Effects of Deletion of Disulfide Bonds by Protein Engineering on the Conformation and Functional Properties of Soybean Proglycinin

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Glycinin, one of the dominant storage proteins of soybean seeds, has two disulfide bonds in each constituent subunit: Cys12–Cys45 and Cys88–Cys298 in the proglycinin $A_{1a}B_{1b}$ subunit. To examine the effects of disrupting disulfide bonds on the formation and maintenance of structure and on the functional properties of proglycinin, we replaced the cysteine residues (Cys12 and Cys88) by oligonucleotide-directed mutagenesis, giving mutant proglycinins Gly12, Ser88, and Gly12Ser88. The mutant proglycinins overproduced in *Escherichia coli* cells accumulated as soluble proteins and self-assembled into trimers like the native proglycinin. The functional properties of proglycinins Gly12 and Ser88 purified to near homogeneity were examined as models of modified glycinins. Proglycinin Ser88 formed a harder gel than native glycinin and unmodified expressed proglycinin even at the protein concentrations at which native glycinin did not form a hard gel. On the other hand, proglycinin Gly12 formed a gel only at higher protein concentrations (>6%), the hardness of which was similar to that of the native glycinin. Both proglycinins Gly12 and Ser88 exhibited emulsifying activity similar to that of unmodified expressed proglycinin. These results suggest that the number and topology of free sulfhydryl residues are closely related to the heat-induced gel-forming ability and the gel properties of glycinin but not to its emulsification.

INTRODUCTION

Improvement of functional properties and nutritional value of soybean proteins is a major objective in the food industry (Kinsella, 1979). Glycinin is a suitable target for the improvement of the functional properties, since it is the dominant storage protein in soybean seeds. Protein engineering is a powerful approach to attain this goal. Previously we succeeded in creating novel proglycinins designed to improve their nutritional and functional properties (heat-induced gelation and emulsification) by protein engineering based on the structural characteristics of glycinin and the relationships between its structure and the functional properties (Kim et al., 1990b). For this purpose, we utilized the following relationships: (i) Heat instability of the constituent subunits of glycinin is related to the heat-induced gel-forming ability (Nakamura et al., 1984). (ii) Hydrophobicity is an important factor in the emulsifying properties (Nishimura et al., 1989; Utsumi and Kito, 1991). (iii) The surface properties of a protein depend on the conformational stability—the more unstable, the higher the emulsifying properties (Kato and Yutani, 1988).

On the other hand, disulfide exchange plays an important role in the formation of heat-induced gel (Mori et al., 1982), and the number and the topology of free sulfhydryl residues are closely related to the heat-induced gel-forming ability and the gel properties of glycinin (Nakamura et al., 1984). These facts indicate that the substitution of a sulfhydryl residue involved in disulfide bond formation for another amino acid may result in a change of the gel-forming ability, the gel properties of glycinin, and also increase of its emulsifying ability. Protein-engineered glycinin should be able to assume the correct conformation and accumulate in the protein bodies of soybeans at high levels similar to that of native glycinin.

Two disulfide bonds, one at residues 12–45, which is in the acidic polypeptide region, and the other at 88–298, which is between the acidic and the basic polypeptide regions, are identified in soybean proglycinin $A_{1a}B_{1b}$ subunit (Staswick et al., 1984; Nielsen, 1985; Utsumi et al., 1987; Wright, 1988; Utsumi, 1992). Here, we have examined the effects of disrupting each disulfide bond individually and both bonds simultaneously in proglycinin $A_{1a}B_{1b}$ subunit on conformation and functional properties of soybean proglycinins.

MATERIALS AND METHODS

Bacterial Strains, Medium, and Plasmids. *Escherichia coli* strain JM105 was used as the host cell (Yanisch-Peron et al., 1985). LB medium (pH 7.5) used consisted of 1% bactotryptone (Difco), 0.5% yeast extract (Difco), and 1% NaCl. Plasmids employed were pAM82 (Miyanohara et al., 1983), M13mp18 (Yanisch-Perron et al., 1985), pGST4-2-11-10 carrying the cDNA encoding preproglycinin $A_{1a}B_{1b}$ prepared according to the method of Okayama and Berg (1982) (Utsumi et al., 1987), and $pKGA_{1a}B_{1b}$-3 (Utsumi et al., 1988). In the latter expression plasmid the ATG codon under the control of the *trc* promoter in the expression vector pKK233-2 (Pharmacia) was joined to the fourth codon in the $A_{1a}B_{1b}$ cDNA (Utsumi et al., 1988). The expressed proglycinin from $pKGA_{1a}B_{1b}$-3 was termed $A_{1a}B_{1b}$-3, in which N-terminal methionine was retained (Utsumi et al., 1988).

