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Food Chemistry 89 (2005) 325–332
Food Chemistry
www.elsevier.com/locate/foodchem

Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants

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Received 21 February 2003; received in revised form 25 February 2004; accepted 25 February 2004

Abstract

The crosslinking of wheat flour proteins results in significant improvements in the functional properties of baked products. In this research, the enzyme glucose oxidase was investigated for its crosslinking effect on the dough proteins of bread and croissants. The macroscopic effects resulting from the addition of glucose oxidase to the dough formulation were compared to changes seen at the molecular level in individual protein fractions. Treatment with glucose oxidase produced slight improvements in crumb properties but no increase in product volume. At the molecular level, crosslinking occurred mainly in the water-soluble (albumin and globulin) fraction and was demonstrated to involve both disulfide and non-disulfide linkages. The SDS-soluble and -insoluble glutenins, which make up much of the gluten network, were crosslinked to a much lesser extent, with mainly non-disulfide linkages. These findings corroborate our theories on the relationship of wheat protein crosslinking and functional properties. Specifically, we conclude that (i) crosslinking of albumin and globulin proteins enhances crumb properties and (ii) changes in croissant volume require crosslinking of the glutenin fraction of wheat proteins.

1. Introduction

In wheat flour doughs and baked products, proteins play a major role in product quality and determine, to a large degree, the measured rheological and functional properties of these systems (Wrigley et al., 2000). Wheat-based products thus provide an ideal model system within which to explore the potential of crosslinking agents to alter the properties of foods (Gerrard, 2003). Our research has previously demonstrated that crosslinking of wheat proteins by the enzyme transglutaminase can have a dramatic influence on the properties of bread (Gerrard et al., 1998) and croissants (Gerrard et al., 2000, 2001). Recently, we have also demonstrated the potential of Maillard chemistry to influence the properties of doughs and baked goods via the introduction of covalent crosslinks (Gerrard, Brown, & Fayle, 2002a, 2002b, 2002c). In particular, the reactive crosslinking molecule glutaraldehyde was found to be an effective crosslinking agent in situ.

A comparison of the action of the transglutaminase and glutaraldehyde at both the macroscopic and molecular levels provided some insight into the relationship between the functional properties of bread and croissants, and the particular class of wheat proteins crosslinked (Gerrard et al., 2002c). Both transglutaminase and glutaraldehyde influenced dough properties and had a favourable impact on the texture of bread and croissants but, whilst transglutaminase led to a dramatic improvement in the volume of croissants, glutaraldehyde did not have a significant effect on croissant volume compared to controls. We sought an explanation for this difference in macroscopic behaviour in terms of the protein crosslinking patterns of the two crosslinking reagents. Transglutaminase crosslinked both the albumin and globulin fraction and the SDS-insoluble glutenin fraction of wheat proteins. Glutaraldehyde, on the other hand, crosslinked only the albumin and globulin fraction, in situ. This led us to the hypothesis that the

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high performance liquid chromatography.

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doi:10.1016/j.foodchem.2004.02.052

albumins and globulins play a role in determining dough and crumb properties, whilst the SDS-insoluble fraction, consisting largely of HMW glutenin subunits, is important in determining the characteristics of croissant pastry. In this paper, we seek to test this hypothesis that specific crosslinking patterns correlate with particular macroscopic properties of food using another crosslinking agent – glucose oxidase.

