

# Molecular Improvement of Food Functional Properties of Soybean Glycinin by Protein Engineering

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## Abstract

A study was made to elucidate the three dimensional structure of soybean glycinin which is one of the dominant storage proteins of soybean seeds. Previously, the two disulphide bonds Cys12-Cys45 and Cys88-Cys298 in the proglycinin A<sub>1a</sub>B<sub>1b</sub> subunit were deleted and Cys residues were replaced by Gly and Ser by Oligonucleotide-directed mutagenesis. The mutant proglycinins Gly12, and Ser88 showed to have better gelation and emulsifying properties. The mutant proglycinins were crystallised along with the normal proglycinin (A<sub>1a</sub>B<sub>1b</sub>-3) and subjected to X-ray structure analysis in an attempt to determine their structure-function relationships. The crystals diffracted X-ray to a resolution limit of 2.9 - 3.4Å on still photographs and belong to the tetragonal system, space group P4<sub>1</sub> or P4<sub>3</sub> with cell dimensions of  $a = b = 114.3 - 115.2\text{Å}$  and  $c = 145.7 - 147.1\text{Å}$  with 3 protomers per asymmetric unit. Further refinement data for the crystals of normal proglycinin were obtained by multiple isomorphous replacement and solvent flattening techniques. The trimer dimensions of the normal proglycinin as determined at 6Å were 93Å by 93Å with the thickness of 36Å.

Key words: Crystallisation, Proglycinin, Protein Engineering, Soybean glycinin, X-ray Crystallography

## Introduction

The proteins that form part of human foods are mainly derived from both animal and plant sources. Of the plant protein sources, soybean seeds are no doubt the most widely used in human food formulations. It has been estimated that soybean seeds account for over 70% of the world production of oilseed proteins (Bodwell and Hopkins, 1989) and are utilised in a variety of forms including tempeh, tofu, shoyu and infant formulars (Friedman *et al.*, 1989 Mtebe and Bangu 1990). This flexibility in application is undoubtedly due to better nutritional qualities and food functions of soybean proteins as compared to other plant protein sources (Utsumi *et al.*, 1982).

Soybean proteins are composed of two major components; glycinin and -conglycinin (Derbyshire *et al.*, 1976). Of these two compo-

nents, glycinin accounts for 60-70% of the soybean globulins (Kinsella 1979; Shortwell and Larkins 1989; Thanh and Shibasaki 1978) and it contains more sulphur-containing amino acid residues than -conglycinin (Millerd 1975). Due to its high level of expression and accumulation, glycinin determines the nutritional and functional qualities of soybean proteins and as such is a suitable target for creating ideal food proteins by protein engineering.

In order to make an optimal use of soybean glycinin as a functional ingredient in food systems, better insight into its structure at molecular level is important and this can be accomplished by determining the three dimensional structure of soybean glycinin. This is important since it would provide a solid framework both for understanding its function- structure relationships and in formulating strategies for designing means of improving its nutritional and

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functional qualities even better for wider food applications. Protein engineering coupled with X-ray crystallography and computer graphics provide powerful means of achieving such objective.

As the crystallisation of soybean glycinin from seeds has proved difficult due to the polymorphism of its primary structure and heterogeneity of its molecular species (Utsumi *et al.*, 1981), attempts have been made to elucidate its structure by crystallizing recombinant proglycinins expressed in *Escherichia coli* (Gidamis *et al.*, 1993; Utsumi *et al.*, 1996). Previous studies conducted by Utsumi *et al.*, (1986) have indicated that soybean glycinin cDNAs encoding proglycinins expressed in *Escherichia coli* could assemble into trimers as proglycinins which were not processed to a mature form and had similar secondary structure to that of glycinin. These expressed proglycinins exhibited intrinsic functional properties similar to those of soybean glycinin. In the present study the expression system of soybean proglycinin was therefore employed in *Escherichia coli* to express recombinant soybean proglycinins.

