

Polymorphism and Expression of cDNAs Encoding Glycinin Subunits

Shigeru UTSUMI, Chan-Shick KIM, Mitsutaka KOHNO
and Makoto KITO*Research Institute for Food Science, Kyoto University,
Uji, Kyoto 611, Japan*

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The nucleotide sequence of cDNA encoding the glycinin A_2B_{1a} subunit from var. Shiotsurunoko was determined and compared with that in the case of var. Bonminori. The comparison showed six nucleotide substitutions in the coding sequence, one of which results in one amino acid replacement, and three in the 3'-noncoding region. These differences indicate the occurrence of polymorphism of the glycinin A_2B_{1a} subunit gene between the cultivars. The present data together with the previous results indicating the polymorphism of the $A_{1a}B_{1b}$ subunit gene [Utsumi *et al.*, *J. Agric. Food Chem.*, **35**, 210 (1987)] suggest that the polymorphism is a general property of glycinin subunit genes. The expression of cDNAs encoding the A_2B_{1a} and $A_{1a}B_{1b}$ subunits was examined. The results obtained in both *in vivo*- and *in vitro*-expression experiments indicate that the resultant products were readily degraded.

Glycinin, 11S globulin, is one of the predominant storage proteins of soybeans (*Glycine max* L.). It is composed of six subunits, each of which consists of an acidic and a basic polypeptide, that are linked by a disulfide bridge in a 1:1 ratio.^{1~4)} A single-polypeptide precursor consisting of covalently linked acidic and basic polypeptides is synthesized initially and then processed posttranslationally to form both acidic and basic polypeptides.^{5~7)} At present, the following subunits have been identified: $A_{1a}B_{1b}$ ($A_{1a}B_x$), $A_{1b}B_2$ ($A_{1b}B_y$), A_2B_{1a} , A_3B_4 and $A_5A_4B_3$.^{4,8~12)} We determined the nucleotide sequence of full-length cDNAs encoding the $A_{1a}B_{1b}$ subunit from var. Shiotsurunoko and compared it with that in the case of var. Bonminori.^{10,12)} The comparison showed eight nucleotide substitutions, five of which resulted in four amino acid replacements.¹²⁾ This indicates the polymorphism of the $A_{1a}B_{1b}$ subunit gene between soybean cultivars. However, it is still unknown whether such polymorphism is a general property of other subunits.

Enhancement of the functional properties and nutritive value of soybean proteins in-

creases the utilizability of soybean proteins in various foods, although soybean proteins have relatively superior functionality and nutritive value among plant protein resources. Techniques for the enhancement include chemical (including enzymatic), physical and biological methods. Recent progress in gene manipulation and elucidation of the interrelationship between structure and functionality of soybean proteins^{13~16)} has made it possible to adopt protein engineering for the enhancement of soybean protein qualities. For this purpose, it is necessary to develop an expression system of protein-engineered soybean proteins with a microorganism and to evaluate their functionality.

In this study, in order to establish the generality of the polymorphism of glycinin subunit genes among cultivars, we determined the nucleotide sequence of glycinin A_2B_{1a} cDNA from var. Shiotsurunoko and compared it with that in the case of var. Bonminori.¹⁰⁾ In addition, we examined the expression of the cDNAs encoding the $A_{1a}B_{1b}$ and A_2B_{1a} subunits.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Toyobo and Takara Shuzo. The expression vector (pKK233-2) was from Pharmacia. The proto immuno kit was from Promega Biotec. The *in vitro* expression system was from New England Nuclear. The nitrocellulose filter was from S & S. The protein A-bacterial adsorbent was from Bio-Yeda. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma. Isopropyl- β -D-thiogalactopyranoside (IPTG) was from Nakarai Chemicals. Other chemicals were the same as described previously.¹²⁾

