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# Changes in proteins induced by heating gluten dispersions at high temperature

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**Abstract**

Aqueous dispersions of gluten (1.6 mg/ml) were heated for varying times at temperatures from 30 to 120 °C and the proteins characterized by size exclusion-high performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. At temperatures below 100 °C at pH 5.0, only increases in the molecular size of the glutenins were observed. At 120 °C, gliadins as well as glutenins polymerized as seen by a progressive shift of the SE-HPLC profile to lower elution times with time of heating. The gliadins appear to cross-link only with glutenins, as no peaks were observed at elution times expected for gliadin oligomers. The changes in the chromatographic profiles were accompanied by increasing amounts of protein becoming unextractable even with sonication. ω-Gliadins did not participate in the changes, suggesting that disulfide bonding was involved. After polymerization at 120 °C, the kinetics of reduction of the protein by mercaptoethanol were monitored by SE-HPLC. The activation energy of the reduction process was calculated to be 35 kJ/mol.
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*Keywords:* Gluten; High temperature heating; Sulphydryl-disulphide interchange

## 1. Introduction

The behavior of gluten proteins subjected to relatively high temperatures has been studied by a number of workers. It was shown (Booth et al., 1980; Schofield et al., 1983) that the baking performance of gluten decreased progressively on heating and that most of its functionality was destroyed at 75 °C. As the extractability of the gluten proteins decreased, simultaneously the molecular size was shifted to higher molecular weights. At temperatures up to 75 °C, only glutenins appeared to react but, at 100 °C, gliadins were also affected in a similar way. It was postulated that the effect of heat was to induce sulfhydryl-disulfide interchange reactions. Weegels et al. (1994a,b) have questioned this, as their results showed that in heated gluten, free sulfhydryl groups decreased and disulfide bonds increased in heated gluten, consistent with oxidation of sulfhydryl groups. Heat-induced changes in properties of gluten proteins as a function of moisture content were also reported in these studies. Spectroscopic and fluorescent techniques were used by Guerrieri and Cerletti (1996) and Guerrieri et al. (1996) to study heat-induced modifications of gluten. They observed that after heating at 110 °C for 18 h, the insolubility was not reversed by dithiothreitol, suggesting that changes other than disulfide bridging were involved.

Apart from its fundamental interest, the effects of heating gluten proteins are relevant to practical processes such as the drying of rain-damaged wheat, drying of gluten from starch/gluten manufacturing plants, and in relation to effects on gluten proteins during baking and extrusion and the potential for producing modified glutens with unique technological properties.

Recently size exclusion-high performance liquid chromatography (SE-HPLC) has emerged as a rapid and precise method for quantifying wheat proteins and their fractions. This technique was used to examine the behavior of gluten proteins subjected to high temperature. The aim was to follow the polymerization of the different gluten protein fractions and to monitor the changes in molecular size distribution with time. In addition, the kinetics of reduction

*Abbreviations:* MWD, molecular weight distribution; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SE-HPLC, size exclusion-high performance liquid chromatography; TFA, trifluoroacetic acid.

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of the polymerized protein was measured to throw further light on the phenomenon by enabling a calculation of the activation energy for bond breaking.

## 2. Experimental

### 2.1. Gluten preparation

Gluten was prepared from the wheat variety Karl 92 by hand-washing, freeze-drying and grinding in a coffee grinder. The gluten powder was used after passing through a 132 μm sieve. Gluten protein was dispersed in deionized water to the concentration required and the pH was adjusted by addition of 0.1 M NaOH or 0.1 M HCl. Dispersions were heated in a water bath up to 100 °C or in an autoclave at 120 °C.

