gluten (Figure 2A). In GF batters, hydrocolloids contribute
102 to: (i) improve viscoelastic properties, (ii) act as water binders, (iii) improve texture by
103 forming gels and (iv) stabilize gas cells. Their contribution to the structuring process in
104 GF batters depends on their rheological and flowing properties and their interfacial and
105 gel forming properties (Lazaridou et al., 2007; Hüttner and Arendt, 2010), which greatly
106 depend on their origin and chemical structure. The rheological properties imparted to
107 the GF batter by the hydrocolloids largely determine their baking quality. A strong
108 correlation between rheological parameters such as the elastic modulus $G'$ and the ratio
109 of viscous to elastic behavior tan $\delta$, and final bread quality have been reported
110 (Lazaridou et al., 2007; Crockett et al., 2011a). In fact, a balance between elastic
111 properties (film formation and gas retention) and viscous properties (protein absorption
112 to the liquid lamella and flexibility for gas expansion) is required to achieve optimal
113 baking quality in GF breads (Lazaridou et al., 2007; Crockett et al., 2011a; Matos and
114 Rosell, 2013; Matos and Rosell, 2015).
115 Among the hydrocolloids, HPMC and xanthan gum are most frequently used because
116 they most successfully replace gluten in GF breads within a wide spread of formulations
117 (Anton and Artfield, 2008). In particular, HPMC is capable of stabilizing gas bubbles
118 by accumulating at the gas liquid interface, forming an elastic microgel (Schober,
119 2010). When a solution of HPMC in water is mixed at high speed, the surface active
120 properties of HPMC enable the formation of stable and well aerated foams similar to
121 whipped egg white while the same is not achieved with xanthan gum (Schober et al.,
122 2008). Consequently, the resulting GF bread shows high specific volume and low
123 crumb hardness (Crockett et al., 2011a; Mezaize et al., 2009; Sabanis, and Tzia, 2011).
124 Microstructure analysis suggests that hydrocolloids alone are not sufficient to fully
125 replace gluten in GF breads. Proteins from GF cereal flours generally lack the ability to

126 form a protein network upon baking (gel) (Figure 2A) and that the supplementation with
127 functional proteins is therefore necessary. Scanning electron microscopy of GF breads
128 demonstrated that a low-protein starch formulation including HPMC and xanthan gum
129 lacked of matrix development (Ahlborn et al., 2005). On the contrary, a fibrous, web-
130 like structure more similar to wheat bread could be achieved when supplementing with
131 eggs and milk proteins.
132 Interactions among the main structure building elements in GF formulations, i.e.
133 hydrocolloids, proteins and starches, should be carefully considered. Nowadays several
134 GF grains, legumes, seeds and nut flours are used as they offer increased variety, high
135 nutritional quality and palatability of the GF formulation (Zannini et al., 2012). These
136 ingredients strongly diverge in their chemical composition and certain components may
137 interact to different extents with the hydrocolloids (Hager and Arendt, 2013), thus
138 resulting in GF batter microstructures and baking functionalities which are strongly
139 dependent on the specific formulation used (El-Sayed, 2009; Hüttner and Arendt, 2010,
140 Matos and Rosell, 2013). Special care should be taken with the hydrocolloids-starch
141 interactions since those are specific and greatly dependent on the type of hydrocolloid
142 (Gularte and Rosell, 2011). Protein source (e.g. soy, egg, milk) can affect hydrocolloid
143 functionality by altering water distribution within the batter, weakening interactions
144 with the starch matrix and reducing foam stability (Crockett et al., 2011b; Nunes et al.,
145 2009). However, the negative effects might be overcome when the protein becomes the
146 primary scaffolding element in the batter (Crockett et al., 2011b; Schober et al., 2008).
147 Minor components such as soluble fibers can also strongly affect batter structure by
148 creating a homogeneous phase with hydrocolloid and water which coats starch and flour
149 particles, resulting in a more stable batter during proofing and baking (Martinez et al.,
150 2014).

## 3. Enzyme technology

In the last decade, there have been an increasing number of studies focusing on enzymatic processing of GF batters, with particular focus on enzymes which could enhance the functionality of proteins either originating from GF flours or added as supplements to the formulation. A number of protein modifying enzymes are available for which their action mechanism can be classified as direct cross-linking, indirect cross-linking and proteolysis (Table 1).

### *3.1 Crosslinking enzymes in GF baking applications*

The formation of linkages within proteins originating from GF flours and supplemented was initially considered the most plausible way to mimic gluten functionality in GF batters (Rosell 2009). For that purpose, the use of transglutaminase and different oxidases has been proposed.

#### *3.1.1 Transglutaminase action in GF applications*

Transglutaminase (TGase) is a protein-glutamine γ-glutamyl-transferase (EC 2.3.2.13), which catalyses an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues and a variety of primary amines (Motoki and Seguro 1998). When the ε-amino group of a peptide bound lysine residue acts as substrate, the two peptide chains are covalently linked through an ε-(γ-glutamyl)-lysine bond (Folk and Finlayson 1977). Thus, the enzyme is capable of introducing covalent cross-links between proteins (Nonaka et al. 1989), building up new inter- and intramolecular bonds. In the absence of primary amines, water becomes the acyl-acceptor and the γ-carboxy-amide groups of glutamine residues are deamidated, yielding glutamic acid residues, which decrease the hydrophobic environment (Gerrard et al. 1998). Therefore, transglutaminase activity depends on the accessibility of glutamine and lysine residues in the proteins (Gerrard 2002, Houben, Hochstotter, and Becker 2012).

