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Improvement of the Quality of Soya Bean Seed Storage Proteins by Genetic Engineering cDNA Encoding G2 Subunit

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تحسين نوعية البروتينات المخزنة ببذور فول الصويا من خلال هندسة الـــ"دنا" (المكمل) المشفر لتحت وحدة الجليسنين "جى٢"

رضـــا سمـــور

قسم النبات، كلية العلوم ، جامعة طنطا، مصر

يهـدف هـذا البحـث إلى تحسين نوعية بروتينات بذور فول الصويا، من خلال تغير الـــ"دنا" (المكمل) المشفر لتحت وحدة الجليسنين "جى٢"، مما يجعلها قادرة على التجمع في المعمل. لإحداث ذلك، استخدمت تقنيات البيولوجيا الجزيئية التالية: الترجمة والنسخ في المعمل، النسخ في المعمل، النسخ والتجميع الذاتي في المعمـــل، الـــتفريد المتدرج على وسائد من السكر، تحديد تتابعات الـــ"دنا"، الترسيب بحامض الخليك ثلاثي الكلور، التفريد الكهـــربائي. تـــم بنـــاء بـــلازميدين؛ سمى أحدهما "pSP65/G4SacG2"، و سمى الأخر "pSP65/G4SacG2HG4". و قـــد احتوى كل بلازميد من البلازميدين على الشظية "Sac" (الشظية التي تحـــتوي على الأحد والعشرين حامض أميني الأول من السلسلة القاعدية) من الجين "جى واى ٤"، المسئولة عن التجمع الذاتي إلى بروتين ثلاثي البناء الفضائي. كذلك، يشمل البلازميد ""pSP65/G4SacG2HG4"، إضـــافة إلى الشظية "Sac" من الجين "جى واى ٤"، السلسلة الحامضية من الجين "جى واى ٤" التي تتحمل أيـــلاج ثلاث جزيئات ميثونين. لهذا، فهذا البلازميد لديه القدرة على التجمع في المعمل، كما أن لديه ـــ في نفـــس الوقت ـــ القدرة على إيواء عدد من جزيئات الميثونين. تم اختبار قدرة هذه البلازميدات على التجمع في تجمعات صغيرة مشابهة لتلك التي توجد في البذور.

***Key Words**: *Cloned cDNA; Glycinin subunit G2; In vitro transcription; In vitro translation; Mutagenesis; SDS-polyacrylamide gel*

ABSTRACT

The goal of this work is to improve nutritional quality of soybean seed proteins by altering cDNA-encoding G2 subunit of Soybean glycinin to be capable of self-assembly *in vitro.* Two plasmids were constructed; one designated pSP65/G4*Sac*G2 and the other pSP65/G4*Sac*G2HG4. The observation that basic chain did not tolerate modification and that a 21 amino acids deletion in the basic chain of G4 proglycinin inhibited self-assembly into trimers [1], make the introduction of *Sac* fragment of GY4 to GY2 is a possible key factor in getting G2 self-assemble. pSP65/G4*Sac*G2HG4 construct includes, in addition to G4 *Sac* fragment, the acidic chain of GY4 which was proved to tolerate an introduction of three Met residues. This construct, therefore, had the ability to self-assemble *in vitro* and in the mean time the ability to harbor a number of Met residues. The ability of these constructs to assemble into oligomer similar to those that occur in the seed was tested.

Introduction

Glycinins are the predominant storage proteins in soybean seeds. They account for more than 20% of the seed dry weight in some cultivars, have no known catalytic activity, and are thought to function as a reserve for carbon and nitrogen to be used upon seed germination [2]. Glycinin proteins are produced primarily in cotyledons where they are sequestered within subcellular organelles called protein bodies. As isolated from seed extracts, the glycinins are an oliogomer of six similar subunits [3]. The properties of these subunits have been reviewed extensively [3 – 5], and five major subunits have been identified on the basis of differences in their primary structures [6]. Each glycinin subunit is composed of two disulphide-linked polypeptides. One polypeptide has an acidic isoelectric point, and the other is basic. The two-polypeptide chains result from post-translation cleavage of proglycinin precursors [7], a step that occurs after the precursor enters the protein bodies [8].

