

In Vitro Polymerization of Wheat Glutenin Subunits with Inorganic Oxidizing Agents. II. Stepwise Oxidation of Low Molecular Weight Glutenin Subunits and a Mixture of High and Low Molecular Weight Glutenin Subunits

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ABSTRACT

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High and low molecular weight glutenin subunits (HMW-GS and LMW-GS) were isolated from wheat flour. LMW-GS and a 1:2 (w/w) mixture of HMW-GS and LMW-GS were polymerized in vitro at pH 3.0 by stepwise oxidation with different oxidants (KBrO₃, KIO₃, H₂O₂). In analogy with the oxidation of HMW-GS, KBrO₃ produced smaller polymers for LMW-GS than KIO₃ or H₂O₂. While similar average sizes were found with flow-field flow fractionation for LMW-GS polymerized with KIO₃ and H₂O₂, multilayer SDS-PAGE and size exclusion HPLC indicated that size distributions were different. Significantly fewer

monomers remained after polymerization of LMW-GS than with HMW-GS. The higher polymerization efficiency of LMW-GS was also obvious during polymerization of a mixture of HMW-GS and LMW-GS. Mixed polymerization of HMW-GS and LMW-GS had no observable effect on the polymer size. Multilayer SDS-PAGE of remaining monomers during polymerization revealed differences in polymerizing efficiency between structurally different glutenin subunits, with *x*-type HMW-GS and B-LMW-GS showing higher degrees of incorporation than *y*-type HMW-GS and C-LMW-GS, respectively.

The importance of wheat glutenin for breadmaking is well recognized (Weegels et al 1996). Wheat glutenin consists of a heterogeneous mixture of polymers with molecular weights of up to several million (Wrigley 1996). Two main classes of glutenin subunits, high molecular weight glutenin subunits (HMW-GS) (MW 80,000–120,000, based on SDS-PAGE) and low molecular weight glutenin subunits (LMW-GS) (MW 30,000–55,000, based on SDS-PAGE) join in glutenin polymers through interchain disulfide (SS) bonds (MacRitchie 1992). More than 20 different HMW-GS (Payne and Lawrence 1983) and 40 different LMW-GS (Gupta and Shepherd 1990) have been detected so far. The numbers of different HMW-GS and LMW-GS in a hexaploid bread wheat cultivar are 3–5 (Payne 1987) and 7–16 (Gupta and Shepherd 1990), respectively. HMW-GS can be subclassified as *x*-type and *y*-type HMW-GS. Apart from a difference in size (*x*-type is larger), both types also have differences in structure (Shewry et al 1992). LMW-GS were originally subclassified as B-, C-, and D-LMW-GS, based on differences in mobility on SDS-PAGE (Payne and Corfield 1979, Jackson et al 1983). The major fraction of LMW-GS are B-LMW-GS with MW 40,000–50,000 (Payne and Corfield 1979). C-LMW-GS have MW 30,000–40,000 (Payne and Corfield 1979). D-LMW-GS, present in some wheat cultivars, represent a very minor fraction of LMW-GS with SDS-PAGE mobilities slightly below those of the B-LMW-GS (Masci et al 1993). Lew et al (1992) hypothesized that most C-LMW-GS are probably mutated gliadins with an additional cysteine residue, while the majority of B-LMW-GS would form a more distinct class of LMW-GS with two cysteine residues not involved in intrachain SS bonds. Thus, while most B-LMW-GS may act as chain-extendors, most C-LMW-GS may act as chain terminators in glutenin polymerization (Lew et al 1992).

The importance of glutenin size distribution for glutenin quality (reviews by MacRitchie 1992, Weegels et al 1996) explains recent studies on the in vitro polymerization behavior of native (Werbeck and Belitz 1993, Schropp et al 1995, Szabó et al 1995, Veraverbeke et al 2000) and mutant GS (Shani et al 1992, Thompson et al 1994). To increase insight in the polymerization behavior of

different types of GS within a mixture, we studied the polymerization behavior of GS isolated from wheat flour with different inorganic oxidants. In an accompanying article (Veraverbeke et al 2000), we described the in vitro polymerization behavior of HMW-GS, isolated from wheat flour. In this study, the LMW-GS fraction was polymerized in vitro using the stepwise oxidation procedure (Veraverbeke et al 2000). Furthermore, a mixture of HMW-GS and LMW-GS was polymerized as well.