Site-Directed Mutagenesis and Construction of Expression Plasmids. A *Cfr*13I–*Pvu*II fragment (1.9 kbp) containing the $A_{1a}B_{1b}$ cDNA was obtained by cutting pGST4-2-11-10 with *Cfr*13I (partially) and *Pvu*II and then treated with the large (Klenow) fragment of *E. coli* DNA polymerase I in the presence of all four deoxynucleotides. The *Cfr*13I–*Pvu*II fragment was ligated with the large XhoI (filled-in)–*Pvu*II fragment of pAM82 to generate $pAMA_{1a}B_{1b}2$, where *Xho*I (filled-in)–*Cfr*13I (filled-in) and *Pvu*II–*Pvu*II were joined, and *Xho*I and *Pvu*II sites were regenerated. A *Xho*I (filled-in)–*Pvu*II fragment (1.9 kbp) from $pAMA_{1a}B_{1b}2$ was inserted into the *Sma*I site of M13mp18, and single-stranded DNA was isolated for mutagenesis. Site-directed mutagenesis using a synthetic oligonucleotide primer containing the desired substitution and selection of mutant proteins were performed using a site-directed mutagenesis kit by Takara Shuzo based on the method of Kunkel (1985). The specific primers used were as follows: 5′-TTGGATCTGGCCCTCGTTTT-3′ for Cys12–Gly12 (M13mp18Gly12) and 5′-TGCTAGGAGAGC-

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CCGGGTA-3′ for Cys88–Ser88 (M13mp18Ser88). As a result of mutation, the restriction sites *Hae*III and *Ban*II were generated, respectively.

The expression plasmids for the mutant proglycinins Gly12, Ser88, and Gly12Ser88 were constructed as follows. A *Dde*I–*Eco*T22I fragment (610 bp) from M13mp18Gly12, and fragments *Bam*HI–*Dde*I (300 bp) and *Bam*HI–*Eco*T22I (5130 bp) from $pKGA_{1a}B_{1b}$-3 were ligated to generate $pKGA_{1a}B_{1b}$GLY12 for the mutant proglycinin Gly12. A *Sma*I–*Eco*81I small fragment (1140 bp) from M13mp18Ser88 was ligated with a large *Sma*I–*Eco*8II fragment (4900 bp) from $pKGA_{1a}B_{1b}$-3 to generate $pKGA_{1a}B_{1b}$-Ser88 for the mutant proglycinin Ser88. A *Sma*I–*Eco*81I small fragment (1140 bp) from $pKGA_{1a}B_{1b}$Ser88 was ligated with a *Sma*I–*Eco*81I large fragment (4900 bp) from $pKGA_{1a}B_{1b}$Gly12 to generate $pKGA_{1a}B_{1b}$Gly12Ser88 for the mutant proglycinin Gly12Ser88.

Expression and Detection of the Mutant Proglycinins from *E. coli*. LB medium (300 mL) containing ampicillin (25 μg/mL) was inoculated with 3 mL of a full-grown culture of MJ105 harboring individual expression plasmids and cultured as described (Kim et al., 1990a). At A_{600} = 0.3, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After cultivation for 20 h at 37 °C, the induced cells were harvested by centrifugation. The cells were disrupted by sonication, and the cell debris and the supernatant were fractionated by centrifugation (Kim et al., 1990a). The analysis of the total cells, the cell debris, and the supernatant by $NaDodSO_4$–polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) was performed as described previously (Kim et al., 1990b). The amounts of the expressed proglycinins were measured by densitometric analysis following Coomassie brilliant blue staining and immunoblotting of $NaDodSO_4$ gels.

Purification of the Mutant Proglycinins from *E. coli*. *E. coli* cells from 12 L of culture were disrupted by sonication, and the cell debris and unbroken cells were removed by centrifugation as described previously. The mutant proglycinins were then purified by ammonium sulfate fractionation, Q-Sepharose column chromatography, and cryoprecipitation as described (Kim et al., 1990a).