On wheat-based baked goods TGase application reduces the required work input, decreases water absorption of the dough (Gerrard et al. 1998), increases dough stability (Gottmann and Sproessler 1992), increases volume, improves structure of breads, strengthens bread crumb (Gerrard et al. 1998), and baking quality of weak wheat flours (Basman, Koksel, and Ng 2002). Electrophoretic analysis revealed that the effect was due to the crosslinking within gliadins and glutenins (Rosell et al. 2003). Furthermore, water soluble proteins, generally considered as non-dough-forming proteins, would be also involved in the formation of covalent bonds catalyzed by TGase (Bonet, Blaszczak, and Rosell 2006).

Gujral and Rosell (2004a) initially exposed the hypothesis that the enzymatic creation of a protein network in GF doughs might mimic gluten functionality. The addition of increasing amounts of TGase (0.5, 1.0 or 1.5% w/w) to rice flour induced a progressive enhancement of the viscous ($G''$) and elastic ($G'$) moduli, but the highest bread volume and softer crumb was obtained with 1.0% TGase. The protein fractionation of rice doughs indicated that albumins and globulins fractions were mostly affected, and the electrophoresis analysis confirmed the intermolecular crosslinking leading to high molecular weight proteins, which would result in a more continuous protein phase (Marco et al. 2007).

Nonetheless, flour source has great influence on the resulting TGase induced effect, likely due to their amino acid composition, since lysine and glutamine are required for the enzyme activity. In fact, Renzetti, Dal Bello, and Arendt (2008) observed significant differences when comparing the action of TGase on six different gluten-free cereals (brown rice, buckwheat, corn, oat, sorghum and teff). The presence of protein complexes was confirmed by three-dimensional confocal laser scanning micrographs. Batter fundamental rheological analysis and bread quality confirmed the improving

effect of TGase on buckwheat and brown rice batters and breads, which was explained by protein crosslinking and formation of large protein complexes for both buckwheat and brown rice breads (Renzetti et al., 2012; Renzetti et al., 2008a; Renzetti et al., 2008b). Conversely, TGase addition had a detrimental effect on the elastic-like behavior of corn batters but yielding higher specific volume and lower crumb hardness on corn breads. TGase was not effective to obtain breads from oat, sorghum or teff (Renzetti, Dal Bello, and Arendt 2008). However, Onyango et al. (2010) reported a decrease in the resistance to deformation and an increase in the elastic recovery of TGase treated batters composed of sorghum blended with pregelatinized cassava starch, leading to an improvement in the final breads.

Protein crosslinking seems to be an effective alternative to create internal networks in the GF systems. However, excessive crosslinks may result in a tight structure that impedes the expansion during proofing. In order to optimize TGase treatment of GF flours, the enzyme dosage should be carefully considered depending on the specific formulation, since availability and accessibility of lysine and glutamine varies among GF flours. In fact, studies carried out with bug damaged wheat flour, which has higher number of free amino acids, revealed that as the level of TGase increases it does augment the crosslinks and simultaneously the number of disulfide bonds. Although an increase in the level of crosslinks is not directly related to flour functionality improvement. Indeed, rheological studies combined with calorimetric and biochemical analysis confirmed that bug damaged wheat flour requires higher level of TGase than sound wheat flour for obtaining an optimum functional response (Bonet et al., 2005; Caballero et al., 2005). Certainly, the amount and nature of the proteins present on those flours, and more specifically the level of lysine and glutamic acid, must explain differences encountered among flours.

To solve the possible protein deficiency, protein supplementation was proposed to increase the amount of substrate available for the enzyme (Marco et al. 2008, Marco and Rosell 2008a, b, Marco et al. 2007). Studies carried out in wheat flour confirmed that TGase was able to form homologous polymers within water-soluble, salt-soluble, and glutenin proteins. Scanning electron micrographs of the doughs made from blends of wheat and protein sources doughs showed the formation of heterologous structures in the wheat-lupin blends (Bonet et al., 2006). Marco and Rosell (2008a) reported the effect of transglutaminase on rice flour functionality when it was blended with protein isolates from different sources (pea, soybean, egg albumen and whey proteins). A decrease in the amount of free amino acids confirmed the crosslinking action of TGase in the case of soybean and whey proteins blended with rice flour, although it was not possible to identify whether the crosslinking was between homologous or heterologous protein chains. Viscoelastic moduli of the rice dough were significantly modified by the action of TGase, but whereas the presence of pea and soybean increased $G'$ and $G''$, egg albumen and whey protein decreased them. It seems that vegetable proteins added to rice flour interconnected by inter or intra linkage due to TGase, whereas some antagonistic effect was observed with the animal proteins, likely genetic aspects might be involved in their differences.

Derived from the complexity of the GF systems, different experimental designs have been proposed for optimizing the nature and levels of proteins and the amount of TGase (Storck et al. 2013, Bojana et al. 2012). An experimental design was recommended for obtaining better structured protein network from a combination of soybean and pea protein (Marco and Rosell 2008b). Electrophoretic studies confirmed that TGase action resulted in the formation of isopeptide and disulfide bonds. In the case of pea proteins, major pea proteins extracted in the glutelin and in albumin–globulin fractions

underwent the greatest crosslinking, consequently large aggregates between pea and rice proteins were formed (Marco et al. 2007). Similarly, soybean proteins were crosslinked with rice proteins through the formation of new intermolecular covalent bonds catalysed by transglutaminase and the indirect formation of disulfide bonds among proteins, mainly involving β-conglycinin and glycinin of soybean and the glutelins of the rice flour, although albumins and globulin also participated (Marco et al. 2008). The strategy of creating a protein network by TGase treatment of protein supplemented GF formulations, became effective after optimization of water and supplemented proteins amounts and of enzyme dosage. HPMC was also included in the optimization process to provide additional structural strength and a more open aerated structure included (Marco and Rosell 2008c). Although soybean proteins reduced the specific volume of the bread, scanning electron micrographs confirmed the participation of those proteins in the network created by the TGase. Moore et al. (2006) also showed by confocal laser-scanning microscopy (CLSM) that it is possible to form a protein network in GF bread with the addition of TGase and proteins like skim milk powder, soya flour and egg powder. However, the effectiveness of the enzyme is dependent on both the protein source and the enzyme concentration.

