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Crystal Structures and Structural Stabilities of the Disulfide Bond-Deficient Soybean Proglycinin Mutants C12G and C88S

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The constituent subunits of seed storage protein 11S globulin have two disulfide bonds that are common among 11S globulins from legume and nonlegume seeds. In the case of the A1aB1b subunit of soybean 11S globulin, glycinin, Cys12−Cys45 and Cys88−Cys298 are observed by X-ray crystallography. The significance of these two disulfide bonds for structural stability was investigated by mutagenesis of Cys12 to Gly and of Cys88 to Ser. The disulfide bond-deficient mutants C12G and C88S could form the correct conformations identical to that of the wild-type proglycinin except in the vicinities of the mutation sites C12 and C88 as shown by their crystal structures. Thermal stability monitored by differential scanning calorimetry of the mutants indicated that the contribution of these disulfide bonds to the thermal stability of proglycinin A1aB1b is low, although there is a small difference in the extent of the contribution between the two disulfide bonds (Cys12−Cys45 > Cys88−Cys298). The contribution of Cys88−Cys298 to the resistance of proglycinin A1aB1b to proteinase digestion is higher than that of Cys12−Cys45. Possible effects of structure on the different properties of C12G and C88S are discussed.

KEYWORDS: Crystal structure; disulfide bond-deficient; proglycinin; soybean; structural stability

INTRODUCTION

Storage proteins of legume seeds accumulate at a high level (20−50% of seed weights). The major components are 7S and 11S globulins (*1*, *2*), which have compact tertiary structures similar to one another (*3*−*8*). The compact structure may be required for the high-level accumulation in seeds. The storage proteins of legume seeds are important protein resources for humans. In addition, the storage proteins especially of soybean are important protein materials for the production of processed foods. Soybean proteins have functional properties such as gel-forming and emulsifying abilities (*1*).

Soybean 7S and 11S globulins are called β-conglycinin and glycinin, respectively. The constituent subunits of β-conglycinin and glycinin contain zero and two or three disulfide bonds, respectively (*1*). The two disulfide bonds of glycinin are conserved among all 11S globulins sequenced thus far from various legume and nonlegume seeds. One of them connects the acidic and basic polypeptides of the constituent subunits, forming a hexameric structure, and the other is in the acidic polypeptide. In the case of the glycinin A1aB1b subunit, the two disulfide bonds are C12−C45 and C88−C298, and the third disulfide bond, C271−C278, was earlier suggested (*1*). The two conserved disulfide bonds are observed by X-ray crystallography of proglycinin (a precursor to the mature form) A1aB1b homotrimer, but not the third one, because this is located in the disordered region (*7*).

Disulfide bonds generally play an important role in the folding and structural stability of disulfide-containing proteins through the reduction of the number of conformations that the peptide can form in the unfolded state (*10*, *11*). Analysis using the disulfide bond-deficient mutants C12G and C88S (formally named Gly12 and Ser88, respectively) produced in *Escherichia coli* suggested that both disulfide bonds are not required for the correct folding and the assembly into the proglycinin trimeric structure (*9*). However, the contribution of the conserved disulfide bonds to the structural stability of glycinin is not known at all except that simultaneous deletion of the two conserved disulfide bonds induces loss of resistance against proteolysis at a low ionic strength (*9*). Elucidation of their contribution is valuable in designing glycinin with improved functional properties and nutritional value because structural stability is an important factor in functional properties such as gel-forming and emulsifying abilities (*1*) and digestibility.

Proglycinin has a trimeric structure in the endoplasmic reticulum (ER) and is processed to the acidic and basic polypeptide as it is sorted in protein storage vacuoles (*1*).