Protein Measurement. Proteins in the samples were determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Analysis of Self-Assembly of the Mutant Proglycinins. The soluble extract (~5 mg/mL) of JM105 cells harboring individual expression plasmids was dialyzed against 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 1.5 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM EDTA. After dialysis, assembly was analyzed by sucrose density gradient centrifugation (Utsumi et al., 1988). The 2S, 7S, and 11S fractions purified from soybean according to the method of Thanh and Shibasaki (1976) were run in parallel as size markers.

Preparation of Glycinin from Soybean. Purified glycinin was prepared from sobyean (*Glycine max* L. var. Tsuru-no-ko) seeds according to the procedure of Mori et al. (1979).

Preparation and Measurement of Protein Gels. The protein samples were thoroughly dialyzed against 3.5 mM potassium phosphate buffer (pH 7.6) (buffer A) just before gel preparation use. After dialysis, the samples were concentrated to 5–10% protein by ultrafiltration and then diluted to the desired protein concentration with buffer A. The protein gels were prepared by boiling in a water bath for 30 min according to the micromethod of Utsumi et al. (1982). Gel hardness was measured with a rheometer (Yamaden, RE-3305, Rheoner) using a plunger 8 mm in diameter at a speed of 1 mm/s. Force required to compress 0.4 mm into the gels was recorded as hardness.

Emulsifying Activity. The emulsifying activity of the protein samples was measured according to the method of Pearce and Kinsella (1978). To prepare the emulsion, 0.5 mL of soybean oil and 1.5 mL of 0.05% protein solutions in buffer A were sonicated at 25 °C with a sonicator (Insonator Model 200 M, Kubota) at 100 W for 30 s. Aliquots (30 μL) of the emulsions were taken from the bottom of the container immediately after sonication and diluted in 20 mL of 0.1% (w/v) $NaDodSO_4$. The turbidity of the diluted emulsion was then measured in a 1-cm path length cuvette at a wavelength of 500 nm. Absorbance of duplicate aliquots of duplicate emulsions from each protein sample was measured.

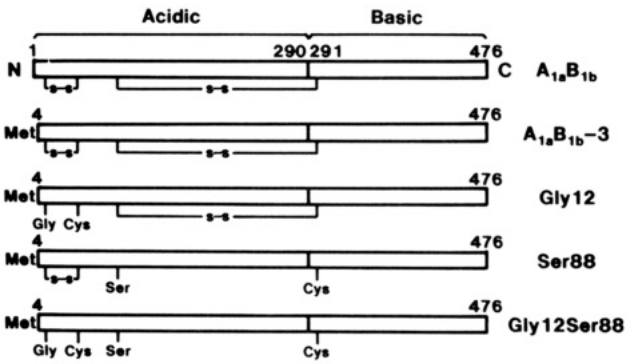

Figure 1. Schematic representation of the proglycinin $A_{1a}B_{1b}$, unmodified expressed proglycinin $A_{1a}B_{1b}$-3, and disulfide bond-deleted mutant proglycinins. Acidic and basic refer to the acidic and basic polypeptide regions of the mature glycinin, respectively. The numbers of the residues from the N terminus are described for the acidic and basic polypeptide regions.