## Materials and Methods

### Construction of Expression Plasmids and Mutant Proglycinins

Plasmids employed were PAM82 (Miyano-hara *et al.*, 1983), PGST4-2-11-10 with cDNA encoding preproglycinin A<sub>1a</sub>B<sub>1b</sub> (Okayama and Berg 1982), M13mp18 (Yanisch-Resson *et al.*, 1985), and pKGA<sub>1a</sub>B<sub>1b</sub>-3. The construction of expression plasmids and site directed mutagenesis involved were performed as explained by Utsumi *et al.*, (1993). The expressed proglycinin from plasmid pKGA<sub>1a</sub>B<sub>1b</sub>-3 was termed A<sub>1a</sub>B<sub>1b</sub>-3 in which N-terminal methionine was retained. The expressed proglycinins resulting from the expression plasmids pKGA<sub>1a</sub>B<sub>1b</sub>Gly12 and pKGA<sub>1a</sub>B<sub>1b</sub>Ser88 were termed Gly12 and Ser88 respectively. Expression of mutant proglycinins

*Escherichia coli* JM105 was used as the host cell and was grown on Luria-Bertani (LB) medium (pH 7.5) containing 1% bactotryptone, 0.5% yeast extract, 1% NaCl and ampicillin (25g/ml). The medium was inoculated with 3ml

of a full-grown *E. coli* JM105 culture harbouring individual expression plasmids and cultured as explained by Kim *et al.*, (1990). Isopropyl-D-thiogalactopyranoside (IPTG) was added to the culture at A<sub>600</sub> = 0.3 to the final concentration of 1mM. The culture was cultivated for 20 hours at 37°C and cells were harvested by centrifugation at 10,000g for 20 minutes at 4°C.

### Purification of the recombinant proglycinins

The harvested *E. coli* cells were disrupted by sonication, and cell debris and unbroken cells were removed by centrifugation. The recombinant proglycinins were then purified by ammonium sulphate fractionation, Q-sepharose column chromatography, and cryoprecipitation as described by Kim *et al.*, (1990). Purified glycinin from soybean seeds was prepared as described by Mori *et al.*, (1979). Protein contents in the samples were determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard. The purity of expressed mutant recombinant was measured by NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis (PAGE). The amounts of the expressed proglycinins were measured by densitometric analysis following Coomassie brilliant blue staining and immuno-blotting of NaDodSO<sub>4</sub> gels.

### Crystallisation of recombinant proglycinins

Expressed purified proglycinin A<sub>1a</sub>B<sub>1b</sub>-3 (Normal) was crystallised by following the dialysis equilibrium method. The proglycinin solution at a protein concentration of 6.6mg/ml was dialysed against 0.1M Tris-HCl (pH 7.6) containing 1.5mM PMSF, 1mM EDTA, 0.02% sodium azide and 10mM 2-Mercaptoethanol (2-ME) at 4°C. The crystals appeared after 48 hours and grew to more than 1mm in length within seven days. The crystals of expressed purified mutant proglycinin Gly12 were obtained by dialysis equilibrium method in 0.125M Tris-HCl buffer (pH 7.6) containing 1.5mM PMSF, 1mM EDTA, 10mM 2-ME and 0.02% sodium azide at 6°C. Expressed purified proglycinin Ser88 crystals were obtained by a hanging drop vapour diffusion method at 8°C

in 35mM potassium phosphate buffer (pH 7.6) containing 9% polyethylene glycol (PEG) in addition to other components as in the case of crystallisation of recombinant proglycinin Gly12.

### Measurement of crystal density

The density of the crystals was measured in a Ficoll 400 density gradient (1.12-1.24gm/ml) calibrated with water saturated mixtures of carbon tetrachloride and toluene of known densities as described by Bode and Schirmer (1985).

### X-ray crystallographic analysis of recombinant proglycinins

Proglycinins crystals larger than  $0.2 \times 0.2 \times 0.2$  mm were mounted in quartz capillaries and subjected to X-ray diffraction using a Nonius precession camera with Ni-filtered CuK radiation generated by a Rigaku X-ray generator operating at 35kV and 20mA. Further X-ray diffraction data for normal proglycinin crystals were collected on Siemens X-1000 multiwire area detector for both native and heavy atom derivative crystals. Heavy atom derivatives were prepared by soaking native crystals separately to the mother liquor containing 1mM methyl mercury 1mM p-chloromercuribenzenesulphonate (PCMBs) and 5mM gold chloride for 15-20 hours at 20°C and then subjected to

X-ray diffraction as explained previously.

## Results and Discussion

### Construction of recombinant proglycinins and their ability to assume correct conformation

The constructed mutant proglycinins following deletion of disulphide bonds are as shown in Figure 1. The amino acid sequences in the vicinity of the translation initiation site and the promoter of each expression plasmid constructed are the same as that of pKA<sub>1a</sub>B<sub>1b</sub>-3. In this respect identical efficiencies of transcription and translation are expected in each case, and the expressed proglycinins retain initiation methionine.

