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Engineering two mutants of cDNA-encoding G2 subunit of soybean glycinin capable of self-assembly *in vitro* and rich in methionine

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Abstract: The main goal of this work was to make the cDNA-encoding subunit G2 of soybean glycinin, capable of self-assembly *in vitro* and rich in methionine residues. Two mutants (*pSP65/G4SacG2* and *pSP65/G4SacG2HG4*) were therefore constructed. The constructed mutants were successfully assembled *in vitro* into oligomers similar to those occurred in the seed. The successful self-assembly was due to the introduction of *Sac* fragment of *Gy4* (the codons of the first 21 amino acid residues), which reported to be the key element in self-assembly into trimers. The mutant *pSP65/G4SacG2HG4* included the acidic chain of *Gy4* (*HG4*), which was previously molecularly modified to have three methionine residues. This mutant will be useful in the efforts to improve the seed quality.

Key words: soybean; *Gy2*; glycinin; self-assembly; G2 subunit.

Introduction

Glycinin is the predominant storage proteins in soybean seeds. It accounts for more than 20% of the seed dry weight in some cultivars, has no known catalytic activity, and is thought to function as a reserve for carbon and nitrogen to be used upon seed germination (Nielsen et al. 1989). As isolated from seed extracts, the glycinin was found to be an oliogomer of six similar subunits (Badely et al. 1975). The properties of these subunits were reviewed extensively (Wolf 1976; Larkins 1981; Nielsen 1984), and five major subunits were identified on the basis of differences in their primary structures (Moreira et al. 1979). Each glycinin subunit is composed of two disulfide-linked polypeptides. One polypeptide has an acidic isoelectric point, whereas the other is basic. The two polypeptide chains result from post-translational cleavage of proglycinin precursors (Turner et al. 1982), a step that occurs after the precursor enters the protein bodies (Chrispeels et al. 1982).

Nielsen et al (1989) characterized the structure, organization, and expression of genes that encode the soybean glycinins. It was found that the predominant glycinin subunits found in soybean seeds were encoded by a family of five genes. These genes diverged into two subfamilies that are designated as Group-1 and Group-2 glycinin genes (Nielsen 1984). The genes in Group-1, including Gy1, Gy2, and Gy3, have nucleotide sequences that are more than 80% homologous to one another (Nielsen et al. 1989). The nucleotide sequences for members of Group-2, which includes Gy4, Gy5, are likewise more than 80% identical with one another, but are less than 60% homologous with those in Group-1 (Cho et al. 1989). Beilinson el al. (2002) identified two new genes: a glycinin pseudo-gene, Gy6, and a functional gene, Gy7. Even though the amino acid sequence of the glycinin subunit G7 is related to the other five soybean 11S subunits (glycinin subunits), it does not fit into either the Group-1 (Gyl, Gy2, Gy3) or the Group-2 (Gy4, Gy5) glycinin subunits.

Dickinson et al. (1987) developed an *in vitro* system that allows the self-assembly of Group-2 proglycinin subunits into the form that resembles those found naturally in the endoplasmic reticulum. This system showed that Group-2 subunits were capable of self assembly into trimers similar to those formed in endoplasmic reticulum. However, they found that the Group-1 subunits were unable to assemble in the absence of Group-2 subunits.

Group-1 subunits were initially considered to be the best candidate into which to engineer additional sulfur amino acid residues, because Group-1 had higher sulfur content than the other glycinin subunits. However, the observation that Group-1 subunits did not self-assemble *in vitro* could mean that a protein engineered from it and expressed at high level might not assemble efficiently in protein bodies. The aim of this work, therefore, was to adopt a better strategy to improve the nutritional quality of soybean seed proteins through alteration of Group-1 subunits to be capable of self-assembly *in vitro* and to harbor more methionine residues.

Material and methods

Material

Plasmids *pSP65/248* and *pMP18/MG2H* served as the first step in the construction of the plasmids *pSP65/G4SacG2* and *pSP65/G4SacG2HG4*. The isolation of *pG27*, a full-length *Gy2* cDNA was described in Scallon et al. (1985).

