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Concentrations of Low and High Molecular Weight Thiols in Wheat Dough As Affected by Different Concentrations of Ascorbic Acid

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Different amounts of ascorbic acid (AA) were added to flour, and the concentrations of low and high molecular weight thiols in the dough were determined. For the determination of the low molecular weight thiols, glutathione, cysteine, and the corresponding disulfides, an isotope dilution assay with a ^{14}C-labeled internal standard was used. For the determination of the high molecular weight thiols, a method was developed that involved derivatization of dough with Ellman's reagent, removal of excess reagent by dialysis, micro-Osborne fractionation, release of the label by reduction, and determination of reduced Ellman's reagent by reversed-phase high-performance liquid chromatography. Mixing of flour without AA led to a decrease of the glutathione and an increase of the cysteine concentration. Addition of AA reduced the concentration of both thiols to a minimum when 125 mg of AA/kg of flour was applied. Furthermore, the concentrations of high molecular weight thiols in the glutenins of flours from different wheat cultivars were determined. The values ranged from 5.6 to 8.2 μmol/kg of protein and showed a correlation between flour quality and SH concentration. On addition of AA and mixing of a dough, the concentrations of the protein thiols in the glutenins isolated from the dough increased to a maximum when 100 mg of AA/kg of flour was added. Higher concentrations of AA led to a decrease of the SH concentration. The last results are not in accordance with previously published data or with current hypotheses about the mechanism of the AA improver action.

KEYWORDS: Ascorbic acid; glutathione; high molecular weight thiols; wheat; gluten; Ellman's reagent

INTRODUCTION

In many countries L-threo-ascorbic acid (AA) is used as a flour improver. Its addition to flour causes reduced stickiness of the dough, an increase in loaf volume, and an improvement in crumb structure. Typical concentrations applied in bread-making are in the range of 20−150 mg/kg of flour. The amount of AA depends on the wheat cultivar, the type and the storage time of the flour, and also on the processing technology and the type of bread.

The improver effect of AA was discovered by Jørgensen (*1*), and since that time several studies have been carried out to establish the mechanism of action of AA. The results of these studies have been reviewed by several authors (*2*−*5*) and led to two major hypotheses about the mechanism of action. One hypothesis has been postulated by the group of Grosch (*4*, *6*, *7*) and the other by Every (*8*, *9*). It is commonly accepted by both hypotheses that only the L-threo isomer of AA is rheologically active and that added AA is quickly converted to L-threodehydroascorbic acid (DHA) by ascorbate oxidase (AOX), which is present in wheat flour. The hypothesis of Every proposes direct oxidative cross-linking of protein thiols (PSH) to interprotein disulfides (PSSP) by DHA during proofing

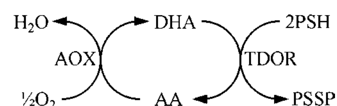

Figure 1. Proposed mechanism of action of AA according to Every et al. (*8*, *9*).

(**Figure 1**) and recovery of AA. This reaction requires a thiol disulfide oxidoreductase (TDOR) as a catalyst, which has not been identified in flour until now. On the other side, the approach of Grosch postulates a rapid conversion of endogenous glutathione (GSH) to its oxidized form (GSSG) and simultaneous reduction of DHA to AA (**Table 1**) at the early stages of dough mixing. For this reaction the enzyme glutathione dehydrogenase (GSH-DH) is required, which has been identified in wheat flour by Kuninori and Matsumoto (*10*, *11*). It has been shown that the enzyme exclusively uses GSH as H donor, and not cysteine (CSH). The best H acceptor is L-threo-DHA; the other DHA isomers were only weakly active (*12*−*14*). As a result of the formation of GSSG on addition of AA, GSH is not available to cleave disulfide bonds of glutenin by thiol/disulfide interchange reactions. Depolymerization of glutenin and therefore weakening of dough are inhibited.

