

# In Vitro Polymerization of Wheat Glutenin Subunits with Inorganic Oxidizing Agents. I. Comparison of Single-Step and Stepwise Oxidations of High Molecular Weight Glutenin Subunits

Wim S. Veraverbeke,<sup>1,2</sup> Oscar R. Larroque,<sup>3</sup> Frank Békés,<sup>3</sup> and Jan A. Delcour<sup>1</sup>

## ABSTRACT

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High molecular weight glutenin subunits (HMW-GS) were isolated from wheat flour and polymerized in vitro at pH 3.0 with different oxidizing agents (KBrO<sub>3</sub>, KIO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>). An oxidation protocol with single addition of oxidant (single-step oxidation) was compared with a set-up in which the oxidant was added in multiple steps (stepwise oxidation). Changes in size distribution were evaluated with size-exclusion HPLC, multilayer SDS-PAGE, and flow-field flow fractionation (flow-FFF). Flow-FFF is particularly suitable for measuring changes in glutenin size in the very high size ranges. In order of increasing sizes of the resulting polymers, the different oxidizing agents could be ranked as KBrO<sub>3</sub> <

KIO<sub>3</sub> < H<sub>2</sub>O<sub>2</sub>. However, none of the oxidation conditions allowed for a complete polymerization of HMW-GS. Interestingly, it was found that high concentrations of KIO<sub>3</sub> negatively affect the degree of polymerization. A similar observation was not made with KBrO<sub>3</sub> or H<sub>2</sub>O<sub>2</sub>. SDS-PAGE showed that  $\gamma$ -type HMW-GS particularly failed to incorporate in glutenin polymers. Simultaneously, these HMW-GS displayed higher mobilities on SDS-PAGE that can be ascribed to the formation of intrachain SS bonds. Possible explanations for the incomplete polymerization of HMW-GS are given.

Gluten proteins impart viscoelastic properties to wheat flour dough and are, therefore, of major importance in determining breadmaking quality. They can be classified as monomeric gliadin or polymeric glutenin. Gliadin is responsible for the viscous properties of dough, while glutenin contributes to dough strength and elasticity. Glutenin consists of polymers built from low molecular weight glutenin subunits (LMW-GS) (MW 30,000–55,000, based on SDS-PAGE) and high molecular weight glutenin subunits (HMW-GS) (MW 80,000–120,000 based on SDS-PAGE) linked by interchain disulfide (SS) bonds (MacRitchie 1992).

Several studies stress the importance of the polymeric glutenin for breadmaking. As reviewed by MacRitchie (1992) and Weegels et al (1996), wheat breadmaking quality positively correlates with the amount of the more insoluble or larger glutenin polymers. Furthermore, differences in HMW-GS composition can be related to differences in quality, as inferred from an empirical breadmaking quality score based on HMW-GS composition (Payne et al 1987). More than 20 different HMW-GS have been detected so far. The structures of HMW-GS, including the distributions of cysteine residues, have been well characterized (reviews by Shewry et al 1992, Shewry and Tatham 1997). Two structurally distinct types of HMW-GS exist: the slightly higher molecular weight  $x$ -type (generally containing four cysteine residues) and the slightly lower molecular weight  $y$ -type (generally containing seven cysteine residues) (Shewry et al 1992). Hexaploid bread wheat cultivars contain two or three different  $x$ -type and one or two different  $y$ -type HMW-GS (Payne 1987). Despite the progress in understanding the structure of HMW-GS, knowledge on the intra- and interchain SS bonding patterns of HMW-GS is still incomplete (review by Shewry and Tatham 1997). Furthermore, the biochemical basis for differences in HMW-GS quality remains unclear.

Based on the current insights in glutenin quality and in the importance of glutenin size distribution in particular, one can assume that differences in GS quality may result from the impact on the size distribution of the glutenin polymers. One approach to test this hypothesis is to compare the intrinsic polymerization behavior

of different GS in vitro. In recent years, preliminary work on the in vitro polymerization of single HMW-GS and  $x$ -type or  $y$ -type pairs of HMW-GS was reported by Szabó et al (1995) and Candler et al (1996). Single native and mutant HMW-GS (Shani et al 1992) or LMW-GS (Thompson et al 1994), obtained by heterologous expression, were polymerized by oxidative refolding of reduced and denatured monomers. Furthermore, in vitro polymerization of HMW-GS mixtures (Werbeck and Belitz 1993, Schropp et al 1995) and of LMW-GS enriched fractions isolated from flour (Werbeck and Belitz 1993) have been described. In the in vitro polymerization experiments with individual HMW-GS,  $x$ -type HMW-GS generally polymerized more readily than  $y$ -type HMW-GS (Szabó et al 1995). Furthermore, fewer monomers remained and larger homopolymers were formed with  $x$ -type HMW-GS. Clear differences between different HMW-GS were observed in polymerization rate, level of remaining monomers, and size distribution of the resulting polymers. Interestingly, a synergistic effect was observed when a mixture of one  $x$ -type and one  $y$ -type HMW-GS was polymerized (Szabó et al 1995). On the other hand, in vitro polymerization of HMW-GS mixtures isolated from wheat flour showed that different mixtures of HMW-GS, generally assumed to have different qualities, polymerized in a very similar way and resulted in polymers with similar size distribution (Schropp et al 1995) and functional properties (Schropp and Wieser 1996).

