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Cloning and Expression of Methionine-enriched Glycinin GY4-Genes in *E. Coli*

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إنسال وتعبير جينات الجليسين (GY4) المخصبة بالمثونين في بكتريا القولون

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أجري عدد من الطفرات في الحامض النووي الناسخ لإحدى تحت وحدات الجليسين، لزيادة محتواها من المثونين (حامض أميني)، وقد قيمت هذه الطفرات في المعمل قبل نقلها إلى نبات فول الصويا باستخدام نظام الطبع والنسخ. ومن الجدير بالذكر، أن هذه الطفرات قد أجريت في الجزء الحامضي من الجين، الذي ثبت معملياً نجاح التحورات النيكليوتيدية - أياً كانت - فيه، وتحتوي كل طفرة من الطفرات الثلاثة، التي خلقت معملياً في هذه الدراسة، ما بين واحد وثلاث جزيئات من الميثونين، وقد سميت هذه الطفرات pSP65/248 Met1,2,3 , pSP65/248 Met2,3 ,pSP65/248,Met1,pSP65/248. وفي نهاية الدراسة، قيمت قدرة هذه الطفرات على التجمع في صورة مماثلة لتلك التي تحدث في البذور.

Key Words : *Clond cDNA; Glycinin subunit GY4; In vitro transcription; In vitro translation; Mutagenesis; SDS-polyacrylamide gel electrophoresis.*

ABSTRACT

Cloned cDNAs encoding glycinin subunit GY4 was modified to increase methionine content. The effect of the modification was evaluated using an *in vitro* transcription / translation system. The modifications were carried out in the acidic region; the region which tolerates conservative point mutations. Three bacterial mutants that contain increased amounts of methionine were constructed. These mutants named pSP65/248 Met1, pSP65/248 Met2,3 and pSP65/248 Met1,2,3. Their ability to assemble into oligomer similar to those that occur in the seed, were tested. By putting these modified genes into soybean lines that lack certain glycinin subunits considerable improvements in seed quality can be made.

Introduction

Glycinins are the predominant storage proteins in soybean seeds. They account for more than 20% of the seed dry weight in some cultivars, being produced primarily in cotyledon cells where they are sequestered within subcellular organelles called protein bodies [1]. As isolated from seed extracts, the glycinins are an oliogomer of six simillar subunits [2]. The properties of these subunits have been reviewed extensively [3 - 8].

Glycinin subunits accumulate rapidly during embryogenesis and this accumulation is associated with dramatic changes in the prevalence of glycinin mRNAs [1]. Glycinins mRNAs begin to accumulate early in embryogenisis, are highly prevalent during the midmaturation stage and they decay prior to seed dormancy [9-11]. The accumulation stage and decay of these mRNAs is regulated in part by transcriptional processes similar to those that regulate other seed proteins mRNAs [9 , 12].

Nielsen *et al.* [1] characterized the structure, organization, and expression of genes that encode the soybean glycinins. Five genes encode the predominant glycinin subunits found in soybeans. The five genes have diverged into two subfamilies that are designated as Group-I and Group-II glycinin genes. The genes in Group-I include Gy1, Gy2, and Gy3, while genes in Group-II include GY4, Gy5 [1, 12].

Dickinson *et al.* [13] developed an *in vitro* system that results in the self assembly of subunit precursors into that resemble those found naturally in the endoplasmic reticulum. The assays have also been used to show that post-translational cleavage of Group-II proglycinin subunits in those trimers is required before they are assembled into hexamers similar to those isolated from the proteins bodies of mature seeds.

Because of the high concentration of the glycinin, its major contribution to the nutritional properties of soybean proteins, and its effect on the functional properties of food products made from beans has been targeted for genetic manipulation to improve soybean nutritional quality. This work, therefore, was carried out to improve the nutritional value of glycinin proteins by using genetic engineering. Three bacterial mutants contain an increased amount of methionine (one to three residues) were constructed. The mutants were tested for *in vitro* assembly prior to introducing them into soybean varieties.

Materials and Methods

Mutagenesis Procedure

The mutagenesis was carried out according to the method of Marotti & Tomich [14].

DNA Sequence Analysis

Nucleotide sequence analysis was carried out by the chemical method of Maxam & Gilbert [15]. Synthetic ligonucleotides 5'GCGAGACAAGAAACGGGGTTGAGG and 5'GAGAACATTGCTCGCCCTTCGCGC were used as primers for sequencing across the GY4 regions.

Plasmid Construction.

