Effect of Cysteine on the Molecular Weight Distribution and the Disulfide Cross-Link of Wheat Flour Proteins in Extrudates

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The changes in solubility, molecular size, and free sulfhydryl and disulfide contents of wheat proteins as affected by additions of cysteine during wheat flour extrusion processing were investigated. An increase in the concentration of added cysteine resulted in increases in the solubility and sulfhydryl and disulfide contents of wheat proteins in the wheat flour extrudates. The addition of cysteine as a reducing agent resulted in a decrease in the molecular size and therefore an increase in the solubility of the cross-linked wheat proteins by weakening the extensive disulfide-mediated cross-linking of wheat proteins during extrusion processing. The oxidation of two SH groups into an S−S bond between the protein and the added cysteine resulted in an increase in the disulfide content of the wheat proteins. The interchange between an SH group of an added cysteine and an S−S bond of a protein resulted in an increase in SH content in wheat proteins. A schematic pathway to illustrate the mechanism of all these changes is proposed.

Keywords: *Extrusion; wheat protein solubility; molecular size; disulfide cross-linking*

INTRODUCTION

Wheat, one of most widely cultivated plants (Autran, 1993), is unique among cereal and other proteinaceous plant in its ability to form a dough with viscoelastic properties ideally suited to making bread, biscuits, pasta, and cereal products (Autran, 1993; Kokini et al., 1994). The average protein content of wheat is 12% and can vary greatly from 7.5 to 18.5% (Kokini et al., 1994). Studies on wheat protein interactions have shown that disulfide cross-links play a significant role in the protein network formation in a dough (Schofield et al., 1983; Schofield and Chen, 1995; Weegels et al., 1993). Physical processes affect the breakdown and reassembly of the disulfide structure. Glutenins partly depolymerize during mixing and then repolymerize during resting (Weegels et al., 1993). Gliadins and glutenins interact by noncovalent forces (notably hydrogen bonds and hydrophobic interactions) in addition to covalent disulfide bonds during dough resting (Tatham et al., 1990; Weegels et al., 1996).

The role of sulfhydryl groups in dough chemistry has attracted the attention of many cereal chemists. The main premise is that these sulfhydryl groups are potentially able to undergo a disulfide−sulfhydryl interchange, which involves the cleavage or re-formation of disulfide bonds mediated by endogenous sulfhydryl-containing components (e.g. proteins, reduced glutathione) or by exogenous sulfhydryl-containing compounds (e.g. cysteine, reduced glutathione) (Dong and Hoseney, 1995; Dreese et al., 1988; Schofield and Chen, 1995). Many different experimental approaches have been used to study the contributions of disulfide cross-links to the bread-making potential of wheat flour and the rheological properties of the dough. Oxidizing and reducing agents are often used as bread improvers to affect the sulfhydryl/disulfide interchanges. Disulfide reducing agents (e.g. cysteine, glutathione, dithioerythritol, sulfite), sulfhydryl oxidizing agents (e.g. bromate, iodate), and sulfhydryl blocking agents (e.g. *N*-ethylmaleimide) change the physical properties of a dough considerably (Bloksma and Bushuk, 1988; Dong and Hoseney, 1995; Dreese et al., 1988; Gaines, 1990; Kim and Bushuk, 1995; Sarwin et al., 1993). It has been shown that cysteine cleaves disulfide bonds, resulting in a decrease in gluten elasticity and an increase in cookie diameter (Bloksma and Bushuk, 1988; Gaines, 1990).

Extrusion, one of the most versatile food processes, is widely used in the food and feed industries today to make products such as snacks, cereals, pastas, textured vegetable proteins, pet foods, and animal feeds (Rizvi et al., 1995). Extrusion processing can alter protein structure and thereby change its physical and functional properties by heat, shear force, pressure, and the presence of oxygen (Li and Lee, 1996a; Phillips, 1989; Ummadi et al., 1995). It has been reported that during extrusion processing, protein cross-linking is responsible for protein polymerization (Ledward and Tester, 1994; Prudêncio-Ferreira and Arêas, 1993; Strecker et al., 1995;). Disulfide-mediated cross-links have been identified as the major covalent bonding force for wheat protein polymerization during extrusion processing (Koh et al., 1996; Li and Lee, 1996a,b, 1997; Strecker et al., 1995). The extensive disulfide-mediated cross-linking of wheat proteins results in the formation of a protein network, which results in an increase in molecular weight and, subsequently, a decrease in the solubility of wheat proteins (Li and Lee, 1997). A significant correlation has been found between the extrusion temperature and the solubility and the disulfide content of wheat flour proteins (Li and Lee, 1997).

Earlier, we reported that the addition of cysteine significantly affected the physical, functional, and mi-

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crostructural properties of wheat flour extrudates (Li and Lee, 1996b). To obtain more information on the effect of added cysteine on the disulfide-mediated cross-linking of wheat proteins during extrusion processing, we extended our study to investigate the changes in the solubility, molecular size, and free sulfhydryl and disulfide contents of wheat flour proteins in the extrudates.