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Table 1. Statistics of Data Collection and Refinement

	C12G	C88S
data collection		
no. of crystals used	1	1
space group	$P4_1$	$P4_1$
cell dimension		
a, *b*	115.2	114.3
c	147.4	145.8
resolution range (Å)	31.4–3.2	28.9–3.1
no. of unique reflections	30085	34980
R_{sym} (%)	14.1 (52.3)[a]	12.0 (38.8)
completeness (%)	97.4 (95.6)	100 (100)
multiplicity	3.5 (3.3)	4.7 (4.2)
$<I/\sigma I>$	8.75 (2.46)	9.15 (2.28)
refinement		
resolution range (Å)	8.0–3.2	8.0–3.1
no. of reflections used[b]	23305	26670
no. of residues	1101	1098
average B-factor (Å^2)	16.7	18.4
rms deviation		
bond length (Å)	0.012	0.012
bond angle (°)	3.0	3.0
R factor	0.180 (0.212)	0.193 (0.226)
free *R* factor[c]	0.257 (0.311)	0.253 (0.278)

[a] Statistics for outer shells from 3.3 to 3.2 Å (C12G) and from 3.2 to 3.1 Å (C88S). [b] $F > 2\sigma F$. [c] Free set contains 10% of reflections.

Asparaginyl endopeptidase is responsible for the processing *(12, 13)*. This enzyme recognizes a specific asparagine residue in the junction between the acidic and basic polypeptides. The junction and even the following conserved five residues are in the disordered region (*7*). Although asparagine residues are present in the other disordered regions 3 and 3′, they are located on the edges of the disordered regions. This may be why only the junction is cleaved by the enzyme. In fact, proglycinin A5A4B3, which has an extra asparagine residue in the center of the disordered region 2 (*7*), is cleaved by the enzyme, resulting in A5 and A4 polypeptides (*14*). Therefore, there is a possibility that the susceptibility of the disulfide bond-deficient mutants to this enzyme and even other proteolytic enzymes might be different from that of the wild type. Increased susceptibility to proteolytic enzymes brings about enhanced nutritive value.

For this paper, crystal structures, thermal stabilities, and susceptilities to proteases of the disulfide bond-deficient mutants C12G and C88S were studied, and the contribution of the disulfide bonds to structural stability is discussed.

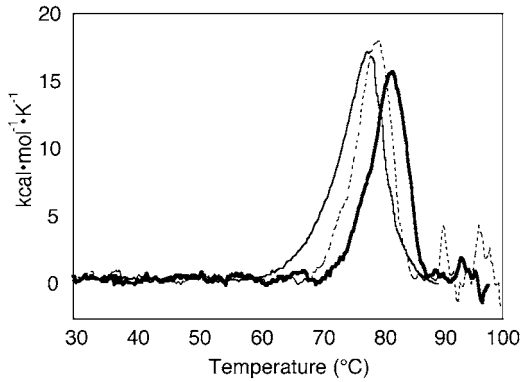

Figure 2. DSC profiles for the wild-type and mutant proglycinins. The thick, thin, and dashed lines indicate the wild type, C12G, and C88S, respectively.

MATERIALS AND METHODS

Bacterial Strains, Medium, and Plasmids. We used *E. coli* strain JM105 as the host cell (*15*) and LB medium for culture. Expression plasmids employed here were pKGA1aB1b-3 (*16*) for wild-type proglycinin, pKGA1aB1bGly12 (*9*) for C12G, and pKGA1aB1bSer88 (*9*) for C88S. In these expression plasmids 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 A1aB1b cDNA coding the mature region (*16*).

Expression in *E. coli* and Purification of Wild-Type and Mutant Proglycinins. The wild-type and disulfide bond-deficient mutant proglycinins were expressed in *E. coli* and extracted by buffer A [35 mM potassium phosphate (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride] from the cells as described (*17*). Each extract was subjected to ammonium sulfate fractionation. The fractions of 35−70% saturation containing recombinant proteins were disolved in and dialyzed against buffer B [35 mM potassium phosphate (pH 7.6), 0.15 M NaCl, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride]. The dialysate was applied to a HiLoad 26/10 Q-Sepharose high-performance column (Amarsham Pharmacia Inc.) equilibrated with buffer B. The recombinant proteins were eluted by a linear gradient of NaCl (0.15−0.3 M). The fractions containing recombinant proteins were subjected to gel filtration using a Hi-Prep 16/60 Sephacryl S-200 HR column equilibrated with buffer A.