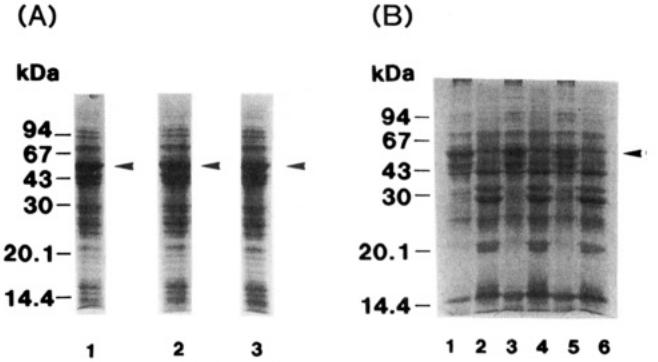

Figure 2. $NaDodSO_4$–PAGE analysis of the expressed proglycinins from individual expression plasmids. (A) Extracts from *E. coli* cells harboring $pKGA_{1a}B_{1b}$Gly12 (lane 1), $pKGA_{1a}B_{1b}$-Ser88 (lane 2), and $pKGA_{1a}B_{1b}$Gly12Ser88 (lane 3) were applied to $NaDodSO_4$ gels and stained with Coomassie brilliant blue. (B) The soluble (lanes 1, 3, and 5) and insoluble (lanes 2, 4, and 6) fractions of the extracts from the *E. coli* cells harboring $pKGA_{1a}B_{1b}$Gly12 (lanes 1 and 2), $pKGA_{1a}B_{1b}$Ser88 (lanes 3 and 4), and $pKGA_{1a}B_{1b}$Gly12Ser88 (lanes 5 and 6) were analyzed as described in (A). Arrowheads indicate the position of the expressed proglycinins. The numbers on the right denote MW.

RESULTS

Construction of the Disulfide Bond-Deleted Proglycinins. We mutated the Cys12 and Cys88 codons in $A_{1a}B_{1b}$ cDNA and constructed the expression plasmids for the mutant proglycinins Gly12, Ser88, and Gly12Ser88 as described under Materials and Methods. The constructions of the mutant proglycinins are as shown in Figure 1. The nucleotide sequences in the vicinity of the translation initiation site and the promoter of each expression plasmid constructed here are the same as that of $pKGA_{1a}B_{1b}$-3. Therefore, the efficiencies of transcription and translation are expected to be identical to each other, and the expressed proglycinins retain the initiation methionine.

Abilities of the Mutant Proglycinins To Assume the Correct Conformation. Production of the mutant proglycinins in JM105 cells harboring individual expression plasmids was assessed by $NaDodSO_4$–PAGE (Figure 2). The densitometric scanning of the gels indicates that all of the mutant proglycinins accumulated in the cells at the level of ~20% of the total *E. coli* proteins (Figure 2A) as compared to the scan of the extract from cells harboring the expression vector pKK233-2 alone [data not shown;

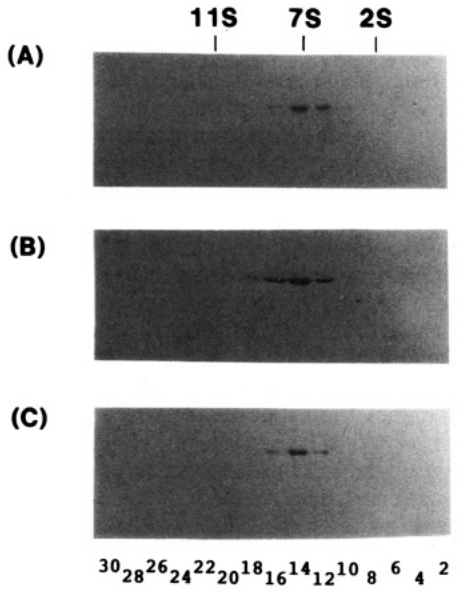

Figure 3. Self-assembly of the disulfide bond-deleted mutant proglycinins. Assembly was assayed by centrifugation on a 12-mL 10–30% (w/v) linear sucrose density gradient. (A) Gly12; (B) Ser88; (C) Gly12Ser88. Sedimentation is from right to left. Sedimentation standards are given.

see Kim et al. (1990a,b)]. The expression levels correspond to that of $A_{1a}B_{1b}$-3 (Kim et al., 1990a). After the disruption of the cells harboring individual expression plasmids by sonication, the debris and the soluble fractions were subjected to $NaDodSO_4$–PAGE (Figure 2B). Most (>85%) of the mutant proglycinins were present in the soluble fractions. This was confirmed by immunoblotting (data not shown).

To determine whether the mutant proglycinins are able to self-assemble into trimers like the native proglycinin, the soluble fractions of the cells harboring individual expression plasmids were subjected to sucrose density gradient centrifugation. After fractionation, proteins in each fraction were analyzed by $NaDodSO_4$–PAGE and immunoblotting. All of the mutant proglycinins predominantly sedimented in fraction 14, which corresponds to a size of 7–9S (trimer) (Figure 3), which was similar to that observed with unmodified proglycinin $A_{1a}B_{1b}$-3 (Utsumi et al., 1988).