### 2.2. Size-exclusion HPLC

SE-HPLC was conducted using a HP 1100 system (Hewlett Packard) with automatic injection. All samples were sonicated (unless otherwise stated) at 15 W for 45 s, centrifuged at 3000*g* for 15 min and loaded on a Biosep-SEC-S4000 column (Phenomenex, Torrance, CA). The eluting solvent was acetonitrile/water (1:1,v/v) containing 0.05% trifluoroacetic acid (TFA). Two elution protocols were used, one using a slow flow rate of 0.5 ml/min (Batey et al., 1991) and the other using a faster flow rate of 2.0 ml/min (Larroque and Bekes, 2000).

The SE-HPLC column was calibrated using protein standards with a range of molecular weights (kDa) as follows: ribonuclease A (13.7), chymotrypsinogen (25.0), ovalbumin (43.0), bovine serum albumin (67.0), aldolase (158), catalase (232), ferritin (440) and thyroglobulin (669). The molecular weights were calculated from the line of best fit through the calibration points, given by: log molecular weight $= 8.1448 - 0.1995 \times$ elution time (min).

### 2.3. SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was run on a mini gel system (Novex, San Diego, CA). Protein was dissolved in reduction buffer at pH 6.8 and separated on 10% SDS-PAGE minigels in a discontinuous system. The minigels were run at a constant voltage of 200 V for 1 h. Gels were stained according to the procedure of Neuhoff et al. (1988).

## 3. Results and discussion

### 3.1. Preliminary experiments

In preliminary experiments, gluten dispersions (1.6 mg/ml, pH 5.0) were heated at 30, 45, 70, 90, and 100 °C for different times up to 120 min. At pHs below 4.0, protein solubility increased and changes in the SE-HPLC profile with time were much less than at higher pHs. It is possible that deamidation is occurring at the lower pHs, as noted by Wu et al. (1976). SE-HPLC of samples heated at pH 5.0 showed no changes in the gliadin peak, but above 70 °C, the peaks in the chromatograms in the region of the intermediate-size glutenins showed a shift towards lower elution times. This is consistent with the results reported by Schofield et al. (1983) in which glutenins were shown to be more susceptible to heat than gliadins. On the basis of these results, a more detailed study was made of gluten heated at a higher temperature (120 °C) in an autoclave.

### 3.2. Effects of heating at 120 °C

Dispersions of gluten (1.6 mg protein/ml) were made up to pH 5.0 and held in an autoclave at 120 °C for times up to 60 min and agitated on a vortex mixer. Changes in the elution profile from SE-HPLC are shown in Fig. 1. As the time of heating increased, in addition to the apparent shift of intermediate-size glutenins, there was a progressive decrease in the main gliadin peak and a corresponding increase in the peak at the void volume. The simplest interpretation is that gliadins are being cross-linked into the glutenin structure. This is implied by the absence of gliadin oligomers which would be expected to appear in the elution time range of approximately 13–17 min. There may be two types of reaction occurring. The polymerization of glutenins at temperatures below 100 °C may involve oxidation of thiol groups as postulated by Weegels et al. (1998a,b) based on their finding of a decrease in free sulphydryl groups. On the other hand, the incorporation of gliadins into the glutenin structure, which occurs at higher temperatures, may result from a sulphydryl-disulphide interchange. Most gliadins have an even number of cysteine residues (Kasarda, 1989), which participate in intramolecular disulfide bonds. The failure of ω-gliadins, with no cysteine residues, to react is strong evidence that the polymerization occurs through disulfide bonds.

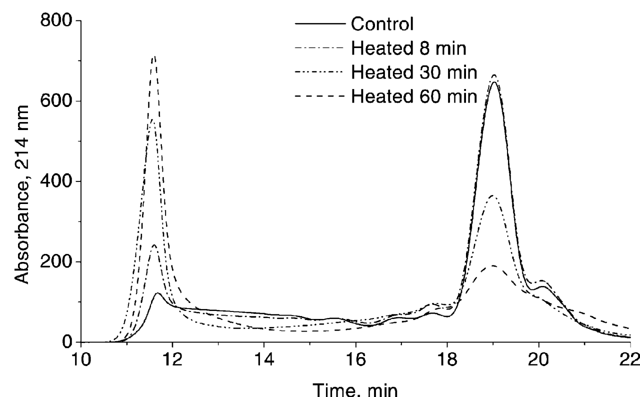


Fig. 1. SE-HPLC profile showing changes in gluten protein after heating at 120 °C for different times.