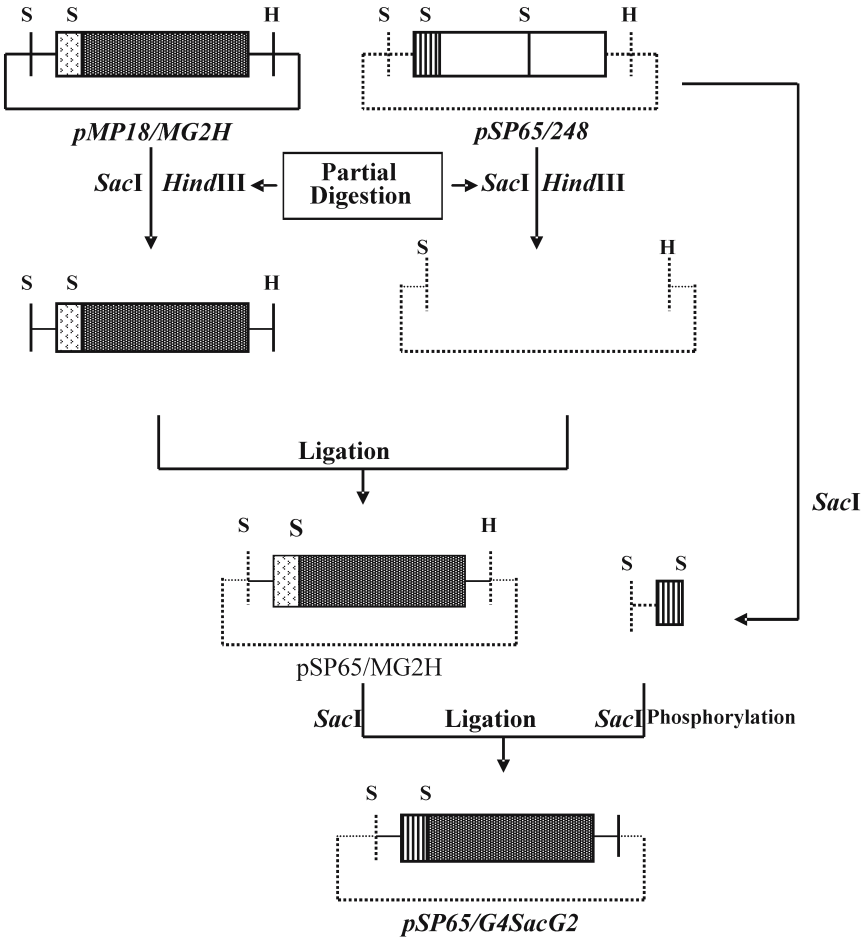

Fig. 1A. Construction map of *pSP65/G4SacG2*

The vectors *pSP65* and *pMp18* (Melton et al. 1984) was purchased from Promega Biotec (Madison, WI).

Construction of pSP65/G4SacG2

To construct this plasmid, the *pSP65/MG2H* and *pSP65/248* were separately partially digested at *Sac*1 and *Hind*III sites in the polylinker (Fig. 1A). The 0.9 kb polylinker *Sac*1/*Hind*III fragment of *pSP65/248* substituted *Sac*1/*Hind*III fragment, which included *MG2H* of *pSP65/MG2H* to form *pSP65/MG2H*. However, this plasmid lacked *Sac*1 fragment. Therefore, *pSP65/248* was digested with *Sac*1 and the 0.16 kb *Sac*1 fragment was isolated and then inserted at *Sac*1 site in *pSP65/MG2H*. The plasmid obtained was denoted pSP65/G4*Sac*G2 (Fig. 1A).

Construction of pSP65/G4SacG2HG4

pSP65/G4SacG2HG4 was constructed by separately digestion of *pMP18/MG2H* and *pSP65/248* with *BamH*1 and *Hind*III. *BamH*1/*Hind*III of *pMP18/MG2H* was substituted for the corresponding fragment from cDNA clone *pSP65/248* to form the plasmid *pSP65/G2HG4* (Fig. 1B). The plasmid denoted *pSP65/G2HG4* and the plasmid *pMP18/G2Sac* were separately digested with *Sam*1. The *Sam*1 fragment of *pMP18/G2Sac* was trade with *Sam*1 fragment of *pSP65/G2HG4* to construct the plasmid *pSP65/G2SacG2HG4*. Both *pSP65/G2SacG2HG4* and *pSP65/248* were separately digested with *Sac*1 and *Xho*1. *Sac*1/*Xho*1 fragment of *pSP65/G2SacG2HG4* was ligated in the same sites of *pSP65/248* to form *pSP65/G2HG4*. *pSP65/G2HG4* and *pSP65/248* were separately digested with *Sac*1 and then *Sac*1 fragment of *pSP65/248* was inserted in *Sac*1 site of *pSP65/G2HG4* to form the plasmid *pSP65/G4SacG2HG4*.