The level of expressed recombinant proglycinins in *E. coli* was assessed by NaDodSO<sub>4</sub>-PAGE. The densitometric scanning of the gels indicated that both mutant proglycinins accumulated in the cells at the level of approximately 20% of the total *E. coli* proteins.

The correct confirmation of mutant proglycinins was assessed by subjecting the soluble fractions of the cells harbouring individual expression plasmids to sucrose density gradient centrifugation and after fractionation proteins in each fraction were subjected to NaDodSO<sub>4</sub>-PAGE and immunoblotting. All recombinant

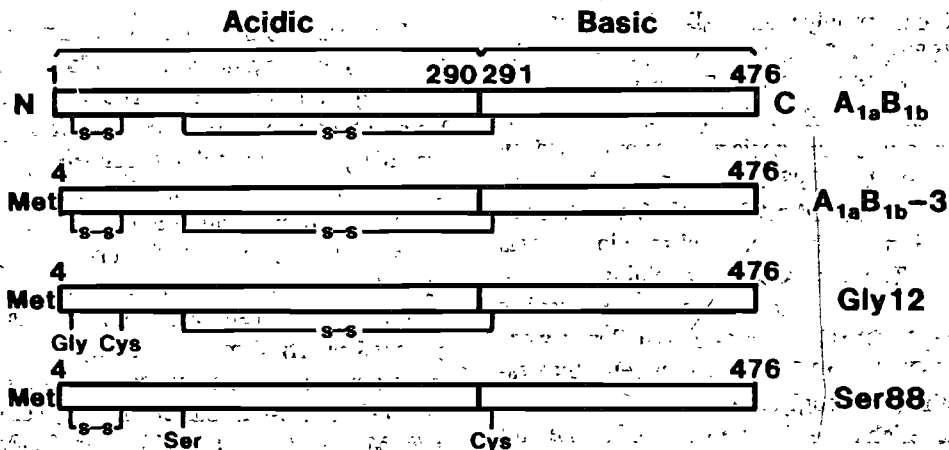


Figure 1: Schematic representation of proglycinin

proglycinins predominantly sedimented in a fraction that corresponds to a trimer size of 7-9s (Utsumi *et al.*, (1996). These observations show that the two disulphide bonds are not necessary for the formation and maintenance of the proglycinin trimer structure.

### X-ray crystallographic analysis of soybean recombinant proglycinins

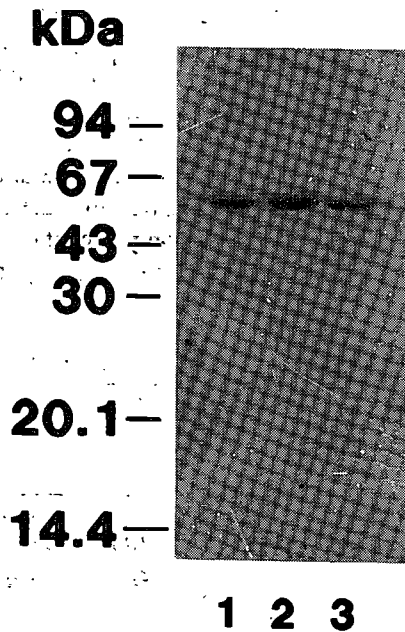


Figure 2: NaDodSO<sub>4</sub>-PAGE analysis of proglycinins

In order to understand the relationships between the structural conformation and functional properties of soybean proglycinins at molecular level, the recombinant proglycinins were crystallised and subjected to X-ray crystallographic analysis. The crystals suitable for X-ray crystallographic analysis were obtained, each under different suitable crystallisation conditions (Table 1).

The crystals of recombinant A<sub>1a</sub>B<sub>1b</sub>-3 proglycinin (figure 3(a)) diffracted X-rays to a resolution limit of at least 2.9Å on still photographs. The systematic absence of reflection in *h0l* and *hk0* zones of precession photograph (figure 3(b)) indicates that the crystals belong to tetragonal system, space group P4<sub>1</sub> or P4<sub>3</sub> with unit cell dimensions of  $a = b = 115.2\text{\AA}$  and  $c = 147.1\text{\AA}$ . The unit cell volume of the crystals is  $1.95 \times 10^6 \text{\AA}^3$  and density of  $1.16 \text{ g cm}^{-3}$  at 25°C. The number of protomers per asymmetric unit was calculated to be 3.17 using a partial specific volume of proteins of  $0.722 \text{ cm}^3 \text{ g}^{-1}$  as estimated from the amino acid composition of soybean proglycinin.