To get evidence for the second hypothesis, sensitive methods for the determination of the low molecular weight thiols GSH

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Table 1. Proposed Reactions To Explain the Improver Effect of AA According to the Hypothesis of Grosch (*4*, *6*, *7*)[a]

reaction	educts		products
1	AA + $^1/_2O_2$	$\xrightarrow{AOX}$	DHA + H_2O
2	DHA + 2GSH	$\xrightarrow{GSH\text{-}DH}$	AA + GSSG
3	PSH + GSSG	→	PSSG + GSH
4	GSH + PSSP	→	GSSP + PSH
5	GSH + CSSC	→	GSSC + CSH
6	CSH + PSSP	→	CSSP + PSH
7	PSH + CSSC	→	PSSC + CSH

[a] AOX, ascorbate oxidase; GSH-DH, glutathione dehydrogenase.

and cysteine (CSH), for their reaction products GSSG and cystine (CSSC), and for protein-bound glutathione (PSSG) and cysteine (PSSC) in flour and dough have been developed (*6*, *15*, *16*). Concentrations of 10−120 μmol of GSH and 8−15 μmol of CSH per kilogram of flour have been found. A positive correlation between the ash content of the flour and the GSH concentration and a negative correlation between storage time of the flour and the concentration of GSH and CSH have been established (*6*, *17*, *18*). The concentrations of the disulfides GSSG, CSSC, PSSG, and PSSC were substantially higher than those of the corresponding thiols.

Furthermore, it has been shown that GSSG produced by the redox reaction with DHA reacts with free thiol groups of gluten proteins (*7*, *19*). In contrast to the reaction of GSH with interprotein disulfide bonds, which leads to depolymerization of glutenin, binding of GSSG does not cause depolymerization because reaction takes place only with free thiol groups of gluten proteins. The rheological properties of AA-containing doughs are therefore comparable with those of doughs to which SH-blocking agents such as *N*-ethylmaleimide have been added (*20*).

Whereas the direct cleavage of interchain disulfide bonds by GSH (**Table 1**, reaction 4) has already been established (*21*), direct experimental proof for the reaction of GSSG with free thiol groups of the glutenins (**Table 1**, reaction 3) and formation of GSH and a mixed disulfide PSSG is still missing.

The present study is the first part of a series of investigations to gain further insight into the mechanism of action of AA. The aim of this part of the study was to get more information about the concentration of the low and high molecular weight thiols in dough on addition of increasing amounts of AA prior to mixing. The concentrations of low molecular weight thiols were determined in relation to AA concentration by an isotope dilution assay. For the quantification of reactive thiol groups in the glutenins, an HPLC method based on Ellman's reagent has been developed.

MATERIALS AND METHODS

Chemicals. [2,3-^{14}C]Maleic acid anhydride, GSH, GSSG, CSH, CSSC, dithioerythritol (DTE), and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) were from Sigma (Taufkirchen, Germany), trifluoroacetic acid (TFA) was from Fluka (Taufkirchen, Germany), and all other chemicals were from Merck (Darmstadt, Germany). The quality was "pro analysi" or better.

Flour. Kernels of the German and French wheat cultivars Apollo, Astron, Contra, Flair, Glockner, Kanzler, Monopol, Rektor, and Soissons and of the Canadian wheat class Canadian Western Red Spring (CWRS) were used. They had different qualities ranging from the high-quality wheat class CWRS and the high-quality cultivars Astron, Glockner, Kanzler, Monopol, Rektor, and Soissons to the medium-quality cultivar Flair and the poor quality cultivars Apollo and Contra. The kernels were milled into flour at 14% moisture by means of a Quadrumat Junior mill (Brabender, Duisburg, Germany), sieved (∅ = 0.2 mm), and stored for a minimum of 2 weeks prior to use. The moisture of the flours was determined according to AACC method 44-19 (*22*), the ash content according to ICC method 104/1 (*23*), and the nitrogen content on an FP328 nitrogen analyzer (Leco, Moenchengladbach, Germany). The protein content was calculated from the nitrogen content by using a conversion factor of 5.7. The analytical data of the flours are shown in **Table 2**.

Dough Preparation. Ten grams of flour (8.6 g of dry mass) and 0.2 g of sodium chloride were mixed with a solution of ascorbic acid (1 mL) and water (x − 1 mL) in a microfarinograph (Brabender, Duisburg, Germany) at 22 °C and 60 rpm. The amount of water (x mL) was adapted to get a maximum peak consistency of 550 BU. AA was dissolved in water to obtain solutions containing 0, 0.2, 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 mg of AA/mL corresponding to concentrations of 0, 20, 50, 75, 100, 125, 150, and 200 mg of AA/kg of flour. At peak consistency (550 BU) the dough was removed from the farinograph, immediately frozen in liquid nitrogen, lyophilized, and powdered in a mortar.

