Molecular manipulation and modification of the genes encoding the G2 and G4 glycinin subunits

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Abstract

The genes encoding the glycinin subunits G2 and G4 were molecularly manipulated and modified to test the possibility of increasing the nutritional value of soybean seed proteins. The recombinant DNAs pSP65/G2HG4, pSP65/G4HG2, pSP65/248 Metl, pSP65/248 Met2,3 and pSP65/248 Metl.2,3 were used in in vitro translation to produce (i) chimeric proteins consisting of reciprocally exchanged acidic and basic G2 and G4 domains and (ii) Gy4 point mutants with an increased number of methionine residues. The ability of the recombinant proteins to assemble into proper quaternary structures was investigated using sucrose gradient fractionation. The data produced by this study could provide valuable clues for the potential improvement of genetically modified crops.

Key words: cDNA, glycinin subunit G4, in vitro translation, mutagenesis, soybean.

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Introduction

Glycinins are the predominant storage proteins in soybean seeds where they account for more than 20% of the seed dry weight in some cultivars and are primarily produced in cotyledon cells where they are sequestered within sub-cellular organelles called protein bodies (Nielsen et al., 1989; Nielsen and Nam, 1999; Vitale and Jurgen, 1999). As isolated from seed extracts, the glycinins are an oligomer of six similar subunits (Badley et al., 1975; Nielsen, 1996; Wang et al., 2003), the properties of which have been extensively reviewed (Moreira et al., 1979; Larkins, 1981; Turner et al., 1981; Chrispeels et al., 1982; Nielsen, 1984; Nielsen et al., 1996; Jung et al., 1998).

Glycinin subunits accumulate rapidly during embryogenesis and this accumulation is associated with dramatic changes in the prevalence of glycinin mRNAs (Nielsen et al., 1989) which begin to accumulate early in embryogenesis, are highly prevalent during the mid-maturation stage and decay prior to seed dormancy (Walling et al., 1986; Goldberg et al., 1989a; Goldberg et al., 1989b). The accumulation and decay of these mRNAs is in part regulated by transcriptional processes similar to those regulating other seed protein mRNAs (Cho et al., 1989; Goldberg et al., 1989a; Beilinson et al., 2002).

The predominant glycinin subunits found in soybeans are encoded by five genes (Gy) which have diverged into two subfamilies designated as group-1 (Gy1, Gy2, and Gy3) and group-2 (Gy4, Gy5) glycinin genes (Cho et al., 1989 and Nielsen et al., 1989). More recently, Beilinson et al. (2002) identified two new genes, a glycinin pseudogene (Gy6) and a functional gene (Gy7). Even though the amino-acid sequence of the glycinin G7 subunit is related to the other five soybean 1 IS subunits (soybean legumin like protein), it does not fit into either the group-1 (G1, G2, G3) or the group-2 (G4, G5) glycinin subunit families.

Dickinson et al. (1989) developed an in vitro system that allows the self-assembly of group-2 proglycinin subunits into complexes resembling those found naturally in the endoplasmic reticulum. The self-assembly of group-2 proglycinin into trimers requires post-translation cleavage before they are assembled into hexamers similar to those isolated from the protein bodies of mature seeds (Nielsen et al., 1996; Jung et al., 1998; Kinney et al., 2001). The ability to synthesize and assemble glycinin complexes in vitro is a useful tool for attempts to engineer subunits which can be assembled into proteins with improved nutritional qualities (Kirn et al., 1990; Utsumi et al., 1993; Katsube et al., 1994; Katsube et al., 1998) and for the identification of regions in the subunits crucial for oligomer formation (Dickinson et al., 1987; Dickinson, 1988; Nielsen, 1990).

Because of the high concentration of glycinin in soybean seeds and its major contribution to the nutritional properties of soybean proteins, combined with the effects of glycinin on the functional properties of food products made from beans this protein has been targeted for genetic manipulation to improve soybean nutritional quality. The
observation that the G2 subunit, rich in sulfur amino acids, does not self-assemble in vitro could mean that a modified G2 expressed at high level will not assemble efficiently in protein bodies (Dickinson 1988). It also remains to be demonstrated that in vitro and in vivo glycinin assembly occur in the same way, so an alternate and better strategy for improving the nutritional quality of glycinin may be to alter the G4 subunit which is capable of self-assembly.

In the study reported in this paper we constructed three bacterial mutants containing an increased amount of methionine (one to three residues), substitute the acidic subunit of G4 with that of G2 to determine structural features contributing to the different assembly behaviors of the group-1 and group-2 glycinins and test their self-assembly in vitro. Moreover it will shed light on the possibility of engineering transgenic soybean seed with high nutritional quality.