Despite the usefulness of microbial TGase for improving GF systems functionality, some concern has been raised suggesting (i) its homology to tissue TGase that mediates in the coeliac disease, and (ii) higher reactivity of IgA of celiac patients sera against prolamins from TGase treated breads (Cabrera-Chavez et al. 2008, Dekkings et al. 2008). Currently, no further studies have been reported supporting those hypothesis.

### *3.1.2 Oxidases action in GF applications*

Different oxidases (lipoxygenase, sulphydryl oxidase, glucose oxidase, polyphenoloxidase and peroxidase) have been used for its beneficial effect on bakery

276 applications due to their action on dough strengthening and stabilization (Oort 1996),
277 and as dough bleaching agents (Gelinas et al. 1998), improving the quality of fresh
278 breads.
279 Glucose oxidase (EC 1.1.3.4) (GO) catalyzes the conversion of β-D-glucose to δ-D-1,5-
280 gluconolactone, which is spontaneously converted into gluconic acid and hydrogen
281 peroxide. The hydrogen peroxide ($H_2O_2$) interacts with the thiol groups of the proteins
282 resulting in disulphide bonds and promotes the gelation of water-soluble pentosans,
283 changing the rheological properties of wheat dough (Hoseney and Faubion 1981,
284 Primo-Martin, Valera, and Martinez-Anaya 2003). It must be stressed that side activities
285 present in glucose oxidase commercial preparations might have a substantial effect on
286 those changes (Hanft and Koehler 2006). From a molecular standpoint, high
287 performance capillary electrophoresis and cryo-scanning electron microscopy indicated
288 that glucose oxidase modified gluten proteins (gliadins and glutenins) through the
289 formation of disulfide and non-disulfide crosslinks. The reducing action of the peroxide
290 mainly affected high molecular weight glutenin subunits (Bonet et al. 2006b), resulting
291 in an increased content of gluten macropolymer (Steffolani et al. 2010). Nevertheless,
292 some protein disruption was observed when analyzing dough ultrastructure, which
293 could facilitated the enfolding of starch granules by the gluten matrix (Indrani et al.
294 2003). GO action was not limited to gluten proteins. In fact, a decrease in sulfhydryl
295 (SH) groups has been observed in soluble and insoluble protein fractions during the
296 initial stage of mixing where a high consumption of the $H_2O_2$ was observed, without
297 further significant SH changes after mixing (Pescador-Piedra, Farrera-Rebollo, and
298 Calderon-Dominguez 2010). Nevertheless, over-dosage of glucose oxidase produces
299 excessive crosslinking in the gluten network with dramatic effect on the breadmaking
300 properties.

When GO was supplemented to rice dough, bread specific volume increased with a simultaneous reduction of the crumb hardness (Gujral and Rosell 2004b). The GO action resulted in an increase of the dough consistency and the elastic and viscous moduli, leading to doughs which were more resistant to deformation. From a molecular standpoint, the effect was ascribed to protein crosslinking and gelation of water soluble pentosans in the rice flour. Protein crosslinking resulted from the ability of hydrogen peroxide to form disulfide bonds, as indicated by the decrease in free SH groups (Gujral and Rosell 2004b). Simultaneously, a decrease in the amount of free amino acids was reported, which implied the formation of additional covalent crosslinks (Gujral and Rosell 2004b). The action of GO on other GF (corn, sorghum, brown rice and teff) was tested by Renzetti and Arendt (2009a), showing that enzyme effect was dependent on the type of flour and enzyme concentration. GO improved the specific volume and crumb structure of breads made with corn or sorghum flour, but crumb softening was only observed in corn. The observed changes in baking quality were associated with increased elastic-like behavior, viscosity and resistance to deformation (i.e. increased $G^*$) of the GO treated batters. On the contrary, none or minor effects were reported for brown rice or teff flour. Overall, GO offers an alternative to promote rapid dough or batter crosslinks in GF systems, but the primary protein structures greatly determines the final effect on GF batters and breads.

Polyphenoloxidases that catalyze the polymerization of the phenolic compounds such as catechol, pyrogallol, and gallic acid to quinones by molecular oxygen are designated, based on their substrate specificity, as tyrosinase (EC 1.14.18.1), catechol oxidase (EC 1.10.3.2) and laccase (EC 1.10.3.1). Free radical generated in these reactions are mainly responsible for the protein-protein cross-linking, ferulic acid mediated protein-

arabinoxylan interactions and diferulated oxidation of arabinoxylans. Laccase is able to stabilize the dough structure by cross-linking proteins and proteins with arabinoxylans, resulting in a strong arabinoxylan network by oxidative dimerization of feruloyl esters through ferulic acid. In wheat bread applications, laccase has been reported to decrease arabinoxylans extractability, increase oxidation of sulfhydryl groups and the rate of protein depolymerization during mixing (Labat, Morel, and Rouau 2000). These specifically catalyzed actions are mainly responsible for the improvement of wheat flour dough properties (Houben, Hochstotter, and Becker 2012, Labat, Morel, and Rouau 2000). Laccase supplemented wheat dough has higher strength and stability and lower stickiness, improving its machinability and leading to softer crumb in baked products (Selinheimo et al. 2006, Caballero, Gómez, and Rosell 2007). Consequently, increased loaf bread volume and improved crumb structure and softness have been reported (Goesaert et al., 2005; Labat, Morel, & Rouau, 2000).