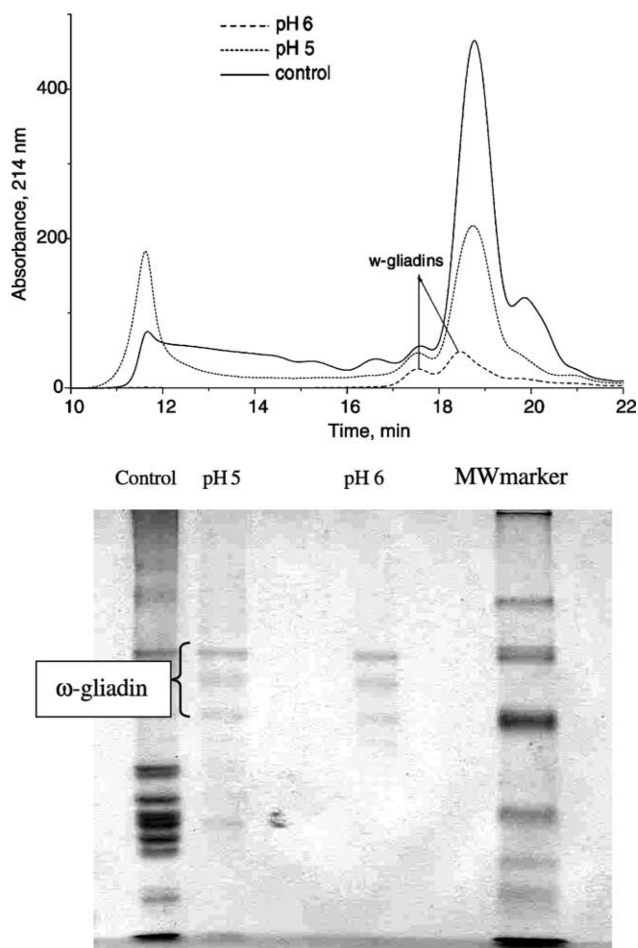


Fig. 2. Upper. Changes in SE-HPLC profiles after heating gluten dispersions for 20 min at pH 5.0 and 6.0. All samples were agitated on a vortex mixer before chromatography. Lower. SDS-PAGE of fractions collected from column in the elution time range 16.5–22 min. Stained with Coomassie Brilliant Blue.

### 3.3. Heating at different pHs

Preliminary experiments showed that, as the pH was lowered, the rate of change of the elution profile during heating markedly decreased. Fig. 2 (upper panel) shows the effect of heating for a constant time (20 min) at 120 °C at two pHs, 5.0 and 6.0. In this experiment, the protein was solubilized using sonication. At pH 5.0, the area of the gliadin peak decreased and there was a corresponding increase in the intermediate-size glutenin peak. Part but not all, of the gliadin peak is recovered in the peak at the void volume. Evidently, the loss of protein is due to the formation of very large polymers, which are not soluble, even after sonication. At pH 6.0, there is a much greater loss of gliadin and there is no appearance of protein in the glutenin region, i.e. elution times lower than 16 min. The fraction eluting at times above 16.5 min was collected at both pH 5.0 and 6.0 and run on SDS-PAGE. The patterns are shown in Fig. 2 (lower panel). The fraction collected from the sample that was heated at pH 5.0 shows ω-gliadins together with other gliadins. However, the fraction from the pH 6.0 sample shows only γ-gliadins. The small peaks appearing in the elution profile (Fig. 2) have elution times corresponding to ω-gliadins. ω-Gliadins do not contain cysteine residues which strongly suggests that the cross-linking of the α-, β- and γ-gliadins is occurring through disulfide bonds in agreement with the conclusions reached by Schofield et al. (1983).