Protein Measurement. The protein content of samples was determined using the Bradford method (*18*) with bovine serum albumin as standard.

X-ray Crystallography. C12G and C88S were crystallized by hanging drop vapor diffusion method as described (*7, 19*). Data collection of mutant proteins was carried out at 20 °C on a Bruker multiwire HI-STAR detector mounted on a MAC Science M18XHF

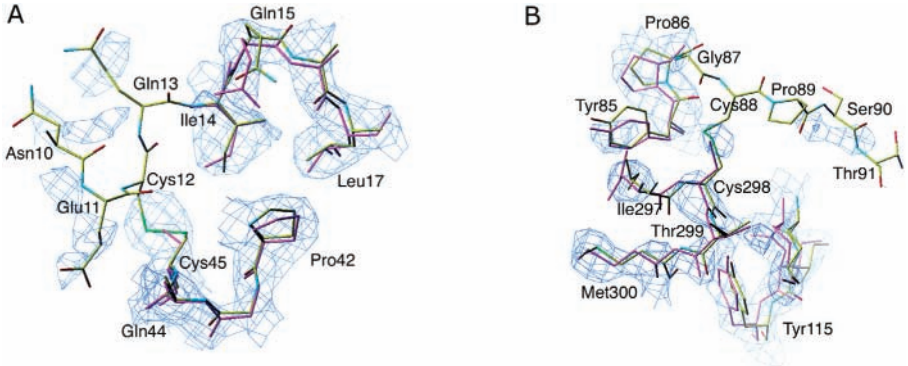

Figure 1. Schematic drawing of backbones around the disulfide bonds with electron density maps of the mutants. Protein models are represented in $2|F_o|-|F_c|$ electronic density maps contoured at 1.0σ generated using the program TURBO–FRODO (BioGraph). The coordinates of the wild-type proglycinin were superimposed on the maps of the mutants C12G (A) and C88S (B) shown in blue. The bond models of mutants are shown in violet. Carbon, oxygen, nitrogen, and sulfur atoms of wild-type proglycinin (PDB: 1FXZ) are yellow, red, cyan, and green, respectively.

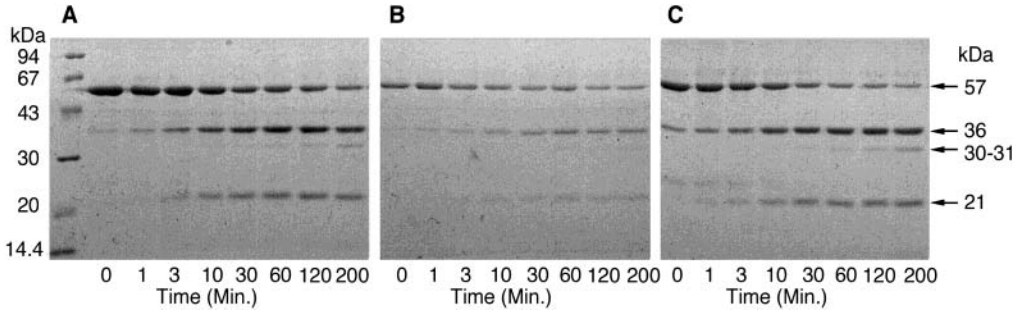

Figure 3. SDS-PAGE analysis of susceptibilities of the wild-type and mutant proglycinins to proteinase B: (A) wild type; (B) C12G; (C) C88S. Protein samples (0.2 mg/mL) were incubated with proteinase B (10 μg/mL) at 25 °C for 1–200 min. Two microgram proteins of the wild type and C88S were loaded per well. In the case of C12G, 1.0 μg of proteins was loaded. Molecular markers are indicated on the left.