Studies on laccase applications in GF breads are limited. Renzetti et al. (2010) reported the increased specific volume and softening crumb effect of preparations of laccase containing endo-β-glucanase side activity for making GF oat flour. Authors explained the improvement by the increase in batter softness, deformability and elasticity, in part due to the β-glucan depolymerisation. Flander et al. (2011) also reported high specific volume of oat bread combining *Trametes hirsute* laccase and xylanase, although crumb softness remained unaltered.

### *3.1.3 Further considerations on cross-linking enzymes in GF applications*

From a rheological standpoint, GF batters treated with TGase or GO show a considerable increase in elastic-like behavior and in the resistance to deformation, which results from the promotion of large protein aggregates in comparison to a

dispersed protein phase of the non-treated batters. Protein polymerization may enhance the continuity of protein networks by strengthening those already present in the floury endosperm (Renzetti et al., 2008a) or by promoting the formation of supramolecular aggregates within the native GF proteins (Renzetti et al., 2008a; Renzetti et al., 2012)(Figure 3A,B). When GF batters are supplemented with functional proteins from other sources (e.g. soy and whey protein isolate, egg), protein networks can be the result of heterologous protein complexes. The changes in the rheological and microstructural properties of the batters are reflected in the breadmaking performance of the GF system, resulting in significant improvements especially in terms of crumb structure (Renzetti et al., 2008a; Moore et al., 2006; Marco and Rosell, 2008c). The effect of the observed changes in rheology and microstructure have not been unanimous, with some authors reporting negative influences on specific volume and crumb hardness (Renzetti, Dal Bello, Arendt, 2008; Moore et al., 2006; Marco and Rosell, 2008c), and others reporting high volumes and soft crumbs (Gujral and Rosell, 2004a; Gujral and Rosell 2004b). As stated earlier, variations in the GF formulations in terms of water amounts, enzyme dosage and protein source and amount may modulate considerably the effects on baking quality. Furthermore, hydrocolloids such as HPMC has been used in some of the reported formulations, while others have relied only on the breadmaking properties of the GF flours. Synergistic interactions between enzymatic induced molecular and rheological changes with HPMC should therefore be carefully considered. An overview of successful GF formulations with TGase or GO application is provided in Table 2.

*3.2. Proteases in GF baking applications*

Proteases (EC 3.4), which include proteinases and peptidases, are enzymes capable of hydrolyzing the peptide bonds in proteins. In standard baking applications, proteases are

generally used to weaken gluten strength, reduce mixing time, decrease dough consistency, improve machinability and extensibility of the dough, ensure dough uniformity, regulate gluten strength in bread, control bread texture and also to improve flavor (Goesaert et al., 2005; Di Cagno et al., 2003; Mathewson, 1998). In addition, proteases have largely replaced bisulfite, which was previously used to control consistency through reduction of gluten protein disulfide bonds, while proteolysis breaks down peptide bonds. In both cases, the final effect is a similar weakening of the gluten network (Linko et al., 1997). Apart from direct baking applications, proteases can also be applied to improve the functional properties of cereal proteins (Xiangzhen Kong et al., 2007; Celus et al., 2007) in order to develop functional ingredients.

The application of proteases to improve GF bread quality have been first proposed by Renzetti and Arendt (2009b), which reported a 1.3 fold increase in specific volume and 0.3 fold decrease in crumb hardness for brown rice bread treated with a commercial protease (Neutrase from *Bacillus amyloliquefaciens*). The study was performed on a simple formulation based on brown rice flour and water without the addition of hydrocolloids. Therefore, the gas retention capability and the structure forming properties were mainly relying on the functionality of the rice flour constituents, i.e. proteins and starch. From a rheological standpoint, improved batter expansion was related to a decrease in the resistance to deformation of GF batters (decrease in complex moduli $G^*$), while maintaining a similar ratio of the viscous to elastic behavior (i.e. tan $\delta$), which favored film formation and gas retention. Similar effects on batter rheology were confirmed in a later study at both small and large deformations by application of Neutrase in oat breads (Renzetti et al., 2010). The increase in batter deformability and elasticity obtained with protease treatment were related to increased stability of the batter film during expansion of the gas cells. The improved film stability prevented

401 premature gas cell rupture and collapsing of dough during proofing and oven spring, as
402 suggested by a considerable increase in the maximum height of batter during proofing.
403 A similar rheological mechanism was also observed with rice bread supplemented with
404 whey proteins and it was related to specific protein functionality among those of
405 varying dairy sources (Nunes et al., 2009).
406 Gas cell stabilization in protease treated rice bread was further elucidated by Hamada et
407 al. (2013), which showed the retention of many small bubbles during fermentation as
408 compared to large and irregular air bubbles in the collapsing control batter. The
409 improved gas retention with yeast fermentation was related to a considerable reduction
410 in sedimentation of the flour particles for the protease treated batter.
411 From a molecular standpoint, the rheological behavior of the protease treated batters
412 could not be entirely explained by changes in the water holding capacity of hydrolyzed
413 proteins, as further addition of water to untreated rice batters would not provide with
414 similar rheological effects (Renzetti and Arendt, 2009b). Instead, protease induced
415 changes in protein-protein and protein-starch interactions may explain such effects
416 (Renzetti and Arendt, 2009b; Amemiya and Menjivar, 1992). Microscopic analysis of
417 rice batters showed a fine network of interlinked protein-starch aggregates after
418 inducing protein degradation (Hatta et al., 2015; Hamada et al., 2013), thus confirming
419 the relationship between the changes in batter rheology and the observed molecular
420 interactions (Figure 2B). When such molecular structures are achieved, a cellular
421 microstructure is predominantly observed in the GF bread crumb compared to untreated
422 bread (Hatta et al., 2015; Hamada et al., 2013; Kawamura-Konishi et al., 2013).
423 Fine network of protein-starch aggregates were observed with metallo, serine, cysteine
424 proteases and with a protease derived from *Aspergillus oryzae* (Hatta et al., 2015).
425 These enzymes showed almost complete degradation of the α- and β- glutelin subunits