All plasmids were derived from pSP65/248 which originated from a fusion between pSP65 and pG248 (Dickinson [16] and Dickinson *et al.*, [13]). 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/GY4 Met1 (Fig. 1. Lanes 1,2 &7 and Fig. 3A).

Plasmid pSP65/248 Met2,3 was constructed by deleting the sequence between *Hind* III site in pSP65/248. This deletion was filled with the sequence between the *Hind* III in pMP18/GY4Met2,3 and *Hind* III in the polylinker of pMP18 (Fig. 1. Lanes 3,4 &8 and Fig. 3B).

Pasmid pSP65/248 Met1,2,3 was made by deleting the sequence between the *Hind* III site in pSP65/248 Met1 and *Hind* III in pSP65 polylinker. This deleted sequence was filled with the sequence between the *Hind* III in pMP18/GY4 Met 2,3 and *Hind* III in the pSP65 polylinker (Fig. 1. Lanes 5,6 &9 and Fig. 3C).

In Vitro Synthesis and Assembly

In vitro transcription with SP6 RNA polmerase followed by translation with rabbit reticulocyte lysate were accomplished as described by Dickinson *et al.* [13]. Self-assembly of proglycinin of the three bacterial mutants constructed in this study were done as reported by Dickinson [16].

Sucrose Gradient Fractionation.

Assembly was assayed by layering 100 ul samples of the in vitro synthesis reaction onto 11 ml linear 7-25 % sucrose density gradient as described by Dickinson *et al.* [16].

Trichloroacetic (TCA) Precipitations

TAC was carried out according to the method reported by Dickinson et al. [17]. In this method the samples of assembly (100 ul each) of each mutant were mixed with a 25 ml of 1.5 NaOH, 2-25% 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).

SDS- Polyacrylamide Gel Electrophoresis

SDS- polyacrylamide gel electrophoresis was performed in 12% gels [18]. 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%-mercaptoethanol / 2.5M urea / 10% glycerol), boiled for 2 min before loading and then electrophoretically separated. After electrophoresis the gel was stained with coomassie blue, and when appropriate, treated with EN3HANCE (DU Pont-New England Nuclear), and dried, and the proteins visualized fluorographically.

Results and Discussion

The expression plasmid pSP65/248 of GY4 was the starting point in this study. Although GY4 is not rich in methionine, it was the choic for this study because of its assembly in vitro and its expression in large quantities. On the other hand, although GY2 has a higher methionine than the other glycinin subunits, it is not the best candidate for this purpose because it does not self-assemble efficiently in proteins bodies [16].

The construction of expression plasmid, and its restriction map were reported elsewhere [13]. The construction of the expression plasmide of GY4 (pSP65/248) was based on the stepwise deletion of the cDNA

region encoding the signal sequence and N-terminal region of the preproglycinin. An *in vitro* system as well was developed that results in the self assembly of subunits precursors into that resembles those found naturally in the endoplasmic reticulum [13]. Subunits of glycinin, the predominant seed protein soybeans, were synthesized from modified cDNAs using a combination of the SP6 transcription and rabbit reticulocyte translation system.

It was reported that the acidic chain of GY4 can tolerate deletion and inseration [17]. Therefore, this encoding chain was chosen to generate a number of mutants named pSP65/248Met1, pSP65/248 Met1,2 and pSP65/248 Met1,2,3. It is worthwhile to mention that the basic chain of GY4 can not tolerate any modification. The deletion of a 21 N-terminal amino acids in the basic chain of GY4 proglycinin (the codes for the first 21 amino acid residues) inhibits the assembly into trimers [17]. This indicates very clearly the importance of the basic chain for proglycinin assembly *in vitro*.

The procedures described in " Materials and Methods" were used to generate a number of mutants in GY4 encode one of glycinin subunits. The design of the mutants was described in " Methods section". A mutation in GY4 with methionine (ATG) replacing the lysine (AAG) was accomplished by changing a single nucleotide at position 1163 (Fig. 2). A mutagenic oligonucleotide of 18 bases in length (#1 in Table 1) was used to this change, and the template used was plasmid pMPB. The mutagenized plasmid was transformed into *E. Coli* 1190, and the transformants were screened with the mutagenesis oligonucleotide GCACCTTAGGCTTCACG using colony hybridization. The filters 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 idenity of the mutant was confirmed by dideoxy sequenecing of the double stranded plasmid. The frequency of mutagenesis was about 2%.