Determination of the Concentration of Free GSH and CSH and Total GSH and CSH (GSS and CSS) in Flour and Dough. In flours and lyophilized doughs free GSH, CSH, GSS, and CSS were determined by means of an isotope dilution assay on the basis of ^{14}C-labeled internal standards (*7*, *15*). *N*-Phenylmaleinimide was used as the alkylation reagent for GSH and CSH; the internal standards were *S*-(*N*-phenyl-[2,3-^{14}C]succinimido)-L-cysteine and -glutathione.

Determination of the Concentration of Free Thiol Groups of the Glutenins (Figure 1). *UV−Vis Spectra of Ellman's Reagent (DTNB) and Reduced Ellman's Reagent (NTB).* Spectra of DTNB [0.2 mmol/L in 0.1% (v/v) TFA] and NTB [DTNB 0.1 mmol/L in 0.1% (v/v) TFA/0.05 mol/L DTE] solutions were recorded between 200 and 600 nm at a path length of 1 cm. The reference solutions were 0.1% (v/v) TFA for DTNB and 0.1% (v/v) TFA/0.05 mol/L DTE for NTB, respectively.

Praparation of NTB Flour and NTB Dough. A buffer solution containing 6 mol/L urea/0.5% (w/v) triethylamine/0.1 mol/L sodium chloride/0.05 mol/L Tris/0.323 mmol/L DTNB/pH 8.0 with acetic acid/dissolved in nitrogen-saturated 50% (v/v) aqueous 1-propanol was flushed with nitrogen for 1 min. Flour or lyophilized dough (3.5 g) was added to the buffer solution (150 mL) under magnetic stirring. Stirring was continued for another 2 h at room temperature (22 °C), and the suspension was then dialyzed at room temperature for 3 days and lyophilized (NTB flour, NTB dough). Three changes per day of 2.5% (v/v) 2-propanol (day 1) and water (days 2 and 3) were used for dialysis, respectively.

Osborne Fractionation of NTB Flour and NTB Dough. NTB flour or NTB dough was separated by extraction as previously described (*24*) into an albumin/globulin fraction and a gliadin fraction. Fifty

Table 2. Analytical Data of the Flours[a]

	APO	AST	CON	CWR	FLA	GLO	KAN	MON	REK	SOI
harvest	1986	1998	1998	1991	1996	1996	1995	1991	1991	1998
quality	low	high	low	high	medium	high	high	high	high	high
moisture[a] (%)	10.8.	14.0	14.0	13.2	14.7	13.4	n.d.	13.4	13.9	14.0
ash (dry mass)[a] (%)	0.51	0.56	0.52	0.80	0.52	0.43	n.d.	0.54	0.55	0.51
protein content[a] (%)	12.0	11.6	8.2	13.0	8.3	11.1	n.d.	10.4	12.7	10.7

[a] Mean values of duplicate determinations. APO, Apollo; AST, Astron; CON, Contra; CWR, CWRS; FLA, Flair; GLO, Glockner; KAN, Kanzler; MON, Monopol; REK, Rektor; SOI, Soissons; nd, not determined.

Table 3. Effect of AA on the Concentrations of GSH, CSH, GSS, and CSS in Four Different Flours and in Doughs Prepared from These Flours[a]