which constitute the main protein fraction of rice (Van Den Borght et al., 2006; Renzetti et al., 2012). On the contrary, the hydrolytic activity of aspartyl proteases did not result in a similar degradation of rice glutelins and neither a similar microstructure (Hatta et al., 2015). Therefore, the improvements in baking quality of rice bread were specifically related to the extended degradation of the α- and β- glutelin subunits, which almost disappeared as protein bands in the SDS electrophoresis gel (Hatta et al., 2015). The glutelin subunits are linked by an intermolecular disulphide bond and further polymerize by disulphide bonding and hydrophobic interactions to form large macromolecular complexes (Utsumi, 1992). Partial degradation of the macromolecular protein structures resulted in opening up of the protein complexes, resulting in an increase in the α- and β- glutelin subunits extracted from batters under reducing conditions and the release of low molecular weight proteins (Renzetti and Arendt 2009b). Similar results were observed also when dissociation of the disulphide linkages between α- and β- subunits of rice glutelins was obtained by addition of glutathione (Yano, 2010). In both cases, the treatments resulted in improved baking quality of rice batters (Renzetti and Arendt, 2009b; Yano, 2010; Yano et al., 2013). Therefore, it remains to be further explored the exact mechanism and the identity of the protein subunits that play a decisive role in the formation of the fine network responsible for improving the expansion of rice batters. Extensive degradation of globulins, which constitute oat main protein fraction, as well as albumins and prolamins were also associated with improved baking performance of batters from oat flour (Renzetti et al., 2010). Overall, improvements in GF bread quality were achieved with protease processing of flours which considerably differed in their protein profile. Hence, the technological functionality provided by proteolytic actions may be derived from varying protein structures and should be further investigated in the future.

Additional to the effects on batter rheology and gas holding properties, a secondary mechanism for the observed improvements in baking quality of GF batters have been ascribed to changes in flour pasting properties (Renzetti and Arendt, 2009b; Renzetti et al., 2010; Yano, 2010; Schober et al. 2007), independently of the flour source used, i.e. rice, oat or sorghum. In general, a decrease in peak viscosity and breakdown of the starch paste were observed with protease treatment (Derycke et al., 2005, Hamaker and Griffin, 1993; Xie et al., 2008). These changes were associated to an improved ability of the starch paste to expand while maintaining the textural integrity of the crumb during baking (Renzetti and Arendt, 2009b; Renzetti et al., 2010; Yano, 2010). Changes in the pasting profiles of the GF batters were related to modifications in protein–starch interactions resulting from the proteolytic activity (Ragaee and Abdel-Aal, 2006; Renzetti and Arendt 2009b). In the concentrated regime conditions of the RVA test, starch granules cannot swell to their maximum because of space restrictions (Derycke et al., 2005). In such conditions, protein structures surrounding the starch granules confer rigidity to the paste, and the rheology of the system is dictated by the rigidity of the suspended particles (Steeneken, 1989). By disrupting the paste rigidity, protein hydrolysis decreases RVA viscosity (Derycke et al., 2005). The improvements in baking performance of the GF batters could not be explained by α-amylase treatment (Hamada et al., 2013; Hatta et al., 2015) and the side α-amylase activity had none or little effect on the pasting curves (Renzetti and Arendt, 2009b; Renzetti et al., 2010).

The extent of protease activity on the GF flour proteins is dependent on the treatment conditions, i.e. temperature and time of incubation. Improvements on baking quality of GF batters were reported for short incubation times, i.e. 30 minutes (Renzetti and Arendt, 2009b; Renzetti et al., 2010), as well as long incubation times, 12-18 hours (Hatta et al., 2015; Hamada et al., 2013; Kawamura-Konishi et al., 2013), with

476 temperature ranges of 23-55˚C. Incubation times have been shown to be a determinant
477 factor for improving GF batter functionality for a specific protease (Hamada et al.,
478 2013). However, the information provided is still very limited and further research
479 should be conducted to relate optimal incubations times to the molecular,
480 microstructural and rheological changes in GF batters and finally link them to baking
481 quality.
482 The de-polymerization mechanism exerted by proteases, whilst proved beneficial for
483 rice and oat batters, has been showed to be detrimental for the baking performance of
484 GF batters based on sorghum and buckwheat, while no effects were observed with corn
485 flour (Renzetti and Arendt, 2009a) . From a rheological perspective, the reason for the
486 detrimental effect may be related to the loss of elastic properties (increase in tan $\delta$),
487 which was associated with the decrease in the resistance to deformation of batters, i.e.
488 $G^*$ (Renzetti and Arendt, 2009a). From a molecular standpoint, buckwheat proteins
489 form web-like structures, which contribute to the textural and baking quality of bread
490 (Renzetti et al., 2008b). TGase treatment improves crumb texture by reinforcing such
491 protein network (Renzetti et al., 2008a), while protease disrupts its continuity resulting
492 in crumb defects. In these type of breads, the integrity of the protein structures may be
493 fundamental to ensure textural quality, unless other structuring ingredients are
494 supplemented, e.g. hydrocolloids (Schober et al., 2007). On the other hand, the
495 information reported is still limited and more extensive research should be conducted on
496 the application and optimization of protease treatment to a wide variety of GF flours.
497

498 **Conclusions**

499 Overall, enzymatic treatment of GF batters is a promising processing technology for
500 improving the breadmaking performance of GF flours. The technology demonstrates the

ability to impart rheological and microstructural changes to GF batters, which enable a substantial improvement in the gas holding and textural properties of GF batters and breads. From the molecular standpoint, the role of the proteins results crucial whenever applying crosslinking enzymes or proteases. The different type of proteins structure determines the effectiveness of the enzymatic treatment, because of that the global effect of the enzymatic treatments are greatly dependent on the flour type and the level of enzyme added. Consequently, each GF system requires a specific optimization of the type of enzymes and the effective levels.