In this study, HMW-GS were purified from wheat flour and polymerized in vitro using different inorganic oxidizing agents. This was done to further increase our insight into glutenin polymerization. In vitro polymerization as a function of time with addition of oxidant in one single step (single-step oxidation) was compared with a novel set-up in which the level of oxidant was increased gradually in time (stepwise oxidation). For the evaluation of glutenin size distribution, in addition to size-exclusion HPLC, two recently proposed techniques were also used: multilayer SDS-PAGE (Khan and Huckle 1992, Békés et al 1996) and flow-field flow fractionation (flow-FFF) (Stevenson and Preston 1996, Wahlund et al 1996). The latter two promised to offer a better resolution in the high molecular weight range of the very large glutenin polymers. In addition, multilayer SDS-PAGE allows simultaneous evaluation of changes in proportion of different GS during polymerization.

## MATERIALS AND METHODS

### Isolation of HMW-GS

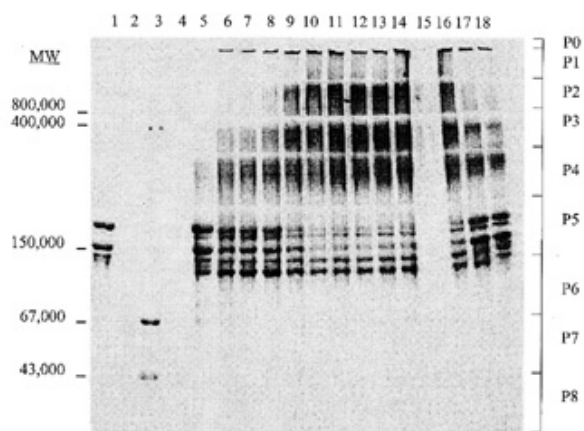
Flour was milled (extraction rate 64%) from wheat cultivar Minaret (HMW-GS composition: 1Ax1, 1Bx7, 1By9, 1Dx5, and 1Dy10)

<sup>1</sup> Laboratory of Food Chemistry, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium.

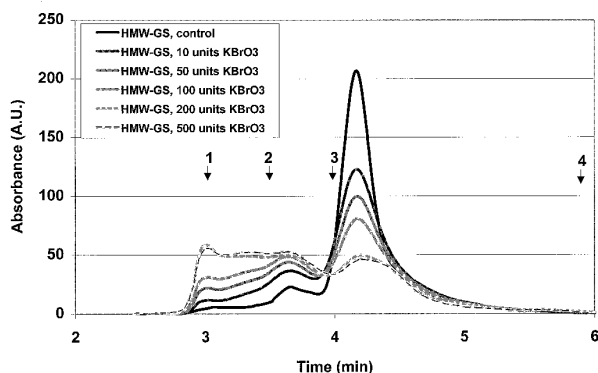
<sup>2</sup> Corresponding author. E-mail: wim.veraverbeke@agr.kuleuven.ac.be Phone: +32-16-321634. Fax: +32-16-321997.

<sup>3</sup> Grain Quality Research Laboratory, CSIRO Plant Industry, P.O. Box 7, North Ryde, NSW 1670, Australia.

(nomenclature according to Payne and Lawrence [1983]) using a Bühler MLU-202 laboratory-scale mill (Uzwil, Switzerland). Flour (500 g) was defatted four times for 1 hr at 20°C with chloroform (1.0 L). Air-dried defatted flour (100 g) was extracted three times (60 min with 500 mL, 30 min with 300 mL, and 30 min with 200 mL) with 50% (v/v) *n*-propanol (*n*-PrOH) under continuous shaking at 20°C to remove all monomeric protein (Fu and Sapirstein 1996). Intermediate centrifugations (10 min, 20°C) were at 1,500 × *g*. GS were subsequently extracted for 60 min at 20°C from the preextracted flour with 50% (v/v) *n*-PrOH containing 5% (v/v) β-mercaptoethanol (333 mL). After centrifugation (30 min, 10,000 × *g*), the extraction was repeated. Supernatants of both extractions were pooled, and HMW-GS were precipitated by dropwise addition of *n*-PrOH containing 5% (v/v) β-mercaptoethanol under magnetic stirring until a final concentration of 60% *n*-PrOH was obtained (Marchylo et al 1989). Suspensions were left for 1 hr at 7°C and centrifuged 30 min at 10,000 × *g*. Precipitated HMW-GS were suspended under nitrogen in 0.1% (v/v) acetic acid containing 0.2% (w/v) dithiothreitol (200 mL) and dialyzed three days under nitrogen against 0.1% (v/v) acetic acid. Finally, the dialyzed protein was freeze-dried.



