Improved Bile Acid-binding Ability of Soybean Glycinin A1a Polypeptide by the Introduction of a Bile Acid-binding Peptide (VAWWMY)

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We have previously identified a potential bile acid-binding peptide sequence (VAWWMY) in acidic polypeptide A1a of the soybean glycinin A1aB1b subunit (Choi, S. K., et al., Biosci. Biotechnol. Biochem., 66, 2395–2401 (2002)). In this study, we introduced the nucleotide sequence encoding this peptide in the coding DNA which corresponds to amino acids between 251 and 256, and 282 and 287 into the A1a polypeptide by replacement to respectively give modified versions A1aM1 and A1aM2. A fluorescence analysis demonstrates that their bile acid-binding ability was improved compared to A1a. Moreover, modified proglycinin A1aB1b with the VAWWMY sequence at the same sites as those of A1aM1 and A1aM2 was judged to assume the correct conformation. These results suggest the possibility of developing transgenic crops to accumulate the modified glycinin.

Key words: soybean; glycinin; bile acid-binding peptide

Many studies on the hypocholesterolemic effects of soy proteins1,2) have supported the hypothesis that a peptide with high bile acid-binding ability could inhibit the reabsorption of bile acid in the ileum, or decrease the micellar solubility of cholesterol in the small intestinal epithelial cells and decrease the blood cholesterol level. Soybean glycinin (11S globulin) has five subunits which are classified into two groups according to their homology in amino acid sequences (group I, A1aB1b, A1bB2 and A2B1a; group II, A3B4 and A5A4B3). We have reported from the previous study3) that acidic polypeptide A1a of A1aB1b had higher bile acid-binding ability than that of A3 of A3B4 and also identified the potential bile acid-binding peptide, VAWWMY, in the A1a polypeptide.

Bioactive peptides derived from food proteins sometimes have beneficial effects on human health. It is therefore reasonable to assume that food protein quality can be improved by introducing a favorable oligopeptide sequence through site-directed mutagenesis of the genes. Makino and co-workers4,5) have reported that acidic polypeptides A1a and A2 had bile acid-binding abilities, and we have confirmed this.6) In the present study, we introduced the VAWWMY sequence into glycinin A1a polypeptide to improve its bile acid-binding ability and also investigated attainment of the correct conformation for proglycinin A1aB1b with the VAWWMY sequence.

To introduce the VAWWMY sequence by replacement, we selected the regions from 251 to 256 and 282 to 287 amino acids in variable region IV, which is longest among the five variable regions,6) because this region has been proven to have high tolerance to modification.7) To construct the expression plasmids (pEA1aM1, pEA1aM2, pEA1aB1bM1 and pEA1aB1bM2) for A1aM1, A1aM2, A1aB1bM1 and A1aB1bM2 (Fig. 1), six synthetic oligonucleotide primers (5'-TGGATGTACCCCCAGGAAGAGGAAGAAGAGGATGAG-3' and 5'-CCACGCAACCGGTGTTTTATCACGCTAGACCTCC-3' for A1aM1 and A1aB1bM1; 5'-TGGATGTACAGCAGAAATTAGAATTCCGGATCCGAATTC-3' and 5'-CCACGCAACGGGGCGTTGGCAGTGTTTGTCTTTACCCTTG-3' for A1aM2; and 5'-TGGATGTACGCAGAAGAAATGGCATTGACGAGACCATATG-3' and 5'-CCACGCAACCGGTGTTTTATCACGCTAGACCTCC-3' for A1aB1bM2) were designed.

The DNAs for modified versions of A1a and A1aB1b were amplified by the polymerase chain reaction (PCR), using pEA1a3) and pEA1aB1b8) as templates. After phosphorylation with T4 polynucleotide kinase (Toyobo), the PCR products were self-ligated with a DNA ligation kit (Takara). The sequences of the DNA regions corresponding to the coding regions were confirmed by using a 310 automated DNA sequencer (Applied Biosystems).

Five hundred ml of a TB medium containing 50 μg/ml of carbenicillin was inoculated with 4 ml of a fully grown culture of BL21(DE3) (Novagen) harboring pEA1a, pEA1aM1 and pEA1aM2, and of AD494(DE3) (Novagen) harboring pEA1aB1b, pEA1aB1bM1 and pEA1aB1bM2, and the culture was incubated with shaking at 37°C. When the OD600 value of the culture...
had reached about 0.5, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 1 mM and cultivation continued for a further 16 hr at 37°C for normal A1a and its modified versions, and at 25°C for normal A1aB1b and its modified versions. The cells were then harvested by centrifugation and stored at −80°C until their further use.

Frozen cells containing normal A1a and its modified versions were resuspended in 10 ml of a buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM NaCl, 10 mM EDTA, 0.1 mM (p-amidinophenyl)methylsulfonyl fluoride, 1 μM pepstatin A and 2 μM leupeptin) per gram of cells and disrupted by sonication on ice and centrifuged at 10,000 g for 10 min at 4°C (Fig. 2A). We used the insoluble fraction of each for purification to ensure simplicity. The precipitate was washed twice with the foregoing buffer to remove the soluble proteins. The pellet was dissolved in 4 M urea and clarified by centrifugation at 14,000 g for 40 min at 4°C. The concentration of protein in the sample was determined with a protein assay kit (Bio-Rad) according to the manufacturer’s instructions. The purity of A1a, A1aM1 and A1aM2 (Fig. 2B) was respectively calculated to be 90%, 88% and 92% densitometrically by using NIH image program.