MATERIALS AND METHODS

Isolation of GS

HMW-GS were isolated from Minaret flour (containing HMW-GS 1Ax1, 1Bx7, 1By9, 1Dx5, and 1Dy10) as previously described (Veraverbeke et al 2000). LMW-GS were isolated from the 60% (v/v) *n*-propanol (*n*-PrOH), 5% (v/v) β -mercaptoethanol supernatant remaining after precipitation of HMW-GS by precipitation at 85% (v/v) *n*-PrOH (Verbruggen et al 1998). Precipitated GS were then treated as previously described (Veraverbeke et al 2000).

Analytical Methods

Determination of protein content and free sulfhydryl (SH) contents, size-exclusion HPLC and flow-field flow fractionation (FFF) were as previously described (Veraverbeke et al 2000).

SDS-PAGE and multilayer SDS-PAGE were performed as outlined elsewhere (Veraverbeke et al 2000). The proportion of monomers, the size distribution of polymers (relative proportion represented by the different layers of the gel), and the relative proportion of the individual HMW-GS and B- and C-LMW-GS were estimated by scanning densitometry (Veraverbeke et al 2000).

Reversed-phase HPLC was performed with a Vydac C18 column (250 × 4.6 mm, 300 Å particle size) (The Separations Group, Hesperia, CA) at 70°C with a flow rate of 1.0 mL/min. A linear gradient from 24 to 48% (v/v) acetonitrile in 55 min was used. Solvents were Milli-Q water (Millipore Water Purification System, Millipore Corp., Bedford, MA) containing 0.07% (v/v) trifluoroacetic acid (sequanal grade from Pierce, Rockford, IL) and acetonitrile (190 grade from Ajax Laboratory Chemicals, Auburn, Australia) containing 0.05% (v/v) trifluoroacetic acid. Samples (2.5 mg of protein) were incubated for 1 hr at 60°C in 0.1M Tris-HCl (pH 6.67) buffer containing 50% (v/v) *n*-PrOH, 2.0M urea, and 1.0% (w/v) dithiothreitol (500 μ L), alkylated (15 min at 60°C) with 4-vinylpyridine (5.0 μ L), filtered (0.45 μ m), and injected (20 μ L) on the column.

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Oxidation of GS

LMW-GS and a 1:2 (w/w) mixture of HMW-GS and LMW-GS were oxidized by stepwise addition of oxidizing agent (KIO₃, KBrO₃, H₂O₂) as described previously for HMW-GS (Veraverbeke et al 2000). Oxidizing agent levels were 0–500 units (Veraverbeke et al 2000). Effects of stepwise oxidation were compared with those of single-step additions of 500 and 1000 units of oxidizing agent. Furthermore, the effect of a single-step addition of 500 units of oxidizing agent to the products obtained by stepwise oxidation with 500 units was investigated.

RESULTS AND DISCUSSION

Isolation of HMW-GS and LMW-GS

The HMW-GS and LMW-GS separation protocol, based on selective precipitation at different *n*-PrOH concentrations, resulted in clear-cut HMW-GS and LMW-GS fractions (RP-HPLC) (Fig. 1) (SDS-PAGE, not shown). In line with earlier observations (Verbruggen et al 1998), a concentration of 85% (v/v) *n*-PrOH yielded a nearly complete precipitation of LMW-GS. Protein contents of HMW-GS and LMW-GS preparations were 91.7 and 91.5% (as-is basis), respectively, and free SH contents were 50 and 196 μmol/g of protein, respectively.

Oxidation of LMW-GS

Minaret LMW-GS were oxidized stepwise at pH 3.0 with different levels of KIO₃, KBrO₃, and H₂O₂ (0–500 units). The free SH content and the proportions of the different multilayer SDS-PAGE glutenin size fractions are shown in Table I for different levels of

oxidation with KIO₃. Results obtained with KBrO₃ and H₂O₂ (not shown) were comparable. Under similar conditions, LMW-GS obviously polymerized more efficiently than HMW-GS (Veraverbeke et al 2000), with a smaller fraction of monomers remaining at 500 units (Table I, Fig. 2). It should be noted that, although a level of 500 units of oxidizing agent was more than sufficient to reach a maximal oxidation of free SH in HMW-GS (Veraverbeke et al 2000), this was not the case for LMW-GS. This may be ascribed to higher initial free SH content (Table I). Nevertheless, multilayer SDS-PAGE shows that the degree of polymerization had reached a maximal level at 500 units (compare 400 and 500 units in Table I), suggesting that only intrachain SS are formed on further oxidation. On the other hand, FFF reveals a further increase in average molecular size when going from 400 to 500 units of oxidizing agent (Table II). Therefore, it is likely that further interchain SS bonds were formed between glutenin polymers that were too large to enter the multilayer SDS-PAGE gels.