MATERIALS AND METHODS

Materials. Commercial wheat flour (Bouncer flour OS2530), purchased from Bay State Milling Co., Quincy, MA, was used for all experiments. The protein content of the wheat flour was 14%, determined by using the semimicro Kjeldahl method (AACC, 1983, method 46-13). Ellman's reagent and bicinchoninic acid (BCA) protein assay kit were purchased from the Pierce Co. (Rockford, IL). The prestained molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA). L-Cysteine, α-amylase (EC 3.2.1.1, from *Bacillus* species, 2100 units/mg), and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Springfield, NJ).

Extrusion and Sample Preparation. L-Cysteine was mixed thoroughly with wheat flour to concentrations of 0, 0.25, 0.50, 0.75, 1.0, and 1.5% (w/w). The extrusion was carried out on a ZSK-30 corotating twin-screw extruder (Werner Pfleiderer Corp., Ramsey, NJ). The extruder composition and screw configuration were the same as reported earlier (Li and Lee, 1996b). Wheat flour was fed into the unit with a K-Tron series 7100 volumetric feeding system (K-Tron Corp., Pitman, NJ). A metering pump (U.S. Electric Motors, Millford, CT) was used to add the water. The following conditions were used: 16% moisture content (wet basis), 185 °C die temperature, 500 rpm screw speed, and 225 g/min mass flow rate.

Approximately 2 kg of the extrudate with various concentrations of added cysteine was collected after the extruder had reached equilibrium conditions, as indicated by the steady die temperature and torque. The extrudates were allowed to cool to room temperature and were then ground with a model 700B Waring blender (Waring Products Corp., New Hartford, CT) to pass through a 40 mesh sieve. Ground samples were sealed and stored at 4 °C in glass bottles for further analysis. The moisture content of each extrudate was determined immediately after extrusion according to AOAC method 934.01 (AOAC, 1990). Approximately 2 g of each sample was placed in a 282A Isotemp vacuum oven (Fisher Scientific) at 100 ± 2 °C and 300 mmHg for 16 h. The moisture content was calculated as the loss in weight.

Sonication and Protein Extraction. Li and Lee (1996b) reported that after extrusion, ≈40% of the added cysteine remained in the wheat flour extrudates (e.g. 0.6% of added cysteine remained in the extrudate after the mixture of wheat flour with a concentration of 1.5% added cysteine was extruded). The loss of cysteine may be due to the degradation of cysteine and the formation of the disulfide bond during extrusion (Li and Lee, 1996b). To identify the effect of cysteine on wheat protein during extrusion and to equalize the effect of residual cysteine in the extrudates on wheat proteins during protein extraction, 2.0 g of the ground extrudate was dry-mixed with various amounts of cysteine to a final concentration of 0.6% (w/w) cysteine before extraction. The mixture was then dispersed in 40 mL of a 50 mM sodium phosphate buffer (pH 6.9) containing 4 units/mL α-amylase and shaken for 6 h to digest gelatinized starches. SDS (0.4 g) was then added to the above mixture to a final concentration of 1.0% (w/w). The mixture was shaken for an additional 2 h and was then transferred into a 50-mL centrifuge tube for sonication. The sonication was performed according to the method described by Singh et al. (1990) with some modifications. A model XL2020 sonifier (Heat Systems Inc., Framingdale, NY) was used with a macrotip probe (12.7-mm in diameter) for large-scale sonication (40 mL of the extracting buffer and 2 g of the sample in a 50-mL polycarbonate centrifuge tube). The sonifier generated ultrasonic vibrations with a frequency of 20 kHz. The sonication power was set at 5 on the output control knob, which was appropriate for breaking the noncovalent bonding of proteins (Singh et al., 1990). The sonication was performed with a timed/pulsed program, 20 cycles of process for 1 min and a 30-s pause. A 0 °C water bath was used to prevent heat buildup in the processed sample. After sonication, the mixture was shaken for a further 4 h.

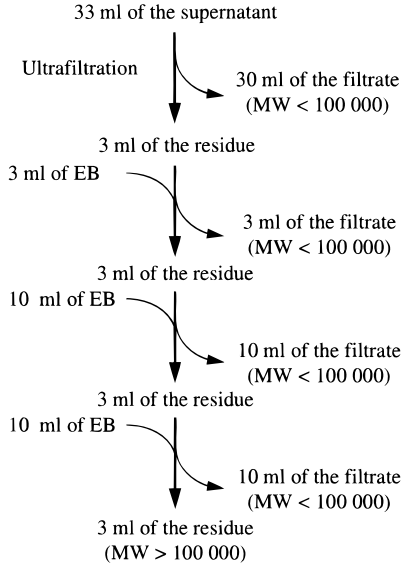

Figure 1. Flowchart for sequentially fractionating soluble proteins in the unextruded wheat flour and extrudates by using a pressure-modified ultrafilter equipped with a macroporous membrane of molecular weight cutoff at 100 000. EB, extracting buffer (1% SDS in 50 mM sodium phosphate buffer, pH 6.9).

The soluble proteins in the wheat flour control and the extrudates were extracted with and without the application of sonication. The mixture was centrifuged at 20000*g* for 40 min at 4 °C, and the supernatant was collected. The protein content in the supernatant was determined by using the BCA protein assay reagent, which was compatible with detergents (up to 1.0% SDS) (Pierce Catalog and Handbook, 1994−95, p 0−65).