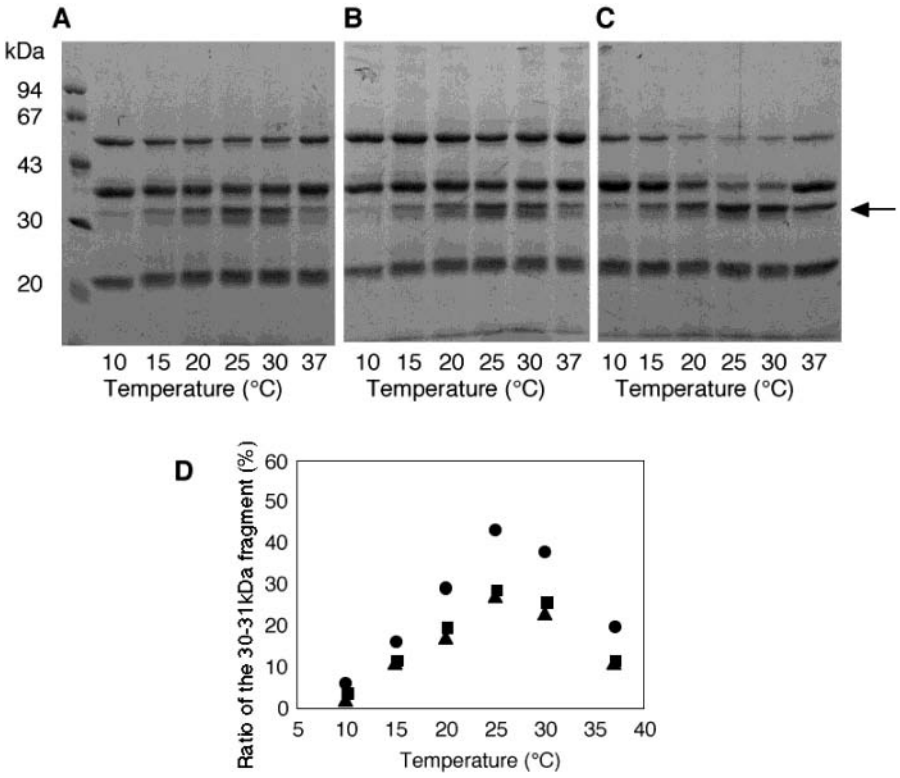

Figure 4. Effect of temperature on susceptibilities of the wild-type and mutant proglycinins to proteinase B: (A) wild type; (B) C12G; (C) C88S. Cleavage was carried out for 12 h at different temperatures (10–37 °C) and pH 5.8. Two microgram proteins were loaded per well. The fragment of 30–31 kDa is indicated by an arrow at the right side of the gels. (D) The amount of 30–31 kDa fragment was estimated by densitometric analysis of SDS-PAGE profiles, and its ratio to total amount of protein was plotted against temperature: (■) wild type; (▲) C12G; (●) C88S.

rotating anode generator. The structure coordinates of the wild type (*7*) were used as an initial model. The models were built using the program TURBO−FRODO (BioGraphics) and refined with the program X-PLOR.

Differential Scanning Calorimetry (DSC) Measurement. DSC experiments were carried out on a Microcal MC-2 ultrasensitive microcalorimeter (Microcal Inc.) as described (*20*). All DSC experiments were performed with a protein concentration of 0.5 mg/mL in buffer A, and the DSC scan rate was 1 °C/min.