Preparation of RNA and double stranded cDNA. Total RNA was extracted from frozen mid-maturation stage soybean (var. Shiroturunoko) cotyledons in the presence of the ribonucleoside-vanadyl complex as described previously.¹²⁾ Glycinin mRNA-rich fractions of polyadenylated RNA purified by oligo(dT)-cellulose column chromatography¹⁷⁾ were obtained by sucrose density gradient centrifugation as described previously.¹²⁾ Double stranded cDNA was synthesized from the glycinin mRNA-rich fractions according to the procedure of Okayama and Berg¹⁸⁾ as described in the previous paper.¹²⁾

Identification of cDNA encoding the A_2B_{1a} subunit. In the case of var. Bonminor, the nucleotide sequence of the A_2B_{1a} subunit is similar to that of the $A_{1a}B_{1b}$ subunit.^{10,11)} Therefore, an approximately 250bp *Sau*3AI-EcoRI restriction endonuclease fragment of pGST8, which codes a part of the $A_{1a}B_{1b}$ subunit sequence,^{10,12)} was 3'-end-labeled with ³²P-dideoxyATP¹⁹⁾ and used as a probe for colony-hybridization to screen cDNA encoding the A_2B_{1a} subunit. Plasmids from the positive clones were isolated by the alkali-SDS method,²⁰⁾ and their sizes were analyzed in 0.7% agarose gel. Plasmids in which the sizes of the inserts were longer than 1800bp were analyzed by EcoRI digestion. We selected pGST4-3-1-4 as a possible candidate containing A_2B_{1a} cDNA. This plasmid was purified according to the procedure of Maniatis *et al.*²⁰⁾ and subjected to nucleotide sequence analysis.

Nucleotide sequence analysis. The nucleotide sequence of the cDNA insert of pGST4-3-1-4 was determined by the dideoxy-sequencing method²¹⁾ employing a sequencing kit from Takara Shuzo.

Construction of expression plasmids pKG2 ($A_{1a}B_{1b}$) and pKG4 (A_2B_{1a}). The cDNA inserts in pGST4-2-11-10¹²⁾ and pGST4-3-1-4 were cut out with *Nco*I and *Pvu*II, this site is located in the vector DNA adjacent to the poly(dA:dT) join, and inserted into the *Nco*I and filled-in *Hind*III sites of pKK233-2 to form pKG2($A_{1a}B_{1b}$) and pKG4(A_2B_{1a}).

In vivo-expression and immunological detection by western blotting. 5 ml of M9 medium supplemented with 0.2% casamino acids, 0.2% glucose, 1 mM thiamine and 25 μ g/ml

of ampicillin was inoculated with 0.1 ml of a full-grown culture in LB medium of *E. coli* JM105 harboring pKG2($A_{1a}B_{1b}$) or pKG4(A_2B_{1a}), followed by culturing at 37°C. At $A_{600}=0.3, 0.8$ or 1.5, IPTG was added to a final concentration of 2 mM. After incubation for 15 min, 30 min, 1 hr, 3 hr, 6 hr or 15 hr, the induced cultures were harvested by centrifugation, and the cell pellets were suspended and boiled for 5 min in 50 to 200 μ l of sample buffer at 100°C according to Laemmli²²⁾ in the presence of 10 mM EDTA and 4 mM PMSF. 50 μ l per well was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated on the SDS gels were transferred to nitrocellulose filters and then analyzed with anti-glycinin serum and alkaline phosphatase-conjugated goat anti-rabbit IgG. Prior to the analysis, the nitrocellulose filters were blocked with non fat dry milk,²³⁾ and the anti-glycinin serum was treated with the boiled lysate²⁴⁾ of JM105 cells.