The crystals of recombinant proglycinins Gly12 and Ser88 (figure 3(a)) diffracted X-ray to a resolution limits similar to that of normal proglycinin and belong to the same tetragonal system space group P4<sub>1</sub> or P4<sub>3</sub>. The unit cell dimensions of these crystals were  $a = b = 114.3 - 114.9\text{\AA}$  and  $c = 145.7 - 146.1\text{\AA}$  which are similar to each other and to those of normal proglycinin (Table 2). The number of protomers per asymmetric units of these crystals as

Table 1: Crystallization conditions and maximum crystal length of the normal and modified proglycinins

Proglycinins	Suitable temperature (°C)	Maximum crystal length (mm)							
		Concentration of Tris-HCl <sup>a</sup> (M)							
		0.05	0.1	0.125	0.135	0.15	0.16	0.175	0.2
A <sub>1a</sub> B <sub>1b</sub> -3	8	ND	1.4	0.2	-	-	ND	ND	ND
Gly12	6	ND	0.5	2.0	ND	ND	ND	ND	ND
Ser88	variable	ND	-	-	ND	-	ND	-	-

Key:

a : pH 7.6

- : No crystals

ND : Not done

calculated using same parameters was about 3 which is consistent with the fact that the soybean proglycinins are trimers. The ratio of cell volume to unit protein mass,  $V_m$  of these crystals ranged from  $2.98 - 3.05 \text{ \AA}^3 \text{ Da}^{-1}$  which is within the range of typical protein crystals ( $V_m = 1.7-3.5 \text{ \AA}^3 \text{ Da}^{-1}$ ) (Mathews 1968).

As there is no structural information on proglycinin-like proteins, it has been necessary to solve the phase problem by Multiple Isomorphous Replacement (MIR) method. After analysing many derivative crystals, phase ambiguity was resolved by using two Hg derivatives and one Au derivative. The two Harker sections of the Patterson maps for Hg derivative, PCMBS are shown in Figure 3. From the space group of these crystals, each section contains four asymmetric units. There are three Hg sites in each section corresponding to the number of protomer per asymmetric unit of the crystals.

Positions of these Hg sites were determined from these maps and used for further phase calculations together with gold sites determined in the same way.

The refinement statistics for the three heavy atom derivatives at 6 Å resolution indicated three sites for each derivative corresponding to the number of protomers per asymmetric unit of the crystals. Positions of these heavy metal atoms were determined from the Patterson maps and used for further phase calculations. Table 3 summarises the final refinement statistics of the heavy metal atoms parameters at 6 Å resolution as refined by using PHASIT programme. Both R-factors calculated for centric and for all reflections are within the acceptable limits. The overall figure of merit was 0.607 for 4348 reflections indicating that the refinement statistics are reasonably acceptable. The figure of merit was further improved by solvent

**Table 2: Preliminary X-ray crystallographic data for the normal and modified proglycinins**

Proglycinins	Maximum resolution (Å)	Crystal System	Space Group	Unit Cell Dimensions (Å)			Density ( $\text{gcm}^{-3}$ )	Protomers /asymmetric unit	$V_m^a$ ( $\text{Å}^3 \text{ Da}^{-1}$ )
				a	b	c			
Normal	2.9	Tetragonal	P4 <sub>1</sub> /P4 <sub>3</sub>	115.2	115.2	147.1	1.16	3.17	3.05
Glyl2	3.4	Tetragonal	P4 <sub>1</sub> /P4 <sub>3</sub>	114.9	114.9	146.1	1.16	3.13	3.02
Ser88	3.0	Tetragonal	P4 <sub>1</sub> /P4 <sub>3</sub>	114.3	114.3	145.7	1.16	3.09	2.98

<sup>a</sup> Ratio of unit cell volume to unit protein mass

**Table 3: Final results of the refinement statistics of the heavy atom parameters at 6 Angstrom resolution**

Heavy Atom Deriv.	Site X	Fractional Coordinates			B (Å <sup>2</sup> )	Occ (%)	RC (%)	RK (%)	Pp
		Y	Z	B					
PCMBS	Hg1-1	0.347	0.867	0.152	10.25	0.32			
	Hg1-2	0.988	0.782	0.100	8.27	0.34	58.6	5.8	1.80
	Hg1-3	0.13	0.537	0.000	46.05	0.32			
CH <sub>3</sub> HgOH	Hg2-1	0.344	0.863	0.161	10.44	0.31			
	Hg2-2	0.991	0.779	0.110	7.94	0.30	58.6	5.5	1.68
	Hg2-3	0.126	0.533	0.003	8.18	0.26			
KauCl <sub>4</sub>	Au1-1	0.350	0.869	0.164	31.19	0.32			
	Au1-2	0.986	0.780	0.110	15.62	0.29	63.9	5.6	1.55
	Au1-3	0.128	0.537	0.011	19.23	0.31			