Protein Fractionation. The protein fractionation was conducted on a pressure-modified ultrafilter (Stirred Cells, model 8050, Aminco Inc., Beverly, MA) equipped with a macroporous membrane with a molecular weight cutoff at 100 000 (Aminco Inc.). The supernatant obtained with 1% SDS in 50 mM sodium phosphate buffer was separated into two fractions on the basis of molecular weight of protein−SDS micelles: >100 000 and <100 000. The fractionation procedures are shown in Figure 1. The protein content in each fraction was determined by using the BCA protein assay reagent.

Sulfhydryl Group and Disulfide Bond Determination (in the Soluble Fraction). The total soluble sulfhydryl content (including free sulfhydryl and disulfide) was determined according to the method of Thannhauser et al. (1987). The soluble free sulfhydryl content was determined by using Ellman's reagent according to the Pierce method (Pierce, Immunotechnology Catalog, 1993, p E-56). The detailed procedures were the same as described by Li and Lee (1996a). The soluble disulfide content was calculated from the difference between total and free sulfhydryl contents.

Gradient Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). The SDS−PAGE of proteins was performed according to the method of Bollag and Edelstein (1991) on 3−20% (w/v) gradient polyacrylamide gels. One hundred microliters of the supernatant obtained with the 1% SDS in 50 mM sodium phosphate buffer was mixed with 20 μL of the SDS−PAGE sample buffer (5×). The mixture was heated in a 100 °C water bath for 10 min and then

Table 1. Protein Content in the Soluble Fraction of the Unextruded Wheat Flour and Extrudates in the Extracting Buffer of 1% (w/v) SDS in 50 mM Sodium Phosphate Buffer (pH 6.9)

	protein content (mg/g of extrudate on dry basis) in each soluble fraction			
	without sonication		with sonication	
sample ID	>100 000	<100 000	>100 000	<100 000
unextruded flour	103.8 ± 0.3	19.1 ± 0.1	103.9 ± 0.7	26.0 ± 0.9
EWSH-0[a]	15.8 ± 0.1[b]	23.4 ± 0.5	66.8 ± 0.4	19.6 ± 0.3
EWSH-0.25[a]	23.1 ± 0.2	28.4 ± 0.5[c]	89.9 ± 0.5	24.2 ± 0.5[c]
EWSH-0.50[a]	28.6 ± 0.3	32.8 ± 0.7[c]	90.0 ± 0.9	28.0 ± 1.0[c]
EWSH-0.75[a]	29.7 ± 0.2	37.8 ± 0.5[c]	88.4 ± 1.1	33.3 ± 0.5[c]
EWSH-1.00[a]	28.5 ± 0.1	39.7 ± 0.7[c]	90.7 ± 0.6	39.3 ± 0.7[c]
EWSH-1.50[a]	33.0 ± 0.2	47.1 ± 0.4[c]	93.8 ± 0.7	50.8 ± 1.8[c]

[a] EWSH-0, EWSH-0.25, EWSH-0.50, EWSH-0.75, EWSH-1.00, EWSH-1.50: wheat flour extrudates with concentrations of 0, 0.25, 0.50, 0.75, 1.00, 1.5% added cysteine, respectively. [b] Values are averages of triplicate measurements ± standard deviations. [c] Values are questionable, because the residual added cysteine (reducing reagent) interferes with the BCA protein assay.

centrifuged at 13 000 rpm for 4 min. After that, the sample solution was introduced into a well of the gel. The sample buffer (5×) contained 0 or 14.4 mM β-mercaptoethanol for unreduced and reduced protein SDS−PAGEs, respectively. Prestained molecular weight markers for gradient SDS−PAGE were as follows: myosin, 207 000, blue; β-galactosidase, 139 000, magenta; bovine serum albumin, 84 000, green; carbonic anhydrase, 41 700, violet; soybean trypsin inhibitor, 32 000, orange; lysozyme, 17 900, red; aprotinin, 8 600, blue.

RESULTS AND DISCUSSION

Effect of Cysteine on the Solubility of Wheat Proteins in the Extrudates. As shown in Table 1, the solubility of wheat proteins with a molecular weight >100 000 decreased dramatically after extrusion. As compared to the soluble protein content in the unextruded flour, approximately 84.8% and 35.7% of the proteins with a molecular weight >100 000 became insoluble in the control extrudate without and with sonication to solubilize proteins, respectively. The added cysteine in the wheat flour significantly affected the solubility of wheat proteins during extrusion processing. The soluble protein content in the MW >100 000 fraction increased from 15.8 to 33.0 mg/g of extrudate, as the concentration of added cysteine was increased from 0 to 1.5% (Table 1). However, when sonication was used to enhance the solubilization of wheat proteins, the soluble protein content in the MW >100 000 fraction increased dramatically, from 66.8 to 93.8 mg/g of extrudate, as the concentration of added cysteine was increased from 0 to 1.5% (Table 1). These results show that the added cysteine reduced the decrease of wheat protein solubility during extrusion processing. Approximately 7−17 mg of additional proteins was soluble without using sonication, while 22−27 mg of the additional proteins was soluble after sonication. Similar results were reported by Koh et al. (1996), who found that the soluble protein content in a 1.5% SDS aqueous solution increased with each increment of added cysteine (0, 0.5, 1.0, and 2.0%) in the wheat flour extrudates.