**Material and Methods**

**Glycinin purification, in vitro synthesis, assembly and labeling**

Glycinin was purified according to previously published procedures (Moreira et al., 1979). The in vitro transcription with SP6 RNA polymerase followed by translation with rabbit reticulocyte lysate was accomplished as described by Dickinson et al. (1989). Self-assembly of the proglycinin produced by the bacterial mutants (recombinant DNAs) constructed in this study was carried out as reported by Dickinson (1988).

For re-assembly with labeled protein 8 μL of 5X extraction buffer (0.175 M phosphate, pH 7.5, 2 M NaCl) was vortexed with 40 μL of [3H]-labeled glycinin protein which had been freshly synthesized in reticulocyte lysate after which 40 μL of dissociated glycinin (8 mg mL⁻¹) was added and the mixture again vortexed before quickly adding a further 8 μL of 5X extraction buffer and re-vortexing the mixture which was then dialyzed overnight against the acidic mixture containing 35 mM phosphate (pH 7.5), 0.4 M NaCl and 10 mM 2-mercaptoethanol. After dialysis the solution was diluted with 100 μL of 1X extraction buffer without 2-mercaptoethanol and the re-assembly products loaded onto a sucrose density gradient. For re-assembly with proglycinin trimers the 9S sucrose gradient fraction was dialyzed against 1X extraction buffer and then mixed directly with dissociated glycinin, the subsequent steps being performed as described above.

**Construction of the pSP65/G4HG2 and pSP65/G2HG4 plasmids**

To constrict plasmids encoding the G2 and G4 chimeric subunits it was necessary to introduce a HindIII site at position 928 (numbering from the initial ATG methionine codon in Gy2 cDNA (Nielsen et al. 1989) into the pMP 18/27 plasmid which contains Gy2 cDNA but lacks the portion encoding the signal sequence. Mutagenesis was performed using the method of Kunkel (1985) and the oligonucleotide pGGCGAACCTTTCACTTGTC. The resulting plasmid was denoted pMP18/MG2H and contained a HindIII site at the appropriate position. A HindIII site exists at an analogous position on the Gy4 cDNA clone pSP65/248 plasmid.

Two plasmids were created encoding chimeric glycinin subunits. The pSP65/G2HG4 plasmid encoded the G2 acidic polypeptide fused to the G4 basic polypeptide and the pSP65/G4HG2 plasmid encoded the G4 acidic polypeptide fused to the G2 basic polypeptide. To create pSP65/G2HG4, pSP65/248 was digested with BamHI and partially digested with HindIII. The 3.7 kb fragment which resulted from this treatment was ligated to the 0.9 kb Bam/HindIII fragment of pMP18/MG2H (Figure 1A). To create pSP65/G4HG2, the 0.5 kb HindIII fragment of pMP18/MG2H was ligated into the 4.1 kb HindIII fragment of pSP65/248. Recombinants were screened by restriction digest (Figure 1B). The identities of both constructions were verified by sequencing the region encoding the junction between the glycinin acidic and basic chains (Chen and Seeburg, 1985).

**Construction of pSP65/248 Met1, pSP65/248 Met2,3 and pSP65/248 Met1,2,3 plasmids**

All plasmids were derived from pSP65/248 which originated from a fusion between pSP65 and pG248 (Dickinson, 1988; Dickinson et al., 1989). Mutagenesis was carried out according to the method of Marotti and Tomich (1989) and the synthetic oligonucleotides 5′GCCA CTTTGAAGCTTTCAAC3′, 5′GATTGAATTCC3′ and 5′CAGTGCTATCTGACTCGAG3′. The underlined bases represent changes, and the template used was the pMPB plasmid. The mutated plasmid was transformed in E. coli 1190 and the transformants screened with the mutated oligonucleotides using colony hybridization. After transformation the transformation membranes were washed in 5 X SSC and 0.1 % SDS at 45 °C and the transformants giving strong hybridization signals were selected as putative mutants. The identity of mutants was confirmed byideoxynucleotide sequencing of the double stranded plasmid. The frequency of mutagenesis was about 2%. The first mutation was at position 1163 where methionine (ATG) replaced lysine (AAG), the second was at position 1311 where methionine (ATG) replaced isoleucine (ATT), and the third was at position 1353 where methionine (ATG) replaced valine (GTG) (Figure 2).