Improvements in GF systems could be obtained without the need of hydrocolloid addition and further research should be conducted in order to understand whether these technologies could be combined to provide synergistic effects. As earlier discussed, molecular interactions between the hydrocolloids and GF flour components should be carefully considered in order to ensure the correct functionality to the GF batter. On the contrary, the use of enzymes in replacement of hydrocolloids could be beneficial to reduce the costs of GF breads as well as the list of additives in view of current market trends towards consumer's friendly, clean label formulations.

As the reported achievements relied on a biochemical modification of GF flours, a further understanding of the molecular mechanisms may open new opportunities for the milling and ingredient supplier industry in the development of GF flours, which have been functionalized by biochemical or physical modification processes. Furthermore, alternative technologies, such as sourdough or gluten-degrading enzymes, could be successfully applied in GF bread not solely to degrade gluten contaminant (Di Cagno et al., 2004), but also to increase the breadmaking functionality of the GF flours. Therefore, although up to now enzymes were considered processing aids, these further applications could allow promoting the term healthy aids.

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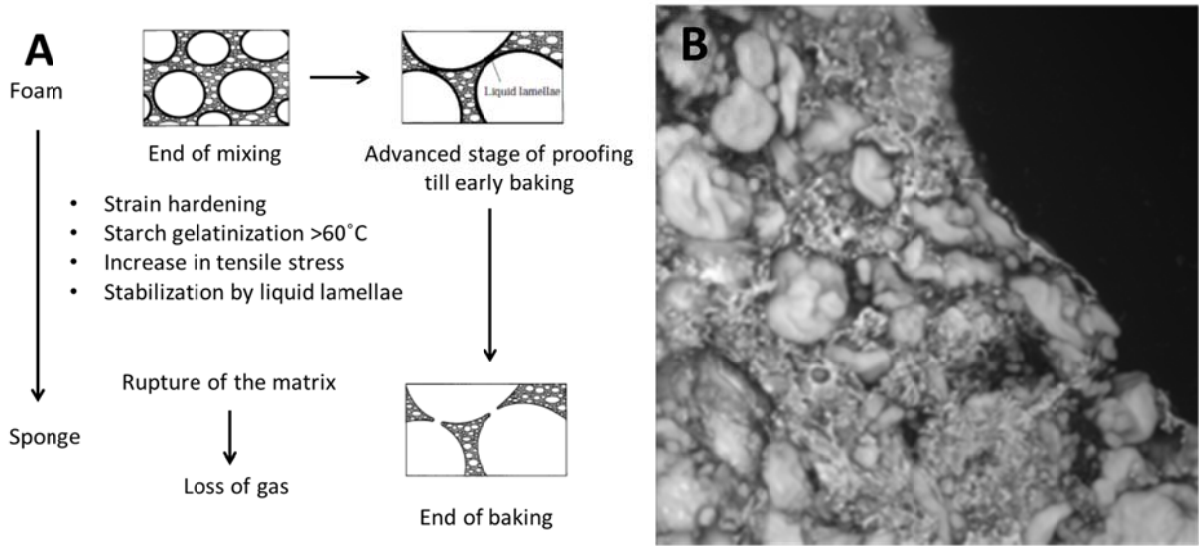


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824 Figure 1. (A) Wheat dough microstructure and mechanisms of expansion and cellular
825 strcture formation during proofing and baking (Adapted from Gan et al., 1995); (B)
826 Confocal laser scanning microscopy image of wheat bread crumb showing the gluten-
827 starch matrix: gelatinised starch granules embedded in the gluten network (Adapted
828 from Zannini et al., 2012).

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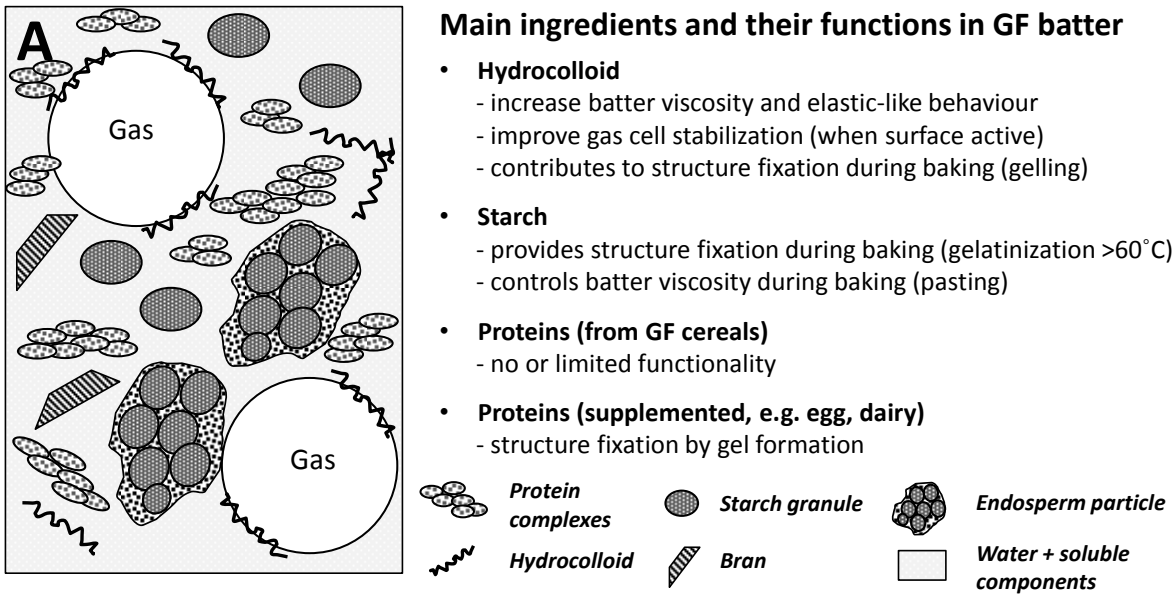