In vitro-expression and detection. pKG2($A_{1a}B_{1b}$) and pKG4(A_2B_{1a}) purified by *CsCl*₂-centrifugation were subjected to *in vitro*-expression according to the protocol of New England Nuclear. After the reaction, the expressed samples were precipitated by the addition of 5 vol of cold acetone, stored at -20°C for 16 hr, centrifuged and then washed two times with 83% acetone. The pellet was dried for 30 min at room temperature under vacuum, dissolved in the SDS-sample buffer and then subjected to SDS-PAGE according to Laemmli.²²⁾ After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 in 10% acetic acid containing 30% methanol and destained with 7% acetic acid containing 30% methanol. The destained gel was treated with Enlightening (NEN) and then fluorographed. In the case of immunoprecipitation, the expressed sample (8 μ l) was mixed with 22 μ l of H₂O, 3 μ l of 2.75% NP-40 and 1 μ l of non-immunized rabbit serum, followed by incubation for 15 min at 25°C. After the incubation, 20 μ l of a 10% (v/v) suspension of protein A-bacterial adsorbent was added, and the mixture was incubated for 15 min at 25°C and then centrifuged for 10 min at 14,000 rpm. To the supernatant, 1 μ l of anti-glycinin serum was added, and then the mixture was incubated for 20 min at 25°C, mixed with 50 μ l of protein A-bacterial adsorbent and finally incubated for 20 min at 25°C. After the incubation, the mixture was centrifuged for 10 min at 14,000 rpm. The pellet was washed 4 times with 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA and 0.25% NP-40, and then dissolved in the SDS-sample buffer.

Preparation of S-100 of JM105 cells harboring plasmid. 20 ml of LB containing ampicillin was inoculated with full-grown culture cells (0.1 ml) of JM 105 harboring pKK233-2 or pKG2($A_{1a}B_{1b}$), followed by culturing at 37°C until $A_{600}=0.3$. Then, IPTG was added to a final concentration of 2 mM. After 2 hr incubation, the cells were harvested and suspended in 10 mM Tris-HCl buffer (pH 7.5). The

cell suspension was lysed by sonication and then centrifuged for 1 hr at 100,000 G. The supernatant was called S-100 and used to examine the effect of S-100 on the degradation of the expressed products.

RESULTS AND DISCUSSION

Isolation and sequencing of cDNA encoding the A_2B_{1a} subunit

Clones from the glycinin-rich cDNA library were screened with the ^{32}P -3'-end-labeled Sau3AI-EcoRI fragment of pGST8 to obtain a plasmid containing a cDNA encoding the A_2B_{1a} subunit, as described under MATERIALS AND METHODS. Then, we selected one clone (pGST4-3-1-4), which contains the longest insert, as a possible candidate containing A_2B_{1a} cDNA, on the basis of the results of EcoRI digestion, since the A_2B_{1a} cDNA of var. Bonminori has two EcoRI sites but $A_{1a}B_{1b}$ has only one site.^{10,11)} The sequencing strategy is shown in Fig. 1. The nucleotide sequence and the deduced amino acid sequence of the insert of pGST4-3-1-4 are shown in Fig. 2. The cDNA insert contained the entire coding region of mRNA and adjacent portions of its untranslated sequences. The length of the cDNA insert was 1803 bp, containing 95 b poly(A). The deduced amino acid sequence completely corresponds to the NH_2 -terminal sequences of A_2 and B_{1a} .^{6,25)}

The nucleotide sequence of the A_2B_{1a} subunit from var. Bonminori has been reported by Momma *et al.*¹¹⁾ Comparison of the nucleotide sequence of the A_2B_{1a} subunit from var. Shiotsurunoko with that in the case of var. Bonminori shows that one, three and two nucleotide substitutions occur in the signal peptide region, the acidic polypeptide region and the basic polypeptide region, respectively (Table I). One of the three substitutions in the acidic polypeptide region results in one amino acid replacement, *i.e.*, Asp in var. Shiotsurunoko was changed to Gly in var. Bonminori. In addition, there are two insertions of nucleotide and one nucleotide substitution in the 3'-untranslated region (Table I). On the other hand, Marco *et al.* reported the partial nucleotide sequence of a genomic

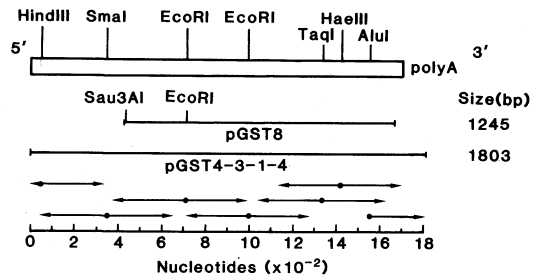


FIG. 1. Size and Restriction Endonuclease Map of the A_2B_{1a} cDNA Clone and the Sequencing Strategy.