**Fig. 1.** Profiles of multilayer SDS-PAGE under nonreducing conditions (lanes 5–14) of oxidation products for stepwise oxidation of Minaret HMW-GS with  $\text{KIO}_3$ . Lane 5: HMW-GS starting material; lanes 6–14: HMW-GS oxidized stepwise with 0, 10, 50, 100, 150, 200, 300, 400, and 500 units of  $\text{KIO}_3$ , respectively. Lane 1: reduced Minaret HMW-GS; lane 3: reduced MW markers; lane 16: HMW-GS, oxidized stepwise with 500 units of  $\text{KIO}_3$  followed by single-step oxidation with 500 units of  $\text{KIO}_3$ ; lanes 17 and 18: HMW-GS oxidized by single-step oxidation with 500 and 1,000 units of  $\text{KIO}_3$ , respectively.



**Fig. 2.** Size-exclusion HPLC profiles of oxidation products of stepwise oxidation of Minaret HMW-GS with different levels of  $\text{KBrO}_3$ . Elution times indicated by arrows. MW markers: (1) thyroglobulin (MW 669,000); (2) bovine serum albumin dimer (MW 135,000); (3) bovine serum albumin monomer (MW 67,000); (4) vitamin B-12 (MW 1,375).

## Protein Determination

Protein was determined according to the Dumas total combustion method using an elemental analyzer (CHN-1000, Leco Inc., St. Joseph, MI). The amount of protein was estimated as  $\text{N} \times 5.7$ .

## Free Sulfhydryl (SH) Determination

Free SH groups were determined spectrophotometrically after reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Ellman 1959). Samples (0.5–2.0 mg of protein) were shaken for 1 hr in 1.0 mL of sample buffer (0.05M sodium phosphate buffer, pH 6.5, containing 2.0%, v/v, SDS; 3.0M urea; and 1.0 mM tetrasodium ethylene diamine tetraacetate). DTNB reagent (0.1%, w/v, DTNB in the sample buffer) (100 μL) was mixed with the samples and the absorbance at 412 nm was read after 45 min. Absorbance values were converted to amounts of free SH using a calibration curve with reduced glutathione (0–0.10 μmol).

## SDS-PAGE

SDS-PAGE (Laemmli 1970) was conducted with slab gels (135 × 140 × 1.5 mm) using vertical electrophoresis equipment (SE 600 Hoefer Pharmacia Biotech, San Francisco, CA). A 4% (w/v) acrylamide stacking gel (0.4%, w/v, bisacrylamide) 1.0 cm high was used. For regular SDS-PAGE, a 10% (w/v) acrylamide (0.1%, w/v, bisacrylamide) separating gel (12.5 cm high) was used. For multilayer SDS-PAGE, the separating gel consisted of layers with acrylamide concentrations increasing from top to bottom. The different layers used were 5 and 6% (w/v) acrylamide gels (0.4%, w/v, bisacrylamide) 1.0 cm high; 7, 8, 9, and 10% (w/v) acrylamide gels (0.3%, w/v, bisacrylamide) 1.5 cm high, and a bottom layer (4.5 cm) of 12% (w/v) acrylamide (0.3%, w/v, bisacrylamide). Samples (0.5 mg of protein) were dissolved in 0.125M Tris-HCl buffer (pH 6.8) containing 4.0% (w/v) SDS, 30% (w/w) glycerol, and 0.002% (w/v) bromophenol blue (160 μL) and 20 μL was loaded on the gel. For SDS-PAGE under reducing conditions, the protein samples were incubated (1 hr, 60°C) in the buffer with 1.0% (w/v) dithiothreitol. A constant current (30 mA/gel) was applied until the dye front reached 1 cm from the bottom of the gel. Gels were stained with deionized water containing 40% (v/v) methanol, 7.0% (v/v) acetic acid, and 0.025% (w/v) Coomassie brilliant blue R-250 and destained with deionized water containing 5.0% (v/v) methanol and 7.0% (v/v) acetic acid. The proportion of monomers, the size distribution of polymers (proportion of the polymers present on top of the stacking gel [P0] and in the first [P1], second [P2], third [P3], and fourth [P4] layer of the gel), and the relative proportions of the individual HMW-GS were estimated by densitometry using a Sharp JX 330 scanner (Pharmacia Biotech). Densitometry data were processed with Image Master 1D, version 2.0 (Pharmacia Biotech). The standard proteins used for MW calibration of multilayer SDS-PAGE (Gradipore, Sydney, Australia) were

**TABLE I**  
Size Distribution<sup>a</sup> of Oxidation Products from 24-hr Single-Step Oxidations of Minaret High Molecular Weight Glutenin Subunits (HMW-GS) with Different Levels of  $\text{KBrO}_3$  and  $\text{KIO}_3$

Oxidant (units) <sup>b</sup>	$\text{KBrO}_3$			$\text{KIO}_3$			
	0	50	100	500	50	100	500
P0	0	2	3	3	3	6	3
P1	0	3	2	4	3	6	2
P2	0	8	7	14	8	16	6
P3	3	11	16	23	16	18	12
P4	10	22	23	23	22	21	21
HMW-GS	87	54	49	33	48	32	56

<sup>a</sup> Relative proportion (%) of multilayer SDS-PAGE size fractions. P0–P4 = polymer on top, in the first, second, third, and fourth layer of multilayer SDS-PAGE gel, respectively. HMW-GS = monomer in the fifth and sixth layer of multilayer SDS-PAGE gel.