Multilayer SDS-PAGE further revealed that not all LMW-GS incorporated into polymers with the same efficiency. As shown in Table I, B-LMW-GS incorporated to a larger extent than C-LMW-GS during oxidation with KIO₃. A similar trend was observed with KBrO₃ and H₂O₂. Current views on the structure of LMW-GS distinguish LMW-GS that contain two cysteine residues available for interchain SS bonds (chain extenders) and those that do only contain one (chain terminators) (Shewry and Tatham 1997). It appears that most of the former type of LMW-GS are B-LMW-GS, while the latter are mostly C-LMW-GS (Lew et al 1992). Apart from differences in number of cysteine residues available for interchain SS, differences were found in position, and thus possibly accessibility

TABLE I
Free Sulfhydryl Content^a, Size Distribution^b, and Composition of Remaining Monomers^c
as a Function of Oxidant Level During Stepwise Oxidation of Minaret LMW-GS with KIO₃

	S ^d	Level of Oxidants (units) ^e											
		0	10	50	100	150	200	300	400	500	500+500 ^f	+500 ^g	+1,000 ^h
Free SH content	196	184	171	167	140	115	96	53	14	9	10	13	5
Size distribution													
P0	1	1	3	4	5	5	6	17	12	12	15	16	12
P1	0	1	1	3	1	2	7	10	13	9	13	18	12
P2	0	3	2	3	2	3	9	18	23	22	17	21	20
P3	0	1	1	3	6	9	18	22	30	33	24	20	19
P4	0	1	3	3	8	9	11	7	7	9	7	4	3
P5	1	3	4	3	6	6	5	3	4	3	5	3	2
P6	10	22	23	19	22	22	14	7	4	5	11	7	13
LMW-GS	88	67	62	61	51	44	29	15	7	7	9	10	18
LMW-GS composition													
B-LMW-GS	69	55	61	66	57	59	52	40	29	29	30	30	44
C-LMW-GS	31	45	39	34	43	41	48	60	71	71	70	70	56

^a μmol of SH/g of protein.

^b Relative proportions (%) of multilayer SDS-PAGE size fractions determined by scanning densitometry. P0–P4 = polymer on top in the first, second, third, and fourth layer of multilayer SDS-PAGE gel, respectively. HMW-GS = fifth and sixth layer of multilayer SDS-PAGE gel. LMW-GS represents monomeric LMW-GS in the seventh and eighth layers of multilayer SDS-PAGE gel.

^c Relative proportions (%) of B- and C-LMW-GS in remaining monomers determined by scanning densitometry of multilayer SDS-PAGE gels.

^d S = LMW-GS starting material.

^e One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free SH in Minaret HMW-GS.

^f 500 units added stepwise followed by 500 units added in a single step.

^g 500 units added in a single step.

^h 1,000 units added in a single step.

TABLE II
Flow-Field Flow Fractionation Estimated Average Sizes (kDa) of Stepwise Oxidized Minaret LMW-GS as a Function of Different Oxidant Levels

Oxidant	Level of Oxidant (units) ^a									
	0	10	50	100	150	200	300	400	500	
KBrO ₃	36	55	56	68	137	156	167	163	244	
KIO ₃	39	64	83	154	159	170	241	285	298	
H ₂ O ₂	40	81	99	108	132	156	...	243	...	

^a One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free sulfhydryl in Minaret HMW-GS.

or reactivity, of these cysteine residues (Shewry and Tatham 1997). Our observation that B-LMW-GS polymerized more efficiently probably reflects these differences.