The horizontal arrows show the direction, and distance, in which sequences were obtained. The exact nucleotide length of each cDNA clone was determined by nucleotide sequencing and is shown on the right.

DNA encoding the A_2B_{1a} subunit from var. CX635-1-1-1.²⁶⁾ Comparison of the present data with their data shows that two silent nucleotide substitutions occur in the basic polypeptide region (Table I). These results indicate the occurrence of polymorphism of the A_2B_{1a} subunit gene, similar to that of the $A_{1a}B_{1b}$ subunit gene,¹²⁾ among cultivars. This bears out at the gene level the fact that there are six differences in the amino acid sequences of the A_2B_{1a} subunit between var. Bonminori, of which the amino acid sequence was deduced from the nucleotide sequence of the cDNA, and var. CX635-1-1-1, of which the amino acid sequence was determined at the protein level.²⁷⁾ Thus, the polymorphism among cultivars is probably an inherent property of glycinin subunit genes. Nucleotide substitution was seen to occur between A and G or between C and T among cultivars except at position 1460. This was also the case for the $A_{1a}B_{1b}$ cDNA.¹²⁾

Expression of cDNAs encoding the glycinin $A_{1a}B_{1b}$ and A_2B_{1a} subunits

The cloning vector employed in this study is not an expression vector. For the expression of glycinin subunit proteins in *E. coli*, it is necessary to insert the cDNAs into an expression vector. Fortunately, the translation initiation sites of the $A_{1a}B_{1b}$ and A_2B_{1a} cDNAs fall within an *NcoI* site similarly to in other eu-