The sonication could not make more wheat proteins soluble in the MW <100 000 fractions, indicating that all of these proteins with a molecular weight <100 000 might be soluble in the extracting buffer (Table 1). The value marked with "*c*" in Table 1 is questionable because the residual added cysteine interferes with the BCA protein assay, giving a higher value than the practical value. However, this could indicate that there is less of an effect of the added cysteine on the soluble proteins in the MW <100 000 fraction than in the MW >100 000 fraction of the wheat flour extrudates.

Changes in protein solubility can occur due to changes in its conformation and/or molecular size (Phillips et al., 1994). The solubility of heated proteins was dependent on their molecular sizes (Pomeranz, 1991). The wheat protein network developed during heating at high temperatures was insoluble even in strong dissociating media, but soluble in the presence of a reducing agent, indicating that the disulfide cross-linking of wheat proteins was induced by heat treatment (Lavelli et al., 1996). Schofield et al. (1983) reported that the conformational changes caused by heat treatment rearranged the disulfide bonds of wheat proteins, affecting protein solubility. After extrusion, the decrease in the wheat protein solubility in the extracting buffer containing 1% SDS might be the result of an increase in its molecular size. The wheat proteins aggregated and cross-linked through hydrophobic interactions and intermolecular disulfide bonds, which resulted in an increase in their molecular sizes and, subsequently, a decrease in their solubility (Li and Lee, 1996a, 1997). An increase in the extrusion die temperature (0−160 °C) resulted in an increase in protein molecular size and, therefore, a decrease in wheat protein solubility (Li and Lee, 1997; Ummadi et al., 1995). The reducing agents (e.g. sulfite, β-mercaptoethanol, dithioerythritol) and SDS could enhance the solubilization of wheat proteins (Bloksma and Bushuk, 1988; Kim and Bushuk, 1995; Li and Lee, 1996a, 1997). Reducing agents acted in a direct chemical way by cleaving intermolecular disulfide bonds to reduce the protein molecular weight, which in turn resulted in an increase in the extractability of wheat proteins (Weegels et al., 1996). The addition of redox active components in wheat flour was important as these components modified the structures and functional properties of gluten proteins, affecting product quality (Lavelli et al., 1996; Li and Lee, 1996b). Recently, considerable interest has been expressed in the involvement of reduced glutathione, an endogenous sulfhydryl-containing component in wheat, which was specifically able to cleave protein disulfide bonds (Schofield and Chen, 1995). In our study, the added cysteine reduced the decrease in protein solubility of the wheat flour extrudates (Table 1). This indicated that the added cysteine could partially inhibit an increase in the protein molecular size during extrusion processing, probably by weakening the disulfide-mediated cross-linking and/or by cleaving the original intermolecular disulfide bonds of wheat proteins.

Disulfide Cross-Linking of Wheat Proteins during Extrusion Processing. The soluble protein content in the MW <100 000 fraction consisted of only a small portion of the total protein content in the unex-

Table 2. Free Sulfhydryl and Disulfide Contents in the MW >100 000 Soluble Fraction of the Unextruded Wheat Flour and Extrudates in the Extracting Buffer of 1% (w/v) SDS in 50 mM Sodium Phosphate Buffer (pH 6.9)

	SH or S−S content (nmol/mg of protein) in the MW >100 000 soluble fraction			
	without sonication		with sonication	
sample ID	SS	SH	SS	SH
unextruded flour	151.2 ± 1.1	12.6 ± 1.2	142.7 ± 3.4	9.1 ± 1.2
EWSH-0	nd[b]	39.1 ± 8.7	70.3 ± 1.2	15.6 ± 0.4
EWSH-0.25	6.8 ± 4.3[c]	50.8 ± 9.5	86.7 ± 3.9	22.6 ± 0.6
EWSH-0.50	33.2 ± 0.9	50.2 ± 9.2	103.1 ± 0.6	37.7 ± 3.3
EWSH-0.75	48.0 ± 1.0	41.7 ± 2.6	120.4 ± 7.7	34.0 ± 0.9
EWSH-1.00	46.5 ± 4.8	46.6 ± 1.8	116.2 ± 5.2	35.4 ± 0.4
EWSH-1.50	61.8 ± 1.4	40.9 ± 6.0	118.9 ± 5.3	34.4 ± 0.3

[a] EWSH-0, EWSH-0.25, EWSH-0.50, EWSH-0.75, EWSH-1.00, EWSH-1.50: wheat flour extrudates with concentrations of 0, 0.25, 0.50, 0.75, 1.00, 1.5% added cysteine, respectively. [b] Not detectable. [c] Values are averages of triplicate measurements ± standard deviations.

truded wheat flour and changed little after extrusion (Table 1). The results reported by Li and Lee (1997) show that the proteins in the MW >100 000 fraction are involved in protein cross-linking during extrusion. The study of disulfide cross-linking of wheat proteins is focused on the MW >100 000 fractions.