Susceptibility to Protease Digestion. The susceptibilities of wild-type and mutant proglycinins to proteinase B (*21*) were measured in 50 mM MES buffer (pH 5.8) containing 0.2 M NaCl and 1 mM DTT. Because proteinase B is a cysteine proteinanse and proglycinin is insoluble under the low ionic strength condition at pH 5.8, DTT and 0.2 M NaCl were added to the reaction mixture. The susceptibilities of wild-type and mutant proglycinins to trypsin, α-chymotrypsin, and pancreatin were measured in 35 mM sodium phosphate buffer (pH 7.6). The protein samples (0.2 mg/mL) were incubated with proteinase B (10 μg/mL) at 25 °C for 1−200 min or for 12 h at 10−37 °C. For the susceptibilities to trypsin (10 μg/mL), α-chymotrypsin (200 μg/mL), or pancreatin (100 μg/mL), the protein samples (1.56 mg/mL) were incubated with an enzyme at 25 °C. After incubation, the digestion was stopped by boiling for 5 min. The digested samples were subjected to SDS-PAGE in the presence of 2-mercaptoethanol using 11% (mass/volume) acrylamide (*22*). The gel was stained with Coomassie brilliant blue R250. The intensities of the stained bands were estimated by densitometry using transmitted light and an NIH image program.

N-Terminal Amino Acid Sequencing. N-Terminal amino acid sequences of the fragments due to proteinase digestion were determined using a Protein Sequencer Procise 490 (Applied Biosystems) according to the procedure of Matsudaira (*23*) as described previously (*16*).

RESULTS

Structures of the Disulfide Bond-Deficient Mutants C12G and C88S. The crystal structures of disulfide bond-deficient mutants C12G (PDB:1UCX) and C88S (PDB:1UD1) were determined at 3.2 and 3.1 Å resolution, respectively (**Table 1**). The crystal structures of the mutants show that the overall structures are consistent with that of the wild type (*7*) except at

the vicinities of the mutation sites C12 and C88, where the electron densities were decreased (**Figure 1**). The root-mean-square values between Cα atoms of the wild type and C12G, the wild type and C88S, and C12G and C88S were 0.207, 0.325, and 0.319, respectively. These indicate that the deletion of the disulfide bond does not cause a visible structural fluctuation at the level of the resolution except the disorder in the vicinities of the mutation site. In other words, neither of the two disulfide bonds plays an important role in the formation and maintenance of proglycinin structure.

Thermal Stability Monitored by Differential Scanning Calorimetry. We tested the effect of scan rate on the temperature at the maximum of the heat capacity curve, T_m, by performing some experiments at heating rates of 30, 60, and 120 °C/h. It was observed that the value of T_m varied by as much as 1.0 °C. Therefore, application of equilibrium thermodynamics to the analysis of our data will lead to unfolding parameters close to the true equilibrium value (*24*).

DSC profiles for the wild-type proglycinin and disulfide bond-deficient mutants are shown in **Figure 2**. The T_m value of the wild-type proglycinin was 81.9 °C. Disulfide bond-deficient mutants C12G and C88S exhibited a little lower T_m values, 78.5 and 79.6 °C, respectively. There are only slight differences in ΔH among the wild type and mutants. These facts indicate that the contribution of each of the disulfide bonds to the thermal stability of the proglycinin A1aB1b is low.

Susceptibility to Proteinase B. Proteinase B from germinating vetch (*Vicia sativa* L.) seeds is an asparagine-specific endopeptidase closely related to a processing enzyme responsible for the maturation of proglycinin (*21*). Therefore, we examined the susceptibility to proteinase B of the wild-type and mutant proglycinins.

The time dependence of cleavage of the wild-type and mutant proglycinins by proteinase B was monitored by SDS-PAGE (**Figure 3**). With time, the amounts of the wild-type and mutant proglycinins decreased and those of the two fragments of around 36 and 21 kDa concomitantly increased. The overcleaved fragments of ~31 kDa appeared after 60 min of reaction and slightly increased with time. There is no significant difference in the results obtained for the samples, indicating that the deletion of each disulfide bond does not cause the change of susceptibility of proglycinin A1aB1b to proteinase B at 37 °C for 2 h. This is consistent with the study using mutants similar to C12G and C88S (*25*).