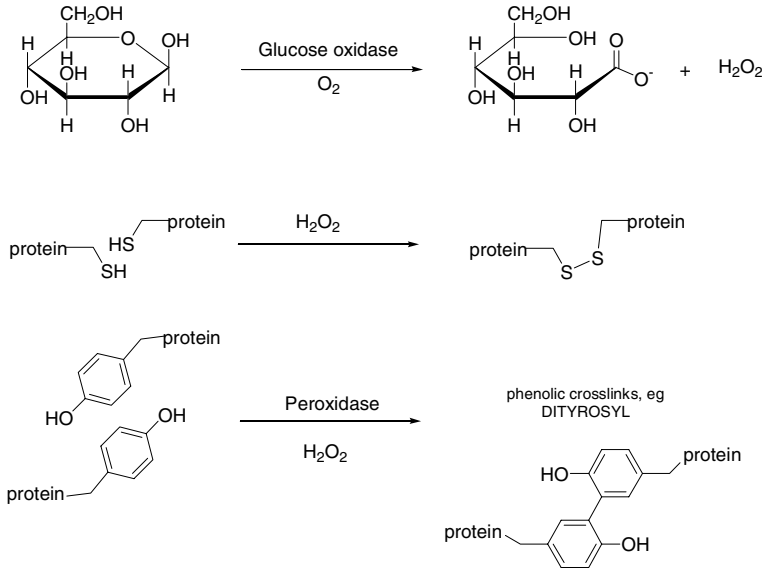
Glucose oxidase has attracted considerable interest in the recent literature, as an improver for wheat flour doughs (Ameille et al., 2000; Dunnewind, van Vliet, & Orsel, 2002; Garcia, Rakotozafy, Telef, Potus, & Nicolas, 2002; Hilhorst et al., 1999; Vemulapalli, Miller, & Hoseney, 1998). Despite this, little detailed work has been carried out as to its mechanism of action, although various theories have been put forward, including the gelative oxidation of pentosans and the strengthening of the protein crosslinking mechanism via the disulfide network. (Garcia et al., 2002, & references therein) The work of Hoseney and co-workers (Miller & Hoseney, 1999; Vemulapalli et al., 1998) suggests that the improvements in the properties of dough and baked bread can be attributed to the introduction of disulfide crosslinks into the gluten network. They propose that glucose oxidase catalyses the conversion of D-glucose, in the presence of oxygen, to D-gluconic acid and hydrogen peroxide (H_2O_2) and that this H_2O_2 oxidises, indirectly, the thiol groups of 2 cysteine residues to form disulfide bonds (Scheme 1). Alternate theories have been discussed by Miller and Hoseney (1999) who point out the possibility of ferulic acid and tyrosine being involved in the crosslinking process. Similarly, Ameille et al. (2000) suggest, instead, that the H_2O_2 produced by the glucose oxidase catalysed reaction acts as a substrate for endogeneous peroxidases in the wheat flour, which catalyses crosslinking via the type of phenolic linkages recently described by Tilley et al. (2001). This is summarised in Scheme 1.

Since glucose oxidase was thought both to introduce protein crosslinks into dough, and to improve dough and bread properties, it was selected for this study to test our theory that certain classes of wheat protein can be specifically manipulated to change the properties of baked products in predictable ways. We also aimed to shed light on which of the two proposed mechanisms of protein crosslinking was operating. We describe herein the macroscopic changes that take place in our test systems on addition of glucose oxidase and examine the nature of the crosslinks introduced by glucose oxidase at the molecular level. These changes are interpreted in the context of alterations in the functionality of baked bread and croissants brought about by other crosslinking agents.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all materials were obtained from Sigma Chemical Company Ltd. (St. Louis, MO, USA). Glucose oxidase was a gift from Enzyme Services (New Zealand) Ltd. The batch used was Ensidase GO WS25, with an activity of 20–30 titrimetric units. Each titrimetric unit oxidises 3 mg of β-D-glucose to gluconic acid in 15 min at 35 °C, pH 5.1. Commercial baking



Scheme 1. Two previously proposed mechanisms of glucose oxidase improvement, via disulfide linkages (Vemulapalli et al., 1998) or phenolic type linkages (Ameille et al., 2000).

flour was purchased from Champion Flourmill and stored at −10 °C.