Nielsen *et al.,* [2] 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 have diverged into two subfamilies that are designated as Group-I (G-I) and Group-II (G-II) glycinin genes [5]. The genes in G-I (GY1, GY2, and GY3) have nucleotide sequences that are more than 80% homologous to one another [2]. The nucleotide sequences for members of G-II, which includes GY4, GY5, are likewise more than 80% identical with one another, but are less than 60% homologous with those in Group-I [9].

Dickinson *et al.* [10] developed an *in vitro* system that results in the self-assembly of subunit precursors resembling to those found *in vivo* in the endoplasmic reticulum. This system shows that G-II glycinin subunits (GY4, GY5) are capable of self-assembly into trimers similar to those formed in

endoplasmic reticulum. However, it was found that the G-I subunits were unable to assemble in the absence of G-II subunits.

G-I is initially considered to be the best candidate into which to engineer additional sulfur amino acid residues can be engineered, because it has higher sulfur content than the other glycinin subunits. However, the observation that G-I does 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, is to adopt a better strategy to improve nutritional quality of soybean seed proteins through altered G-1 subunits, to be capable of self-assembly *in vitro.*

Materials and Methodes

Materials

Plasmids pSP65/248 and pMP18/MG2H served as the first step in the construction of the plasmids pSP65/G4*Sac*G2 and pSP65/G4*Sac*G2HG4. The isolation of the plasmid carrying GY2 cDNA (pG27) was described in Scallon *et al.* [11]. The vectors pSP65 and pMp18 [12] were purchased from Promega Biotec (Madison, WI).

Methods

Plasmid Construction

The two plasmids were derived from pSP65/248, which originated from a fusion between pSP65 and pG248 [10, 13]. Plasmid pSP65/G4*Sac*G2 was made by separately digestion of pSP65/MG2H and pSP65/248 at *Sac*I and *Hind*III sites in the polylinker (Fig. 1A). The 0.98 Kb (Kilobase) polylinker *Sac*I / *Hind*III fragment of pSP65/248 substituted *Sac*I / *Hind*III fragment which includes MG2H of pSP65/MG2H to form pSP65/MG2H. However this plasmid lakes *Sac*I fragment. Therefore, pSP65/248 was digested with *Sac*I, and the 0.16 Kb *Sac*I fragment was isolated, and then inserted at *Sac*I site in pSP65/MG2H. The plasmid obtained is denoted pSP65/G4*Sac*G2 (Fig. 1A).

Plasmid pSP65/G4*Sac*G2HG4 was constructed by separately digestion of pMP18/MG2H and pSP65/248 with *BamH*I and *Hind*III. However, a partial digestion was carried with *Hind*III. The 0.5 Kb *BamH*I / *Hind*III fragment of pMP18/MG2H was substituted for the corresponding fragment from cDNA clone pSP65/248 to form the plasmid pSP65/G2HG4 (Fig. 1B). The plasmide denoted pSP65/G2HG4 and the plasmid pMP18/G2*Sac* were separately digested with *Sam*I. The 0.31 Kb *Sam*I fragment of pMP18/G2*Sac* was trade with *Sam*I fragment of pSP65/G2HG4 to construct the plasmide pSP65/G2*Sac*G2HG4. Both pSP65/G2*Sac*G2HG4 and pSP65/248 were separately digested *Sac*I and

*Xho*I. The 0.72 Kb *Sac*I/*Xho*I fragment of pSP65/G2*Sac*G2HG4 was ligated in the same sites of pSP65/248 to form pSP65/G2HG4. pSP65/G2HG4 and pSP65/248 were separately digested with *Sac*I and then the 0.16 Kb *Sac*I fragment of pSP65/248 was inserted in *Sac*I site of pSP65/G2HG4 to form the plasmid pSP65/G4*Sac*G2HG4.

DNA Sequence Analysis

Nucleotide sequence analysis was carried out by the chemical method of Maxam and Gilbert [14]. Synthetic oligonucleotides **5'GCGAGACAAGAAACGGGGTTGAGG** and **5'GAGAACATTGCTC GCCCTTCGCGC** were used as primers for sequencing across the GY4 regions.

In Vitro Transcription

In vitro transcription with SP6 RNA polymerase followed by translation with rabbit reticulocyte lysate was performed as described by Dickinson *et al.*[1].