cultivar		GSH[b] (nmol/g)	GSS[b] (nmol/g)	CSH[b] (nmol/g)	CSS[b] (nmol/g)
CWRS	flour	80.9 ± 5.3	493 ± 100	15.7 ± 1.0	467 ± 55
	dough	37.3 ± 3.3	387 ± 71	55.9 ± 1.3	506 ± 42
	dough, 20 mg of AA/kg[c]	15.5 ± 1.8	379 ± 20	41.4 ± 1.8	595 ± 49
	dough, 50 mg of AA/kg[c]	14.9 ± 2.5	427 ± 30	36.1 ± 1.4	590 ± 53
	dough, 75 mg of AA/kg[c]	11.3 ± 1.1	370 ± 21	31.5 ± 3.0	649 ± 106
	dough, 100 mg of AA/kg[c]	11.6 ± 2.2	386 ± 31	31.3 ± 1.1	491 ± 37
	dough, 125 mg of AA/kg[c]	8.3 ± 0.9	414 ± 19	37.8 ± 4.3	485 ± 51
	dough, 150 mg of AA/kg[c]	16.1 ± 5.2	390 ± 10	27.4 ± 0.8	568 ± 39
Glockner	flour	40.9 ± 4.0	132 ± 12	11.6 ± 0.9	165 ± 17
	dough	8.5 ± 1.1	156 ± 7	26.5 ± 1.1	221 ± 23
	dough, 20 mg of AA/kg[c]	4.1 ± 1.4	156 ± 9	14.8 ± 0.3	160 ± 19
	dough, 50 mg of AA/kg[c]	5.5 ± 1.2	178 ± 18	13.4 ± 0.7	267 ± 28
	dough, 75 mg of AA/kg[c]	4.5 ± 1.3	142 ± 12	8.2 ± 0.5	163 ± 17
	dough, 100 mg of AA/kg[c]	4.5 ± 0.5	126 ± 14	7.9 ± 0.5	195 ± 18
	dough, 125 mg of AA/kg[c]	4.3 ± 0.4	157 ± 6	2.8 ± 0.7	242 ± 24
	dough, 150 mg of AA/kg[c]	5.1 ± 0.7	178 ± 10	2.5 ± 0.1	259 ± 29
Monopol	flour	43.0 ± 1.6	156 ± 25	12.9 ± 0.3	161 ± 12
	dough	8.9 ± 0.9	250 ± 33	18.6 ± 2.0	164 ± 11
	dough, 20 mg of AA/kg[c]	4.5 ± 0.5	210 ± 9	16.1 ± 1.8	197 ± 15
	dough, 50 mg of AA/kg[c]	3.0 ± 0.4	220 ± 12	12.9 ± 0.4	156 ± 17
	dough, 75 mg of AA/kg[c]	3.1 ± 0.6	190 ± 20	14.6 ± 0.3	152 ± 13
	dough, 100 mg of AA/kg[c]	6.6 ± 0.8	209 ± 8	9.8 ± 0.4	168 ± 13
	dough, 125 mg of AA/kg[c]	5.7 ± 0.5	210 ± 10	6.7 ± 0.2	127 ± 20
	dough, 150 mg of AA/kg[c]	8.0 ± 0.9	214 ± 10	6.0 ± 0.7	198 ± 18
Flair	flour	18.3 ± 1.0	142 ± 12	11.1 ± 0.4	159 ± 11
	dough	12.4 ± 1.1	214 ± 30	23.7 ± 1.8	227 ± 8
	dough, 20 mg of AA/kg[c]	11.5 ± 0.7	171 ± 15	16.7 ± 1.7	235 ± 6
	dough, 50 mg of AA/kg[c]	7.8 ± 0.7	158 ± 8	15.0 ± 1.5	208 ± 19
	dough, 75 mg of AA/kg[c]	7.8 ± 0.3	151 ± 7	14.9 ± 0.4	229 ± 9
	dough, 100 mg of AA/kg[c]	6.5 ± 0.4	155 ± 8	15.2 ± 0.7	275 ± 29
	dough, 125 mg of AA/kg[c]	4.8 ± 0.5	168 ± 9	18.1 ± 0.3	234 ± 5
	dough, 150 mg of AA/kg[c]	7.4 ± 0.3	152 ± 5	12.3 ± 0.8	316 ± 35

[a] Mean values of duplicate determinations ± standard deviation. [b] Concentration in nmol/g of flour. [c] Concentration in mg/kg of flour.

milligrams instead of 100 mg of material and therefore half of the amount of the solvents previously described (*24*) was used. Then, a buffer solution containing urea 2 mol/L/Tris 0.05 mol/L/DTE 1% (w/v)/pH 7.5 with hydrochloroic acid/dissolved in nitrogen-saturated 50% (v/v) aqueous 1-propanol was prepared. To dissolve glutenin subunits and release NTB, the residue of the gliadin extraction (glutenin) was then extracted twice with buffer solution (2 × 0.5 mL) and centrifuged at 4 °C and 16000*g* for 5 min. For extraction, the sample was vortexed in a 2 mL Eppendorf cap for 1 min at room temperature and then extracted for 30 min at 60 °C by magnetic stirring. The supernatants were made up to 1 mL and used for HPLC. To measure residual DTNB, NTB flour or NTB dough was extracted as described above with the exception that DTE was omitted in the solvent for the glutenins.

Quantification of the Amount of Protein Present in the Glutenin Fraction. The glutenin fraction was separated by RP-HPLC (*24*). From the area of the eluted peaks at 210 nm the amount of protein was calculated on the basis of a calibration curve obtained by separation of different amounts of bovine serum albumin (BSA). From a standard solution with a BSA concentration of 1 mg/mL were made injections of 50, 100, 200, 300, 400, and 500 μL, corresponding to 50, 100, 200, 300, 400, and 500 μg of BSA.