To generate a mutation in GY4 with methionine (ATG) replacing isoleucine (ATT) at position 1311 (Fig. 2), a mutagenesis oligonucleotide of 18 bases in length (#2 in Table 1) was used with plasmid pMPB as template. Oligonucleotide has a calculated Tm of 53°C to the mutant sequence. Following mutagenesis, the plasmid was transformed into *E. Coli* 1190 and the mutation was screened for by colony hybridization using the mutagenic oligonucleotide AATGGAATGTACTCTCC as a probe. The filters were washed in 5 X SSC and 0.1 % SDS at 51°C and transformants with the mutation could be detected.

To generate a mutation in GY4 with methionine (ATG) replacing the valine (GTG) at position 1353 (Fig. 2), a mutagenesis oligonucleotide of 24 bases in length (#3 in Table 1) was used with plasmid pMPB as template. Oligonucleotide has a calculated Tm of 51°C to the mutant sequence. After mutagenesis, the plasmid was transformed into *E. Coli* 1190 and the mutation was screened by colony hybridization using the mutagenic oligonucleotide AGTGTGATCTATATGACTCGAGG as a probe. The filters were washed in 5 X SSC and 0.1% SDS at 51 0C and transformants with the mutation could be detected.

A system that enables the assembly of proglycinin synthesized *in vitro* has been described [13]. The main advantage of the system is its speed and the ability to use recombinant DNA techniques to probe for chain 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. Assembly assay results (Fig. 5 A-D) show the distribution of radioactivity in sucrose gradient after self assembly of oligomers using the various mutants. Each of the described mutants was found to be capable of self assembly. Analysis of the produced H^3-Leu proteins in self assembly of each mutant SDS/PAGE showed that the assembled protein had molecular weight of 66 KiloDalton (Fig. 4). It was reported that self-assembly of pSP65/248 gave a 9S trimer peak with molecular weight 180 KiloDalton and subunit molecular weight 66 KiloDalton [13]. In analogy with the plasmide pSP65/248, the assembled proteins of the bacterial mutants constructed in this study are a trimers with subunit molecular weight of 66 KiloDalton.

There are three structural differences between the native glycinin and GY4 proglycinin: 1, hetero or homo, 2, presence or absence of the processed junction, 3, a hexamer or a trimer.

Conclusion

In conclusion, cloned cDNAs encoding glycinin subunit GY4 was modified using single base directed mutagenesis. The modifications were carried out in the acidic chain (the chain which tolerate conservative point mutations). Three mutants contain increased amounts of methionine have been constructed. These mutants named pSP65/248 Met1, pSP65/248 Met2,3 and pSP65/248 Met1,2,3. Their ability to assemble into oligomers 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.

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Table 1

# 1	GCACCTTGAAGCTTCACG
# 2	GAATGGAATTTACTCTCC
# 3	CAGTGTGATCTATGTGACTCGAGG

The sequence is from 5' to 3'. Underlined bases represent changes.