DNA sequence analysis

Nucleotide sequence analysis was carried out by the chemical method of Maxam & Gilbert (1977). Synthetic oligonucleotides 5'GCGAGACAAGAAACGGGGTTGAGG3' and 5'GAGAACATTGCTCGCCCTTCGCGC3' were used as primers for sequencing across the Gy4 regions.

In vitro transcription

Plasmids were linearized with *Pvu*2 and *Pst*1 and used as template for run-off transcription with SP6 RNA polymerase. Transcription reactions were carried out according to Melton et al. (1984), except that the DNA concentration was raised to 0.2 μg/μL. GTP was reduced to 20 μM, and m7GpppG (Pharmacia) was included at 500 μM. After 90 min at 40 ℃, the GTP concentration was raised to 500 μM and the incubation was continued for 30 min at 40 ℃. The RNA was precipitated by the sequential addition of NaOAc (pH 5.2) to 300 mM and 2.5 vol. of absolute ethanol (Dickinson et al. 1987).

In vitro translation and assembly

In vitro translation with rabbit reticulocyte lysates and (^{3}H) leucine were performed according to the manufacturer's (Promega Biotec) specific reactions. After translation, EDTA was added to 2 mM and phenylmethylsulfonylfluoride was added to 250 μM. The mixtures were

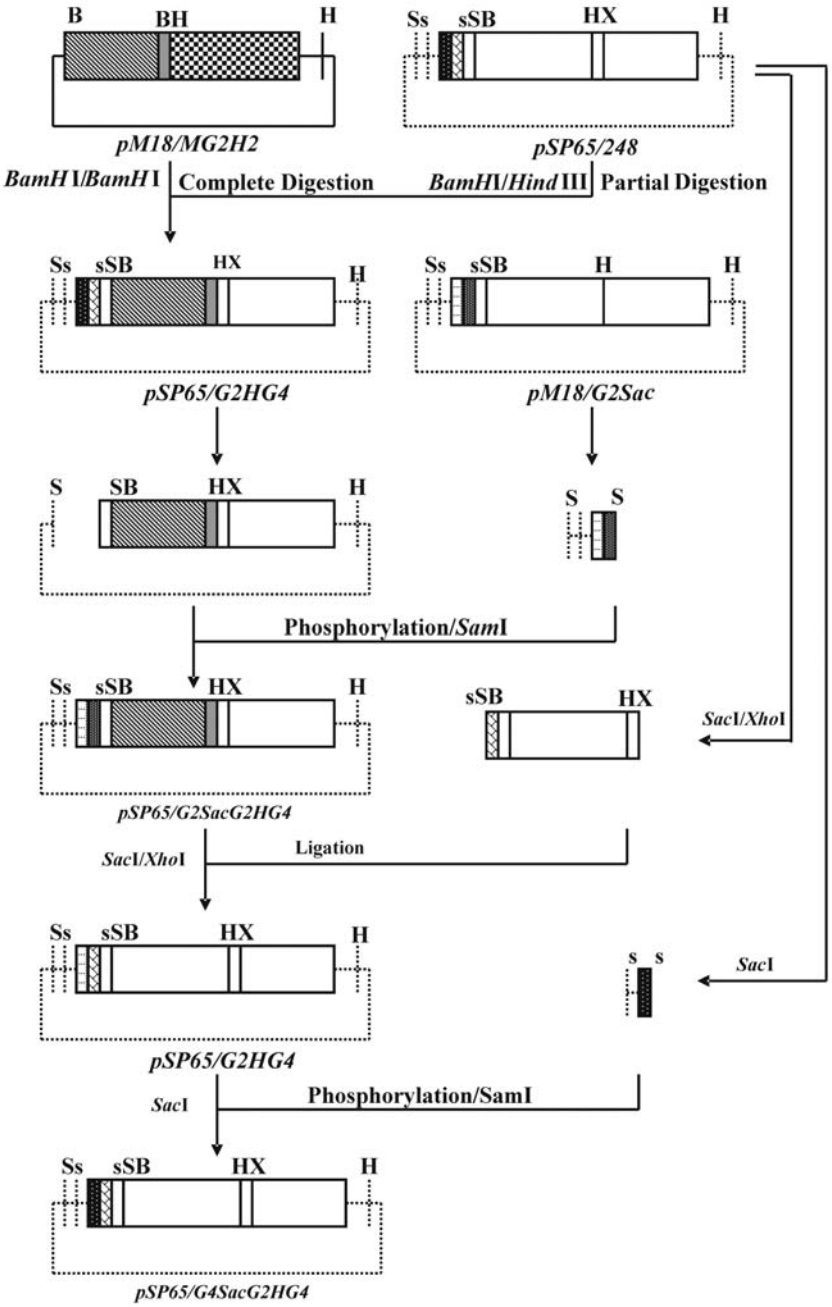

Fig. 1B. Construction map of *pSP65/G4SacG2HG4*.

then incubated for specified times and temperatures and placed on ice.