The soluble disulfide (S−S) and free sulfhydryl (SH) contents in the MW >100 000 fraction of wheat flour extrudates containing various concentrations of added cysteine were determined. Since the molecular weights of cysteine and cystine are 121.2 and 240.3, respectively, after fractionation, the soluble cystine and cysteine were in the MW <100 000 fraction. Therefore, the soluble S−S bonds and SH groups in the MW >100 000 fraction were primarily in the wheat protein molecules. As shown in Table 2, when we did not enhance the solubilization of proteins by sonication, after extrusion, the disulfide content of soluble proteins in the MW >100 000 fraction decreased dramatically from 151.2 nmol/mg of protein to a nondetectable level in the unextruded flour and the control extrudate, respectively. However, the sulfhydryl content of soluble proteins increased from 12.6 to 39.1 nmol/mg of protein in the unextruded flour and the control extrudate, respectively, indicating that the proteins containing high numbers of SH groups were soluble. The added cysteine resulted in an increase in the disulfide content of soluble proteins in the extrudates. The disulfide content of soluble proteins increased from a nondetectable level to 61.8 nmol/mg of protein in the wheat flour extrudates, as the concentration of added cysteine was increased from 0 to 1.5% (Table 2). The sulfhydryl content of soluble proteins varied slightly (Table 2). Koh et al. (1996) reported that in wheat flour extrudates, the sulfhydryl content of soluble proteins increased >10-fold, while the disulfide content of soluble proteins decreased to about half the original level as the concentration of added cysteine was increased from 0 to 2.0%. The difference between Koh et al.'s (1996) results and our group's results may be because they studied whole soluble wheat proteins and we studied the MW >100 000 soluble wheat proteins. When sonication was not used to enhance the solubilization of proteins, more than half of the total soluble wheat proteins in the extrudates were in the MW <100 000 fraction (Table 1); these proteins might contain high numbers of SH groups and low numbers of S−S bonds.

When sonication was used to enhance the solubilization of proteins, in the MW >100 000 fraction, the disulfide content of soluble proteins after extrusion decreased from 142.7 to 70.3 nmol/mg of protein in the unextruded flour and the control extrudate, respectively. The sulfhydryl content of soluble proteins increased from 9.1 to 15.6 nmol/mg of protein in the unextruded flour and the control extrudate, respectively. The added cysteine resulted in an increase in the disulfide content of soluble proteins in the extrudates. The disulfide content of soluble proteins increased from 70.3 to 120.4 nmol/mg of protein in the wheat flour extrudates as the concentration of added cysteine was increased from 0 to 0.75%. However, as the concentration of added cysteine was further increased to 1.5%, there was little additional change. As the concentration of added cysteine was increased from 0 to 0.5%, there was a large increase in the sulfhydryl content of soluble proteins, from 15.6 to 37.7 nmol/mg of protein in the wheat flour extrudates. However, as the concentration of added cysteine was further increased to 1.5%, there was little additional change.

SDS−PAGE of Wheat Proteins in Extrudates. To monitor the molecular weight distribution of wheat proteins on a wider range, the 3−20% gradient SDS−PAGE was done to check the effects of added cysteine on the molecular weight distribution of wheat proteins in the extrudates. The quantitative changes of unreduced soluble wheat proteins in the extrudates as affected by the added cysteine are shown in Table 1. The residual added cysteine in the extrudates is soluble in the extracting buffer, interfering with the BCA protein assay (Brown et al., 1989). The response of the BCA chromophore to cysteine has provided pseudovalues for protein quantitation. Since an equal volume of the supernatant was loaded on each well of the gel for each sample, the changes in the relative amounts of proteins could be compared from the band intensity of the gels. After extrusion, the smeared bands were visible in both the high molecular weight and the low molecular weight regions of the gels (Figures 2 and 3).

When sonication was not used to enhance the solubilization of wheat proteins in the extrudates, the molecular weight of unreduced soluble proteins was lower and primarily <41 700, as shown in the SDS−PAGE (Figure 2A). However, the band intensity of the gel increased as the concentration of added cysteine was increased (Figure 2), indicating that the added cysteine had reduced the decrease in protein solubility by probably inhibiting the cross-linking of wheat proteins during extrusion processing.

When sonication was used to enhance the solubilization of wheat proteins in the extrudates, a large amount of wheat proteins in the extrudates became soluble as indicated by the band intensity of the gels (Figure 3). Most of the unreduced soluble proteins/protein complexes in the extrudates possessed very large molecular weights. They were on the top of the separating gel, indicating that there was the cross-linking of protein−protein and/or protein-nonprotein through covalent bonds (e.g. disulfide bonds, peptide bonds, isopeptide