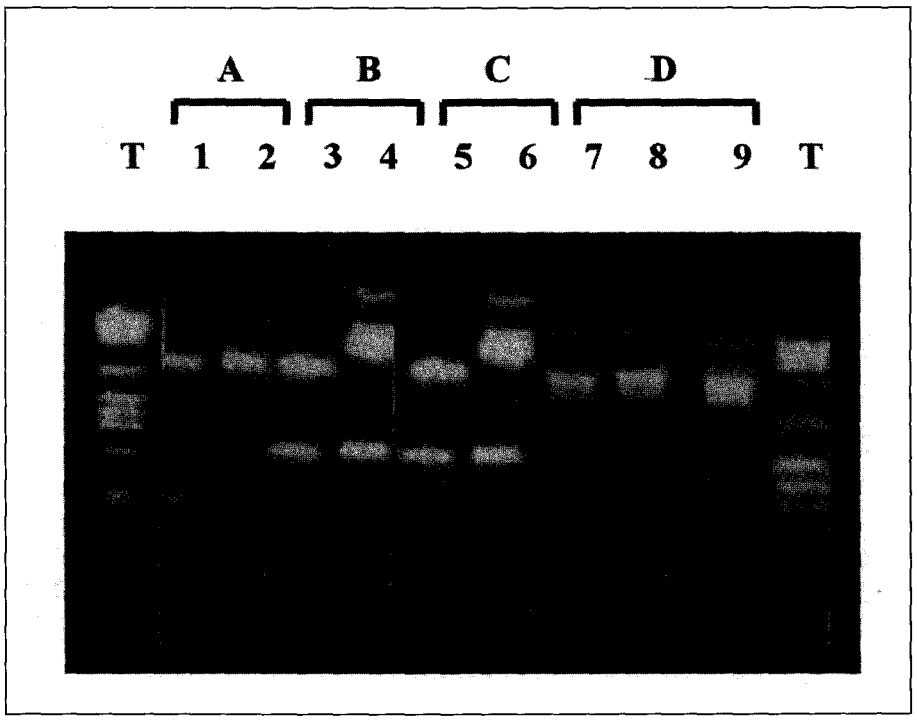

Fig. 1

Fig. 1A. Enzymatic digestion of pSp65/248 and pMp18/G4 Met1 with restication enzymes Acc1 and Xho1. T: DNA ; Lane 1: pSp65/248; Lane 2: , pMp18/G4 Met1.

Fig. 1B. Enzymatic digestion of pSp65/248 and pMp18/G4 Met2,3 with restication enzyme Hind 3. Lane 3: pSp65/248; Lane 4: , pMp18/G4 Met2,3.

Fig. 1C. Enzymatic digestion of pSp65/248 Met1 and pMp18/G4 Met2,3 with restication enzymes Hind 3. Lane 5: pSp65/248 Met1; Lane 6: , pMp18/G4 Met2,3.

Fig. 1D. Ligation of the vector and insertion of the three bacterial mutants; the vectors and insertion were isolated from DNA gels in Fig. 1 A- C. Lane 7: pSp65/248 Met1; Lane 8: pSp65/248 Met2,3; Lane 8: pSp65/248 Met1,2,3.

Fig 2

			1163	T	(Met1)							
385	Cys	Thr	Leu	Lys	Leu	His	Glu	Asn	Ile	Ala	Arg	Pro
+ 1153	TGC	ACC	TTG	AAG	CTT	CAC	GAG	AAC	ATT	GCT	CGC	CCT
	Ser	Arg	Ala	Asp	Phe	Tye	Asn	Pro	Lys	Ala	Gly	Arg
	TCA	CGC	GCT	GAC	TTC	TAC	AAC	CCT	AAA	GCT	CGT	CGC
	Ile	Ser	Thr	Leu	Asn	Ser	Leu	The	Leu	Pro	Ala	Leu
	ATT	ACT	ACC	CTC	AAC	AGC	CTC	ACC	CTC	CCA	GCC	CTC
	Arg	Gln	Phe	Gln	Leu	Ser	Ala	Gln	Tye	Val	Val	Leu
	CGC	CAA	TTC	CAA	CTC	AGT	GCC	CAA	TAT	GTT	GTC	CTC
				1311	G	(Met 2)						
	Tyr	Lys	Asn	Gly	Ile	Tyr	Ser	Pro	His	Trp	Asn	Leu
	TAC	AAG	AAT	GGA	ATT	TAC	TCT	CCA	CAT	TGG	AAT	CTG
				1353	A	(Met 3)						
	Asn	Ala	Asn	Ser	Val	Ile	Tyr	Val	Thr	Arg	Gly	Gln
	AAT	GCA	AAC	AGT	GTG	ATC	TAT	GTG	ACT	CGA	GGA	CAA
	Gly	Lys	Val	459								
	GGA	AAG	GTT	-1377								

Fig. 2. Nucleotide and amino acid sequence comparisons of GY4 genes and proteins. only nucleotides +1153 to -1377 and translated amino acids 385 to 459 are shown. Arrows refer to the nucleotides that have been mutuated in the GY4.