Sucrose gradient fractionation

Assembly was assayed by layering 100 μL samples of the *in vitro* synthesis reaction onto 11 mL linear 7–25% sucrose density gradient that contained 35 mM phosphate, 0.4 M NaCl, 0.01 M 2–mercaptoethanol (pH 7.6). The gradients were centrifuged for 24 h at 35,000 rpm and 4°C in a Beckman SW41 rotor. Fractions of 0.35 mL were collected from the bottom and assayed for radioactivity after trichloroacetic acid precipitations.

Trichloroacetic acid precipitation

Trichloroacetic acid (TCA) precipitation was carried out according to the method reported by Dickinson*et al.* (1989). In this method the samples of assembly (100 μL each) of each mutant were mixed with 25 mL of 25% hydrogen peroxide and incubated at 37°C for 10 min. Then 1.5 mL of 25% TCA, 2% casamino acids were added and mixed, and the mixture was placed on ice for at least 30 min. Samples were collected on glass fiber filters, washed twice with 10 mL of 10% TCA, and subsequently washed with 5 mL of ethanol. The filters were then dried and counted in 10 mL of ACS scintillation fluid (Amersham, USA).

SDS/PAGE

SDS-polyacrylamide gel electrophoresis was performed in 12% gels (Laemmli 1970). The fractions of the 9S peak of assembly of each mutant were pooled and dialyzed against sample buffer (0.03 M Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 2.5 M urea, 10% glycerol), boiled for 2 min before loading and then electrophoretically separated. After electrophoresis the gel was stained with coomassie blue,

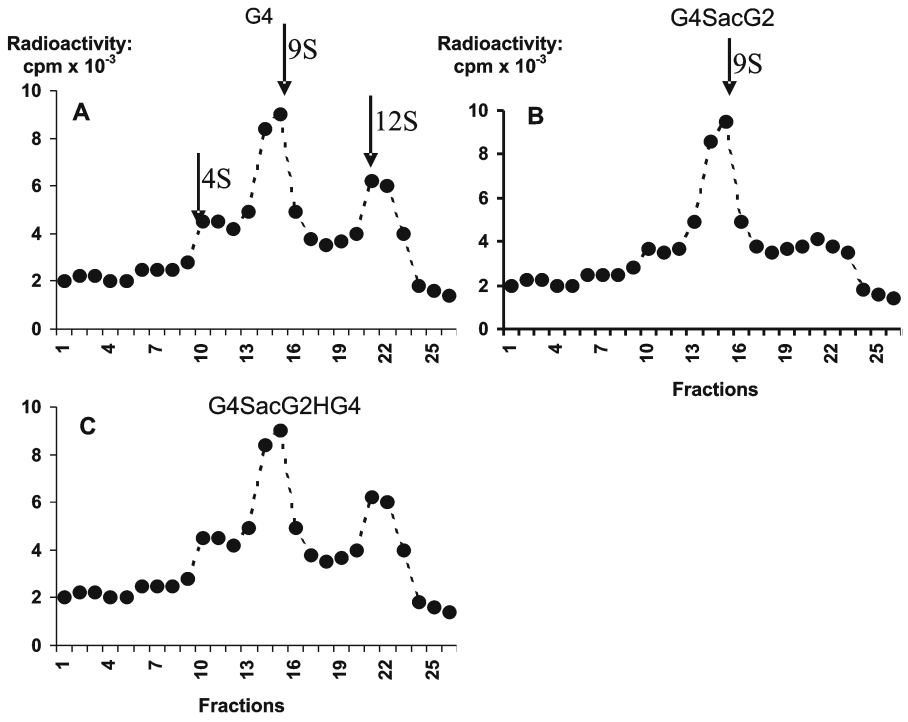

Fig. 2. The results of self-assembly of G4 (A), G4SacG2 (B) and G4SacG2HG4 (C). Radioactive ^{3}H-Leu labeled proglycinins were synthesized *in vitro* using *pSP65/248*, *pSP65/G4SacG2* and *pSP65/G4SacG2HG4*. They were incubated in the translation mixtures for 30 hours at 25 °C to promote self-assembly and then analyzed by sedimentation in sucrose gradients. Sedimentation standards are shown at the top.

treated with EN^{3}HANCE (New England Nuclear, USA) and visualized by fluorography.