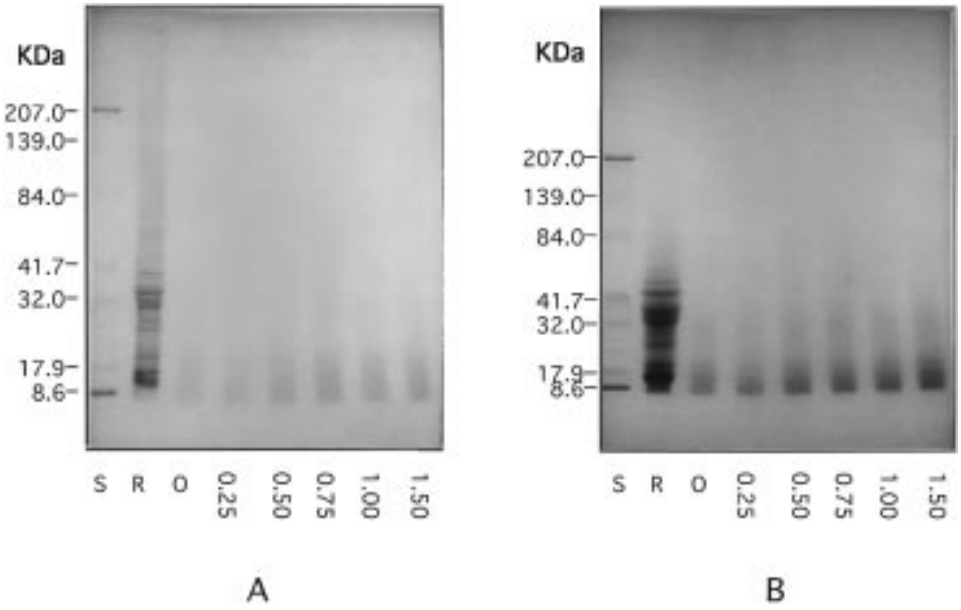

Figure 2. 3−20% gradient SDS−PAGE patterns of soluble proteins from the unextruded wheat flour and extrudates in the extracting buffer (1% SDS in 50 mM sodium phosphate buffer, pH 6.9) *without* sonication to enhance the solubilization of wheat proteins: (A) unreduced proteins; (B) reduced proteins. S, molecular weight standard; R, unextruded wheat flour; 0, 0.25, 0.50, 0.75, 1.00, and 1.5, wheat flour extrudates with concentrations of 0, 0.25, 0.50, 0.75, 1.0, and 1.5% added cysteine, respectively.

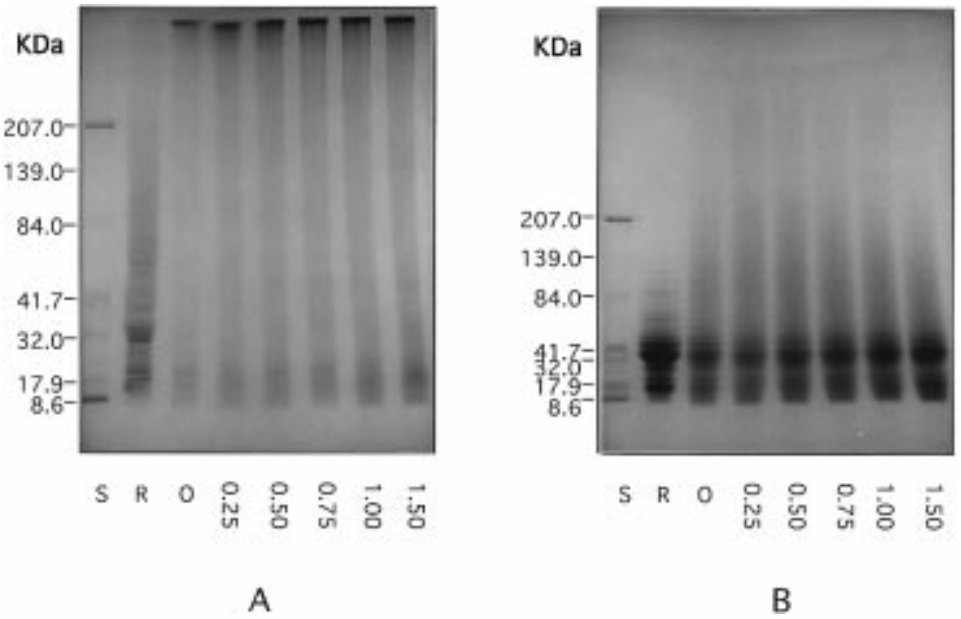

Figure 3. 3−20% gradient SDS−PAGE patterns of soluble proteins from the unextruded wheat flour and extrudates in the extracting buffer (1% SDS in 50 mM sodium phosphate buffer, pH 6.9) *with* sonication to enhance the solubilization of wheat proteins: (A) unreduced proteins; (B) reduced proteins. S, molecular weight standard; R, unextruded wheat flour; 0, 0.25, 0.50, 0.75, 1.00, and 1.5, wheat flour extrudates with concentrations of 0, 0.25, 0.50, 0.75, 1.0, and 1.5% added cysteine, respectively.

bonds, and Maillard derived cross-links). The band intensity of the gels increased with the increased concentration of added cysteine in the extrudates (Figure 3A), reflecting that the added cysteine could reduce the decrease in protein solubility by probably weakening the extensive cross-linking of wheat proteins during extrusion processing. Almost all of these proteins, which were on the top of the separating gel in the unreduced SDS−PAGE (Figure 3A), disappeared in the reduced SDS−PAGE (Figure 3B). This phenomenon demonstrated that the disulfide bond was the primary covalent bonding force for wheat protein cross-linking during extrusion processing. The reduced SDS−PAGE of wheat proteins showed a very clear background in the unextruded wheat flour and a continuous background in the extrudates (Figure 3B). The continuous background meant the continuous distribution of protein molecular weights, indicating that other covalent bonding forces (e.g. Maillard derived cross-links, isopeptide bonds, peptide bonds) might be involved in the protein−protein cross-linking during extrusion processing.