From SE-HPLC (Fig. 3), it appears that, although the LMW-GS polymers have slightly lower sizes than HMW-GS polymerized under the same conditions (Veraverbeke et al 2000), the overall size distribution is similar. SE-HPLC (Fig. 3), multilayer SDS-PAGE (Table I, Fig. 2), and FFF (Table II) all indicate the presence of polymers of high molecular size. The observation that purified LMW-GS can be oxidized with inorganic oxidants at pH 3.0 (in the absence of denaturing agents) to large polymers in the absence of HMW-GS is in line with results obtained with a LMW-GS enriched (>80% LMW-GS) mixture of GS from flour (Werbeck and Belitz 1993) and an individual LMW-GS obtained by heterologous expression (Thompson et al 1994) oxidized at pH 8.0 with oxygen in urea-containing buffers. The ability of LMW-GS to form large polymers with themselves was also demonstrated by Gao and Bushuk (1993) and Gupta et al (1995), who studied glutenin size distributions of a wheat line without HMW-GS.

Size measurements further showed that different oxidizing agents produced polymers with different size distributions. Average size estimates with FFF (Table II) indicated that KBrO_3 produced lower size polymers than KIO_3 and H_2O_2 . KBrO_3 was also less efficient in polymerizing HMW-GS than either KIO_3 or H_2O_2 (Veraverbeke et al 2000). While KIO_3 and H_2O_2 yielded polymers with comparable average size (Table II), size distributions measured with multilayer SDS-PAGE (not shown) or SE-HPLC (Fig. 4) were obviously different.

In our work on the polymerization behavior of HMW-GS, stepwise addition was more efficient than single-step addition, when high levels of KIO_3 were used (Veraverbeke et al 2000). While in case of HMW-GS, this difference was very pronounced at 500 units of KIO_3 , it was not found with LMW-GS at this level (Table I). However, comparison of the polymerization efficiency of single-step oxidation with 1,000 units of KIO_3 with that of single-step or stepwise oxidation with 500 units (Table I) again shows that high

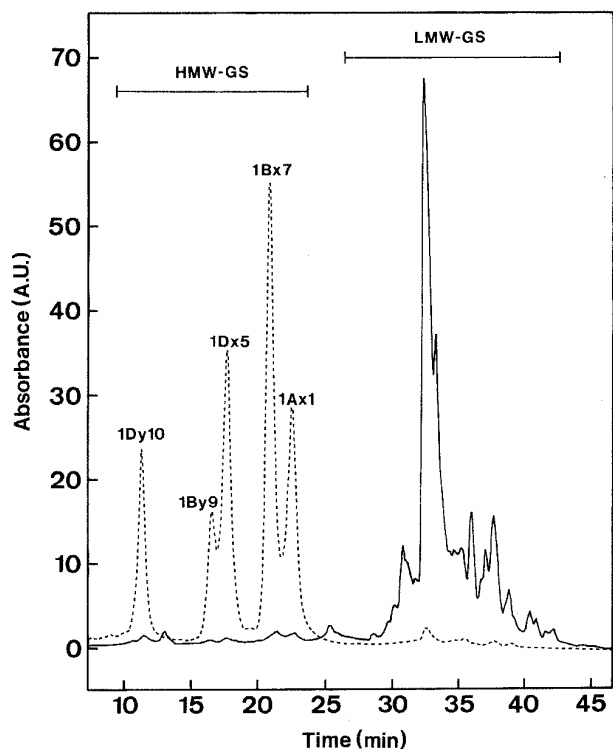


Fig. 1. Reversed-phase HPLC chromatograms of reduced and alkylated HMW-GS (---) and LMW-GS (—) preparations from Minaret flour.

concentrations of KIO_3 lower the polymerization efficiency. Possibly, higher levels of KIO_3 were needed because the level of free SH was also significantly higher for LMW-GS.

Oxidation of a Mixture of HMW-GS and LMW-GS

A 1:2 (w/w) mixture of Minaret HMW-GS and LMW-GS was polymerized by stepwise oxidation to investigate a possible interaction between the two classes of GS during polymerization. Comparison of multilayer SDS-PAGE data of the *in vitro* polymerization of pure HMW-GS (Veraverbeke et al 2000) and pure LMW-GS (Table I) with that of the 1:2 (w/w) HMW-GS-LMW-GS mixture (Table III) reveals no obvious effect on polymer size associated with a possible copolymerization of HMW-GS and LMW-GS. Multilayer SDS-PAGE (Table III) indicated a more efficient incorporation of LMW-GS in glutenin than HMW-GS. This is in line with the behavior of these GS classes when polymerized individually. Changes in relative proportions of individual HMW-GS (Table IV) and in B-LMW-GS to C-LMW-GS ratio (Table V) in the remaining monomers with increasing level of oxidation were very similar