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.* [12], except that the DNA concentration was raised to 0.2μg/μl. GTP was reduced to 20 μM, and m7GpppG (Pharmacia) was included at 500 μM. After 90 min at 40 °C, the GTP concentration was raised to 500 μM and the incubation was continued for 30 min at 40 °C. The RNA was precipitated by the sequential addition of Sodium acetate (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 2mM and phenylmethyl-sulfonylfluoride was added to 250 μM. The ^{3}H-labeled proteins were incubated at 25^{O}C for 30 hours and placed on ice.

Sucrose Gradient Fractionation

Assembly products were assayed by layering 100 μl samples onto 11 ml linear 7-25% sucrose density gradient as described elsewhere [15]. Immunoprecitations were performed according to Turner *et al.* [8].

TCA Precipitations

The sample (100 µl) was mixed with a 25 ml of 1.5 NaOH, 2-25% hydrogen peroxide and incubated at 37 ^{O}C for 10 min. Then 1.5 ml of 25% trichloroacetic acid and 2% Casamino acids were added and mixed. 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 dried and counted in 10 ml of ACS scintillation fluid (Amersham).

SDS/PAGE

SDS/polyacrylamide gel electrophoresis was performed in 12% gels [16]. Samples were diluted 10 fold with sample buffer (0.3 M Tris-HCl, pH 6.8 / 2% 2-mercaptoethanol / 10% glycerol) and boiled for 2 minutes before loading. Gels were stained with coomassie blue to visualize molecular weight standards, En3Hance (New England Nuclear) treated, dried, and subjected to fluorography.

Results and Discussion

The construction of expression plasmid (pSP65/27), and its restriction map are reported elsewhere [10]. It was reported that G-II subunits were able to self-assemble into trimers, but G-I subunits were unable to assemble in the absence of G-II subunits [13]. In addition, it was found that self-assembly of G-I could not be evaluated because the highest concentrations produced from pSP65/27 were still below the threshold level at which GY4 self-assembles into trimers [1]. 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 proved that both G-I and G-II efficiently assemble with G-II proteins. The inability of G-II to self-assemble, therefore, is unlikely to be due to improper folding of the subunits produced *in vitro*. It was also suggested that G-II (and perhaps other group-1 subunits) has lost the ability to self-assemble during evolution. Despite this change, however, the G-I glycinins have remained functional because all members of the glycinin gene family are coordinately expressed [11, 17] and the G-II subunits promote the assembly of G-I subunits into oligomers. The importance of the basic domain for trimer formation is also supported by an earlier observation that a 21 amino acid deletion in the basic chain of GY4 proglycinin inhibited self-assembly into trimers [10]. Therefore, to make GY2 self-assemble a plasmid that contains *Sac*1 fragment (which includes N-terminal of the basic chain of G4) was constructed, and designated pSP65/G4*Sac*G2.

A system has been described that enables the assembly of proglycinin synthesized *in vitro* to be studied [13]. The main advantage of the system is its speed and the ability to use recombinant DNA techniques to probe for region of proglycinin important for self-assembly of trimers. It remains to be

shown, however, that the *in vitro* assembly described here accurately reflects the *in vivo* process. A similar test may be possible *in vivo* with transformed tobacco plants; however, such experiments would consume considerably more time. In addition, the tobacco seed itself has equivalent legumin-like proteins that would complicate interpretations. Assembly assay results of Fig. 2B show the distribution of radioactivity in sucrose gradient after self-assembly of oligomers using pSP65/G4*Sac*G2. The assembled products of pSP65/G4*Sac*G2 were capable of self-assembly. In addition, their analysis after self-assembly on SDS/PAGE showed assembled trimers with molecular weight 66 Kilo Dalton, similar to the trimers produced by plasmid pSP65/27 (Fig. 3). Moreover, the assembly results of pSP65/G4*Sac*G2 expressed a large quantity of proglycinin. The accumulation of large quantities of proglycinin is crucial for adoption of protein engineering to improve quality of soybean proteins and to adopt an assembly system.

Since G-II has higher sulfur content than the other glycinin subunits, it is consider to be the best candidate into which additional sulfur amino acid residues can be engineered. The main obstacle to do that was that G-II did not self-assemble *in vitro*. However, the plasmid pSP65/G4*Sac*G2 that harbor GY2 overcome this obstacle. The successful introduce three Met amino acid residues in the acidic domain of GY4 (unpublished data, R. Sammour) made the author to exchange the acidic chain of GY4.