Quantification of DTNB and NTB in the Glutenin Extracts. The glutenin fraction was passed through a filter membrane (0.45 μm), and 100 μL of the solution was separated by RP-HPLC (Beckman, Muenchen, Germany) by using the following conditions: HPLC software, Beckman System Gold; column, ODS-Hypersil (C_{18}), 5 μm, 10 nm, 250 × 4.6 mm; flow rate, 0.8 mL/min; temperature, 60 °C; detection, UV absorbance at 327 nm; solvent A, TFA 0.1% (v/v); solvent B, TFA 0.085% (v/v) in acetonitrile; elution, linear gradient

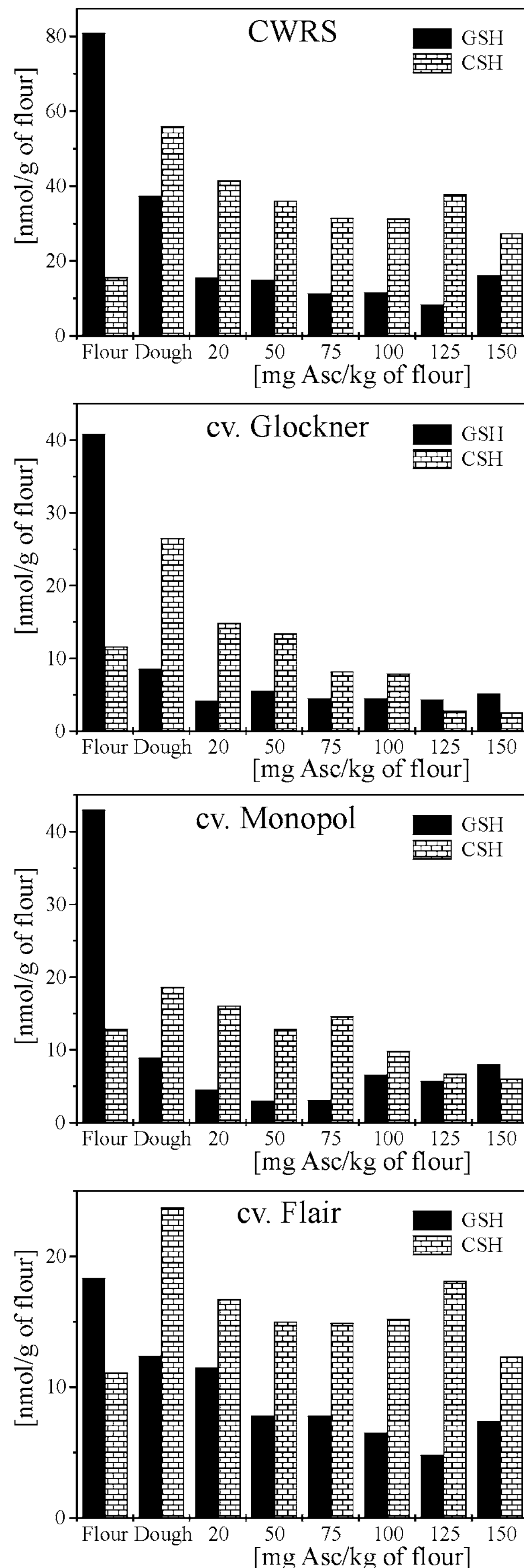

Figure 2. Concentration of GSH and CSH in four different flours and in the corresponding doughs containing increasing concentrations of AA.

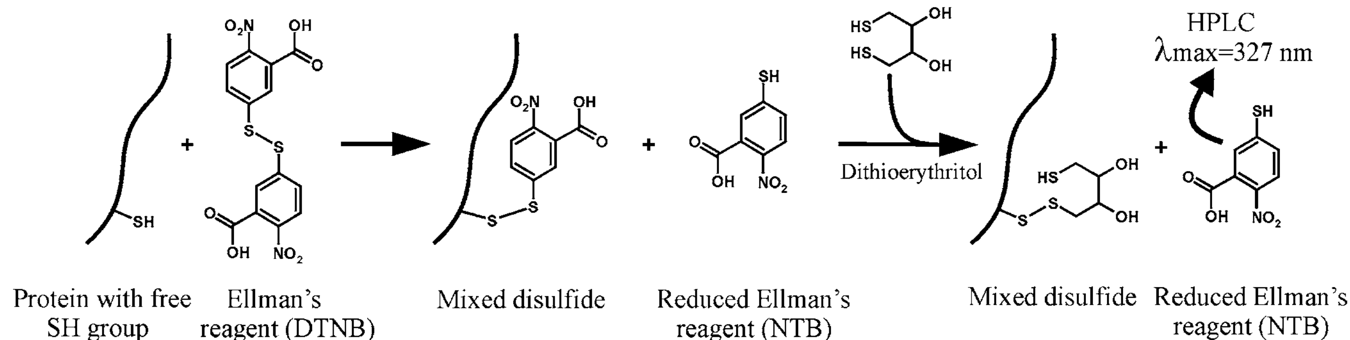

Figure 3. Schematic representation of the determination of free thiol groups in glutenin isolated from flour or dough.