In wheat proteins, glutenins are polymers stabilized by both intramolecular and intermolecular disulfide bonds, and gliadins are monomers, with either no disulfide bonds (ω-gliadins) or intramolecular disulfide bonds (Tatham et al., 1990). During extrusion processing, heating and shearing treatments that destabilize native protein structures and promote unfolding can

facilitate protein interactions and lead to intra- or intermolecular sulfhydryl/disulfide (SH/S−S) interchange or sulfhydryl/sulfhydryl (SH/SH) oxidation reactions (Li and Lee, 1997). The free radicals (RS•) produced during processing might be involved in SH/S−S interchanges and SH/SH oxidations (Dong and Hoseney, 1995; Koh et al., 1996). Both noncovalent (e.g. hydrophobic interactions) and covalent (e.g. disulfide bonds) bonding forces drive wheat protein cross-linking during extrusion processing (Li and Lee, 1996a,b, 1997; Strecker et al., 1995; Ummadi et al., 1995). Environmental factors such as high temperature and high moisture can enhance protein−protein interactions to form a three-dimensional network (Li and Lee, 1997; Monahan et al., 1995; Thomasson et al., 1995). Because solubility properties of processed proteins are primarily related to their molecular sizes (Pomeranz, 1991), an increase in the molecular size of wheat proteins would be expected to cause a decrease in the solubility. Li and Lee (1997) have shown a critical extrusion die temperature of between 120 and 160 °C for the extensive disulfide-mediated cross-linking of wheat proteins under extrusion conditions of 30% moisture content, 200 rpm screw speed, and 275 g/min mass flow rate. The extensive cross-linking of wheat proteins resulted in a dramatic drop in their solubility in the extracting buffer (1% SDS in 50 mM sodium phosphate buffer, pH 6.9) due to a dramatic increase in their molecular size.

The effect of cysteine on the changes in the solubility, molecular weight distribution, and S−S and SH contents of wheat proteins in the extrudates suggested that the added cysteine weakened the disulfide-mediated cross-linking of wheat proteins during extrusion processing. The mechanism can be explained by the following pathways.

Pathways of the Effect of Cysteine on the Changes of Molecular Weight and Sulfhydryl and Disulfide Contents of Wheat Proteins in the Extrudates:

$$Pr_1{-}SH + \overline{Pr_a{-}S{-}S{-}Pr_a} \rightarrow Pr_1{-}S{-}S{-}Pr_a{-}Pr_a{-}SH \quad (0)$$

$$Pr_1{-}SH + Pr_2{-}SH + {}^1/_2O_2 \rightarrow Pr_1{-}S{-}S{-}Pr_2 + H_2O \quad (1)$$

$$nPr_x \rightarrow [Pr_x]_{n*} \quad (2)$$

$$nPr_x \xrightarrow{R-SH} \ldots + [Pr_x]_i + [Pr_x]_j + [Pr_x]_k + [Pr_x]_l + \ldots \quad (3)$$

$$R{-}SH + Pr_3{-}S{-}S{-}Pr_4 \rightarrow R{-}S{-}S{-}Pr_3 + HS{-}Pr_4 \quad (4)$$

$$2R{-}SH + Pr_3{-}S{-}S{-}Pr_4 + {}^1/_2O_2 \rightarrow R{-}S{-}S{-}Pr_3 + R{-}S{-}S{-}Pr_4 + H_2O \quad (5)$$

$$2R{-}SH + \overline{Pr_a{-}S{-}S{-}Pr_a} + {}^1/_2O_2 \rightarrow R{-}S{-}S{-}Pr_a{-}Pr_a{-}S{-}S{-}R + H_2O \quad (6)$$

$$R{-}SH + \overline{Pr_a{-}S{-}S{-}Pr_a} \rightarrow R{-}S{-}S{-}Pr_a{-}Pr_a{-}SH \quad (7)$$

$$R{-}SH + HS{-}Pr_1 + {}^1/_2O_2 \rightarrow R{-}S{-}S{-}Pr_1 + H_2O \quad (8)$$

R− = cysteine molecule; Pr = protein molecule; Pr_a = a part of a protein molecule; Pr_x = various protein molecules; $[Pr_x]_n$ = cross-linked protein; * = cross-linking degree; assumption: $\ldots i < j < k < l < \ldots < n$; cross-linked proteins, $\ldots [P_{rx}]_i$, $[P_{rx}]_j$ are soluble.