Three plasmids were constructed with an increasing number of methionine residues. Plasmid pSP65/248 Met1 was made by deleting the sequence between the AccI and XhoI in pSP65/248 and exchanged with the sequence between AccI and XhoI in pMP18/G4 Met1 (Figure 3A). Plasmid pSP65/248 Met2,3 was constructed by deleting the
sequence between HindIII site in pSP65/248. This deletion was filled with the sequence between the HindIII in pMP18/G4Met2,3 and HindIII in the pSP65 polylinker (Figure 3C).

DNA sequence analysis, sucrose gradient fractionation, trichloroacetic (TCA) precipitation, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Nucleotide sequence analysis was carried out by the chemical method of Maxam and Gilbert (1984). Synthetic oligonucleotides 5’GCGAGACAAGAAACGGGGTTGAGGGG 3’ and 5’GAGAACATTGCTCGCCCTTCGCGC3’ were used as primers for sequencing across the Gy4 regions.

Assembly was assayed by layering 100 µL samples of the in vitro synthesis reaction onto 11 mL linear 7-25% sucrose density gradient as described by Dickinson (1988).

Trichloroacetic precipitation was carried out according to the method reported by Dickinson et al. (1987) in which 100 µL assembly samples of each mutant were mixed with 25 mL of 25% hydrogen peroxide and incubated at 37 °C for 10 min, after which 1.5 mL of 25% TCA and 2% of casamino acids were added and the mixture stirred and placed on ice for at least 30 min. Samples were collected on glass fiber filters, washed twice with 10 mL of 10% TCA and again with 5 mL of ethanol. The filters were then dried and the radioactivity assessed in 10 mL of ACS scintillation fluid (Amersham, USA).
Electrophoresis was performed on 12% (w/v) SDS-polyacrylamide gels (Laemmli, 1970). The 9S assembly fractions from each mutant were pooled and dialyzed against sample buffer (0.03 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% (w/v) mercaptoethanol, 2.5M urea and 10% glycerol), boiled for 2 min and then loaded onto gels and electrophoretically separated. After electrophoresis gels were stained with Coomassie Blue, and when appropriate, treated with ENHANCE (New England Nuclear, USA) and visualized using autoradiography.

Results and Discussion

Two approaches were adopted to increase the number of sulfur amino acid residues in the G4 subunit, the subunit which assembles both in vivo and in vitro. The first approach adopted was to exchange the acidic and basic domains of the G4 and G2 subunits, while the second approach was to make a number of point mutations in the acidic region (which tolerates conservative point mutations) of the cloned cDNAs encoding G4 group-2 proglycinin subunits.

Dickinson et al. (1987) showed that the G4 and G5 group-2 proglycinin subunits were capable of self-assembly into trimers but the group-1 proglycinin G2 unit was not, even though it was capable of reassembling with mature glycinin much like the G4 and G5 subunits (Dickinson, 1988). Because the group-1 proglycinin G2 subunit is rich in sulfur amino acids the fact that this subunit is not subject to in vitro assembly makes the production of transgenic soybean lines rich in sulfur amino acids more difficult, the production of such lines being the most important factor in increasing the nutritional quality of soybean seed proteins. However, understanding the structural features contributing to the different assembly patterns of group-1 and group-2 glycinins will pave the way to engineering transgenic soybean lines producing seed proteins with enhanced nutritional properties.

In this research I constructed chimeric G2 and G4 subunits to determine the structural features contributing to the different assembly patterns of group-1 and group-2 glycinins. A precedent for this approach is the work of Dickinson et al. (1987) who exchanged the acidic and basic chains of the G4 and G5 group-2 glycinins and found that the modified subunits assembled normally, indicating that in this case the acidic and basic polypeptides are functionally interchangeable.

Figure 4 shows that mutant subunits of the expected size were obtained after transcription of the plasmids and translation in a reticulocyte lysate. The concentration of G2HG4 subunits produced in vitro was low, similar to that of G2 produced from pSP65/27 (Dickinson, 1988). This is important because assembly has been shown to be concentration dependent (Dickinson et al., 1987). In both cases, the low subunit concentration was not due to a lack of mRNA (data not shown).
The concentration of fusion G2HG4 was not normally high enough to be tested for self-assembly (0.74 ng/50 µL) but by increasing the concentration of labeled amino acids in the translation reaction a higher concentration of the G2HG4 subunit was achieved and the subunit was capable of self-assembly into complexes which sedimented as if they were trimers, although the high levels of unincorporated label complicate the interpretation of the result (data not shown). In the case of the G4HG2 reciprocal fusion the concentration of subunits produced in vitro was 5 ng per 50 µL, high enough to ensure that self-assembly would occur if it were structurally possible, but no assembled products were observed, suggesting that the G4HG2 fusion product has a structural defect which prevents self-assembly.