2.2. Dough preparation

Bread doughs were prepared according to previously published methods. (Gerrard et al., 1998, 2002b) Where required, glucose oxidase (0.003 g), glucose (0.5%), or H_2O_2 (3.4 ml, 10% solution) were added to the water. Doughs were prepared in duplicate for each treatment. The mixing curve of each dough was monitored and the mixer turned off when the optimum degree of mixing (11.3 Wh/kg) had been reached. Croissant doughs were prepared as described previously (Gerrard et al., 2000). Where required, glucose oxidase (0.003 g), glucose (0.5%), or H_2O_2 (3.4 ml, 10% solution) were added to the water. Five croissant doughs were prepared for each treatment.

2.3. Assessment of product quality

Loaf volume was measured by rapeseed displacement. The texture of the loaf was visually assessed, one day after baking, by a trained operator and rated on a scale of 1–11, using a Crop and Food Research Ltd. in-house method (Gerrard et al., 2002b). Croissants were assessed as previously reported (Gerrard et al., 2000).

2.4. Protein extraction

For protein extraction from doughs, each was submerged in liquid nitrogen immediately upon removal from the mixer. They were then freeze-dried and finely ground using a mortar and pestle. One hundred milligrams of samples of dough was weighed into 1.5 ml Eppendorf tubes. Multiple extractions of each protein group (albumins and globulins, gliadins, SDS-soluble glutenins, SDS-insoluble glutenins) were carried out as described previously (Gerrard et al., 2001, 2002b).

2.5. Analysis of dough proteins

Dough proteins, extracted as described above, were analysed by SDS–PAGE and SE-HPLC, as described previously (Gerrard et al., 2001). SDS–PAGE was carried out under reducing and non-reducing conditions for each extract, using pre-cast Tris-glycine *i*-gels (Gradipore, NSW, Australia) at 8% acrylamide concentration.

3. Results and discussion

3.1. Effect of glucose oxidase on bread properties

To observe the macroscopic effects of glucose oxidase treatment on baked loaves, several bread doughs were prepared and evaluated for overall condition, crumb texture and volume, using our standard methods. Doughs were prepared with and without glucose oxidase, and the effect of glucose supplementation was also examined, in order to test whether the enzyme was substrate-limited. A fifth treatment was included which added a product of glucose oxidase, H_2O_2, in the absence of the enzyme itself, since literature reports suggested that H_2O_2 was responsible for the improver effects (Vemulapalli et al., 1998). A high dose was chosen in order to observe any changes to the wheat proteins once extracted from the dough.

Upon baking, loaves containing extra glucose were found to have a darker crust than those without. This was attributed to Maillard browning caused by the reaction between wheat proteins and the added sugar (Fayle & Gerrard, 2002). Doughs prepared with H_2O_2 were tight and extremely difficult to handle and yielded loaves that were half the size of the controls. This is consistent with effects observed in over-oxidised doughs (Hamer & Hoseney, 1998) and reflects the high dose of H_2O_2 employed.

No significant differences were observed between the loaf volume of glucose oxidase-treated doughs with or without glucose, and controls (Table 1). These results are consistent with those of Vemulapalli et al. (1998). Crumb scoring was carried out for all except the fifth treatment. While there were no large differences in crumb texture, the best overall score was observed with the glucose oxidase + glucose treatment, again consistent with literature reports that glucose oxidase improves the texture of baked goods.

3.2. Effect of glucose oxidase on croissant properties

The same glucose oxidase treatments were employed in our standard croissant trials. Croissants prepared with glucose oxidase did not show a significant increase in volume (Table 2). Furthermore, baked croissants containing glucose oxidase exhibited a tendency to lose shape during baking. Interestingly, the best results, in terms of overall texture and crumb patterns, were obtained with the control + glucose treatment, which

Table 1
Treatments and quality scores of bread loaves

Loaf number	Description	Volume[a]	Texture[a,b]	Total score[b]
1	Control	15	7	22
2	Control + glucose	17	9	26
3	Glucose oxidase	14	9	23
4	Glucose oxidase + glucose	14	9.5	23.5
5	H_2O_2	–	–	–

[a] Means of duplicate measurements by trained operator.
[b] Standard error of this in-house measurement = ±2.