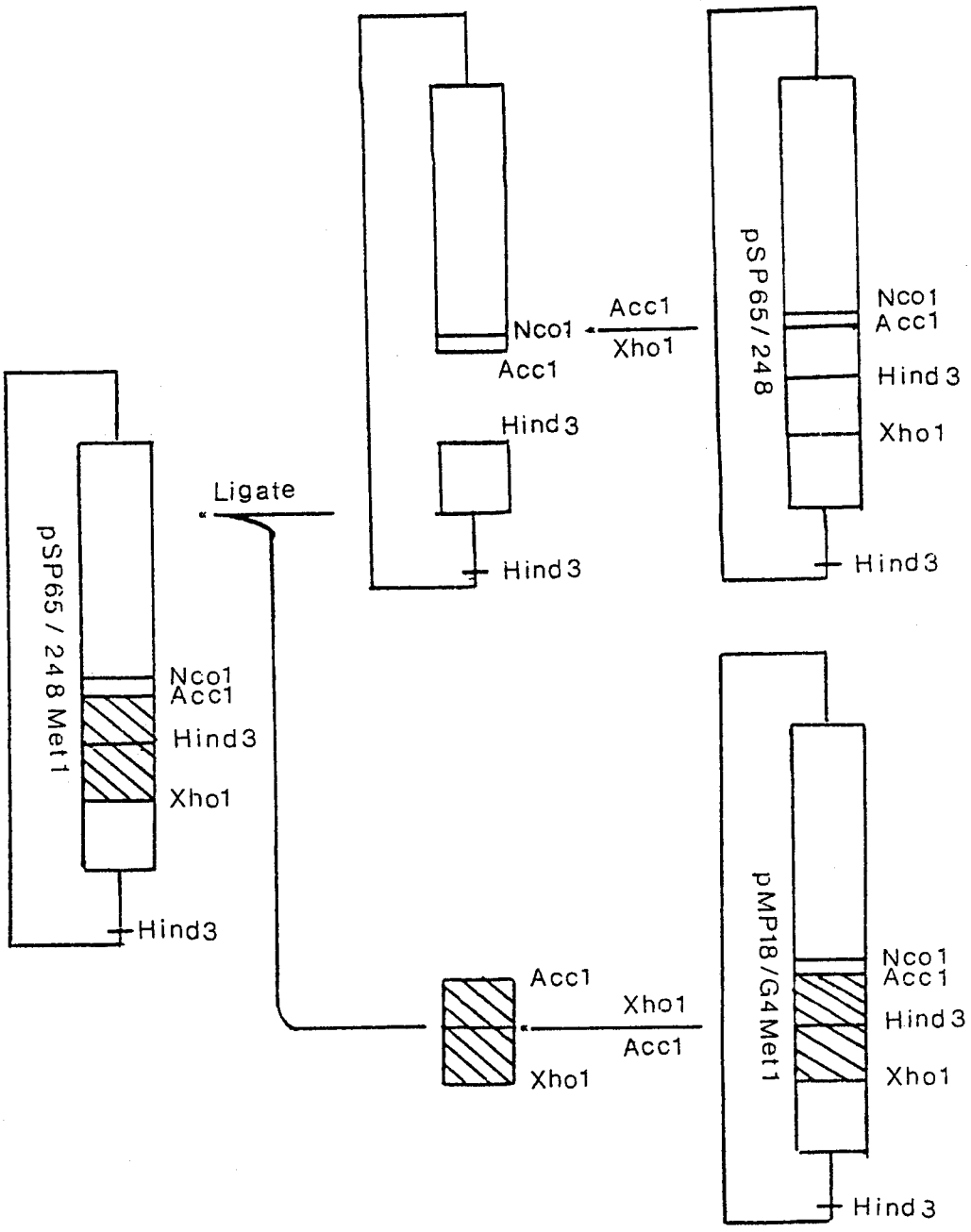

Fig. 3. Construction maps of pSp65/248 Met1 (A).