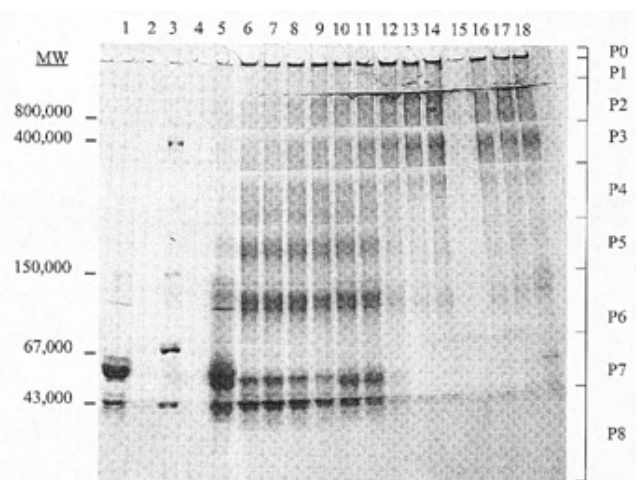


Fig. 2. Profiles of multilayer SDS-PAGE under nonreducing conditions (lanes 5–14) of oxidation products of stepwise oxidation of Minaret LMW-GS with KIO_3 . Lane 5: LMW-GS starting material; lanes 6–14: LMW-GS oxidised stepwise with 0, 10, 50, 100, 150, 200, 300, 400, and 500 units of KIO_3 . Lane 1: reduced Minaret LMW-GS; lane 3: reduced MW markers; lane 16: LMW-GS oxidized stepwise with 500 units of KIO_3 followed by single-step oxidation with 500 units of KIO_3 ; lanes 17 and 18: LMW-GS oxidized by single-step oxidation with 500 and 1,000 units of KIO_3 , respectively.

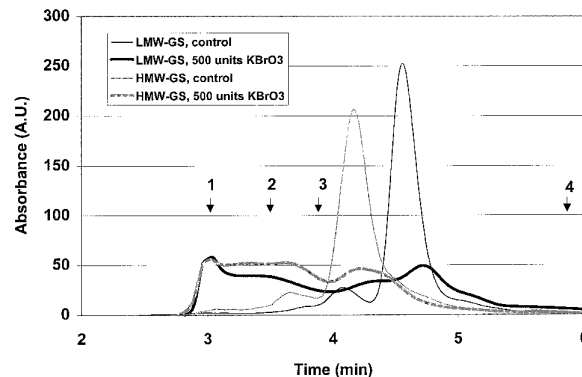


Fig. 3. Comparison of size-exclusion HPLC profiles of polymers formed by stepwise oxidation of Minaret HMW-GS and LMW-GS with KBrO_3 (500 units). Elution times indicated by arrows. MW markers: (1) thyroglobulin (MW 669,000); (2) bovine serum albumin dimer (MW 135,000); (3) bovine serum albumin monomer (MW 67,000); (4) vitamin B-12 (MW 1,375).

TABLE III
Free Sulfhydryl Content^a and Size Distribution^b as a Function of Oxidant Level During Stepwise Oxidation of a Mixture of Minaret HMW-GS and LMW-GS (1:2, w/w) with Different Oxidizing Agents