Table 2
Weight and volume measurements of baked croissants

Treatment	Fresh weight (g)	Baked weight (g)	Volume (ml)
Control	62.1 (0.13)	50.3 (0.12)	927 (26.6)
Control + glucose	62.4 (0.15)	51.5 (0.17)	919 (26.6)
Glucose oxidase	62.4 (0.15)	50.8 (0.18)	937 (47.6)
Glucose oxidase + glucose	62.2 (0.14)	50.9 (0.25)	918 (35.8)

Figures in brackets represent the standard error of the mean of five measurements.

produced the most desirable "honeycomb"-type pattern. Glucose oxidase did produce some improvements over the control, particularly a thicker, moister and more glutenous crumb and an opening of the size of the gas cells. In addition to this, the layers were better formed than the controls, resulting in an increased flakiness of the crust.

Thus the effect of glucose oxidase was small, in both bread and croissants. Product volume was unaffected, in particular contrast to the dramatic effects produced by transglutaminase in our previous studies (Gerrard et al., 2000). The results were, however, comparable to those obtained on glutaraldehyde treatment (Gerrard et al., 2002c). We thus analysed the changes to the individual wheat protein fractions caused by glucose oxidase treatment, by SDS–PAGE and HPLC, in order to see whether the changes were also analogous to glutaraldehyde treatment at the molecular level.

3.3. SDS–PAGE analysis of protein extracts from glucose oxidase doughs

Four protein classes are recognised in wheat flour, according to the solubility-based system of Osborne (1907): the albumins (water-soluble), globulins (dilute saline-soluble), gliadins (aqueous alcohol soluble) and glutenins (soluble in dilute acid or alkali). In this study, proteins were extracted from control and treated bread doughs using a variation of the methods of Hay and Sutton (1990) and of Batey and Gupta (1991), yielding the following fractions: albumins and globulins, gliadins, SDS-soluble glutenins (soluble in SDS–phosphate buffer, pH 6.9) and SDS-insoluble glutenins (dispersed into SDS–phosphate buffer, pH 6.9, by sonication).

The profiles of the different protein fractions from control and glucose oxidase-treated doughs were analysed by SDS–PAGE, using our previously published methodology (Gerrard et al., 2001, 2002b) for monitoring crosslinking by visualising the disappearance of protein bands, the appearance of streaking and the accumulation of crosslinked polymeric material too large to enter the gel. In order to discriminate between disulfide crosslinks and those of a non-disulfide nature (such as phenolic crosslinks, Scheme 1), the analysis was carried out under both reducing and non-reducing conditions. In reducing conditions, disulfide bonds are broken whereas phenolic (and other crosslinks stable to reducing conditions) are not. A comparison of these two treatments thus gave us a simple method with which to distinguish between the mechanisms cited in the literature for the crosslinking of this enzyme (Ameille et al., 2000; Vemulapalli et al., 1998).

Replicate doughs were made for each of the following treatments: (1) control, (2) glucose oxidase, not prooved, (3) glucose oxidase, prooved 23 °C one hour, (4) glucose oxidase plus glucose, not prooved, (3) glucose oxidase plus glucose, prooved and (5) H_2O_2. The prooved dough was included in case a prolonged incubation with the enzyme influenced the degree and nature of the crosslinking. The results of the analysis are shown in Figs. 1–7, and are discussed below.