Purification of the Mutant Proglycinins. The mutant proglycinins Gly12, Ser88, and Gly12Ser88 expressed in *E. coli* strain JM105 were purified following the procedure employed by Kim et al. (1990a) in purifying $A_{1a}B_{1b}$-3. Proglycinins Gly12 and Ser88 exhibited the same behavior as $A_{1a}B_{1b}$-3 during the course of purification. However, proglycinin Gly12Ser88 was easily degraded during dialysis against column buffer prior to the Q-Sepharose column chromatography even in the presence of 1.5 mM PMSF and 1 mM EDTA. We could not therefore obtain a sufficient amount of the purified proglycinin Gly12Ser88 for the investigation of its functional properties. The purified mutant proglycinins (Figure 4) were subjected to sucrose density gradient centrifugation. The results indicate that the purified mutant proglycinins also sedimented in fraction 14, which corresponds to a size of trimers (Figure 5).

Functional Properties of the Mutant Proglycinins. The native proglycinin $A_{1a}B_{1b}$-3 and mutant proglycinins Gly12 and Ser88 at various protein concentrations were boiled for 30 min, and the hardnesses of the gels so formed

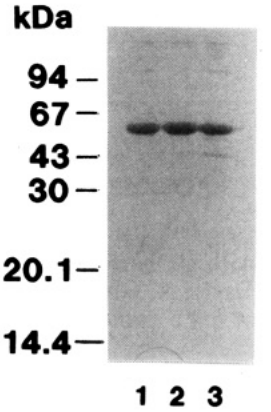

Figure 4. $NaDodSO_4$–PAGE analysis of the purified mutant proglycinins. The purified mutant proglycinins (~2 μg) were subjected to $NaDodSO_4$–PAGE and stained with Coomassie brilliant blue. (Lane 1) Gly12; (lane 2) Ser88; (lane 3) Gly12Ser88.

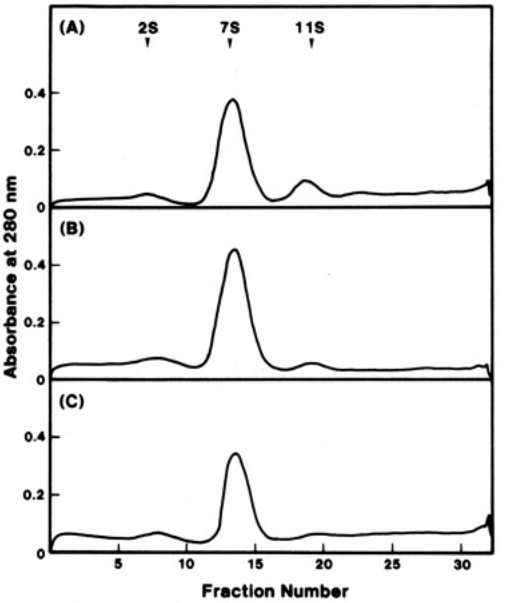

Figure 5. Self-assembly of the purified mutant proglycinins. Assembly of the purified mutant proglycinins (1 mg ~ 0.5 mg/0.5 ml) was analyzed as described in Figure 3. (A) Gly12; (B) Ser88; (C) Gly12Ser88. Sedimentation is from left to right. Sedimentation standards are given.

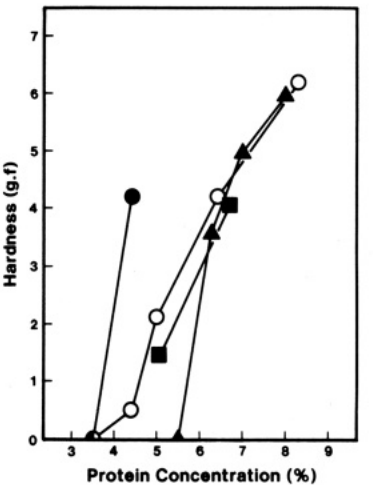

Figure 6. Hardness of the gels from the mutant proglycinins. (○) Native glycinin; (■) $Al_{1a}B_{1b}$-3; (▲) Gly12; (●) Ser88.

were compared as shown in Figure 6. The $A_{1a}B_{1b}$-3 formed gels having a little lower hardness than that of the native glycinin. Gly12 formed gels at protein concentration

Table I. Emulsifying Activity of the Mutant Proglycinins

sample	emulsifying activity,[a] %	sample	emulsifying activity,[a] %
soybean glycinin	100	Gly12	115
$A_{1a}B_{1b}$-3	123	Ser88	133

[a] Emulsifying activity is expressed as relative value compared with the soybean glycinin.

higher than 6% with gel hardness similar to that of the native glycinin. However, Gly12 could not form a gel at a concentration of 5.6% protein, which is sufficient for $A_{1a}b_{1b}$-3 and the native glycinin to form a gel. On the other hand, Ser88 tended to form harder gels than the native glycinin and $A_{1a}B_{1b}$-3.