Oxidant	S ^c	Level of Oxidants (units) ^d												
		0	10	50	100	150	200	300	400	500	500+500 ^e	+500 ^f	+1,000 ^g	
KBrO ₃	Free SH content	147	143	133	124	105	82	72	42	25	21	...	23	12
	Size distribution													
	P0		2	2	4	5	7	7	12	10	12	12	11	11
	P1		2	3	3	2	3	5	8	9	7	8	6	6
	P2		1	1	2	2	2	3	5	6	6	7	6	6
	P3		3	3	5	5	5	7	8	10	13	13	12	14
	P4		11	11	14	13	15	17	17	17	18	17	17	18
	LMW-GS		40	41	42	42	42	42	40	38	37	38	38	37
KIO ₃	Free SH content	147	140	136	121	95	76	58	19	7	7	6	5	2
	Size distribution													
	P0		1	2	3	3	5	8	11	8	11	12	13	14
	P1		1	2	2	2	2	3	6	7	10	9	10	13
	P2		1	1	2	3	4	4	9	17	16	13	13	15
	P3		2	3	6	9	13	15	23	22	20	19	12	10
	P4		10	11	14	17	21	21	19	20	19	19	15	11
	LMW-GS		39	40	41	40	39	37	27	23	21	22	31	31
H ₂ O ₂	Free SH content	147	143	124	101	71	62	36	24	20	14	...	22	14
	Size distribution													
	P0		2	2	2	4	4	6	7	8	8	9	9	9
	P1		2	2	2	3	6	8	8	8	8	8	8	8
	P2		1	1	2	3	11	13	13	13	12	11	13	14
	P3		3	3	6	8	13	16	19	19	18	18	16	16
	P4		10	11	15	15	17	17	14	15	17	18	17	17
	LMW-GS		38	39	40	40	36	33	31	30	29	28	27	26

^a μmol of SH/g of protein.

^b Relative proportions (%) 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 = fifth and sixth layer of multilayer SDS-PAGE gel. LMW-GS represents monomeric LMW-GS in the seventh and eighth layers of multilayer SDS-PAGE gel.

^c S = GS starting material.

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

^e 500 units added stepwise followed by 500 units added in a single step.

^f 500 units added in a single step.

^g 1,000 units added in a single step.

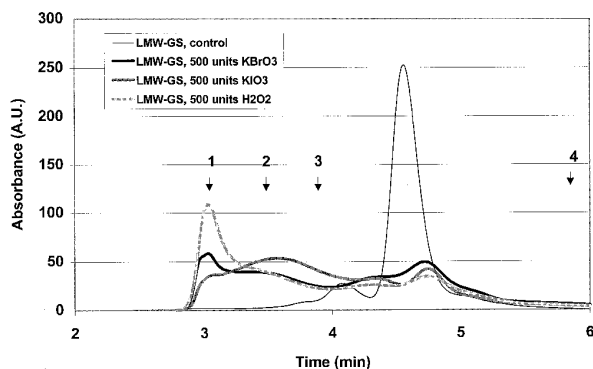


Fig. 4. Comparison of size-exclusion HPLC profiles of polymers formed by stepwise oxidation of Minaret LMW-GS with different oxidizing agents (500 units). Elution times indicated by arrows. MW markers: (1) thyroglobulin (MW 669,000); (2) bovine serum albumin dimer (MW 135,000); (3) bovine serum albumin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

to the changes observed with pure HMW-GS (Veraverbeke et al 2000) and pure LMW-GS (Table I) respectively, showing decreasing x-type/y-type HMW-GS ratios and B-LMW-GS/C-LMW-GS ratios.

The absence of a synergistic effect of HMW-GS and LMW-GS during polymerization is in line with the observations of Gao et al

(1993) with wheat lines containing only HMW-GS, only LMW-GS, or both HMW-GS and LMW-GS. On the other hand, with the same wheat lines, Gupta et al (1995) found that, although large polymers were formed in a wheat line containing only HMW-GS and in a wheat line containing only LMW-GS, glutenin polymers were larger in wheat lines containing both.

As with pure HMW-GS (Veraverbeke et al 2000), the difference in polymerization efficiency between single-step and stepwise oxidation with KIO₃ remained present for the HMW-GS in the mixture of HMW-GS and LMW-GS. A larger fraction of HMW-GS monomers remain when 500 units of KIO₃ is added in a single step compared to stepwise oxidation with 500 units (Table III).

CONCLUSIONS

This study shows that incomplete polymerization observed during in vitro oxidation of pure HMW-GS (Veraverbeke et al 2000) cannot be explained by the absence of LMW-GS because a significant proportion of HMW-GS also remained monomeric when polymerized in a mixture with LMW-GS. Compared with HMW-GS, LMW-GS incorporated to a much larger extent in glutenin polymers, both when polymerized individually and when polymerized in a mixture with HMW-GS. LMW-GS, much as HMW-GS (Veraverbeke et al 2000), were capable of forming large polymers with themselves. No effect on size was observed when HMW-GS and LMW-GS were polymerized together compared with the polymers obtained with the separate classes of GS. Furthermore, during polymerization of the GS mixture, similar