Assembly assay results of Fig. 2 shows the distribution of radioactivity in sucrose gradient after self-assembly of oligomers using the plasmid pSP65/G4*Sac*G2HG4. This plasmid was found to be capable of self-assembly. Analysis of the produced proteins in self-assembly of this plasmid and plasmid pSP65/27 on SDS/PAGE showed that the protein assembled is trimers with molecular weight 66 Kilo Dalton (Fig. 3).

Conclusion

In conclusion, cloned cDNAs encoding glycinin subunit GY2 was modified. The modification was carried out to get G2 self-assembled *in vitro*. Two plasmid contain *Sac*I ,and *Sac*I and acidic fragments of GY4 have been constructed. These plasmids named pSP65/G4*Sac*G2 and pSP65/G4*Sac*G2HG4. Their ability to assembly into oligomer similar to those, which occur in the seed, was confirmed. In addition, electrophoretic separation of the proteins synthesized *in vitro* showed a subunit molecular weight similar to that produced *in vivo* in the endoplasmic reticulum.

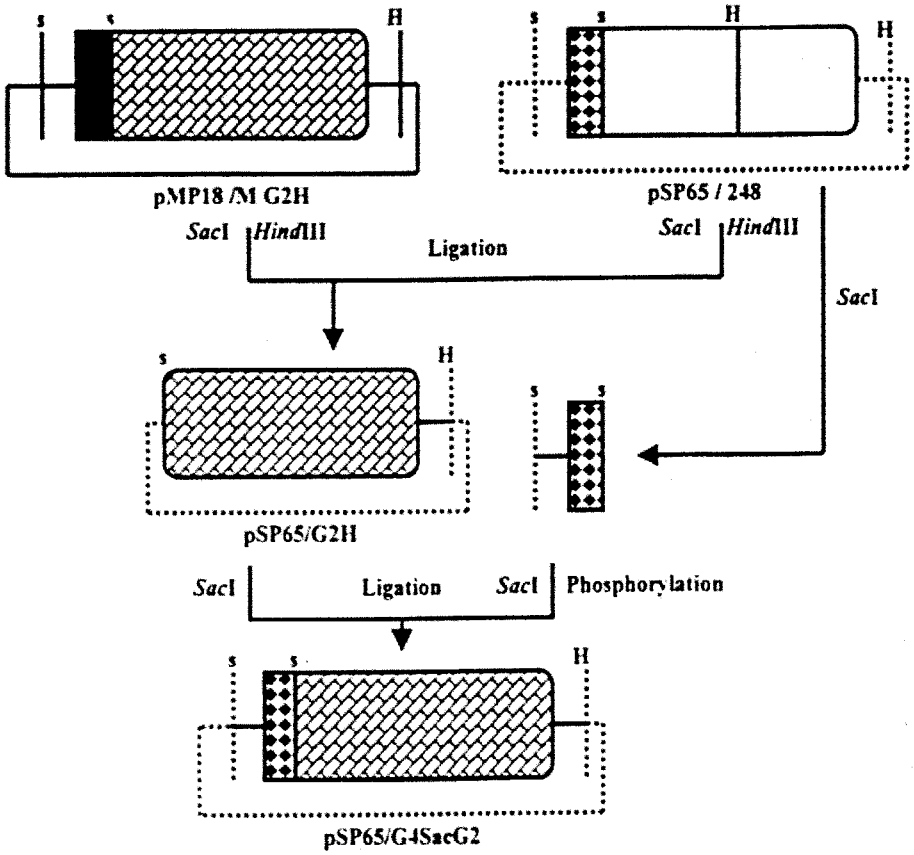

Fig. 1 A. Construction of plasmids pSP65/G4SacG2

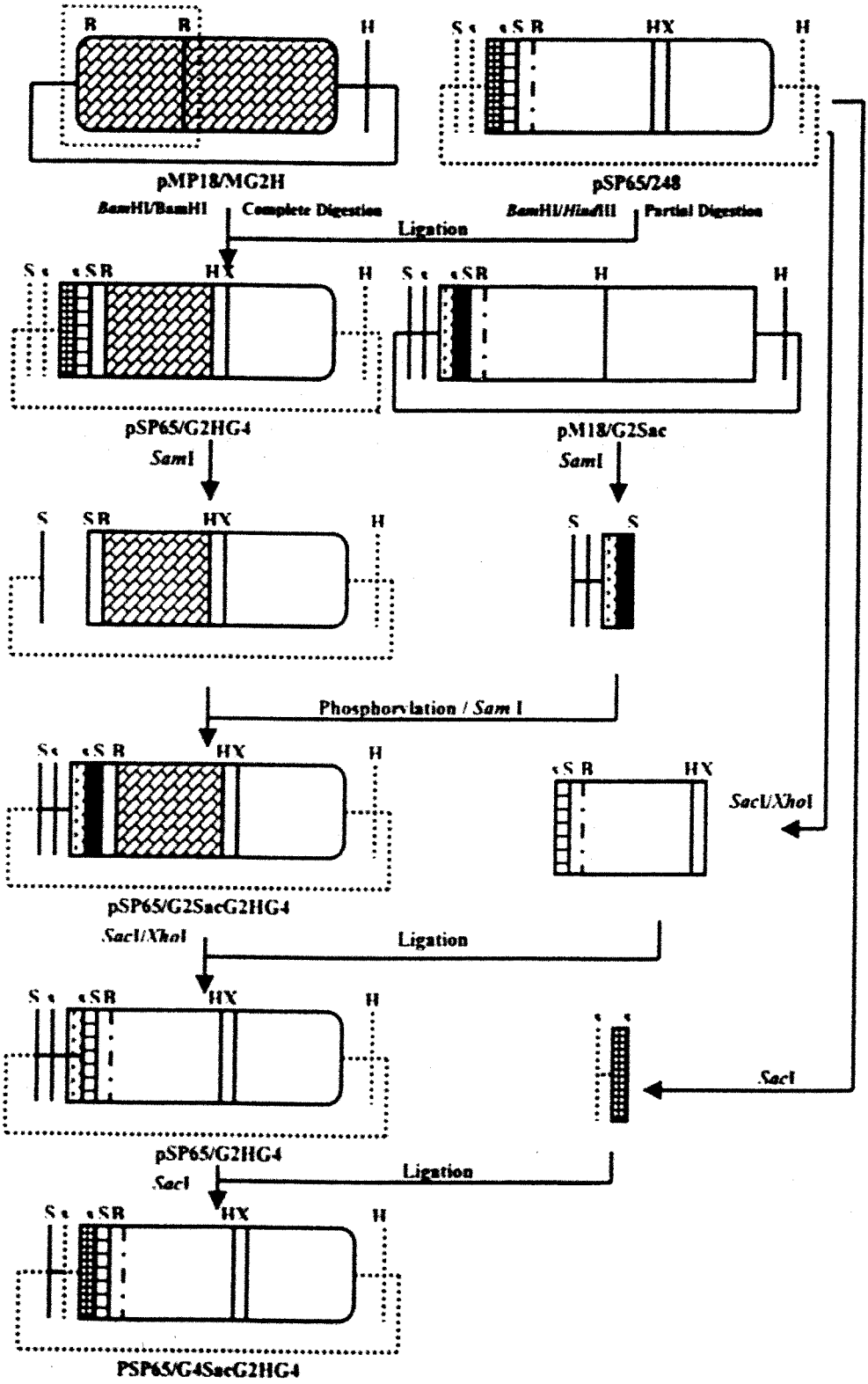

Fig. 1 B. Construction of plasmids pSP65/G4SacG2HG4

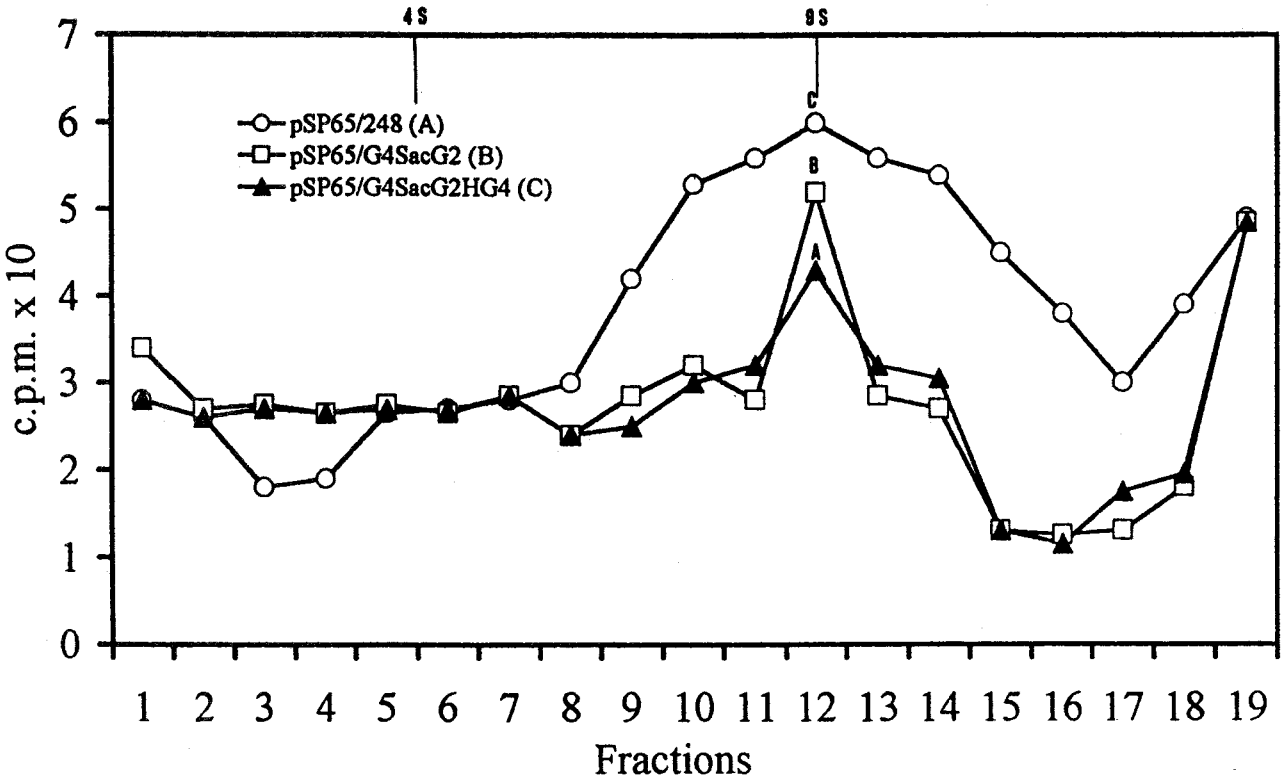

Fig. 2. Self-assembly of ^{3}H-Leu labeled proglycinins produced from pSP65/27, pSP65/G4*Sac*G2, pSP65/G4*Sac*G2HG4. The constructs pSP65/27, pSP65/G4*Sac*G2, pSP65/G4*Sac*G2HG4.The position of sedimentation standards is shown at the top of the figure.