	AACACAAACATTCTCTC	17
CATTGTCTTTGAACACTCATCACCATGGCCAAGCTTGTTCCTTTCCCTTTGTTTCCTTCTTTTCAGTGGCTGCTTCGCT	95	
MetAlaLysLeuValLeuSerLeuCysPheLeuLeuPheSerGlyCysPheAla	-1	
* CTGAGAGAGCAGGCACAGCAAAATGAGTGCCAGATCCAAAAGCTGAATGCCCTCAAACCGGATAACCGTATAGAGTCG	173	
LeuArgGluGlnAlaGlnGlnAsnGluCysGlnIleGlnLysLeuAsnAlaLeuLysProAspAsnArgIleGluSer	26	
GAAGGTGGGTTCATTGAGACCTGGAACCTAACACAAGCCATTCCAGTGTGCCGGTGTTCGCCCTCTCTCGCTGCACC	251	
GluGlyGlyPheIleGluThrTrpAsnProAsnAsnLysProPheGlnCysAlaGlyValAlaLeuSerArgCysThr	52	
CTTAACCGCAATGCCCTTCGTAGACCTTCTACACCAACGGTCCCCAGGAAATCTACATACAACAAGGTAATGGTATT	329	
LeuAsnArgAsnAlaLeuArgArgProSerTyrThrAsnGlyProGlnGluIleTyrIleGlnGlnGlyAsnGlyIle	78	
TTTGGCATGATATTTCCCGGTTGTCTAGCACTTATCAAGAGCCGCAAGAATCTCAGCAACGAGGACGAAGCCAGAGG	407	
PheGlyMetIlePheProGlyCysProSerThrTyrGlnGluProGlnGluSerGlnGlnArgGlyArgSerGlnArg	104	
CCCCAAGACCGTCACCAAAAGGTACATCGCTTCAGAGAGGGTGATTGTATGCGCAGTGCCTACTGGTGTTCATGGTGG	485	
ProGlnAspArgHisGlnLysValHisArgPheArgGluGlyAspLeuIleAlaValProThrGlyValAlaTrpTrp	130	
ATGTACAACATGAAGACACTCTGTGTGTGCGCTTTCTATTATTGACACCAACAGCTTGGAGAACCAGCTCGACCAG	563	
MetTyrAsnAsnGluAspThrProValValAlaValSerIleIleAspThrAsnSerLeuGluAsnGlnLeuAspGln	156	
ATGCCTAGGAGATTCTATCTTGTCTGGGAACCAAGAGCAAGAGTTTCTAAATATCAGCAGCAGCAGCAAGGAGGTTC	641	
MetProArgPheTyrLeuAlaGlyAsnGlnGluGlnPheLeuLysTyrGlnGlnGlnGlyArgGlySerGlnArg	182	
CAAGCCAGAAAGGAAAGCAACAAGAAGAAAACGAAGGAAGCAACATATTGAGTGGCTTCGCCCTGAATTTCTTG	719	
GlnSerGlnLysGlyLysGlnGlnGluGluGluAsnGluGlySerAsnIleLeuSerGlyPheAlaProGluPheLeu	208	
AAAGAAGCGTTCCGCGTGAACATGCAGATAGTGAGAAACCTACAAGGTGAGAACGAAGAGGAGGATAGTGGAGCCATT	797	
LysGluAlaPheGlyValAsnMetGlnIleValArgAsnLeuGlnGlyGluAsnGluGluGluAspSerGlyAlaIle	234	
GTGACAGTGAAAGGAGGTCTAAGAGTCACAGCTCCAGCCAATGAGGAAGCCACAGCAAGAAGAAGATGATGATGATGAG	875	
ValThrValLysGlyGlyLeuArgValThrAlaProAlaMetArgLysProGlnGlnGluGluAspAspGlyIle	260	
GAAGAGCAGCCACAGTGCCTGGAGACAGACAAGGTTGCCAACGCCAAAGCAAAAGGAGCAGAAATGGCATTGATGAG	953	
GluGluGlnProGlnCysValGluThrAspLysGlyCysGlnArgGlnSerLysArgSerArgAsnGlyIleAspGlu	286	
ACCATTTGCACAATGAGACTTCGCCAAAACATTGGTCAGAATTCATCACCTGACATCTACAACCTCAAGCTGGTAGC	1031	
ThrIleCysThrMetArgLeuArgGlnAsnIleGlyGlnAsnSerSerProAspIleTyrAsnProGlnAlaGlySer	312	
ATCACAAACCGCCACCAAGCTTGAATTCACAGCCCTCTGGCTTCTCAAACCTCAGTCCCAGTATGGATCACTCCGCAAG	1109	
IleThrThrAlaThrSerLeuAspPheProAlaLeuTrpLeuLeuLysLeuSerAlaGlnTyrGlySerLeuArgLys	338	
AATGCTATGTTCTGTGCCCACTACACCTGAACGCGAACAGCATAATATACGCATTGAATGGGCGGCATTGGTACAA	1187	
AsnAlaMetPheValProHisTyrThrLeuAsnAlaAsnSerIleIleTyrAlaLeuAsnGlyArgAlaLeuValGln	364	
GTGGTGAATTGCAATGGTGAGAGAGTGTGTGATGGAGAGCTGCAAGAGGGAGGGGTGCTGATCGTTCCACAAAACCTC	1265	
ValValAsnCysAsnGlyGluArgValPheAspGlyGluLeuGlnGluGlyGlyValLeuIleValProGlnAsnPhe	390	
GCGGTGGCTGCAAAAATCCAGAGCGATAAATTTGAGTATGTGTCATTCAAGACCAATGATAGACCTCGATCGGAAAC	1343	
AlaValAlaAlaLysSerGlnSerAspAsnPheGluTyrValSerPheLysThrAsnAspArgProSerIleGlyAsn	416	
CTTGACAGGGCAAACTCATTTTGAACGCATTGGCCAGGAGGAAGTGAATTCAGCACACTTTTAACCTAAAGAGCCAGCAG	1421	
LeuAlaGlyAlaAsnSerLeuLeuAsnAlaLeuProGluGluValIleGlnHisThrPheAsnLeuLysSerGlnGln	442	
GCCAGGCAGGTGAAGAACAACAACCTTTTCAGCTTCTCTGGTTCCACCTCAGGAGTCTCAGAGGAGAGCTGTGGCTTAG	1499	
AlaArgGlnValLysAsnAsnAsnProPheSerPheLeuValProProGlnGluSerGlnArgArgAlaValAla	467	
AGAGCCCTTTTTCATGTGCTACCACTTTTGTCTTTTGGGCTCTAGCTAGTGTCTGGTAGCAGTAAATAAGATGA	1577	
<u>ATAAAAGAAAACAAAGGCTTAGATGCCCTTTGTGTTAAGGTAGAATAACGGGTATAGTAACATGTATCCCTTTGTGTAATG</u>	1655	
AGTCACTCAAGTACCTATGCGGGAAAAGCATAAAATAAAGAAAGAAATTCCAAT-poly(A)	1708	