0−25 min 10−70% solvent B. Quantification was carried out on the basis of the peak areas of DTNB (elution time = 21 min) and NTB (elution time = 14 min). Calibration curves of DTNB and NTB were obtained by separating 100 μL of standard solution, respectively, containing 0.276, 0.552, 2.07, and 2.76 nmol of DTNB and 1.1, 2.2, 5.5, and 11.0 nmol of NTB. In additional experiments, AA (1 mg/mL) was added to the standard solution of the lowest concentration, respectively, to ensure that no reaction took place between AA and DTNB or NTB.

RESULTS AND DISCUSSSION

Effect of AA on the Concentration of GSH, CSH, GSS, and CSS in Dough. These investigations were carried out with the flours of CWRS, Flair, Glockner, and Monopol. Doughs with AA concentrations of 0, 20, 50, 75, 100, 125, and 150 mg of AA/kg of flour were prepared, and the concentrations of GSH, CSH, GSS, and CSS were determined. The results are shown in **Table 3** and **Figure 2**. The concentrations of GSH in the flours ranged from 18 to 81 nmol/g of flour. Mixing of the flour into a dough reduced this concentration clearly, although no AA had been added. This is caused by the capability of GSH to reduce CSSC as the concentration of CSH increased distinctly in this step. Addition of AA in increasing concentrations led to a decrease of the GSH concentration in the dough to 3−8 nmol/g of flour. However, a complete disappearance of GSH was not obtained. Obviously, GSH produced as a product of the reaction of GSSG with protein thiols could not completely be reconverted to GSSG. In most of the cultivars GSH was minimal when a concentration of 125 mg of AA/kg of flour was used. The concentration of CSH in the dough decreased with increasing AA concentration because AA converted GSH to GSSG so that GSH was no longer available for the reduction of CSSC to CSH. The concentrations of GSS and CSS, which represent GSH and CSH after reduction of the flour or dough with DTE, were in the same range for each flour and were not affected by the addition of AA. This shows that by the addition of AA only reactions of thiols and disulfides took place. Reactions leading to compounds with sulfur in higher oxidation states were not present, because those compounds have not been reported to be reducible by DTE until now. Otherwise, the amounts of GSS and CSS would not have remained constant over the whole concentration range of AA.

Development of a Method for the Determination of the Concentration of Free Thiol Groups of the Glutenins. The principle of the method is shown in **Figure 3**. Flour or dough was derivatized with DTNB (Ellman's reagent), excess reagent was removed by dialysis, and NTB flour or NTB dough was obtained. NTB flour or NTB dough was extracted by a micro-Osborne fractionation, the NTB derivatives present in the glutenins were cleaved by DTE, and NTB (reduced Ellman's reagent) was quantified by RP-HPLC. Its concentration was a measure for the concentration of free SH groups in the glutenins after mixing.

Preparation of NTB Flour and NTB Dough. DTNB reacts spontaneously with free SH groups to a mixed disulfide and a thiolate anion (NTB) (*25*). Thus, one part of the reagent gets covalently bound to the substrate. Excess reagent and released NTB as well as other low molecular weight compounds (M_r < 10000) were then removed by dialysis. For the derivatization of flour or dough, a content of 1−1.5 μmol of SH/g of flour was assumed. A 10-fold molar excess of DTNB over SH groups was used for alkylation. During the first part of the dialysis (1 day) a small amount of 2-propanol was added to the medium to assist in removing DTNB, which is only poorly soluble in water. To ensure the effectiveness of the dialysis, NTB flour and NTB dough were suspended in 0.1% (v/v) TFA (50 mg/mL) and the solution was checked for remaining DTNB by RP-HPLC. A small portion of DTNB remained in the flour/dough. Therefore, it was essential to carry out a blank for each sample to determine residual DTNB. Removal of excess reagent was the critical point of the method, as it was very time-consuming and nonquantitative. A potential for improvement and acceleration at this point would be the replacement of dialysis, for example, by centrifugation with devices for ultrafiltration that are commercially available.