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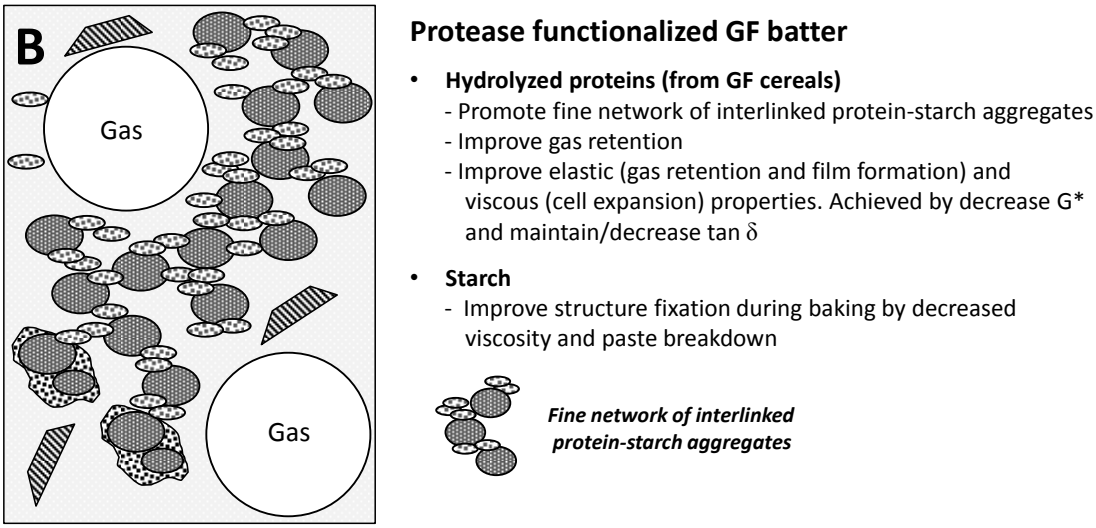


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829 Figure 2. (A) Microstructure of GF batters and main ingredients functionalities (adapted
830 from Schober, 2010); (B) Microstructure of protease treated GF batter and main
831 functionalities provided

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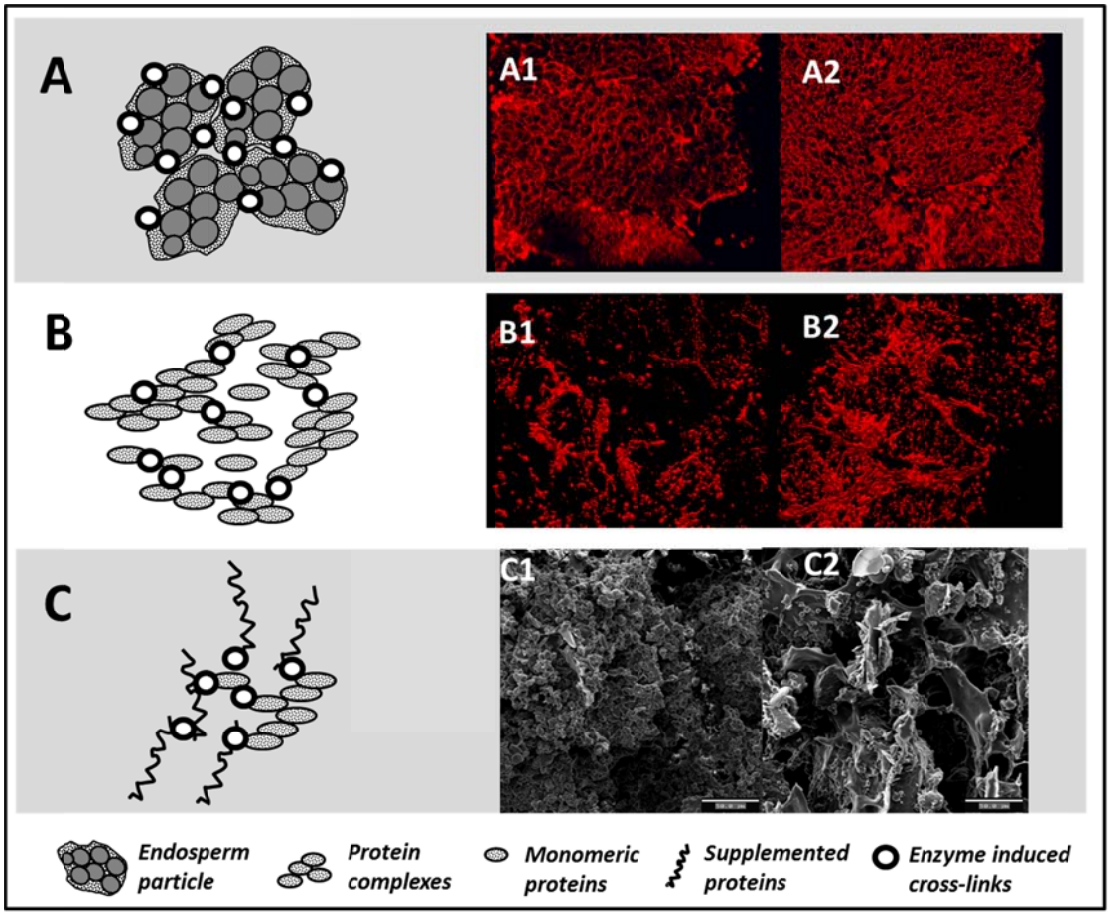