FIG. 2. Nucleotide and Deduced Amino Acid Sequences of the A₂B_{1a} cDNA from var. Shirotsurunoko.

Amino acid residues are numbered in the NH₂ to COOH direction starting with the NH₂-terminal residue of the mature acidic polypeptide. * and ** indicate the NH₂-terminal residues of the mature acidic and basic polypeptides, respectively. The AATAAA sequences in the 3'-untranslated region are underlined. ○ indicates the nonidentical nucleotides between var. Shirotsurunoko and Bonminori.

karyotic genes.^{28,29}) Then, we inserted the cDNA regions of pGST4-2-11-10¹²) and pGST4-3-1-4 into pKK233-2 with an *Nco*I site just downstream from a strong promoter, *trc*, and a ribosome binding site, as

described under MATERIALS AND METHODS. We selected JM105 as a host cell, which is not supE, since the translation stop codons of the A_{1a}B_{1b} and A₂B_{1a} cDNAs are amber codons (TAG). We cultured JM105 harbor-

ing pKG2(A_{1a}B_{1b}) in M9 medium containing casamino acids, glucose, thiamine and ampicillin until the early log phase, log phase or early stationary phase, and then added IPTG to derepress the *trc* promoter (see

TABLE I. DIFFERENCES IN THE NUCLEOTIDE AND AMINO ACID SEQUENCES OF A₂B_{1a} cDNA AMONG VAR. SHIROTSURUNOKO, BONMINORI AND CX635-1-1-1

Region	Nucleotide				Amino acid			
	No.	ST ^a	BM ^a	CX ^a	No.	ST	BM	CX
Signal	95	T	C	?	-1	Ala	Ala	?
Acidic	96	C	T	?	1	Leu	Leu	?
	155	G	A	?	20	Pro	Pro	?
	157	A	G	?	21	Asp	Gly	?
Basic	1265	C	T	T	390	Phe	Phe	Phe
	1460	G	T	A	455	Leu	Leu	Leu
3'—	1511	T	— ^b	T				
	1533	T	—	T				
	1616	T	C	T				

^a ST, BM and CX refer to var. Shiotsurunoko, Bonminori and CX635-1-1-1, respectively.

^b — means deletion.

MATERIALS AND METHODS). After various induction periods, the cells were harvested and lysed in the SDS-sample buffer in the presence of PMSF and EDTA. The proteins were analyzed by immunoblotting as described under MATERIALS AND METHODS. However, we could not detect the glycinin subunit proteins at the sensitivity of 20 ng/ml of culture. There are three possible explanations for the nondetection; 1, no expression; 2, only a small amount of the product due to the formation of a secondary structure during transcription and/or translation, different codon usage and so on; 3, instability of the products in *E. coli*.