3.4. Albumins and globulins

Figs. 1 and 2 clearly demonstrate that the addition of glucose oxidase has resulted in the crosslinking of the albumins and globulins, as seen by the smearing and aggregated material at the top in lanes 3–6 of both gels. This result is in agreement with Vemulapalli et al. (1998) who reported that glucose oxidase affected the water-soluble fraction of wheat flour. Our results suggest that

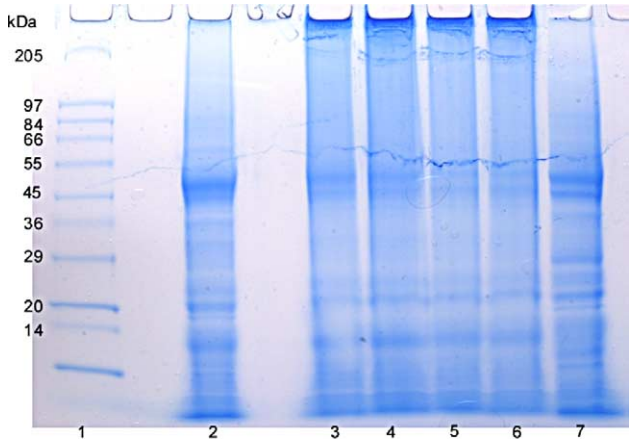


Fig. 1. Non-reduced SDS–PAGE of the albumins and globulins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigma-marker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

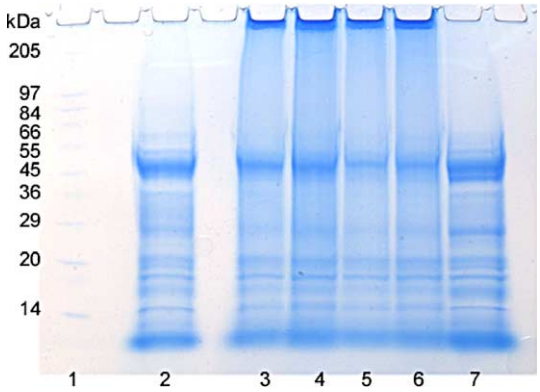


Fig. 2. Reduced SDS–PAGE of the albumins and globulins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigma-marker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

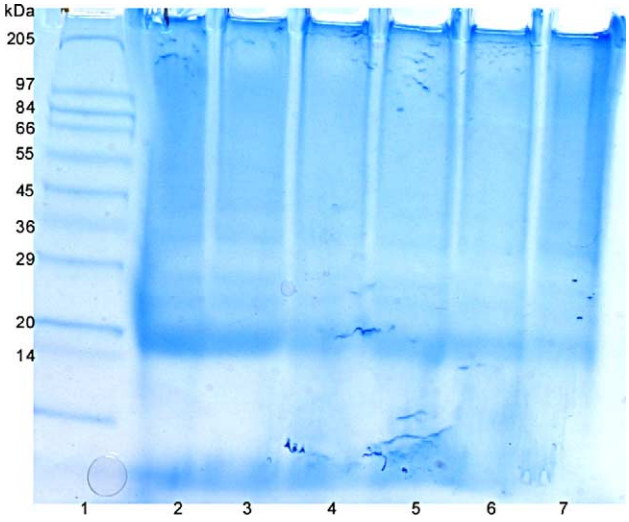


Fig. 3. Non-reduced SDS–PAGE of the SDS-soluble glutenins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigma-marker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

the effect of glucose oxidase on the albumin/globulin fraction is substantial, and that the effect appears to involve both disulfide and some non-disulfide cross-linking. Variables, such as prooving and the addition of glucose, appear to have had no added effect. The minimal loss of the smearing effect upon reduction of the samples (Fig. 2) suggests that, in this fraction of glucose oxidase-treated doughs, the formation of non-disulfide crosslinkages predominates.