<sup>b</sup> One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

$\alpha$ -2 macroglobulin (MW 800,000), a partial breakdown product of  $\alpha$ -2 macroglobulin (MW 400,000), phycoerythrin (MW 150,000), bovine serum albumin (67,000), ovalbumin (43,000), and lactalbumin (14,400).

### SE-HPLC

Protein samples (1.0 mg) in 0.05M sodium phosphate buffer (pH 6.9) containing 0.5% (w/v) SDS (1.0 mL) were centrifuged (17,000  $\times$  g, 10 min), filtered (0.45  $\mu$ m), and loaded (20  $\mu$ L) onto a 300-  $\times$  7.8-mm column (Biosep SEC-4000, Phenomenex, Torrance, CA). Separations were at room temperature with a flow rate of 2.0 mL/min. The mobile phase consisted of a 1:1 (v/v) mixture of acetonitrile (190 Grade, Ajax Laboratory Chemicals, Auburn, Australia) containing 0.05% (v/v) trifluoroacetic acid (Sequal Grade, Pierce, Rockford, IL) and Milli-Q water (Millipore Corp., Bedford, MA) containing 0.05% (v/v) trifluoroacetic acid. Eluted protein was detected at 214 nm with a 166 UV detector (Beckman Instruments, Fullerton, CA). The MW markers used for calibration were thyroglobulin (MW 669,000), bovine serum albumin dimer (MW 135,000), bovine serum albumin monomer (MW 67,000), and vitamin B-12 (MW 1,375) (Gradipore). Chromatograms were divided in three groups representing high MW glutenin polymers (MW > 300,000), low MW glutenin polymers (300,000 > MW > 90,000), and HMW-GS monomers (MW < 90,000), respectively.

### Flow-FFF

Protein samples were treated as for SE-HPLC and loaded (20  $\mu$ L) onto an flow-FFF system consisting of a symmetrical channel (F-1000-FO, FFFractionation, Salt Lake City, UT) fitted with a regenerated cellulose membrane (10,000 MW cutoff). Separations were at room temperature with 0.05M sodium phosphate buffer

(pH 6.9) containing 0.5% (w/v) SDS for both the channel (flow rate 2.0 mL/min) and the cross flow (flow rate 2.0 mL/min). Protein was detected with a 166 UV detector (Beckman Instruments) at 214 nm. A calibration curve of protein standards (Gradipore) including bovine serum albumin (MW 66,000) and thyroglobulin (MW 669,000) was determined to relate elution time to polymer MW. The weight-average MW (Billmeyer 1984) was calculated from baseline-corrected and smoothed elution profiles as:

$$\frac{\sum[\text{abs}(t) \times m(t)]}{\sum[\text{abs}(t)]}$$

where abs(t) is the absorbance at elution time  $t$ , and  $m(t)$  is the MW of the polymer eluting at elution time  $t$ .

### Single-Step Oxidation

One unit of oxidizing agent is defined as the amount theoretically needed to oxidize 1% of the free SH present in the HMW-GS used. The amount of free SH was determined experimentally using Ellman's reagent and it was assumed that 1 mol of KIO<sub>3</sub> or KBrO<sub>3</sub> can oxidize 6 mol of SH (Fitchett and Frazier 1986) and that 1 mol of H<sub>2</sub>O<sub>2</sub> can oxidize 2 mol of SH.

Oxidizing agents (KIO<sub>3</sub>, KBrO<sub>3</sub>) (10, 50, 100, and 500 units) (100  $\mu$ L solutions at pH 3.0) were added to solutions of HMW-GS (1.0%, w/v, protein) in deionized water adjusted to pH 3.0 with 1.0N HCl (5.0 mL). These solutions were stirred continuously at 20°C. Duplicate samples were taken for multilayer SDS-PAGE (50  $\mu$ L) and free SH determination (200  $\mu$ L) at regular time intervals (0–24 hr). Protein was immediately precipitated by adding *n*-PrOH to obtain a level of 85% (v/v) *n*-PrOH. The samples were frozen at –20°C before drying (Speed Vac Concentrator, Savant Instruments, Farmingdale, NY) and determination of free SH and multilayer SDS-PAGE.

TABLE II  
Free SH Content<sup>a</sup> and Size Distribution<sup>b</sup> as a Function of Oxidant Level During Stepwise Oxidation of Minaret High Molecular Weight Glutenin Subunits (HMW-GS) with Different Oxidizing Agents

