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Expression of soybean glycinin subunit precursor cDNAs in *Escherichia coli*

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As the cDNAs encoding $A_{1a}B_{1b}$ and A_2B_{1a} subunit precursors of the glycinin A_2 subfamily contain a unique *Nco*I site sequence, (A)CCATGG, occurring at their translation initiation sites, plasmids were constructed to direct the synthesis of those precursor proteins by inserting *Nco*I/*Pst*I fragments derived from those cDNA clones into the *Nco*I/*Pst*I-pKK233-2 expression vector in *Escherichia coli* MV1190, respectively. The resultant plasmids directed the expression of 57-kDa protein components that have molecular masses in agreement with those of the in vitro translation products directed by glycinin A_2 subfamily mRNAs, by the addition of isopropyl β-D-thiogalactoside. These proteins, which comprised as much as 1% of the total bacterial protein, are immunoprecipitable with rabbit antibodies specific for glycinin subunits. This procedure makes glycinin subunits available as a model for studying structure-function relationships in seed proteins using site-directed mutagenesis. This is the first expression of glycinin-like storage protein in *E. coli*.

Plant storage protein; 11 S globulin; Glycinin A_2 subfamily; *Nco*I site; pKK233-2 expression vector; Immunoblot assay

1. INTRODUCTION

Glycinin, the major seed storage protein of soybean (*Glycine max* (L.) Merr.), is mainly composed of 5 distinct subunit pairs that are classified into two subfamilies on the basis of the extent of homology throughout those precursor molecules [1–5]. These subfamilies are denoted A_2 (A_2B_{1a}, $A_{1a}B_{1b}$, $A_{1b}B_2$) and A_3 (A_3B_4, $A_5A_4B_3$), respectively. In order to elucidate the relationship between primary structures of glycinin subunits and functional properties for food processing, it is of importance to employ protein engineering techniques accompanied by site-directed mutagenesis. As the first step of this procedure, we tried to express cDNAs encoding A_2 subfamily subunit precursors of glycinin in an unfused state. As a unique *Nco*I site sequence occurs at translation initiation sites of A_2B_{1a} and $A_{1a}B_{1b}$ among the glycinin subunit precursors, we used the pKK233-2 expression vector [6] with *Escherichia coli* strain MV1190 as a host. As a result of this experiment, it was found that recombinant plasmids directed the synthesis of approx. 57-kDa proteins.

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2. EXPERIMENTAL

2.1. *Construction of glycinin expression vector*

pKK233-2 expression vector was purchased from Pharmacia. Restriction enzymes were from Nippon Gene and DNA ligase from Takara Shuzo, incubation conditions being as recommended by the vendors. cDNA inserts used for ligation to the vec-

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tor were derived from $pGA_2B_{1a}521$ and $pGA_{1a}B_{1b}325$, respectively [3,5]. Briefly, each recombinant plasmid (approx. 30 μg) was cut by *Nco*I and *Pst*I. The resultant 1.7 kb cDNA fragment was separated electrophoretically on a 1.2% agarose gel, and extracted using an LKB 2014 Extraphor electrophoretic concentrator, followed by passing through a Nensorb 20 column (NEN, DuPont). The purified cDNA fragments were ligated to the *Nco*I/*Pst*I-cut pKK233-2 expression vector, followed by transfection of *E. coli* RR1 as described by Dagert and Ehrlich [7]. Transformants were selected by growth on ampicillin, followed by colony hybridizations using ^{32}P-labeled cDNA inserts derived from A_2B_{1a} and $A_{1a}B_{1b}$ clones [3,5]. After confirmation of the junction region between glycinin cDNA and the expression vector by nucleotide sequencing [8] in those recombinants, the resulting recombinant plasmids, designated $pKGA_2B_{1a}13$ and $pKGA_{1a}B_{1b}6$, were employed for the expression experiment of A_2B_{1a} and $A_{1a}B_{1b}$ subunit precursors of glycinin, respectively. These plasmids were transfected into *E. coli* MV1190, as the tac promoter of the vector may be derepressed at the appropriate time following isopropyl β-D-thiogalactoside (IPTG) induction in a $lacI^Q$ host [9].