Micro-Osborne Fractionation of NTB Flour and NTB Dough. First, NTB flour or NTB dough was extracted with an aqueous salt buffer to remove albumins and globulins, and then the gliadins were extracted with 60% (v/v) ethanol. Residual glutenin was solubilized with 50% (v/v) 1-propanol under reducing conditions at 60 °C. This fraction was made up to a defined volume and was used for the quantification of NTB and DTNB as well as for the determination of the protein content.

Quantitative Determination of DTNB and NTB by RP-HPLC. For the chromatographic separation of the glutenin fraction a solvent system containing water and acetonitrile was used. The classical ion pairing reagent TFA was present in a concentration of 0.1% (v/v), because the glutenin solution was used not only for DTNB and NTB determination but also for protein determination. The pH value of the solvents was 1.75; therefore, the typical yellow color of NTB at neutral pH (absorbance maximum at 412 nm) was not present. To find out the optimal wavelength for detection in 0.1% (v/v) TFA, UV−vis spectra of DTNB and NTB were recorded, which are presented in **Figure 4**. For both compounds a maximum of the absorbance at 327 nm was observed. This wavelength allowed a specific detection of DTNB and NTB without disturbance by proteins that were coeluted in the relevant range of the chromatograms. This specificity is demonstrated by the chromatograms shown in **Figure 5**. A reaction between DTNB or NTB with AA could

Table 4. Concentration of SH Groups in the Glutenins Isolated from Flours of 10 Different Wheat Cultivars[a]

	APO	AST	CON	CWR	FLA	GLO	KAN	MON	REK	SOI
μmol/g of protein	7.3 ± 0.7	6.0 ± 0.5	8.2 ± 0.6	6.3 ± 0.5	6.0 ± 0.3	6.2 ± 0.5	6.5 ± 0.6	5.6 ± 0.5	5.7 ± 0.4	7.5 ± 0.8
μmol/g of flour	0.26 ± 0.026	0.24 ± 0.021	0.22 ± 0.017	0.33 ± 0.026	0.23 ± 0.012	0.31 ± 0.027	0.33 ± 0.030	0.28 ± 0.024	0.26 ± 0.019	0.28 ± 0.029

[a] Mean values of duplicate determinations ± standard deviation. APO, Apollo; AST, Astron; CON, Contra; CWR, CWRS; FLA, Flair; GLO, Glockner; KAN, Kanzler; MON, Monopol; REK, Rektor; SOI, Soissons.

Table 5. Wheat Class CWRS: Concentration of SH Groups in the Glutenins Isolated from Dough As Affected by Different AA Concentrations[a]

	AA concn (mg/kg of flour)				
	0	20	50	100	200
μmol/g of protein	5.2 ± 0.5	5.9 ± 0.4	5.9 ± 0.4	7.1 ± 0.4	6.5 ± 0.5
μmol/g of flour	0.27 ± 0.026	0.31 ± 0.020	0.31 ± 0.022	0.37 ± 0.019	0.34 ± 0.028

[a] Mean values of duplicate determinations ± standard deviation.

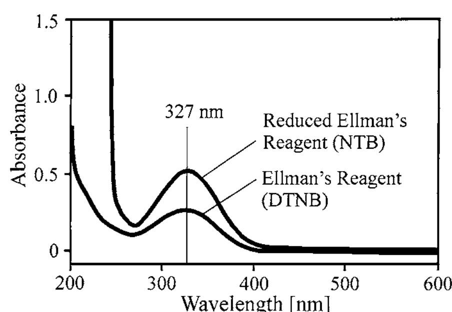

Figure 4. UV–vis spectra of Ellman's reagent (DTNB) and reduced Ellman's reagent in 0.1% (v/v) TFA at pH 1.75.

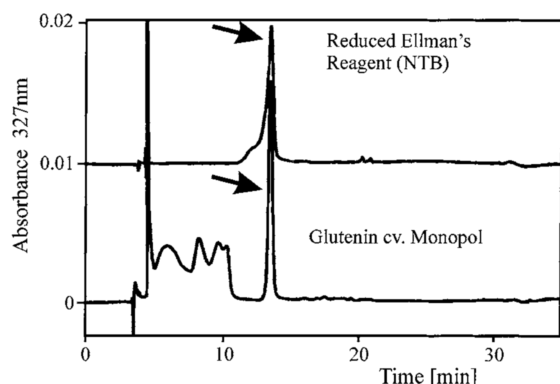

Figure 5. Determination of reduced Ellman's reagent (NTB) by RP-HPLC: (upper line) standard solution of NTB; (lower line) solution of glutenin from cv. Monopol obtained by micro-Osborne fractionation of NTB dough, prepared by addition of 50 mg of AA/kg of flour. The signal of NTB is marked by an arrow.

be excluded by the analysis of mixtures of DTNB and AA as well as NTB and AA.