Figure 3. Protein structures promoted by cross-linking enzymes: (A) cross-linkages induced within and among proteins in endosperm particles such as in buckwheat flour, resulting in strengthened protein networks (A2) which are already partially present in the untreated bread (A1) (Renzetti et al., 2008b); (B) cross-linkages induced among protein complexes and monomeric proteins such as in rice flour, resulting in enhanced continuity of the proteins phase (B2) compared to the untreated bread (B1) (Renzetti et al., 2008b); (C) cross-linkages induced among heterologous proteins including GF flour proteins such as in rice flour and supplemented proteins such as soybean proteins (C2) compared to the untreated dough (C1).

845 **Table 1.** Reaction mechanisms of protein modifying enzymes for GF food applications

846 (adapted from Buchert et al., 2007).

| Type of action | Enzyme | Reaction mechanism | Reactive sites in proteins | Reactive sites in carbohydrates |
|---|---|---|---|---|
| Direct Cross-linking | Tyrosinase EC 1.14.18.1 | Oxidation of mono and diphenols to ortho-quinones | Tyrosine | p-CA and caffeic acid, not FA |
| | Laccase EC 1.10.3.2 | Oxidation of aromatic components to radicals | Tyrosine Cysteine | Phenolic acids: FA, etc. |
| | Peroxidase EC 1.11.1.7 | Oxidation of aromatic components to radicals | Tyrosine Other aromatic AAs | Phenolic acids: FA, etc. |
| | Thiol oxidase EC 1.8.3.2 Glutathione oxidase EC 1.8.3.3 | Oxidation of sulfhydryl groups to disulphides (S-S bonds) | Cysteine (-SH) | - |
| | Protein-glutamine gamma-glutamyltransferase (Transglutaminase) EC 2.3.2.13 | Formation of isopeptide linkage through acyl-transfer reactions | Glutamine Lysine | - |
| Indirect Cross-linking | Glucose oxidase EC 1.1.3.4 Hexose oxidase EC 1.1.3.5 | Production of H2O2 in conjunction with glucose oxidation | Cysteine (-SH) | Phenolic acids: FA, etc. |
| Proteolysis | Peptidases EC 3.4 Cysteine endopeptidase EC 3.4.22 Serine endopeptidase EC 3.4.21 Threonine endopeptidase EC 3.4.25 Aspartic endopeptidase EC 3.4.23 Metalloendopeptidase EC 3.4.24 | Hydrolysis of peptide bonds | | - |

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848 p-CA: para-coumaric acid.

849 FA: Ferulic acid.

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853 **Table 2**. Overview of GF formulations with promising enhancement of breadmaking
854 functionality by cross-linking enzymes
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| Main structure forming GF ingredients | Enzyme used | Batter rheology | Bread properties | Molecular effect/ microstructure | References |
|---|---|---|---|---|---|
| Buckwheat flour | TGase | Increased $G^*$<br>Decreased δ | Improved crumb texture<br>Lower specific volume | Cross-linking of major protein fractions<br>Strengthened protein network (web-like) | Renzetti et al., 2008<br>Renzetti et al., 2009 |
| Brown rice flour | TGase | Increased $G^*$<br>Decreased δ | Improved crumb texture<br>Lower specific volume | Cross-linking of glutelins into macromolecular complexes.<br>Entrapment of LMW proteins.<br>Promotion of protein network | Renzetti et al., 2008<br>Renzetti et al., 2012 |
| Corn flour | TGase | Decreased $G^*$ | Higher specific volume<br>Lower crumb hardness | Possibly deamidation of (α-) zein | Renzetti et al., 2008 |
| Rice flour | TGase | Increased $G^*$ | Higher specific volume<br>Lower crumb hardness | Cross-linking of proteins.<br>Reduction of free amino groups and –SH groups. | Gujral and Rosell, 2004a |
| Rice flour | TGase | Increased $G^*$ | Higher specific volume<br>Lower crumb hardness | Cross-linking of proteins.<br>Reduction of free amino groups and –SH groups. | Gujral and Rosell, 2004 |
| Rice flour soybean proteins | TGase | Increased dough consistency | Higher specific volume<br>Higher crumb hardness | Cross-linking β-conglycinin and glycinin of soybean and the glutelins of rice flour.<br>Cross-linking of albumins and globulins. | Marco and Rosell, 2008c;<br>Marco et al. 2008 |
| Rice flour | GO | Increased $G^*$ | Higher specific volume<br>Lower crumb hardness | Cross-linking of glutelins.<br>Reduction of free amino groups and –SH groups. | Gujral and Rosell, 2004b |
| Rice flour, corn flour, potato starch, xanthan gum, egg powder | TGase | Not determined | Lower specific volume<br>Higher hardness<br>Finer crumb structure | Enhanced continuity of egg protein network | Moore et al. 2006 |

| | | | | | |
|---|---|---|---|---|---|
| Rice flour, corn flour, potato starch, xanthan gum, skim milk powder | TGase | Not determined | Lower specific volume<br>Higher hadrness<br>Finer crumb structure | Enhanced continuityof egg protein netwrok | Moore et al. 2006 |

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