2.2. *Expression of glycinin subunit precursors in* E. coli

In order to express glycinin subunit precursors, overnight-cultured *E. coli* MV1190 harboring $pKGA_2B_{1a}13$ (or $pKGA_{1a}B_{1b}6$) in 0.1 ml Luria broth was inoculated into 2 ml of the broth and cultivated at 37°C until early log phase. After being harvested and suspended in 2 ml M9 minimum medium, bacteria harboring the expression plasmids were grown until the late log phase and then IPTG was added to the culture at a final concentration of 2 mM. After cultivation for 0 min, 20 min, 1 h and 2 h, cell pellets were suspended in 0.125 M Tris-HCl, pH 6.8, 2% SDS, 1 M 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 mM ϵ-amino-*n*-caproic acid and 30% (v/v) glycerol. Lysates were boiled for 5 min before loading on the gel. All the proteins of the lysates were transferred and bound to the nitrocellulose membrane electrophoretically, following separation of the proteins in a 12.5% polyacrylamide gel containing 0.1% SDS, according to Laemmli [10]. The membrane with bound proteins was immersed into a blocking solution which consisted of 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 20% (v/v) fetal calf serum for 2 h at room temperature, followed by washing in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% Nonidet P-40. It was then incubated with anti-glycinin serum (1:480 dilution) [2]. After washing, the membrane was incubated with antibodies conjugated with horseradish peroxidase (Bio-Rad immun-blot assay kit), and immersed into development solution according to the manufacturer's directions as in [2]. Some gels were directly stained with Coomassie brilliant blue without immunoblotting.

3. RESULTS AND DISCUSSION

As the digestion with *Nco*I exposes the start codons for glycinin A_2B_{1a} and $A_{1a}B_{1b}$ subunit precursors, we employed the expression vector pKK233-2, designed with the tac promoter (trp-lac promoter) and the lac Z ribosome-binding site followed by an ATG initiation codon which is contained within an *Nco*I site. The procedure followed to express directly the cDNA inserts of glycinin subunits in *E. coli* is outlined in section 2. *E. coli* cells harboring $pKGA_2B_{1a}13$ or $pKGA_{1a}B_{1b}6$ were tested for expression. Lysates extracted at the appropriate time (0–2 h) after IPTG addition were electrophoresed on SDS-polyacrylamide gels. Coomassie blue staining of the gel indicated that an approx. 56800-Da protein is produced in cells containing the expression plasmids (fig.1A). Immunoblot analysis revealed that the 56800-Da protein is recognized by the glycinin-specific antibodies (fig.1B). No proteins from the control *E. coli* cells containing expression vector pKK233-2 alone interacted with these antibodies (fig.1). The recombinant glycinin production was also enhanced remarkably at 1 h by addition of 2 mM IPTG as evident from fig.1. The molecular masses of these in vivo products are in agreement with those of the in vitro translation products directed by glycinin A_2 subfamily mRNAs in a micrococcal nuclease-treated rabbit reticulocyte lysate (not shown). Densitometric scans of the gels stained with Coomassie blue indicate that glycinin precursor proteins represent approx. 1% of the total protein in bacterial lysates (fig.1A).

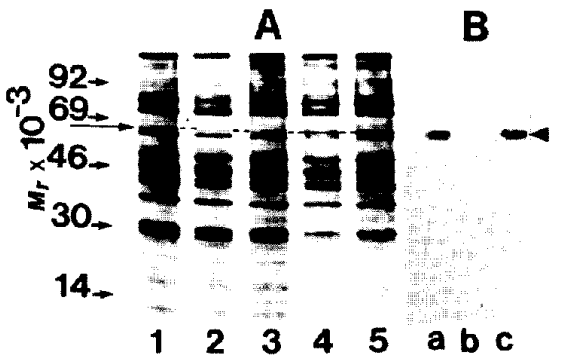

Fig.1. (A) SDS-polyacrylamide gel analysis of soybean glycinin subunit precursors in *E. coli*. Samples of lysates were loaded on a 12.5% polyacrylamide gel containing 0.1% SDS. Lanes: (1) *E. coli* MV1190 harboring $pKGA_{1a}B_{1b}6$ grown at 1 h following IPTG induction; (2) cells containing the expression vector pKK233-2 alone; (3–5) cells harboring $pKGA_2B_{1a}13$ grown at 0 min, 20 min, and 2 h following IPTG induction (2 mM), respectively. (B) Immunoblot analysis of soybean glycinin synthesis in *E. coli*. After SDS-polyacrylamide gel electrophoresis, whole protein of the lysate was transferred to a nitrocellulose membrane. The membrane was interacted with anti-glycinin serum, followed by incubation with antibodies conjugated to horseradish peroxidase as described in section 2. Lanes: (a,b) samples and conditions for IPTG induction were identical to lanes 1,2 in (A), respectively; (c) cells harboring $pKGA_2B_{1a}13$ grown at 1 h following IPTG induction (2 mM). M_r markers: phosphorylase *b* (92500), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000), and α-lactalbumin (14000). The long arrow (A) and arrowhead (B) indicate the positions of glycinin A_2 subfamily subunit precursors, whose M_r values are approx. 57000, respectively.

It is clear from these pieces of evidence that glycinin subunit precursors are synthesized in *E. coli* and that their production is increased on induction with IPTG. This may imply that the protein engineering technique is available for studying the relationship between the primary structure of glycinin and functional properties. Further studies on the purification of the glycinin subunit precursor produced in *E. coli* and site-specific mutagenesis for this storage protein are in progress.

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