Cleavage of the wild-type and mutant proglycinins by proteinase B was also carried out for 12 h at different temperatures (10−37 °C) and monitored by SDS-PAGE (**Figure 4**). By increasing the reaction time from 2 to 12 h, the overcleaved fragments became broad (the size was ~30−31 kDa) and much more intense. The intensities of the overcleaved fragments were variable among reaction temperatures and among the samples. To make this point clear, the ratio of the band of the 30−31 kDa fragment to the sum of all protein bands in the cleaved sample was estimated densitometrically (**Figure 4D**). The amount of the 30−31 kDa fragment from C88S was larger than that of C12G, which was equivalent to that of the wild type. No distinct fragment other than the 30−31 kDa fragment was observed in any samples. These results suggest that the structure of C88S is locally a little bit looser than those of C12G and the wild type.

The N-terminal amino acid sequence of the 30−31 and 36 kDa fragments was Met-Arg-Glu-Gln-Pro-Gln-, corresponding to the N-terminal sequence of proglycinin A1aB1b. This result indicates that a small fragment containing the C terminus of the acidic polypeptide was cleaved.

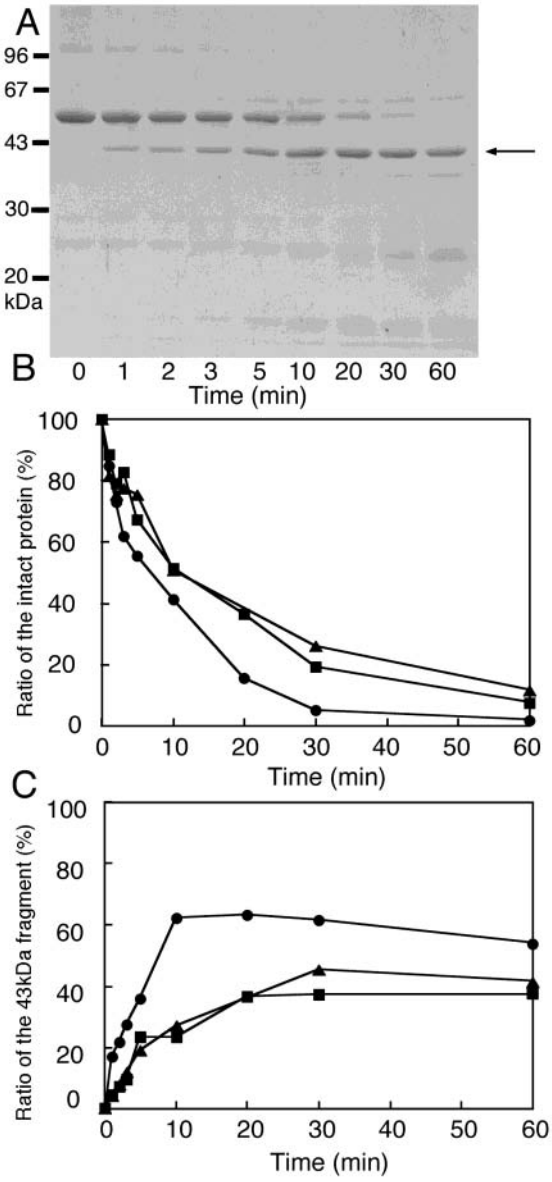

Figure 5. SDS-PAGE analysis of susceptibilities of the wild-type and mutant proglycinins to α-chimotrypsine: (A) SDS-PAGE profile of C88S (1 μg of protein was loaded per well; molecular markers are indicated on the left; the 43 kDa fragment is indicated by an arrow at the right side of the gels); (B) ratio of amounts of proteins with intact sizes to their initial amounts and (C) ratio of amounts of 43 kDa fragments to those of the total proteins (symbols are the same as in **Figure 4**).

The extent of overcleavage of the wild type, C12G, and C88S depended on temperature. In any case, the extent of overcleavage was highest at 25 °C. The reason for this observation is not clear, but probably due to structural rigidity of these proteins, their structure at 25 °C is more flexible than that at the lower and higher temperatures.