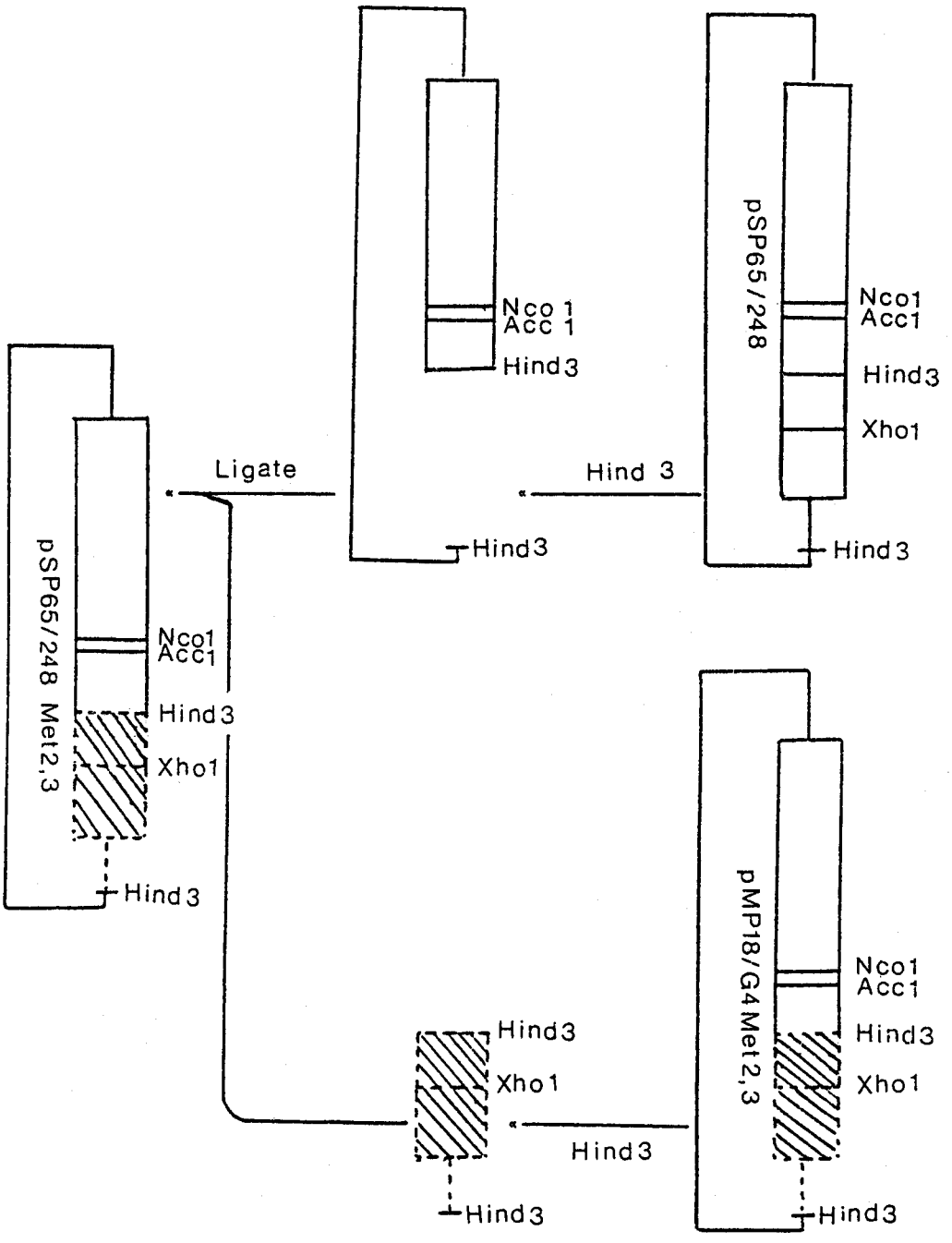

Fig. 3 : Construction maps of pSp65/248 Met2,3, (B).