It was important to determine whether or not the chimeric proglycinin monomers would re-assemble into hexamers together with mature glycinin subunits that had been dissociated. However, this type of assay is less sensitive to subunit concentration because the large excess of mature dissociated glycinin subunits promotes assembly. Figure 5 shows the results of a typical experiment and it can be seen that after re-assembly with G2HG4 the distribution of radioactivity in the sucrose gradient was similar to that observed when the G4 proglycinin was used. By contrast, the distribution of radioactivity observed when G4HG2 monomers were used was different in that most of the radioactively labeled G4HG2 subunits remained in the unassembled 3S form, indicating that G4HG2-proglycinin subunits were less capable of re-assembly with mature glycinin than the G2HG4 proglycinin monomers.

Dickinson et al. (1987) reported that the acidic chain of G4 can tolerate deletion and insertion in the hyper-variable region and the mutant subunits produced by such modifications can assemble and re-assemble in a similar manner to that of the G4 subunit. Based on this I chose the acidic chain of G4 to generate a number of mutants (pSP65/248Met1, pSP65/248 Met2,3 and pSP65/248 Met1,2,3, see Figure 3). It is important to note, however, that the basic chain of G4 cannot tolerate any modification because deletion of any of the 21 N-terminal amino residues in the basic chain of the G4 proglycinin subunit inhibits the assembly into trimers (Dickinson et al., 1987) indicating very clearly the importance of the basic chain for in vitro proglycinin assembly.

Each of the mutant subunits described in this paper was found to be capable of self-assembly (Figure 6 A-D) and analysis of the 3H-Leu proteins produced by the self-assembly of each mutant subunit during SDS-PAGE showed that the assembled protein had a molecular weight of 66 kilodaltons (Figure 7).

My study showed that in the re-assembly assays all the glycinin subunits tested assembled efficiently with dissociated purified glycinin, although in the self-assembly assays there were differences in assembly pattern between some of the subunits. It is known that the group-2 G4 and G5 glycinin subunits self-assemble into trimers (Dickinson et al., 1987), although the significance of this observation is difficult to evaluate because a similarly modified group-1 glycinin subunit apparently assembles into 9S oligomers when produced in E. coli (Utsumi et al. 1988). The inability

![Figure 5](image5.png)

**Figure 5** - Radioactively labeled G4, G2HG4 or G4HG2 proglycinins in 0.4 M NaCl were incubated with dissociated glycinin subunits for 24 h at 25 °C. The distribution of radioactively labeled protein was determined following sucrose density gradient centrifugation. The distribution obtained with G4 is shown as a control and is indicated by a solid line. The position of sedimentation standards in the sucrose gradients is shown at the top of the figure.

![Figure 6](image6.png)

**Figure 6** - Self-assembly of pSP65/248, pSP65/Met1, pSP65/Met2,3, pSP65/248 Met1,2,3. Radioactive H3-Leu labeled proglycinins were synthesized in vitro using pSP65/248, pSP65/Met1, pSP65/Met2,3, pSP65/248 Met1,2,3. They were incubated in the translation mixtures for 30 h 250 °C to promote self-assembly and then analyzed by sedimentation in sucrose gradients.
of the G2 subunit to self-assemble is unlikely to be due to improper folding because these subunits are able to form mixed oligomers when assembled in the presence of either G4 or G5 (Dickinson, 1988). More probably, the failure to self-assemble reflects structural differences between subunits, which cause them to have different conditions for optimal assembly. Self-assembly of the G2 subunit may be possible with accessory proteins or a higher subunit concentration, either of which may be provided in the E. coli system.

One obvious difference between the group-1 and group-2 subunits is the size of the hypervariable region, which is much larger in group-2 subunits than group-1 subunits. This structural difference could be related to differences in the assembly properties of the subunits, although this is unlikely because deletion of the entire hypervariable region from the G4 group-2 subunit does not prevent self-assembly into trimers (Dickinson et al., 1990). This indicates that differences in the size of the hypervariable region between glycinin subunits will have little apparent effect on subunit assembly and that the defect, which prevents group-1 self-assembly, lies elsewhere in the protein.