When sonication was used to disrupt the noncovalent bonding forces in aggregated wheat proteins, the molecular size of aggregated proteins was dominated by covalent bonding forces. During extrusion processing, the interchange of SH/S−S and the oxidation of two SH groups among proteins could result in the cross-linking of wheat proteins and, subsequently, an increase in the molecular weight (eqs 0 and 1). In the control extrudate, the extensive disulfide-mediated cross-linking made wheat proteins possess very large molecular weights, which made them insoluble in a 1% SDS aqueous solution in the absence of reducing agents (e.g. dithioerythritol, β-mercaptoethanol) (eq 2). However, almost all of these cross-linked wheat proteins in the extrudates became soluble in the 1% SDS plus 2% β-mercaptoethanol aqueous system (Li and Lee, 1996a, 1997). As a reducing agent, the added cysteine might weaken the extensive disulfide-mediated cross-linking of wheat proteins during extrusion processing. Therefore, the average molecular size of the cross-linked wheat proteins in the extrudates with an addition of cysteine was relatively lower than that in the control extrudate, and more wheat proteins were soluble as the concentration of added cysteine was increased (eq 3). An SH group in the added cysteine might cleave an intermolecular S−S in the protein, resulting in a decrease of the protein molecular weight and, subsequently, an increase of protein solubility (eqs 4 and 5). The reactions between an SH group in the added cysteine and an intramolecular S−S bond in a protein would have little effect on the protein molecular weight (eq 6 and 7), because the molecular weight of cysteine could be ignored compared to the molecular weight of a protein. The new liberated SH group in a protein (eq 4 and 7) could be further involved in the polymerization or oxidation reaction.

The increase of S−S and SH contents in the MW >100 000 soluble fraction coupled with the increase in the concentration of added cysteine in the extrudates indicated that during extrusion processing, S−S bonds formed between added cysteine and protein instead of between protein and protein and that the S−S bonds in proteins might be reduced by the SH groups in the added cysteine. Since oxygen was not excluded during high-temperature extrusion processing, the oxygen-catalyzed oxidation of two SH groups into an S−S bond resulted in an increase of S−S bonds in wheat proteins (eqs 1, 5, 6, and 8). The interchange between an SH group of the added cysteine and an S−S bond of the protein resulted in an increase of SH groups in wheat proteins (eqs 4 and 7). Therefore, the added cysteine became a part of the protein structure. This is one of the reasons for the decrease in added SH groups (cysteine) after extrusion reported by Li and Lee (1996b).

Wheat proteins appeared to be involved differently in heat-induced aggregation: some S−S bonds were not affected except under extreme conditions, some S−S bonds were moderately affected, and some S−S bonds were readily affected (Guerrieri and Cerletti, 1996; Koh et al., 1996; Lavelli et al., 1996). During extrusion at a die temperature of 185 °C and a moisture content of 16%, proteins completely denatured and unfolded to expose the previously buried S−S bonds and SH groups,

facilitating the disulfide-mediated cross-linking of wheat proteins to form a protein network in extrudates. The SH groups in the added cysteine could react readily with the exposed SH groups and S−S bonds of wheat proteins, which would have weakened the disulfide-mediated cross-linking of wheat proteins. Reduction of only a small portion of disulfide bonds would have a large influence on the cross-linking of wheat proteins (Strecker et al., 1995). No breakdown of the proteins in dough with a low concentration of added DTT (20 μmol/50 g of flour) could be visualized by SDS−PAGE without reduction. However, the farinograph properties of the same dough were significantly affected, suggesting a marked decrease in molecular size of the component(s) responsible for the consistency of dough (Gao et al., 1992). Therefore, the low concentration of added cysteine markedly affected the protein solubility in the extrudates. As a result, a reduction in the extent of protein cross-linking made the extrudate possess a weakened network, which resulted in the marked changes in the physical, functional, and microstructural properties of the extrudates as reported earlier by Li and Lee (1996b).

In fact, the effect of the residual/additional cysteine on protein structure is not avoided during extraction and sonication. The changes in solubility, SH/SS content, and molecular size of wheat proteins in extrudates may be attributed to the effect of added cysteine during extrusion and to the effect of added cysteine during extraction and sonication. On equalizing cysteine content in the wheat flour control and extrudates before extraction and sonication, it is possible that there are equalized effects on the protein structures during extraction and sonication. On the other hand, since the cross-linking degrees of proteins in the wheat flour and extrudates are different, it is not predictable if the reaction of protein with cysteine is equal. However, there is no doubt that the addition of cysteine modified structures of wheat proteins during extrusion, as indicated by the changes of microstructures of the extrudates (Li and Lee, 1996b). A possible way to avoid the effect of residual cysteine on wheat proteins during extraction and sonication is to alkylate the free SH groups of the extrudates with a sulfhydryl-blocking agent such as iodoacetamide.

Protein ultrafiltration depends on its size. In general, SDS binds to protein in a 1.4:1 (w/w) ratio (Singh et al., 1990). In this study, the ultrafiltration of wheat proteins was carried out in the presence of 1% SDS; therefore, the molecular weight of protein−SDS micelles is accounted for as the protein molecular weight. In the MW >100 000 fraction, the molecular weight of some proteins might be <100 000. This is probably why the ratio of proteins in the MW >100 000 fraction and the MW <100 000 fraction of unextruded flour is different from that shown on SDS−PAGEs (Figures 2 and 3) and does not correspond to the general knowledge about wheat protein composition.

Conclusion. In conclusion, the formation of the intermolecular disulfide bond was responsible for the wheat protein cross-linking during extrusion processing. The addition of cysteine reacted with wheat proteins by SH/S−S interchanges and/or SH/SH oxidation during extrusion processing, which weakened the disulfide-mediated cross-linking among wheat proteins. As a result, the average molecular size decreased, and the solubility, the disulfide content, and the sulfhydryl content increased in the wheat proteins in the extrudates with an addition of cysteine.

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