Oxidant	S <sup>c</sup>	Level of Oxidants (units) <sup>d</sup>												
		0	10	50	100	150	200	300	400	500	500 + 500 <sup>e</sup>	+500 <sup>f</sup>	+1,000 <sup>g</sup>	
KBrO <sub>3</sub>	Free SH content	50	45	40	28	16	7	7	4	5	5		2	2
	Size distribution													
	P0	1	3	6	7	5	5	5	6	5	5	4	4	4
	P1	0	0	2	6	5	5	5	5	4	5	5	4	4
	P2	0	0	3	6	9	13	12	13	12	14	14	15	14
	P3	1	6	7	9	14	18	18	19	19	19	21	21	21
	P4	10	23	21	20	22	20	22	21	21	20	20	20	20
HMW-GS	88	69	61	52	45	39	38	36	39	37	36	36	37	
KIO <sub>3</sub>	Free SH content	50	45	40	26	9	3	3	4	5	5		2	2
	Size distribution													
	P0	1	2	3	6	6	8	7	5	5	6	8	4	5
	P1	0	0	1	2	5	9	8	6	7	10	10	3	4
	P2	0	0	3	7	17	22	22	24	24	20	21	8	8
	P3	0	7	10	13	19	20	19	21	22	19	18	13	13
	P4	13	26	25	24	22	21	22	22	21	20	19	19	18
HMW-GS	86	65	58	48	31	21	22	22	21	24	23	53	52	
H <sub>2</sub> O <sub>2</sub>	Free SH content	50	44	37	23	8	4	4	4	3	4		4	2
	Size distribution													
	P0	0	3	5	6	11	12	11	9	8	7	7	7	7
	P1	0	1	3	4	9	13	14	12	11	10	9	9	8
	P2	1	2	8	12	17	20	21	26	24	24	27	27	28
	P3	2	5	9	12	17	14	16	16	15	17	17	17	17
	P4	11	19	22	20	17	16	14	12	14	16	16	17	17
HMW-GS	86	70	54	46	29	25	24	25	28	26	24	23	23	

<sup>a</sup>  $\mu$ mol of SH/g of protein.

<sup>b</sup> Relative proportion (%) of multilayer SDS-PAGE size fractions. P0–P4 = polymer on top, in the first, second, third, and fourth layer of multilayer SDS-PAGE gel, respectively. HMW-GS = monomer in the fifth and sixth layer of multilayer SDS-PAGE gel.

<sup>c</sup> Starting material.

<sup>d</sup> One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

<sup>e</sup> 500 units added stepwise followed by 500 units added in a single step.

<sup>f</sup> 500 units added in a single step.

<sup>g</sup> 1,000 units added in a single step.

### Stepwise Oxidation

HMW-GS (1.0%, w/v, protein solutions [5.0 mL] at pH 3.0) was oxidized at 20°C by stepwise addition of oxidizing agent (KIO<sub>3</sub>, KBrO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>). Every 10 min, 10 units of oxidizing agent (in 20 µL) were added. Duplicate samples were taken at different levels of oxidation (0–500 units) for analysis of free SH (2.0 mg of protein) and size distribution (0.5 mg of protein). For comparison, additional samples were prepared by single-step addition of 500 units oxidizing agent (in 100 µL) to the 500 units stepwise oxidation products, and by single-step addition of 500 units and 1,000 units oxidizing agent (in 100 µL) to the unoxidized HMW-GS solution (1.0%, w/v, protein solutions, 500 µL). All samples were kept overnight at 20°C and then freeze-dried.

## RESULTS AND DISCUSSION

### Isolation of HMW-GS

SDS-PAGE (results not shown) revealed that the HMW-GS preparation was virtually free of LMW-GS. Only minor contaminants with mobilities between those of HMW-GS and LMW-GS were detected. Polymerization during isolation of HMW-GS was limited by removing the excess reducing agent by dialysis under nitrogen. SDS-PAGE under nonreducing conditions (results not shown) showed a minor degree of polymerization. Protein and free SH contents of the HMW-GS preparation were 91.7% (as-is basis) and 50 µmol/g of protein, respectively.

### Single-Step Oxidation

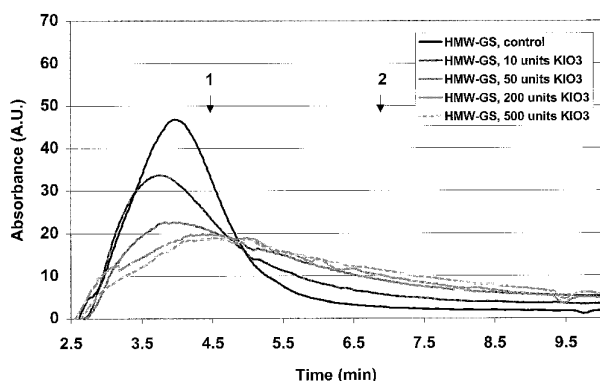
Oxidation of SH groups occurred almost instantaneously on addition of KIO<sub>3</sub>, while oxidation by KBrO<sub>3</sub> was much slower (results not shown). A similar observation was made by Schropp et al (1995) and is in line with the general observation that KIO<sub>3</sub> is a much faster oxidant than KBrO<sub>3</sub> (Fitchett and Frazier 1986). However, multilayer SDS-PAGE gels for both oxidants showed no further disappearance of monomers and no further significant changes in the size distribution of the polymerized material after 5 min of reaction time, suggesting that polymerization was completed with both oxidizing agents after ≈5 min. In the case of KBrO<sub>3</sub>, this contrasts with earlier observations (Schropp et al 1995). Our results indicate that although free SH measurements suggest that oxidation reactions continue after 5 min of oxidation with KBrO<sub>3</sub>, this oxidation does not increase the proportion of interchain SS bonds.