The G2 and G4 glycinin subunits are known to have different in vitro assembly properties, and I evaluated the structural relationships between these subunits by in vitro fusion of the G2 acidic domain and the G4 basic domain or the G4 acidic domain and the G2 basic domain and then tested the fusion products in assembly assays. The self-assembly properties of the chimeric G2HG4 and G4HG2 subunits provided insights into the roles of the acidic and basic polypeptides in subunit interaction. Each fusion protein exhibited assembly characteristics most similar to the protein that donated the basic polypeptide, with the G2HG4 chimera being capable of trimer formation as was the case with the G4 subunit while the G4HG2 chimera was unable to form trimers not as was the G2 subunit (Dickinson, 1988). Dickinson et al. (1987) showed that the same is true for similar fusions between G4 and G5, with G4HG5 assembling more slowly than G5HG4 in the same way as G5 assembled more slowly than G4. These data generally support the hypothesis that the basic polypeptide contains the structural features important in trimer formation (Dickinson et al., 1990).

In my research I produced G2HG4 and G4HG2 monomers in vitro and evaluated them for their ability to assemble with denatured glycinin purified from seeds in a re-assembly assay and found that the majority of G2HG4 monomers re-assembled into trimers and hexamers whereas most G4HG2 subunits remained as monomers. The distribution of radioactively labeled G2HG4 subunits in the sucrose gradient after re-assembly closely resembled the profiles obtained with G2 (Dickinson, 1988) and G4 individually whereas the G4HG2 profile was clearly different. Because the G2 and G4 subunits assembled effectively whereas the G4HG2 subunit did not it is reasonable to infer that the acidic and the basic domains are not independent structural units and that interactions must occur between these domains that are crucial for assembly and possibly for proper folding. This complicates the conclusions drawn from the experiments involving the self-assembly of the G4HG2 subunit, which may fail to self-assemble because it contains the basic domain which prevents self-assembly of the G2 subunit. It is therefore tempting to conclude that the basic polypeptide contains structures which determine the self-assembly behavior of glycinin subunits, although such a conclusion is probably unsound in the light of the re-assembly results obtained with this protein and a more likely explanation of the self-assembly pattern is that the chimeric subunit contains a structural defect as a consequence of the fusion.

Structural differences exist between group-1 and group-2 subunits that influence their functional properties and could have important implications in efforts to engineer glycinin subunits which could be used for improving the nutritional quality of seeds. Because the G2 subunit has a higher sulfur content than the other glycinin subunits it was initially considered to be the best candidate adding additional sulfur amino acid residues. The fact that this subunit does not self-assemble in vitro (Dickinson, 1988) could mean that a modified G2 subunit expressed at high levels will not assemble efficiently in protein bodies. It also remains to be demonstrated that in vitro and in vivo assembly are equivalent and an alternate and perhaps better strategy for improving the nutritional quality of soybean seeds may be to alter those subunits capable of in vitro self-assembly.

These considerations caused me to turn my attention towards modification of the G4 subunit, which self-assembles in vitro and can tolerate modification of its
hypervariable region with minimal detrimental effect upon the structure of the protein (Nielsen, 1996; Utsumi et al., 2002). I created a number of point mutations in cDNA encoding the G4 subunit and found that the mutant subunits produced self-assembled in vitro as expected, although the assembly pattern was not the same for all mutant subunits. The pSP65/248met23 and pSP65/248met23 plasmids produced mutant proglycinins that assembled in a similar manner to proteins produced by the pSP65/248 plasmid which contains Gy4 cDNA but the mutant pSP65/248met23 plasmid produced mutant proglycinin subunits which did not assemble into trimers, from which it can be inferred that the failure of these mutant subunits to form trimers was due to a structural defect as a consequence of miss-folding. The mutations do, however, have wide-ranging effects on the ability of the protein to fold to its native structure, and hence are assumed to affect the stability or structure of a folding intermediate (Chen et al., 1994). It can be concluded that although the acidic subunit of the proglycinin G4 can tolerate deletion and modification without having any effect on assembly, the triple mutation in this domain may disrupt protein folding, such folding being dependent on the presence of acidic and basic subunits. However, the conclusion that limited types of changes targeted at the hypervariable region does not seem to perturb the assembly process (Nielsen 1996) is not supported.

The data presented in this paper suggest that beneficial modification of soybean storage protein subunits might not be as difficult as previously imagined, although all such the modifications must be tested in vivo. The many reports demonstrating that soybeans can be transformed using either disarmed a Ti plasmid or by ballistic methods (Hinchee et al., 1988; Austin and Cress, 1994; Chengsong et al., 1999; Droste et al., 2000.) now make such experiments feasible.

References


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