Susceptibility to Digestion Enzymes. To investigate whether the digestibility of the mutant is different from that of the wild type, the time dependence of digestion of the wild-type and mutant proglycinins by α-chymotrypsin, typsin, and pancreatin was monitored by SDS-PAGE and the intensities of bands were estimated densitometrically. Although there is no significant difference in the extents of digestion by trypsin and pancreatin among the protein samples (data not shown), C88S was digested

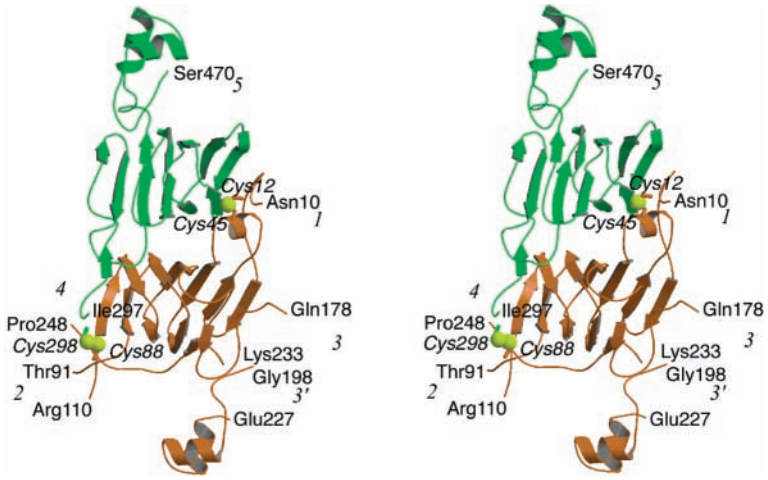

Figure 6. Stereodiagram of proglycinin monomer molecule. Ribbon models of acidic and basic polypeptides are shown in orange and green, respectively. Yellow spheres indicate sulfur atoms forming disulfide bonds of C12–C45 and C88–C298. Terminal residue of fragments and six disordered regions are labeled. The numbers of the disorderd regions are italicized. Coordinates of wild-type proglycinin and program MOLSCRIPT (*35*) were used to generate this ribbon model.

more efficiently by α-chymotrypsin than were C12G and the wild type (**Figure 5**). However, the fragment of ~43 kDa resulting from the digestion of the intact protein samples, even C88S, by α-chymotrypsin accumulated stably. These results correspond to those by proteinase B, strongly suggesting that the structure of C88S is locally looser than those of C12G and the wild type.

The N-terminal amino acid sequence of the ~43 kDa fragment was Ser-Ser-Arg-Pro-Gln-. This corresponds to the amino acid sequence from the 104th residue, which is in the disordered region 2 (*7*).

DISCUSSION

A disulfide bond is suggested to mediate structural stabilization of a protein through the reduction of the number of conformations that the protein can form in the unfolded state (*10*, *11*). The longer the loop between the cysteine residues forming a disulfide bond, the more stable the protein (*26*). This suggests that the structural stability of C88S is lower than that of C12G because the counterparts of the disulfide bonds containing C88 and C12 are C298 and C45, respectively. Although the susceptibility of C88S to proteinases was higher than that of C12G, the opposite was obtained using a thermal stability study. The thermal stability of C12G was lower than that of C88S. Therefore, conformational stabilities of the disulfide bond-deficient mutants as judged by DSC and susceptibility to proteinases were different.