In contrast to the glucose oxidase lanes in both figures, no crosslinking is evident in lane 7, representing the H_2O_2 treatment. Band intensities in this

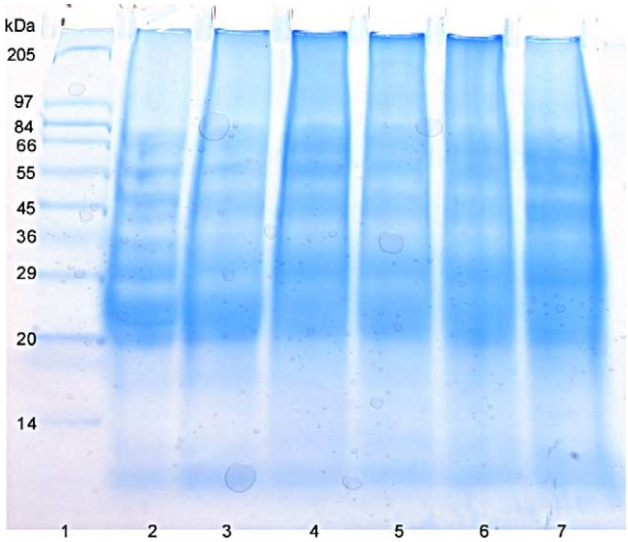


Fig. 4. Reduced SDS–PAGE of the SDS-soluble glutenins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigmamarker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

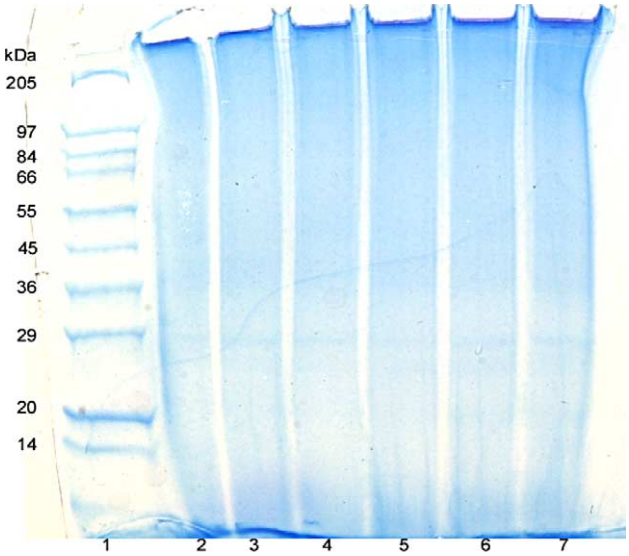


Fig. 5. Non-reduced SDS–PAGE of the SDS-insoluble glutenins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigma-marker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

lane have remained much the same as in the control lane, despite the high dose of H_2O_2 employed. Thus, although H_2O_2 treatment produced dry doughs that were difficult to handle, it does not appear to have affected the albumin/globulin fraction. This is in contrast to the report of Vemulapalli et al. (1998) which

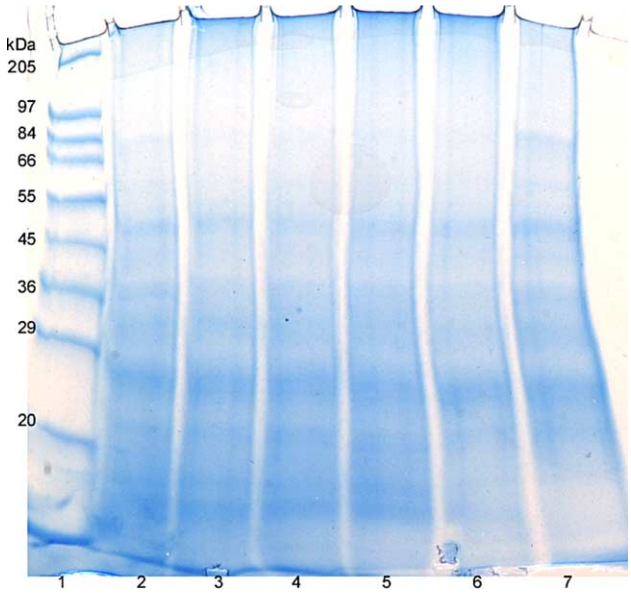


Fig. 6. Reduced SDS–PAGE of the SDS-insoluble glutenins from control, glucose oxidase- and H_2O_2-treated doughs. Lane 1: sigma-marker; lane 2: control; lane 3: glucose oxidase-treated, non-prooved; lane 4: glucose oxidase-treated, prooved; lane 5: glucose oxidase plus 0.5% glucose, non-prooved; lane 6: glucose oxidase plus 0.5% glucose, prooved; lane 7: H_2O_2-treated.