In order to explain why we could not detect the expressed product, the *in vitro*-expression of the cDNAs was examined employing a commercial *in vitro*-expression system. The resultant products of pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) were analyzed by SDS-PAGE, as shown in Fig. 3A. The products of pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) gave bands

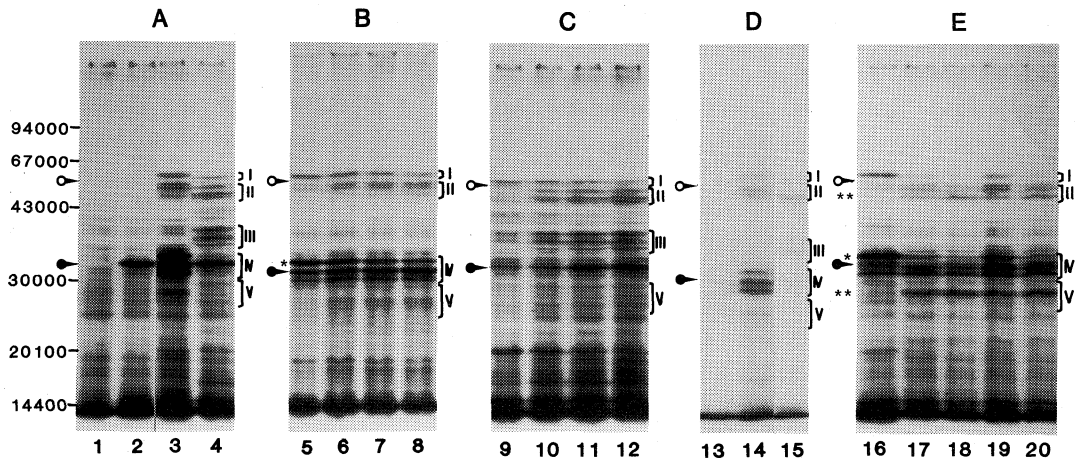


FIG. 3. *In Vitro* Expression of cDNAs Encoding the A_{1a}B_{1b} and A₂B_{1a} Subunits.

A. pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) were expressed for 45 min: Lane 1, no plasmid; lane 2, pKK233-2; lane 3, pKG2(A_{1a}B_{1b}); lane 4, pKG4(A₂B_{1a}). B. Time course of the incubation for the expression of pKG2(A_{1a}B_{1b}): Lanes 5~8 are for 15, 30, 45 and 60 min expression. C. Time course of the incubation for the expression of pKG4(A₂B_{1a}): Lanes 9~12 are for 15, 30, 45 and 60 min expression. D. Immunoprecipitation: Lane 13, pKK233-2; lane 14, pKG2(A_{1a}B_{1b}); lane 15, pKG4(A₂B_{1a}). E. Effect of S-100 of JM105 cells harboring pKG2(A_{1a}B_{1b}) (Lanes 17 and 18) or pKK233-2 (lanes 19 and 20). S-100 was added to the 15 min expressed sample of pKG2(A_{1a}B_{1b}) (lane 16) and then chased for 15 min (lanes 17 and 19) and 45 min (lanes 18 and 20). In order to distinguish the bands, the characteristic bands were divided into regions I~V. ○ and ● indicate the positions of the mature glycinin subunit and β-lactamase, respectively. The numbers on the left denote molecular weights.

in region I, the molecular weights were 59 K and 58 K dalton, which are greater than that of mature glycinin subunits (about 55 K). The difference in the molecular weights of the bands in region I between pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) was one K dalton. This corresponds to the difference in the polypeptide lengths of the A_{1a}B_{1b} and A₂B_{1a} subunit precursors, the former is composed of 495 amino acids and the latter of 485. The bands in region I were not observed in the absence of a plasmid (blank, lane 1) or in the presence of pKK233-2 (control, lane 2). These results clearly indicate that the bands in region I are full size products of cDNAs encoding the A_{1a}B_{1b} and A₂B_{1a} subunits. In other words, pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) can be expressed in *E. coli*.