Contribution of Disulfide Bonds to the Thermal Stability of Proglycinin. In general, a disulfide bond contributes highly to the thermal stability of many proteins such as ribonuclease A (RNaseA) (*27*) and azurin (*11*). RNaseA has four disulfide bonds: two are terminal, and the other two are embedded disulfide bonds. Removing either one of the terminal disulfide bonds decreased the thermal stability by almost 40 °C (*27*). On the other hand, stabilization of some proteins by the introduction of an artificial disulfide bond was observed; thermal stabilities of the mutants were found to be 10−30 °C higher than those of the wild types (*28*−*30*). In contrast to these, the disulfide bond-deficient mutants C12G and C88S are destabilized only by 3.4 and 2.3 °C, respectively, compared to the wild type (**Figure 2**). These results indicate that the contribution of the two disulfide bonds to thermal stability of proglycinin A1aB1b is low.

Although C88S should be destabilized more than C12G according to the change of entropy based on the number of residue between two cysteines, the contribution of mutation on C12 was higher than that on C88. This is probably because the disulfide bond C12−C45 formed on the interface between protomers is located more inside a trimer molecule than C88−C298 and because the N atom of C12 interacts with a side chain of the D157 residue through a hydrogen bond (*7*). In the crystal structure of C12G, the G12 residue was not modeled because of the disorder in the vicinity of the mutation site. On the other hand, C88 interacts only with C298. Perhaps the mutation of C12→G destabilized the structure of proglycinin by the formation of a cavity and lack of a hydrogen bond with D157.

Contribution of Disulfide Bonds to the Susceptibility to Proteinase of Proglycinin. Cleavage of the wild type, C12G, and C88S by proteinase B, which has a function similar to that of an enzyme responsible for the processing of proglycinin to the mature form, and α-chymotrypsin gave similar results: C88S gave the band of 30−31 kDa by proteinase B and the band of 43 kDa by α-chymotrypsin, which are larger amounts than those given by C12G and the wild type (**Figures 3**−**5**). In other words, the susceptibility of C88S to these enzymes is higher than those of C12G and the wild type.

The size of the counterpart of the 30−31 kDa band cleaved from the C-terminal region of the 36 kDa acidic polypepetide is estimated to be ~6 kDa. Cys88 was demonstrated by X-ray crystallography (*7*) to be located near the C-terminal region of the acidic polypeptide (**Figure 6**). Therefore, the higher susceptibility to proteinase B of C88S might be due to the speculation that C88S has a higher flexibility in the vicinity of the 88th residue than the wild type, causing a certain effect on the structure of the C-terminal region of the acidic polypeptide. In fact, N228 is positioned 64 residues from the C terminus of the acidic polypeptide region and is located at the edge of the disordered region 3′ (**Figure 6**). An alternative possibility is that the fragment of 36 kDa was cleaved at D251 and/or D266, located at positions 41 and 26 from the C terminus of the acidic polypeptide, which are positioned in the disordered region 4 (**Figure 6**), because peptide bonds with an Asp residue in the P1 position are also cleaved by plant and animal asparaginyl endopeptidases but more slowly (*21*, *31*−*33*). The latter is more probable, because the broadening with time of the overcleaved

fragments might be due to cleavage of both of the Asp-flanked peptide bonds.

Cleavage by α-chymotrypsin of the wild type, C12G, and C88S occurs dominantly in the disordered region 2, and C88S is more susceptible to α-chymotrypsin than the other two proteins. This is probably because C88 is located near the disordered region 2 (**Figure 6**).

Significance of the Disulfide Bond of Proglycinin in Its Structure. Previously we observed that the disulfide bond-deficient mutant C12G·C88S lacking both disulfide bonds can form the correct conformation, but it is very labile at a low ionic strength, although C12G and C88S, each lacking only one of the two disulfide bonds, are stable under the same conditions (*9*). This means that the deficiency of both disulfide bonds induces a much more critical effect on the structure. This is consistent with the results obtained by Matsumura et al. (*34*), because the effects on thermal stability of disulfide bonds are additive.

Proglycinin is processed to mature form composed of the acidic and basic polypeptides, which are connected by a disulfide bond. Therefore, the two disulfide bonds, especially the one connecting the acidic and basic polypeptides, might play an important role in the conformational stability of mature glycinin.

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