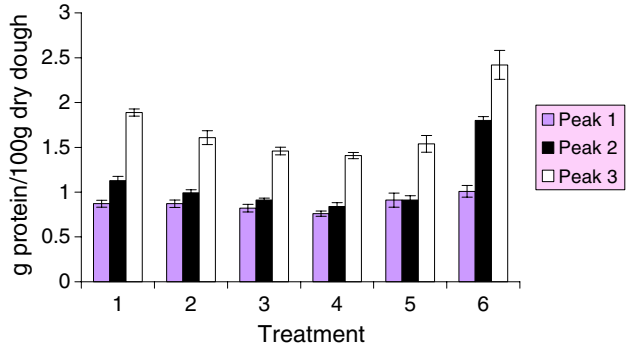


Fig. 7. Changes in amount of protein in the individual peaks on SE-HPLC. Peak 1: aggregated material; peaks 2 and 3: albumins and globulins. Treatments: 1 = control; 2 = GO, non prooved; 3 = GO, 30 min prooved; 4 = GO + glucose, non prooved; 5 = GO + glucose, 30 min prooved; 6 = H_2O_2. Error bars represent standard error of the mean of three measurements.

suggested that the production of H_2O_2 was responsible for the decrease in the sulfhydryl contents of the water-soluble fraction, possibly due to their oxidation into disulfide linkages. Our findings indicate instead, that H_2O_2 production does not cause either type of intermolecular crosslink to form in the albumins and globulins, at least not when added to the dough at the outset of mixing. The possibility of intramolecular crosslinks is not excluded by our analysis.

3.5. Gliadins

Reduced and non-reduced SDS–PAGE revealed negligible crosslinking of the gliadin fraction (data not shown). Gliadin subunits are small, being composed of individual polypeptide chains. The thiol (SH) groups and disulfide crosslinks are internal to the monomeric structure. During dough development, the compact, symmetrical structure resists stretching and linear orientation and the exposure of reactive bonding groups. Therefore, consistent with previous studies, gliadin is not readily susceptible to oxidation and the formation of protein–protein crosslinks (Allen, 1999).

3.6. SDS-soluble glutenins

Figs. 3 and 4 illustrate the effect of glucose oxidase or H_2O_2 treatment on the SDS-soluble glutenin fraction. Not surprisingly, for these large polymeric materials, non-reduced samples (Fig. 3) are heavily smeared in all lanes, including the control. Upon reduction (Fig. 4), the picture shows slightly more evidence of crosslinking in the glucose oxidase lanes than in the control. However, this is much less obvious than the occurrence of non-disulfide crosslinking in the albumins and globulins. The H_2O_2 lane also has some indication of non-disulfide crosslinking, in contrast to the corresponding lanes in the reduced samples of the albumin/globulin and gliadin fractions. Thus glucose oxidase added a limited number of non-disulfide crosslinks to the SDS-soluble glutenins of dough, consistent with the observations of Vemulapalli et al. (1998), who reported a small decrease in the solubility of the SDS-soluble group following treatment with glucose oxidase, with an accompanying reduction in the SH content. Our results suggest that the effect on this fraction may also to be due to the formation of non-disulfide crosslinks, in contrast to the lack of effect of H_2O_2 on the other fractions.