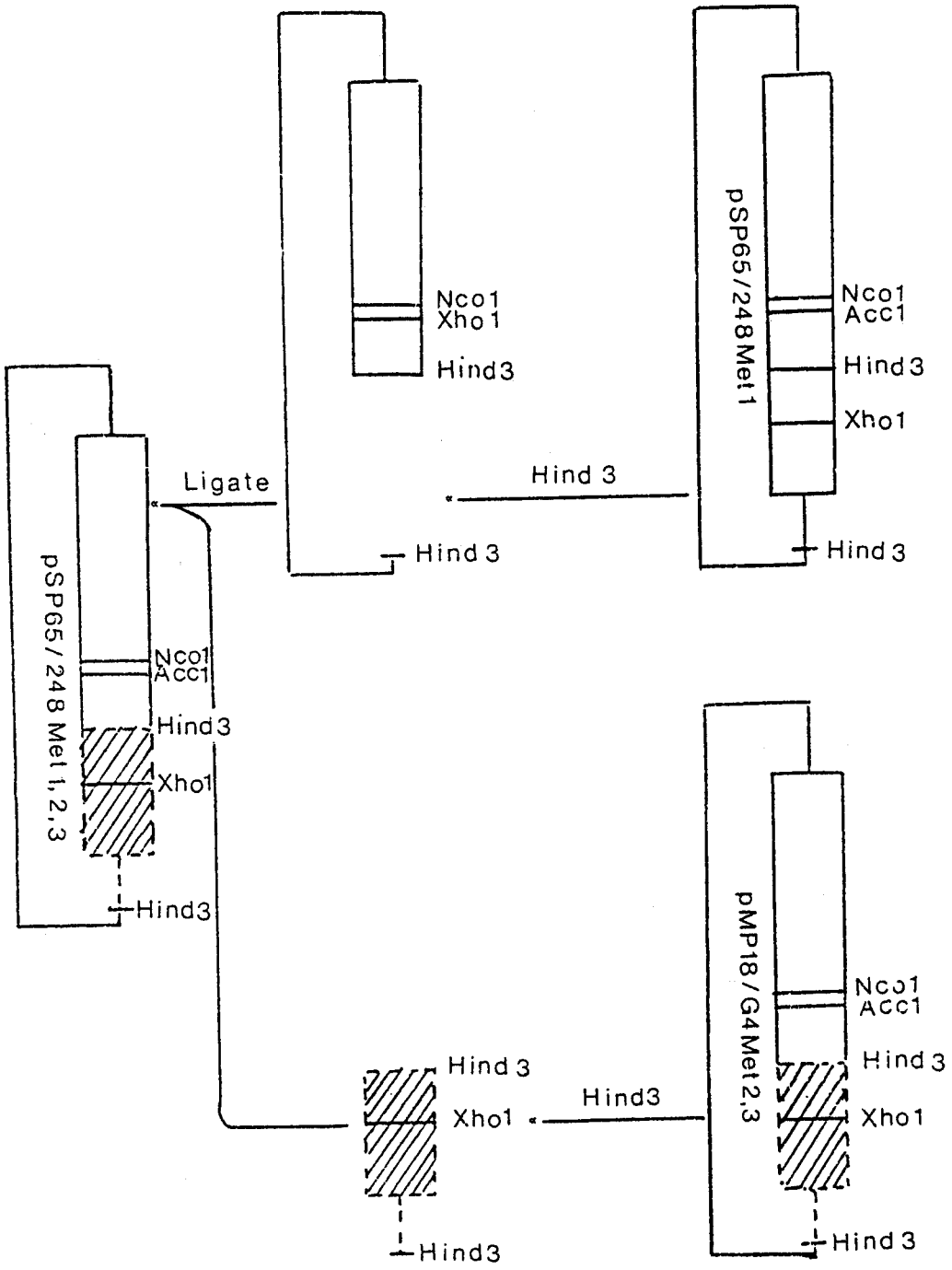

Fig. 3. Construction maps of pSp65/248 Met1,2,3 (C).

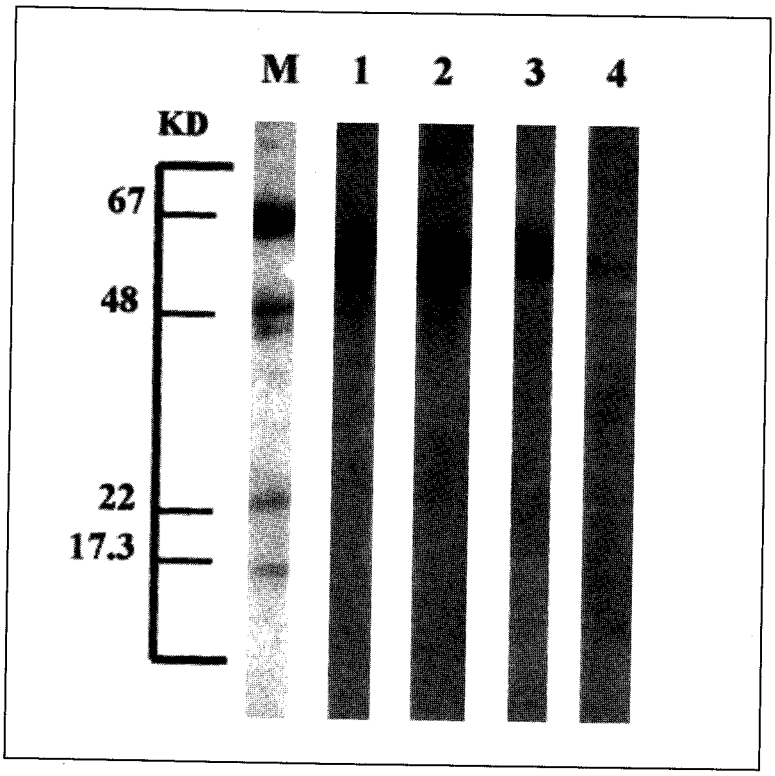

Fig. 4. Electrophoretic separation of G4, Mutant Met1, Mutant Met2,3 and Mutant Met1,2,3 synthesized *in vitro*.

M: protein markers;

Lane 1 : G4 synthesized protein using plasmid pSP65/248;

Lane 2 : Met1 synthesized protein using plasmid pSP65/248 Met1;

Lane 3 : Met 2,3 synthesized protein using plasmid pSP65/248 Met2,3;

Lane 4 : Met1,2,3 synthesized protein using plasmid pSP65/248 Met1,2,3.