Table I shows the emulsifying activity of native glycinin, $A_{1a}B_{1b}$-3, Gly12, and Ser88. The mutant proglycinins Gly12 and Ser88 exhibited higher emulsifying activity than native glycinin, although they both showed similar activity to that of $A_{1a}B_{1b}$-3.

DISCUSSION

For the mutant proglycinins to assume a conformation similar to that of native proglycinin, the following three criteria should be satisfied: (i) high-level expression in *E. coli*, (ii) solubility comparable to that of globulins, and (iii) self-assembly into trimers (Kim et al., 1990b). The mutant proglycinins Gly12, Ser88, and Gly12Ser88 in which disulfide bonds 12–45, 88–298, and both 12–45 and 88–298 were respectively deleted were expressed in *E. coli* as soluble proteins at high level (~20% of total *E. coli* proteins) (Figure 2) and self-assembled into trimers like the native proglycinin (Figure 3). Since all of the mutant proglycinins satisfied the three criteria and the purified mutant proglycinins were also present as trimers (Figure 5), we concluded that all of the mutant proglycinins assumed a conformation similar to that of native proglycinin. This indicates that the disulfide bonds 12–45 and 88–298 are not necessary for the formation and maintenance of the proglycinin trimer structure.

The mutant proglycinins Gly12 and Ser88 could be purified to obtain sufficient amounts for the investigation of their functional properties. However, the mutant proglycinin Gly12Ser88 was easily degraded in the Q-Sepharose column buffer composed of 35 mM potassium phosphate (pH 7.6), 0.15 M NaCl, 10 mM 2-mercaptoethanol, 1.5 mM PMSF, 1 mM EDTA, and 0.02% NaN_3, although it was briefly stable in the same buffer after the ammonium sulfate fractionation. The same mutant proglycinin was also degraded in the cryoprecipitation buffer composed of 60 mM Tris-HCl (pH 6.3), 10 mM 2-mercaptoethanol, 1.5 mM PMSF, 1 mM EDTA, and 0.02% NaN_3. These observations indicate that the conformational integrity of the mutant proglycinin Gly12Ser88 may be susceptible to attack of proteinase and that the proteinase participating in this degradation prefers low ionic strength and may be different from serine or metal proteinases. These observations also suggest that the removal of both disulfide bonds resulted in a molecule that was less compact than native proglycinin, though the overall conformations are similar.

The mutant proglycinin Gly12 did not form a heat-induced gel at low protein concentration (<6%), although the unmodified proglycinin $A_{1a}B_{1b}$-3 and the mutant proglycinin Ser88 did (Figure 6). Mori et al. (1982) reported that disulfide exchange is necessary for the heat-induced gelation of native glycinin. These findings may indicate that the disulfide bond 12–45 plays an important role in the initiation of the disulfide-exchange reaction for gelation. However, it seems that the disulfide bond 12–45 may not be essential for gelation at higher protein concentration, since the mutant proglycinin Gly12 could form a gel at protein concentration >6%. On the other hand, the gel hardness of the mutant proglycinin Ser88 was much higher than those of the native glycinin and the unmodified proglycinin $A_{1a}B_{1b}$-3 (Figure 6). This result supports the above discussion and also the suggestion made by Nakamura et al. (1984) that the number and topology of free sulfhydryl residues are closely related to the heat-induced gel-forming ability and the gel properties of glycinin.

The emulsifying activities of the mutant proglycinins Gly12 and Ser88 were similar to that of $A_{1a}B_{1b}$-3, although they were higher than that of the native glycinin (Table I). This observation is far from our expectation that the destabilization caused by the deletion of disulfide bonds may increase emulsifying ability. This discrepancy may be explained by the disulfide-exchange reaction which proceeded during sonication for making emulsions which made the mutant proglycinins form rigid aggregated structures. This could be further investigated by deleting both Cys12 and Cys45.

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