3.7. SDS-insoluble glutenins

Figs. 5 and 6 show the SDS-insoluble glutenins with and without reduction on SDS–PAGE. Whilst smearing is evident in all lanes, including the control of the non-reduced gel, the reduced gel shows more evidence of non-disulfide crosslinking in the glucose oxidase lanes than in the control. As with the SDS-soluble group, this effect is less obvious than the occurrence of non-disulfide crosslinking in the albumin/globulin fraction. This leads to the novel conclusion that the effect of glucose oxidase on the SDS-insoluble glutenins is mainly in the form of non-disulfide crosslinking.

Vemulapalli et al. (1998) found that, apart from the small effect on the SDS-soluble glutenin group, glucose oxidase did not act directly on the glutenin proteins, as measured by protein solubility or the relative viscosity of

protein solutions. Our results show that although no extra disulfide linkages are added to the SDS-insoluble glutenin group, some non-disulfide crosslinking may take place.

3.8. SE-HPLC analysis of protein extracts from glucose oxidase doughs

Samples from the control and treated bread doughs were analysed by SE-HPLC in order to corroborate the SDS–PAGE analysis and assess the changes in protein extractability. A stronger dough was expected to have a lower extractability of protein (Huang & Khan, 1997). It can be seen from Fig. 7 that most of the differences caused by glucose oxidase were observed in the peak corresponding to the albumin and globulin fraction (peak 3), whereas large proteins (peak 1) were mostly unaffected. Prooving the dough made no significant difference to the results. Hydrogen peroxide leads to a substantial increase in extractability, consistent with a dough that had been weakened through over-oxidation. The SE-HPLC data thus provide further evidence of the small but definite effect of glucose oxidase treatment on the albumins and globulins of dough.

4. Conclusion

The flour-improving action of glucose oxidase has been previously ascribed to the H_2O_2 produced during the oxidation of glucose. Most of the literature reports have noted that the improvements are related to crumb texture and strength, and product volume has not been observed to increase with glucose oxidase treatment.

Our study of glucose oxidase has shown that the macroscopic effects of adding this enzyme to baked products are small. The relatively minor effects to the dough proteins observed at the molecular level add support to this finding. However, most notable of the molecular changes are the crosslinking of the albumin/globulin fraction with both disulfide and non-disulfide bonds, and the slight occurrence of non-disulfide crosslinking in the gluten proteins, both of which are novel findings. These crosslinks are as yet unidentified, but their behaviour is consistent with dityrosine linkages.

Interpretation of these glucose oxidase results, in the light of our findings with TGA (Gerrard et al., 1998, 2000, 2001), as well as with other crosslinking work in our laboratory (Gerrard et al., 2002a, 2002b, 2002c), leads to some interesting conclusions. The reaction catalysed by glucose oxidase was found, in this work, to cause improvements to crumb properties in both bread and croissants. Molecular studies established that it was mainly the albumins and globulins that were crosslinked and that this included both disulfide and non-disulfide crosslinks. A lesser occurrence of non-disulfide bonding was also identified in the glutenin proteins, which did not appear sufficient to affect croissant volume to the same extent as TGA.

It may thus be hypothesised that it is the particular protein group becoming crosslinked, rather than the type of crosslink or the crosslinking agent employed, which is important in the improvement of specific functional properties. Obviously, different agents target different protein groups, with the consequent changes in different functional properties. The crosslinking of the water-soluble albumins and globulins, whether by TGA, glucose oxidase or chemical crosslinkers, results in particular improvements to textural properties in baked products. In contrast, a dramatic increase in the strength of the gluten network, reflected in dough properties in bread or product volume in croissants, correlates with the crosslinking of the SDS-insoluble glutenins of wheat-based dough. These observations are of value to those designing flour improvers to enhance the properties of specific baked products using protein crosslinking technology.

Acknowledgements

This work was funded by Crop & Food Research Ltd as part of contract C02X0001 for the New Economy Research Fund, Foundation for Research Science and Technology, New Zealand. We thank Enzyme Services (NZ) Ltd for donating the glucose oxidase. We thank Marcela Ross and the late Russell Sara for invaluable technical expertise.

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