To compare the bile acid-binding ability of expressed normal A1a and its modified versions, the fluorescence of proteins was measured with an F-3000 fluorescence spectrophotometer (Hitachi), and the dissociation constant (Kd) of each sample for sodium cholate was calculated by the Kaleida graph as previously described. This measurement was done in 4 M urea, since the Kd values of normal A1a in 0, 1, 2, 3 and 4 M urea were

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**Fig. 1.** Schematic Representation of the Modified Versions A1a and A1aB1b.

(A) The modified versions of A1a are named A1aM1 and A1aM2. (B) The modified versions of A1aB1b are named A1aB1bM1 and A1aB1bM2. The replaced amino acid sequence is shown underlined. Closed and open areas are the variable and conserved regions, respectively. The five variable regions are labeled I–V. The numbers in the figure indicate the residue number from the N-terminal, and the letters indicate the amino acid sequence of variable region IV.

**Fig. 2.** SDS-PAGE Analysis of Normal A1a and Its Modified Versions Expressed in *E. coli*.

(A) *E. coli* strain BL21(DE3) cells harboring individual expression plasmids for A1a, A1aM1 and A1aM2 were analysed by SDS-PAGE in 11% gels. T, total fraction of the cells; S, soluble fraction of the cells; I, insoluble fraction of the cells. The numbers on the left denote molecular masses. (B) Partially purified proteins from the insoluble fractions of proteins expressed in *E. coli* were analysed by SDS-PAGE in 11% gels.
very similar to each other. The Kd values of A1aM1 and A1aM2 were 30 and 37 mM, respectively, which are lower than 49 mM of A1a. In other words, both modified A1a versions exhibited higher bile acid-binding ability compared to the normal A1a chain.

In order to learn whether proglycinin A1aB1b could tolerate the modifications without misfolding of the protein structure, we constructed expression plasmids for modified versions A1aB1bM1 and A1aB1bM2 in which the VAWWMY peptide sequence had been respectively introduced at the same site as that of A1aM1 and A1aM2. We have previously proposed that the criteria for judging proper conformation should be as follows: (1) the solubility should be comparable with that of globulins, (2) there must be self-assembly into trimers, and (3) there must be high-level expression (=10% of total E. coli proteins). Densitometric scanning of the gels showed that the expression levels of normal A1aB1b, A1aB1bM1 and A1aB1bM2 were more than 20% of total E. coli proteins, and 85%, 70% and 60% of expressed normal A1aB1b, A1aB1bM1 and A1aB1bM2 were respectively recovered in the soluble fraction (Fig. 3-1). To examine self-assembly of the modified A1aB1b versions into trimers, the soluble fractions of A1aB1bM1 and A1aB1bM2 proteins were applied to a HiPrep 16/60 Sephacryl S-300 HR column (Pharmacia Biotech). Mature glycinin purified from soybean seeds and BSA (Wako) were applied to the same column as size markers. The flow rate was 0.5 ml/min, 2-ml fractions were collected, and the fractions were analysed by SDS-PAGE (A and Western-blotting (B and C). (A), A1aB1b; (B) A1aB1bM1; (C), A1aB1bM2.

Fig. 3. Expression in E. coli and Gel Filtration Analysis of Normal A1aB1b and Its Modified Versions.

(1) E. coli strain AD494(DE3) cells harboring individual expression plasmids for A1aB1b (A), A1aB1bM1 (B) and A1aB1bM2 (C) were analysed by SDS-PAGE in 7.5% gels. T, total fraction of the cells; S, soluble fraction of the cells; I, insoluble fraction of the cells; M, purified normal A1aB1b. (2) Purified normal A1aB1b and the soluble fractions of E. coli cells harboring individual expression plasmids for A1aB1bM1 and A1aB1bM2 were subjected to gel filtration chromatography. The flow rate was 0.5 ml/min, 2-ml fractions were collected, and the fractions were analysed by SDS-PAGE (A) and Western-blotting (B and C). (A), A1aB1b; (B) A1aB1bM1; (C), A1aB1bM2.

ed, we conclude that the modified A1aB1b versions were able to assume the correct conformation, although their folding ability was a little lower than normal A1aB1b. Correct folding of modified glycinin precursors in E. coli has resulted in the accumulation of modified mature glycinins in the protein storage vacuole of tobacco and rice. This suggests that it will be possible to create transgenic crops that accumulate modified glycinin with improved bile acid-binding ability.

Some successful attempts have recently been made to improve the physiological functions of food proteins by introducing a favorable oligopeptide sequence by site-directed mutagenesis of the genes. However, this study is the first report on the improvement of bile acid-binding ability of glycinin by the incorporation of the VAWWMY sequence.

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References

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