Multilayer SDS-PAGE gels further showed that under the experimental conditions a large fraction of HMW-GS remained monomeric even with high levels of oxidizing agent (Table I). Furthermore, high concentrations of oxidizing agent do not necessarily favor polymerization. Indeed, while an increase in the level of KBrO<sub>3</sub>

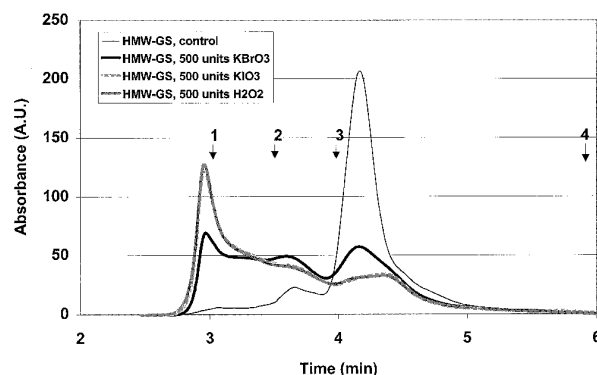
up to 500 units obviously decreased the level of monomers remaining and led to larger polymers, there was an optimal concentration with KIO<sub>3</sub> (Table I). Significantly fewer monomers were incorporated into polymers, and smaller polymers were formed with 500 units of KIO<sub>3</sub> rather than with 100 units (Table I). The conclusion that KBrO<sub>3</sub> is far more efficient than KIO<sub>3</sub> in polymerizing HMW-GS (Schropp et al 1995) is therefore only valid when an excess of oxidizing agent is considered.

A single, well-defined reason for the inability of a certain fraction of HMW-GS to incorporate into glutenin polymers cannot be given. One possibility is that a certain fraction of the SH is not oxidized to SS but to a higher oxidation level (sulphinic or sulphonic acids). It was suggested by Hird and Yates (1961) that such side-reactions occur during oxidation of wheat proteins and thiolated gelatin with oxidizing agents such as KIO<sub>3</sub>. Although it can be calculated from the data by Schropp et al (1995) that at a level of ≈37 units of KIO<sub>3</sub> oxidation to a higher oxidation level than SS does not occur during oxidation of HMW-GS, it is not to be excluded that, at higher levels, other reactions occur as already suggested by the observation that KIO<sub>3</sub> is a less efficient polymerizing agent at higher levels. Interestingly, Hird and Yates (1961) found that at lower SH concentrations more SH oxidized to an oxidation stage higher than SS. A less frequent encounter between free SH might indeed explain a lower probability of SS formation and the consequent occurrence of increased levels of side products. In a similar way, a higher concentration of the fast-acting oxidant KIO<sub>3</sub> might increase the reaction rate of single SH relative to SH that have to diffuse to each other, thereby decreasing the polymerization efficiency.

A second explanation for incomplete incorporation of HMW-GS at full oxidation is the occurrence of alternative SS bond formation. Indeed, formation of an intrachain SS bond between at least one SH that is normally involved in interchain SS bonds inhibits incorporation of the HMW-GS into polymers. Probably, interchain SS bond formation is favored at the initial stages of polymerization. However, as the HMW-GS become polymerized, reaction partners are depleted. Low levels of reacting species (monomers and polymers) favor the formation of the alternative (intrachain) SS bond, because its reaction rate does not depend on the concentration of reacting species. The observation of a higher polymerization efficiency of GS at higher protein concentrations (Beckwith and Wall 1966, Schropp et al 1995) appears to be in line with this view. Furthermore, it might explain why higher concentrations of KIO<sub>3</sub> lead to higher levels of remaining monomers. Indeed, alternative SS formation is expected to occur at an earlier stage of polymerization when the reaction rate of the oxidant is higher.



**Fig. 3.** Flow-field flow fractionation profiles of oxidation products of stepwise oxidation of Minaret HMW-GS with different levels of KIO<sub>3</sub>. Elution times indicated by arrows. MW markers: (1) bovine serum albumin monomer (MW 67,000); (2) thyroglobulin (MW 669,000).



**Fig. 4.** Comparison of size-exclusion HPLC profiles of polymers formed by stepwise oxidation of Minaret HMW-GS with different oxidizing agents (500 units). Elution times indicated by arrows. MW markers: (1) thyroglobulin (MW 669,000); (2) bovine serum albumin dimer (MW 135,000); (3) bovine serum albumin monomer (MW 67,000); (4) vitamin B-12 (MW 1,375).

### Stepwise Oxidation

Because single-step oxidation of HMW-GS showed that high concentrations of oxidizing agent can limit polymerization efficiency, an alternative *in vitro* oxidation (stepwise), in which the level of oxidation was gradually increased by sequential additions of a small amount (10 units) of oxidizing agent was performed. The polymerization efficiencies with  $\text{KBrO}_3$ ,  $\text{KIO}_3$ , and  $\text{H}_2\text{O}_2$  were evaluated by multilayer SDS-PAGE (Fig. 1), SE-HPLC (Fig. 2), and flow-FFF (Fig. 3).