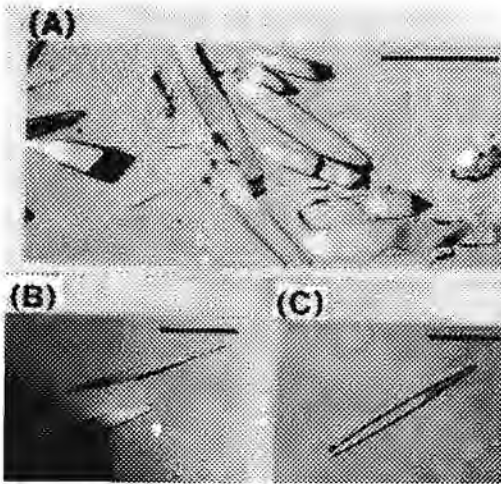


Figure 3a: Photomicrographs-A1aB1b-3(A), Gly12(B) & Ser88(C)

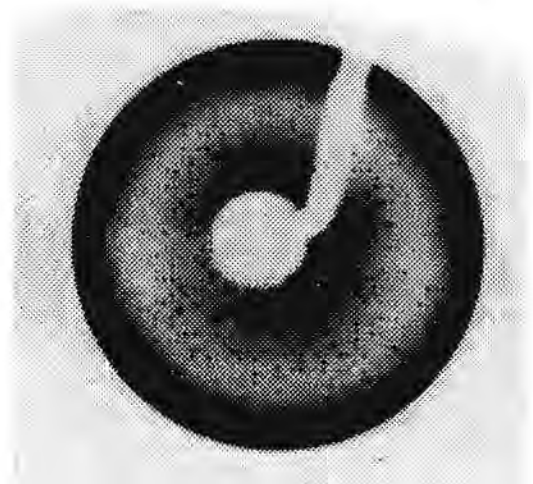


Figure 3b: Reciprocal-lattice planes of proglycinin crystal

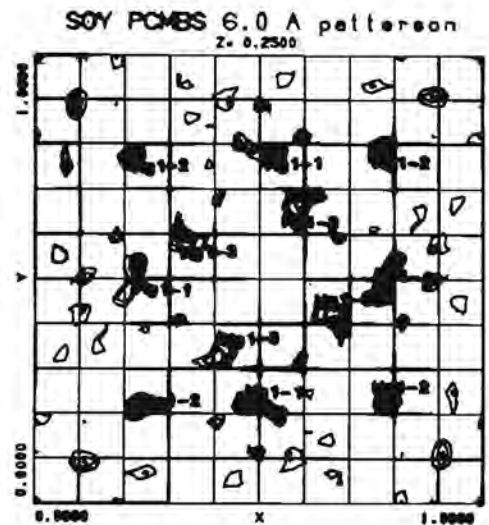


Figure 4: Hacker sections of the difference-Patterson maps

flattening technique to 0.85 for calculating the electron density map.

The three dimensional structure at 6 Å resolution was determined from the electron density map calculated from the data based on the best phases obtained from the heavy metal derivatives. From the electron density map it was possible to clearly distinguish the protein region from that of solvent. The boundaries of the protein region indicate that the proglycinin molecule is composed of three protomers that are related by a three-fold axis symmetry. The three-fold axis symmetry observed corresponds

to axis relating the heavy metal binding sites. The dimensions of the proglycinin trimer are 93Å x 93Å x 36Å, which are quite similar to those of phaseolin, the 7S globulin of kidney beans as reported by Lawrence *et al.*, (1990).

## Conclusions

In this study, attempts have been done to determine the three dimensional structure of soybean proglycinin using a recombinant to a resolution limit of 6Å. Although the soybean proglycinin A1aB1b trimer dimensions were

found to be similar to those of phaseolin of kidney beans, they seem not to be identical molecules. The three dimensional structure of kidney beans phaseolin is known, but it could not be used to determine that of soybean proglycinin by molecular replacement method using the structural data of phaseolin as reported. In this respect, the data collection and processing for refinement at more than 3Å resolution of soybean proglycinin is necessary so as to determine its three dimensional structure directly.

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