Effect of AA on the Concentration of Free Thiol Groups of the Glutenins Isolated from Flour. These investigations were carried out with the whole set of 10 flours (**Table 1**). Free SH groups were present in the glutenins of the flours in concentrations of 0.22−0.33 μmol/g of labeled flour (**Table 4**). Because ∼15% of the mass was lost during dialysis, the concentrations on the basis of the native flours were lower (0.19−0.33 μmol/g). These values are lower than the concentration of SH groups in the entire flours. Several authors found concentrations of 0.7−1.2 μmol/g in the entire flour (*26*−*28*). Therefore, it can be assumed that, in flour, only a small percentage of the SH groups is present in the glutenins. However, the data are more meaningful when they are expressed on a protein basis and not on a flour basis. Therefore, the amount of protein present in the glutenin fraction was determined by RP-HPLC on the basis of the absorbance at 210 nm (*24*). To ensure that the extraction behavior of the protein types during the micro-Osborne fractionation of NTB flour was the same as of the native flour, micro-Osborne fractionations with the two flours were carried out. No difference between the Osborne fractions obtained with and without DTNB was obtained. From the amount of protein present in the glutenin fractions, the concentrations of SH groups per gram of protein were calculated. They were between 5.6 and 8.2 μmol/g of protein as shown in **Table 4**. In the flours of Apollo, Astron, Contra, CWRS, Flair, Glockner, Kanzler, Monopol, and Rektor the bread-making quality was positively correlated with the content of SH groups and vice versa. However, an exception was the high-quality cultivar Soissons, with a content of SH groups in the range of the poor-quality cultivars Apollo and Contra.

Concentration of SH Groups in the Glutenins Isolated from Dough As Affected by Different AA Concentrations. NTB doughs of CWRS with AA concentrations of 0, 20, 50, 100, and 200 mg/kg of flour were analyzed for free SH groups as described for the flours. The results are summarized in **Table 5**. Without the addition of AA, the SH content of the glutenins in the dough was 5.2 μmol/g of protein. Compared to previously published data (*29*, *30*), where 2−4 μmol of SH/g of protein in the whole dough (and not only in the glutenins) was found, the data obtained by using the present method in the glutenins are in the same order of magnitude. The percentage of glutenin in the flour used in this study was 8% on a mass basis and 42.9% on a protein basis. Therefore, it can be calculated that 2−4 μmol of SH/g of protein in the whole dough (*29*) corresponds to 4.7−9.3 μmol of SH/g of protein in glutenin. This means that, in dough, free thiol groups are preferably located in the glutenin fraction. In contrast to previously published data (*29*), where a decrease of the concentration of free SH groups in the dough after the addition of AA was obtained, the concentration of SH groups in the glutenins determined in the present study increased by 35% with increasing AA concentration to a maximum at 100 mg of AA/kg of flour and decreased slightly for 200 mg of AA/kg of flour. This finding is surprising and is not in accordance with either hypothesis about the mechanism of AA (**Figure 1**; **Table 1**). The hypothesis of Grosch (*4*, *6*, *7*) postulates a reduction of the amount of free SH groups on reaction of proteins with GSSG and CSSC (**Table 3**, reactions 3 and 7). The same is true for the hypothesis of Every, in which free SH groups of gluten proteins are directly oxidized to

disulfides by DHA (**Figure 1**). At the moment, no explanation can be given for this effect of AA on the glutenins.

Concluding Remarks. A method for the quantitative determination of high molecular weight thiols in the glutenins isolated from flour and dough has been developed on the basis of the Ellman's reagent. Low molecular weight thiols in flour and dough were determined by an isotope dilution assay (*7*, *15*). Both methods were sensitive enough to cover the concentration range that was required without and with the addition of AA for dough mixing. Concerning the fact that increasing amounts of AA increased the concentrations of high molecular weight thiols of the glutenins, an explanation cannot be given at the moment. Further research has to be done in the field of the AA improver action, as neither existing theory seems to be comprehensive at the moment.

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