TABLE IV
Relative Proportions (%) of the Different HMW-GS in Remaining Monomers as a Function of Oxidant Level During Stepwise Oxidation of a Mixture of Minaret HMW-GS and LMW-GS (1:2, w/w) with Different Oxidants

Oxidant	HMW-GS Composition ^a	S ^b	Level of Oxidants (units) ^c											
			0	10	50	100	150	200	300	400	500	500+500 ^d	+500 ^e	+1000 ^f
KBrO ₃	1Ax1		22	21	21	22	20	19	17	16	15	14	11	15
	1Dx5		10	11	11	11	10	11	10	10	13	12	14	15
	1Bx7		22	20	20	22	24	22	22	19	18	18	19	17
	1By9/1Dy10		46	48	48	45	46	48	51	55	54	56	56	53
KIO ₃	1Ax1		21	21	19	18	18	16	15	14	14	12	19	17
	1Dx5		9	9	10	12	13	14	15	16	18	18	15	14
	1Bx7		22	22	21	20	20	19	17	16	16	15	25	26
	1By9/1Dy10		48	48	50	50	49	51	53	54	52	55	41	43
H ₂ O ₂	1Ax1		22	21	22	19	15	14	10	10	12	11	12	12
	1Dx5		8	9	9	9	10	12	10	11	11	12	12	12
	1Bx7		22	23	22	24	24	23	22	20	18	18	18	17
	1By9/1Dy10		48	47	47	48	51	51	58	59	59	59	58	59

^a Relative proportions of the different HMW-GS in remaining monomers determined by scanning densitometry of multilayer SDS-PAGE gels.

^b S = GS starting material.

^c One unit of oxidizing agent represents the amount that theoretically oxidizes 1% of free sulfhydryl in Minaret HMW-GS.

^d 500 units added stepwise followed by 500 units added in a single step.

^e 500 units added in a single step.

^f 1,000 units added in a single step.

TABLE V
Relative Proportions (%) of B- and C-LMW-GS in Remaining Monomers as a Function of Oxidant Level During Stepwise Oxidation of a Mixture of Minaret HMW-GS and LMW-GS (1:2, w/w) with Different Oxidants

Oxidant	LMW-GS Composition ^a	S ^b	Level of Oxidants (units) ^c											
			0	10	50	100	150	200	300	400	500	500+500 ^d	+500 ^e	+1,000 ^f
KBrO ₃	B-LMW-GS		53	54	53	56	53	50	50	46	37	39	33	38
	C-LMW-GS		47	46	47	44	47	50	50	54	63	61	67	62
KIO ₃	B-LMW-GS		55	54	52	52	28	23	22	29	30	35	46	45
	C-LMW-GS		45	46	48	48	72	77	78	71	70	65	54	55
H ₂ O ₂	B-LMW-GS		54	56	56	56	54	50	33	31	31	32	44	48
	C-LMW-GS		46	44	44	44	46	50	67	69	69	68	56	52

^a Relative proportions of the B- and C-LMW-GS in the remaining monomer were determined by scanning densitometry of multi-layer SDS-PAGE gels.

^b S = GS starting material.

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

^d 500 units added stepwise followed by 500 units added in a single step.

^e 500 units added in a single step.

^f 1,000 units added in a single step.

differences in polymerization efficiency were observed between structurally different GS as during polymerization of HMW-GS and LMW-GS separately, such as more efficient incorporation of *x*-type HMW-GS and B-LMW-GS compared with *y*-type HMW-GS and C-LMW-GS, respectively.

Future research should be directed at further optimizing the oxidation conditions to achieve a complete polymerization of GS. Aspects that are expected to affect correct SS bond formation, and thus deserve further attention, are the pH of the medium and protein concentration during oxidation. From the behavior of the fast-acting oxidant KIO₃ in this study, it may also be inferred that the reaction rate should be controlled to prevent incorrect SS bond formation. Apart from optimizing the oxidation to obtain complete polymerization, it will be necessary to investigate whether the oxidation conditions lead to polymers with a comparable structure and functionality as native glutenin polymers. It is our hope that optimization of the conditions for *in vitro* polymerization of GS will eventually lead to the availability of functional glutenin polymers with a predetermined composition that may be used to study the relationship between glutenin composition and size distribution and its functionality in breadmaking.

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