### 3.4. Change in molecular weight distribution

The changes in amount of protein, measured by the chromatogram area, for the different protein fractions with time of heating at 120 °C is shown in Fig. 3. The elution time ranges were 10–12.5 min (peak 1), 12.5–16.5 min (peak 2) and 16.5–22 min (peak 3). Peaks 2 and 3 show a continuous decrease with time as a result of these proteins being incorporated into high molecular weight polymers eluting in peak 1. Peak 1 initially increases as proteins from peaks 2 and 3 polymerize and are shifted into peak 1. However, as the size of the polymers in peak 1 increase in size, they become insoluble even after sonication. Thus, the net area of peak 1 declines after about 20 min. The total area progressively decreases and the net loss of area of the eluted peaks continuously increases, indicating that the polymerized proteins have increased in size to the point where they are no longer soluble.

A molecular weight distribution (MWD) curve constructed (Fig. 4) from the data in Fig. 3 illustrates how this parameter changes with time of heating at 120 °C. The graphs were drawn by measuring the areas in eight elution time ranges and evaluating the molecular weights from a calibration graph using standard proteins. The final point is arbitrary and is intended to represent the protein that has become insoluble, measured by the net loss of chromatogram area. There is no way of estimating the molecular weight of this insoluble protein so that the final part of the MWD curve is dashed to indicate that it is schematic.

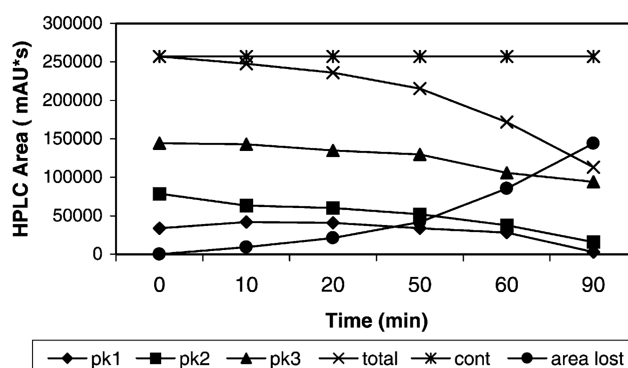


Fig. 3. Changes of area in three elution time ranges, 10–12.5, 12.5–16.5 and 16.5–22.0 min together with area lost as a function of time of heating a gluten dispersion at 120 °C.

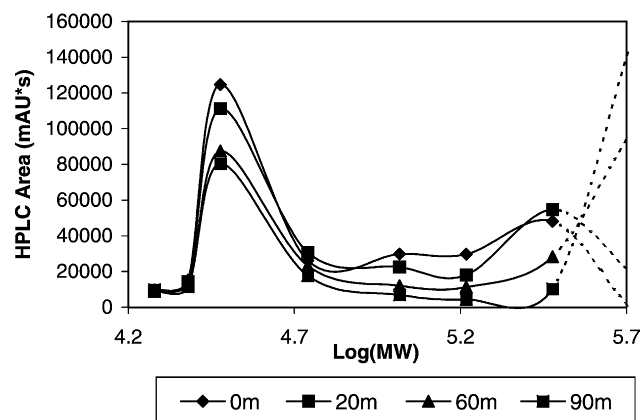


Fig. 4. Change in molecular weight distribution (MWD) with time of heating a gluten dispersion at 120 °C. Dashed line gives an estimate of how the MWD changes, based on the protein that is not solubilized by sonication.