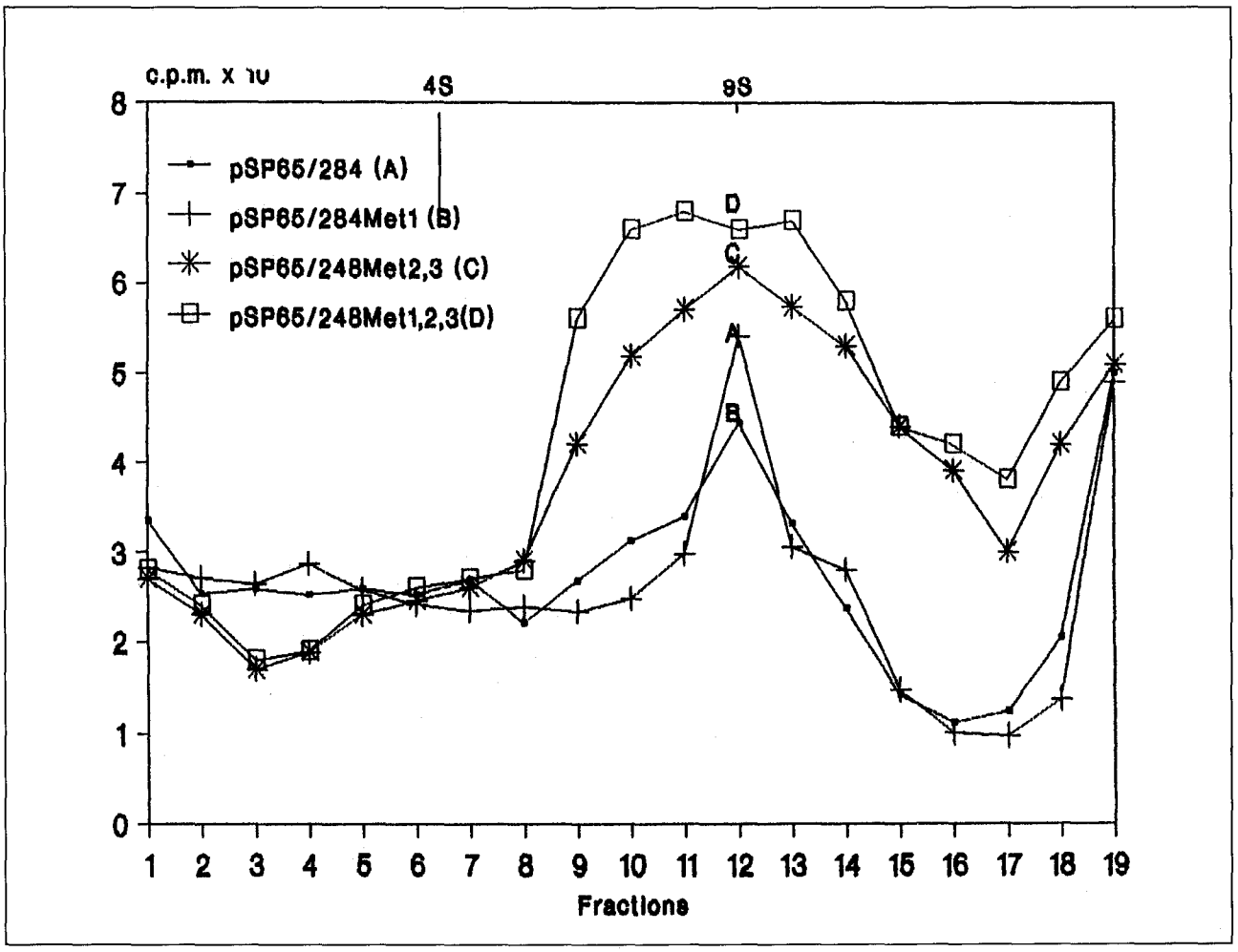

Fig. 5. Self-assembly of pSP65/248. pSP65/Met1, pSP65/Met2,3 pSP65/248 Met1,2,3. Radioactive H3-Leu labeled proglycinis were synthesized *in vitro* using pSP65/248, pSP65/Met1, pSP65/Met2,3, pSP65/248 Met 1,2,3.

They were incubated in the translation mixtures for 30 hours of 25ßC to promote self-assembly and then analyzed by sedimentation in sucrose gradients.

The distribution in sucrose gradients is shown in Fig. 4, and the position of sedimentation standards is shown at the top of the figure.