Cross-linking

Cross-linking was carried out by a modification of the method described by Siezen*et al.* (1980). Fractions from sucrose gradient that contained 9S complexes were pooled, and then samples (80 μL) were mixed rapidly with aliquots of a solution of dithiobis(succinimidylpropionate) (7 mg/mL) in acetonitrile to give a final concentration of 0.016 (w/v) cross-linker. After 30 min at room temperature, each sample was mixed with 20 μL of 6 M urea / 0.15M sodium phosphate buffer, pH7 / 10% $NaDodSO_4$ / 0.02% bromophenol blue, and heated to 100 °C to prevent further cross-linker. Aliquots (20 μl) from each sample were dialyzed against 0.025 M neutral sodium phosphate buffer (electrode buffer) that contained 10% (v/v) glycerol and 0.02% bromophenol blue and were examined by electrophoresis in a 2–10% acrylamide gradient gel. The gels were then stained with commassie blue, treated with EN^{3}HANCE (New England Nuclear, USA), and visualized by fluorography. The gel was calibrated with protein standards. The standards were prepared with iodo[^{14}C]acetamide as described by Crestifield*et al.* (1963).

Results and discussion

The high concentration of glycinin in soybean seeds and its major contribution to the nutritional properties of soybeans proteins, combined with the effects of glycinin on the functional properties of food products processed made this protein a main target to genetic manipulation to improve soybean nutritional quality. Genetic manipulation of glycinin was firstly directed towards the Goup-2 glycinin genes (*Gy4* and *Gy5*). The genes of this group were capable of self-assembly into trimers similar to those formed in endoplasmic reticulum (Dickinson et al. 1987). *Gy4* was manipulated to harbor more methionine residues (Sammour 2005). The members of Group-1 glycinin subunits had higher sulfur content than other glycinin subunits. This made this group the best candidate into which to engineer the additional sulfur amino acid residues. Unfortunately, the members of this group did not self-assemble *in vitro*. Dickinson et al. (1989) found that self-assembly of Group-I glycinin subunits could not be evaluated because the concentration of the protein produced from pSP65/27 (the plasmid harboring *Gy2*) was still below the threshold level at which *Gy4* self-assembled into trimers. These results imply that there are structural differences between the two groups of glycinin subunits that affect their ability to assemble into oligomers. However, it was proven that both Group-1 and Group-2 glycinin subunits efficiently assembled with G-2 proteins. The inability of Group-2 subunits to self-assemble, therefore, is unlikely to be due to improper folding of the subunits produced *in vitro*. It was also suggested that Group-1 sunuits (and perhaps other Group-2 subunits) had lost the ability to self-assemble during evolution (Scallon et al. 1985; Walling et al. 1986). In this report, Gy2 (member of Group-1 subunits) was manipulated to be able to self-assemble *in vitro* and harbor more methionine residues.

Firstly, the designated mutant *pSP65/G4SacG2* was constructed to make Gy2 self-assembly (Fig. 1A). In this plasmid, *Sac*1/*Hind*III fragment of *pSP65/MG2H* substituted *Sac*1/*Hind*III fragment of *pSP65/*

248 which includes *MG2H* of *pSP65/MG2H.* However, this plasmid lacked *Sac*1 fragment. Therefore, *pSP65/248* was digested with *Sac*1. *Sac*1 fragment of *Gy4* which was critical for self-assembly. Therefore, *pSP65/248* was digested with *Sac*1, and the G4 *Sac*1 fragment was isolated and then inserted at *Sac*1 site in *pSP65/MG2H.* This strategy was based on the importance of the basic domain for trimer formation (Dickinson 1988) and on an earlier observation that a 21 amino acid deletion in the basic chain of Gy4 proglycinin inhibited self-assembly into trimers (Dickinson et al. 1987).