For all three oxidizing agents, a level between 100 and 150 units was required for maximal oxidation of Minaret HMW-GS. At

>150 units, no further changes were observed in either the amount of free SH (Table II) or the proportion of monomers as measured with multilayer SDS-PAGE (Table II) or SE-HPLC (Table III). Furthermore, neither multilayer SDS-PAGE nor SE-HPLC revealed significant changes in the size distributions of the polymers of the 150 units oxidation products on further addition of oxidizing agent. On the other hand, although at a lower rate, the average MW of the polymers as estimated by flow-FFF (Table IV) continued to increase with levels of oxidizing agent at >150 units. Apparently, the ability of flow-FFF to resolve glutenin polymers with sizes

**TABLE III**  
Relative Proportion (%) of Different Size-Exclusion HPLC Fractions as a Function of Oxidant Level During Stepwise Oxidation of Minaret High Molecular Weight Glutenin Subunits (HMW-GS) with Different Oxidants

Oxidant	Fractions <sup>a</sup>	S <sup>b</sup>	Level of Oxidants (units) <sup>c</sup>											
			0	10	50	100	150	200	300	400	500	500 + 500 <sup>d</sup>	+500 <sup>e</sup>	+1,000 <sup>f</sup>
$\text{KBrO}_3$	HMW-polymer	2	nd <sup>g</sup>	5	10	14	26	24	25	24	23	21	25	nd
	LMW-polymer	12	nd	20	25	29	35	37	38	39	38	39	39	nd
	HMW-GS	86	nd	75	65	57	39	39	37	37	38	35	36	nd
$\text{KIO}_3$	HMW-polymer	2	nd	7	nd	31	39	nd	39	nd	37	nd	19	23
	LMW-polymer	12	nd	25	nd	33	32	nd	32	nd	33	nd	33	33
	HMW-GS	86	nd	68	nd	36	28	nd	29	nd	31	nd	49	43
$\text{H}_2\text{O}_2$	HMW-polymer	2	nd	9	17	33	35	36	35	nd	nd	nd	35	nd
	LMW-polymer	12	nd	27	33	32	34	34	34	nd	nd	nd	35	nd
	HMW-GS	86	nd	65	50	34	31	29	29	nd	nd	nd	29	nd

<sup>a</sup> High and low molecular weight polymers (MW > 300,000 > MW > 90,000) and monomeric HMW-GS (MW < 90,000), respectively.

<sup>b</sup> S = HMW-GS starting material.

<sup>c</sup> One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

<sup>d</sup> 500 units added stepwise followed by 500 units added in a single step.

<sup>e</sup> 500 units added in a single step.

<sup>f</sup> 1,000 units added in a single step.

<sup>g</sup> Not determined.

**TABLE IV**  
Flow-Field Flow Fractionation Estimated Average MW (kDa) of Stepwise Oxidized Minaret High Molecular Weight Glutenin Subunits (HMW-GS) as a Function of Oxidant Level for Different Oxidants

Oxidant	Level of Oxidants (units) <sup>a</sup>								
	0	10	50	100	150	200	300	400	500
$\text{KBrO}_3$	91	94	131	166	217	233	244		266
$\text{KIO}_3$	91	126	145	252	255	260	307	313	332
$\text{H}_2\text{O}_2$	91	166	275	302	310	344	354	368	384

<sup>a</sup> One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

**TABLE V**  
Relative Proportion (%) of Different High Molecular Weight Glutenin Subunits (HMW-GS) in Remaining Monomers as a Function of Oxidant Level During Stepwise Oxidation of Minaret HMW-GS with Different Oxidants

Oxidant	HMW-GS <sup>a</sup>	S <sup>b</sup>	Level of Oxidant (units) <sup>c</sup>											
			0	10	50	100	150	200	300	400	500	500+ 500 <sup>d</sup>	+500 <sup>e</sup>	+1,000 <sup>f</sup>
$\text{KBrO}_3$	1Ax1	26	19	21	20	17	14	13	13	12	11	9	11	14
	1Dx5	9	13	13	12	14	13	13	12	14	12	13	11	11
	1Bx7	30	20	22	22	24	23	21	19	18	17	18	18	19
	1By9/1Dy10	35	48	44	46	45	50	53	56	56	60	60	60	56
$\text{KIO}_3$	1Ax1	26	17	19	18	13	10	8	9	9	8	12	20	21
	1Dx5	10	15	14	12	14	14	15	14	14	13	13	10	11
	1Bx7	29	21	21	22	21	18	18	17	17	18	19	28	30
	1By9/1Dy10	35	47	46	48	52	58	59	60	60	61	56	42	38
$\text{H}_2\text{O}_2$	1Ax1	24	22	18	17	11	11	11	10	11	11	8	7	8
	1Dx5	8	11	14	14	12	10	10	11	12	13	11	12	13
	1Bx7	31	22	23	20	15	12	13	12	15	14	11	11	12
	1By9/1Dy10	37	45	45	49	62	67	66	67	62	62	70	70	67

<sup>a</sup> Relative proportion of the different HMW-GS in remaining monomer determined by scanning densitometry of multilayer SDS-PAGE gels.

<sup>b</sup> S = HMW-GS starting material.

<sup>c</sup> One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

<sup>d</sup> 500 units added stepwise followed by 500 units added in a single step.

<sup>e</sup> 500 units added in a single step.

<sup>f</sup> 1,000 units added in a single step.