The *in vitro*-expressed products of pKG2(A_{1a}B_{1b}) gave extra bands with the fairly high intensities in regions II, IV and V (lane 3), and those of pKG4(A₂B_{1a}) ones in regions II, III and V (lane 4). These bands might be due to degradation of the full size products and incomplete transcription and /or translation due to the formation of a secondary structure, since these bands were not observed in lanes 1 and 2. To investigate the possibility of proteolysis, the time course of incubation for *in vitro*-expression was investigated, as shown in Fig. 3-B and C. Both pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) gave the most intense bands in region I at the shortest expression time (15 min, lanes 5 and 9). The longer the expression time, the more the intensities of the bands in region I decreased. The band of pKG2(A_{1a}B_{1b}) marked with an asterisk was the most predominant and decreased with the time of incubation (Fig. 3B). Concomitantly, the bands in regions II and V, and the band with the lowest molecular weight in region IV increased in the case of pKG2(A_{1a}B_{1b}), and the bands in regions II, III and V increased in the case of pKG4(A₂B_{1a}). The bands in regions II, III, IV and V as well as those in region I except the band of β -lactamase were precipitated with the glycinin-antiserum (Fig. 3D). It is suggested from these results that the bands in regions II, III, IV and V were derived

on degradation of the full size products. Although the 15 min expression products were treated with the glycinin-antiserum, the patterns and the ratios of the amounts of the bands in regions I, II, III and IV changed after the antiserum treatment (compare lanes 14 and 15 with lanes 5 and 9), especially in the cases of pKG4(A₂B_{1a}) and the band in region IV, marked with an asterisk, of pKG2(A_{1a}B_{1b}), indicating that proteolysis proceeded during the antigen-antibody reaction (see MATERIALS AND METHODS), and that the smaller degradation products (regions III, IV and V) might have weaker reactivity with the antibody than the larger degradation products and the full size products.

After 60 min incubation, we could observe the bands in region I (lanes 8 and 12), whereas the products on *in vivo*-expression were not detected. Proteolytic activities may be revealed more strongly in JM105 cells than in the *in vitro*-expression system. To confirm this, we examined the effect of S-100 of JM105 cells harboring pKK233-2 or pKG2(A_{1a}B_{1b}) on the *in vitro*-expression of pKG2(A_{1a}B_{1b}) (Fig. 3E). After the addition of the S-100 of JM105 cells harboring pKG2(A_{1a}B_{1b}) to 15 min incubated samples of pKG2(A_{1a}B_{1b}), the intensities of the bands in region I and the band marked with an asterisk in region IV decreased dramatically under the conditions used (Fig. 3E, lanes 17 and 18). When we used the S-100 of JM105 cells harboring pKK233-2, they decreased on 45 min incubation. Dramatic changes were not seen after 15 min incubation. The bands marked with two asterisks in regions II and IV increased concomitantly with the decreases of the bands in regions I and IV. Thus, the proteolytic activities of JM105 cells may be stronger than in the *in vitro* system. This suggests that the expression of glycinin subunits in the cells may have induced protease(s) which degrade(s) abnormal proteins (glycinin subunits).

The data obtained here (Fig. 3) indicate that glycinin subunit proteins are readily degraded even in an *in vitro* system after the expression. It is reasonable to assume that glycinin subunit

proteins after the expression in the cell may occur under circumstances where the proteolytic activity is induced or activated. Therefore, the nondetection of the *in vivo*-expression products of pKG2(A_{1a}B_{1b}) and pKG4(A₂B_{1a}) is due to degradation of the products. This is supported by that many eukaryotic foreign peptides are recognized as abnormal proteins in *E. coli* and consequently are degraded.³⁰⁾

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