The concentration of assembly products of *pSP65/G4SacG2* was above the threshold required for self-assembly compared with that of the plasmid which harbors *Gy4* (*pSP65/248*). Therefore, the assembly products of *pSP65/G4SacG2* were efficiently assembled *in vitro* (Fig. 2). In addition, their analysis after self-assembly on SDS-PAGEE showed that the protein assembled was trimers with subunit molecular mass of 66 kDa (Fig. 3) similar to those trimers produced by plasmid *pSP65/248.* To further demonstrate that the 9S complex was indeed trimers, cross-linking experiment were performed. A sucrose gradient was fractionated, and then those fractions that contained the 9S peak were pooled and treated with cross-linking reagent. The cross-linked samples were analyzed on a $NaDodSO_4$/polyacrylamide gel (Fig. 4), which resolved proglycinin monomers (66 kDa), dimmers (130 kDa), and trimers (180 kDa).

Since Gy2 glycinin subunit has a higher sulfur content than the other glycinin subunits, it was considered to be the best candidate into which additional sulfur amino acid residues can be engineered. The main obstacle to do that was that Gy2 subunit was not able to self-assembly *in vitro.* However, the ability of *pSP65/G4SacG2* which harbored *Gy2* for self-assembly, in combination with the successful introduction of three Met residues in the acidic chain of *Gy4* (Sammour 2005) overcome this obstacle and renewed the hope to improve the nutritional quality of glycinin through alter G-1 glycinin genes. The plasmid *pSP65/G4SacG2HG4* was therefore constructed that included both the acidic chain harboring three Met residues and *Sac*1 fragment of Gy4 that is responsible for self-assembly (Fig. 1B).

Assembly products of *pSP65/G4SacG2HG4* were sufficient for self-assembly *in vitro.* The assembly assay results of *pSP65/G4SacG2HG4* showed the distribution of radioactivity in sucrose gradient after self-assembly (Fig. 4). Analysis of the produced proteins in self-assembly of this mutant and plasmid *pSP65/248* on SDS/PAGE showed that the protein assembled was trimers with subunit molecular mass of 66 kDa (Fig. 3) and molecular weight of 180 kDa (Fig. 4).

In conclusion, the cloned cDNAs encoding glycinin subunit Gy2 was modified to be able to self-assemble *in vitro* and to harbor more methionine residues. The ability of self-assembly for the mutants constructed was tested and gave positive results. Transforming these mutants through polyehtyleneglycol, electroporation, microprojectile bombardment, or *Agrobacterium* to soybean is one of the perspectives in our effort to improve the nutritional quality of soybean seed proteins. How-

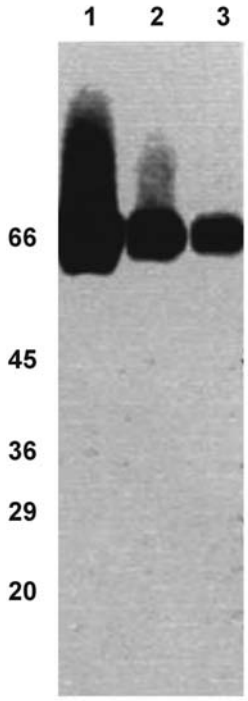

Fig. 3. Fluorogram of SDS/PAGE containing the ^{3}H-labeled products derived from the plasmids *pSP65/248*, *pSP65/G4SacG2* and *pSP65/G4SacG2HG4.* Lane 1: G4 synthesized protein using plasmid *pSP65/248*; lane 2: G4SacG2 synthesized protein using plasmid *pSP65/G4SacG2*; lane 3: G4SacG2HG4 synthesized protein using plasmid *pSP65/G4SacG2HG4.* Molecular weights of protein markers are given in kDa.

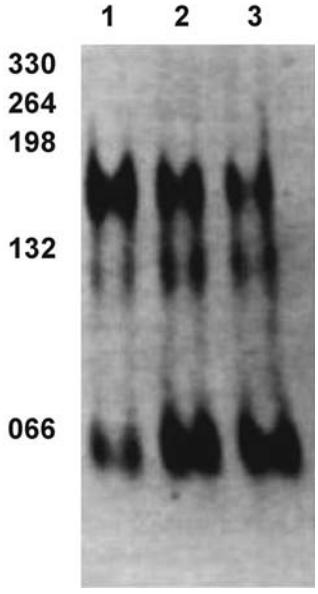

Fig. 4. 9S proglycinins of *pSP65/248*, *pSP65/G4SacG2* and *pSP65/G4SacG2HG4* cross-linked with dithiobis (succinimidyl-propionate) at the concentration 0.16%: Lane 1: *pSP65/248*; lane 2: *pSP65/G4SacG2*; lane 3: *pSP65/G4SacG2HG4.* Protein standards are given in kDa.

ever, the expression of these mutants in a tailor system should have the first priority.

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