## CONCLUSIONS

exceeding the exclusion limits of multilayer SDS-PAGE and SE-HPLC allows measurements of changes in size distribution that could not be detected with the latter two techniques. In this respect, further interchain SS formation between the very large polymers is expected to produce significant increases in average size without affecting the SH content in a detectable manner (Table II). Alternatively, SH-SS interchange reactions may explain the increase in average size without noticeable changes in the free SH content.

When comparing the level of remaining monomers and the size distribution of the polymers resulting from oxidation of HMW-GS with the different oxidizing agents, it was clear that  $\text{KBrO}_3$  is a less efficient polymerizing agent than either  $\text{KIO}_3$  or  $\text{H}_2\text{O}_2$  (Tables II–IV, Fig. 4). While no differences in size distribution were obvious with multilayer SDS-PAGE and SE-HPLC between  $\text{KIO}_3$  and  $\text{H}_2\text{O}_2$ , average size estimates with flow-FFF reveal a higher degree of polymerization with  $\text{H}_2\text{O}_2$ .

In agreement with what was observed for single-step oxidation of HMW-GS, in stepwise oxidation, a large proportion of monomers could not be polymerized. Interestingly, not all HMW-GS in the mixture polymerized to the same extent. Table V shows a decrease in the relative proportion of the  $x$ -type HMW-GS 1Ax1 and 1Bx7 and an increase in the relative proportion of 1Dx5 and the  $y$ -type HMW-GS 1By9 and 1Dy10 in the remaining monomers. The  $y$ -type HMW-GS were especially resistant to incorporation into glutenin polymers.

Visually, hardly any polymerization was observed for the  $y$ -type HMW-GS on multilayer SDS-PAGE gels (Fig. 1). Furthermore, these subunits had a higher mobility than fully reduced forms (Fig. 1). We observed that during isolation a certain fraction of  $y$ -type HMW-GS already adopted a higher mobility (SDS-PAGE, not shown). Apart from the major differences in mobility on SDS-PAGE between the reduced and unreduced  $y$ -type HMW-GS monomers, HMW-GS 1Dx5 displayed three different mobility bands on SDS-PAGE under nonreducing conditions. These different forms apparently result from the presence of more or other intrachain SS bonds. HMW-GS 1Dx5 is the only  $x$ -type HMW-GS with higher SDS-PAGE mobility forms. This may be associated with the presence of an additional cysteine residue in the repetitive domain of HMW-GS 1Dx5 that is not found in other  $x$ -type HMW-GS (Shewry et al 1992).

Intrachain SS bonds increase protein electrophoretic mobility (Goldenberg and Creighton 1984). Formation of an intrachain SS bond in  $y$ -type HMW-GS might impede incorporation of these subunits into glutenin polymers if this bond involves a cysteine residue essential for interchain SS bond formation. Alternatively, partial folding of HMW-GS as a result of intrachain SS bond formation might also make the essential cysteine residues inaccessible.

Increased mobility of  $y$ -type HMW-GS was also observed by Werner et al (1992) after partial reduction of glutenin. Shani et al (1992) suggested that an intrachain SS bond between the two cysteine residues at the C-terminal end of  $y$ -type HMW-GS possibly restricts polymerization. Recently, Shimoni et al (1997) demonstrated the existence of an intrachain bond linking N- and C-terminal domains of a recombinant subunit consisting of the N-terminal part of 1Dy10 and the central and C-terminal domain of 1Dx5. This bond was held responsible for the low incorporation of this construct into polymers.

An interesting observation was made when comparing the effect of stepwise oxidation and single-step oxidation with  $\text{KIO}_3$ . As shown in Fig. 1 and Tables II and III, significantly fewer monomers are polymerized, and the polymers are smaller when a large amount of  $\text{KIO}_3$  (>500 units) is added at once, compared with the situation where the same amount is added in several steps. This again indicates that, depending on the  $\text{KIO}_3$  concentration, different oxidation reactions occur. As in single-step oxidation of HMW-GS, this concentration dependence was not observed for  $\text{KBrO}_3$  and was also not found for  $\text{H}_2\text{O}_2$  (Tables II and III).

None of the oxidation conditions resulted in a complete incorporation of monomeric HMW-GS into polymers. Incomplete incorporation was also experienced by Werbeck and Belitz (1993), Schropp et al (1995), and Szabó et al (1995). Possible explanations for the inability of a certain fraction of the monomeric HMW-GS to become incorporated into polymers include oxidation of the SH to an oxidation stage other than SS and alternative SS bond formation. In this study,  $y$ -type HMW-GS (1By9 and 1Dy10) were particularly resistant to incorporation into polymers. The observation that these subunits have an altered mobility on SDS-PAGE as compared with fully reduced forms indicates the presence of an intrachain SS bridge. In at least two different ways, formation of this intrachain SS may be responsible for the inability of these subunits to become incorporated into glutenin polymers. The intrachain SS bond may link cysteine residues, of which at least one is essential for interchain SS bond formation. Alternatively, it might induce a different conformation that renders the essential cysteine residues inaccessible. Interestingly, high concentrations of  $\text{KIO}_3$  during the oxidation of HMW-GS had a very significant negative effect on the degree of polymerization. Apparently, a high concentration of  $\text{KIO}_3$  favors other reaction pathways that negatively affect the degree of cross-linking.

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