### 3.5. Reduction of heat-polymerized protein

A dispersion of gluten (10%) at pH 5.0 was heated in the autoclave for 20 min, cooled and freeze-dried. The freeze-dried protein (1.6 mg/ml) was sonicated in pH 3 solution (6 W, 30 s), centrifuged at 3000*g* for 15 min and filtered. 2-Mercaptoethanol was added (final concentration 5%) and the vial placed in the injector chamber set at the required temperature. Injections were made on the column at 15 min intervals up to 3 h. Three temperatures were used, 40, 50 and 60 °C. The change in the SE-HPLC profile with time for the reduction at 40 °C is shown in Fig. 5. The void volume peak progressively diminished in area while the amount of protein eluted at times longer than the void volume peak increased. Two main peaks appeared at about 8 and 9 min and these increased in area with time. The peak at about 8 min corresponds to high molecular weight glutenin subunits while the peak at about 9 min corresponds to a mixture of gliadins and low molecular weight glutenin subunits. The rate constants ($k$) for the reduction were

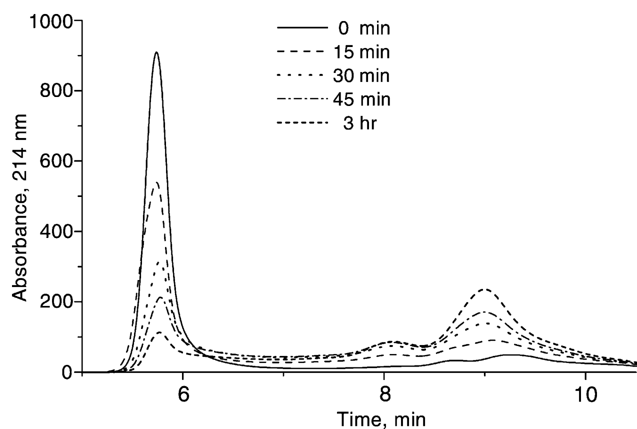


Fig. 5. Change in SE-HPLC profile during reduction of heat-polymerized gluten, measured at 40 °C.

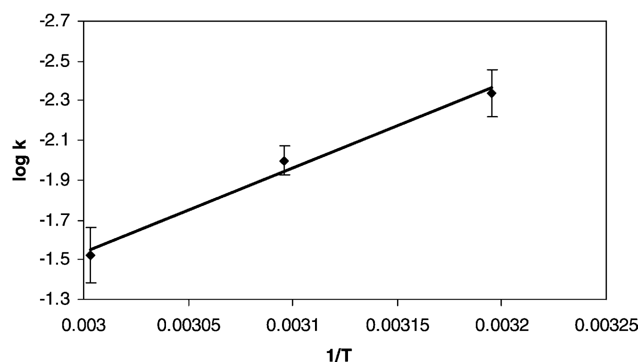


Fig. 6. Log $k$ vs. $1/T$ for kinetics of reduction of heat-polymerized gluten.

calculated from plots of $\log(\mathrm{Area}_{\mathrm{init}} - \mathrm{Area}_t)$ vs. time where $\mathrm{Area}_{\mathrm{init}}$ is the initial area of the void volume peak and $\mathrm{Area}_t$ is the area at time $t$. These plots were linear, consistent with a first-order rate process. Average rate constants were calculated from triplicate measurements. The activation energy for the reduction was estimated from a plot of $\log k$ vs. $1/T$ (Fig. 6) where $k$ is the rate constant and $T$ is the temperature (K).

## 4. Conclusions

At temperatures below 100 °C (pH 5.0), glutenins appear to polymerize but polymerization of gliadins occurs only at higher temperatures. At 120 °C, there is a decrease in the gliadin peak and a corresponding increase in the glutenin peak in SE-HPLC with time. No protein appears at elution times between the two peaks, leading to the conclusion that gliadins are cross-linked into the glutenin structure and do not form gliadin oligomers. The polymerization of glutenin may involve oxidation of sulfhydryl groups whereas sulfhydryl-disulfide interchange may be the mechanism for cross-linking of gliadin. The kinetics of reduction of the polymerized protein were measured at different temperatures from which an energy of activation of 35 kJ/mol was calculated for the reduction process.

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