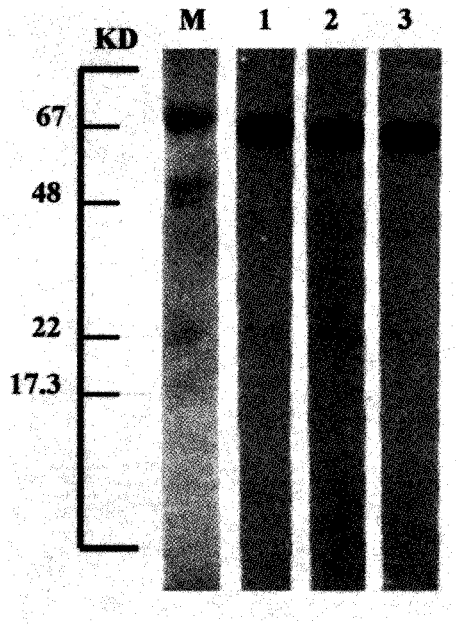

Fig. 3. *In vitro* synthesis of modified proglycinin. Products of *in vitro* transcription and translation of the constructions pSP65/27, pSP65/G4*Sac*G2 and pSP65/G4*Sac*G2HG4 were separated on a 12.5% SDS polyacrylamid gel and visualized by fluorography. Molecular weight standards are given in KD. Lane 1 shows protein markers; Lane 2 shows the translation products derived from plasmid pSP65/27; Lane 3 shows the translation products derived from plasmid pSP65/G4*Sac*G2; Lane 4 shows the translation products derived from plasmid pSP65/G4*Sac*G2HG4.

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