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Molecular and structural analysis of electrophoretic variants of soybean seed storage proteins

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Abstract

Soybean (*Glycine max* L.) storage proteins are composed mainly of two major components, β-conglycinin and glycinin. Electrophoretic variants of the β subunit of β-conglycinin and the A3 polypeptide of glycinin were detected on SDS-PAGE, and designated them as β* and A3*, respectively. β* and A3* exhibited higher and lower mobilities, respectively, than the common β subunit and A3 polypeptide. The N-terminal nine and 10 amino acid sequences of β* and A3* were completely identical to the previously reported sequences of the β subunit and the A3 polypeptide, respectively. Analysis using concanavalin A-horseradish peroxidase and treatment with N-glycosidase indicated that glycans were not responsible for the difference in electrophoretic mobility of β* or A3*. Furthermore, five clones of β* or β and three clones of A3*, respectively, were sequenced but we could not detect deletions and insertions except for a single or a few amino acid substitutions as compared with the common β subunit and A3 polypeptide. These results indicate that a single or a few amino acid substitution affects the electrophoretic mobilities of β* and A3*.

Keywords: Leguminosae; Soybean; Seed storage protein; Glycinin; β-Conglycinin; Electrophoretic variant; *Glycene max*

1. Introduction

Soybean (*Glycine max* L.) storage proteins are composed of two major components, β-conglycinin and glycinin corresponding to 7S and 11S globulins, respectively (Derbyshire et al., 1976; Shewry et al., 1995). Both are stored in vacuoles of seed cells and account for about 80% of storage proteins. β-Conglycinin is a trimeric protein with a molecular mass of 150–200 kDa, being composed of three subunits, α (~67 kDa), α′ (~71 kDa) and β (~50 kDa). Each subunit is processed by co- and post-translational modifications during biosynthesis (Ladin et al., 1987; Utsumi, 1992). For example, all subunits of β-conglycinin are glycosylated and the α and α′ subunits are processed at N-terminal regions (Utsumi, 1992; Doyle et al., 1986). An amino acid sequence of each subunit is variable among cultivars (Maruyama et al., 1998). On the other hand, glycinin is a hexameric protein with a molecular mass of 300–380 kDa. Subunits of glycinin are composed of acidic (A1a, A1b, A2, A3, A4, A5) and basic (B1a, B1b, B2, B3, B4) polypeptides linked by a disulfide bond, and are not glycosylated. Glycinin exhibits polymorphism of the subunit composition among the cultivars (Mori et al., 1981). Moreover, an amino acid sequence of a subunit is variable among cultivars similar to β-conglycinin (Utsumi et al., 1987).

Davies et al. (1985) reported that there are many electrophoretic variants of β-conglycinin having mobilities different from the equivalent common ones on SDS-PAGE. Furthermore, Coates et al. (1985) reported the existence of β′ subunit which migrates faster on

Abbreviations: ConA-HRP, concanavalin A-horseradish peroxidase, DSC, differential scanning calorimetry, *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride, PNGase F, N-glycosidase F, RT-PCR, reverse transcription-polymerase chain reaction.

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SDS-PAGE than the β subunit. However, analysis at the molecular level of the electrophoretic variants remained to be performed. Here, we investigated the protein components of the seed storage proteins in many cultivars of soybean, detected and identified β* subunit of β-conglycinin and A3* polypeptide of glycinin whose electrophoretic mobilities were higher and lower, respectively, than the common ones. To know why their electophoretic mobilities were different from the common ones, we analyzed the glycans, the primary sequences and the protein chemical properties of the electrophoretic variants.

2. Results

2.1. Screening of electrophoretic variants

For screening electrophoretic variants of soybean storage proteins from germplasm lines, protein extracts from 5828 soybean cultivars were analyzed by SDS-PAGE. We observed that the seeds of 45 cultivars including 'Gotenba Zairai' contained β* in addition to the β subunit of β-conglycinin, which migrated faster than the β subunit on SDS-PAGE (Fig. 1, lane a), and that the seeds of 3 cultivars including 'Zairaishu 51-1-2′ contained A3* instead of the A3 polypeptide of glycinin, which migrated slower than the A3 polypeptide on SDS-PAGE (Fig. 1, lane b). To characterize these polypeptides, we analyzed the extracts by 2D electrophoresis. β* gave several spots with different isoelectric points in comparison with the β subunit (Fig. 2A). The proteins were extracted from each spot of the gel and determined their N-terminal sequences. N-terminal nine amino acid sequences of all spots were completely identical to that of the β subunit previously reported (Maruyama et al., 1998). The N-terminal sequences of β* of 3 of 45 cultivars having β* were also identical to that of the β subunit. On the other hand, A3* gave two spots with different isoelectric points (Fig. 2B) in comparison with A3 polypeptide (Fig. 2A). The 10 N-terminal amino acid sequences of both spots were completely identical to the previously reported sequence of the A3 polypeptide (Nielsen et al., 1989).

2.2. Detection of N-glycan

The common β subunit is glycosylated, but the normal A3 polypeptide is not, although some of the A3 polypeptides have a potential N-glycosylation site (Fukazawa et al., 1985). Therefore, there is a possibility that the differences in electrophoretic mobility between the β subunit and β* and between the A3 polypeptide and A3* are due to the presence and absence of N-linked glycans. To assess this point, we analyzed the

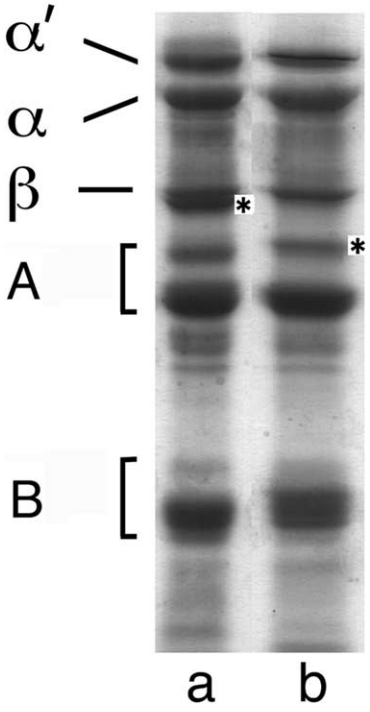

Fig. 1. SDS-PAGE patterns of proteins from unique soybean cultivars. α′, α and β indicate subunits of β-conglycinin. A and B indicate acidic and basic polypeptides of glycinin, respectively. Astarisks indicate β* (lane a) and A3* (lane b). Lane a, 'Gotenba Zairai'; lane b, 'Zairaishu 51-1-2′.

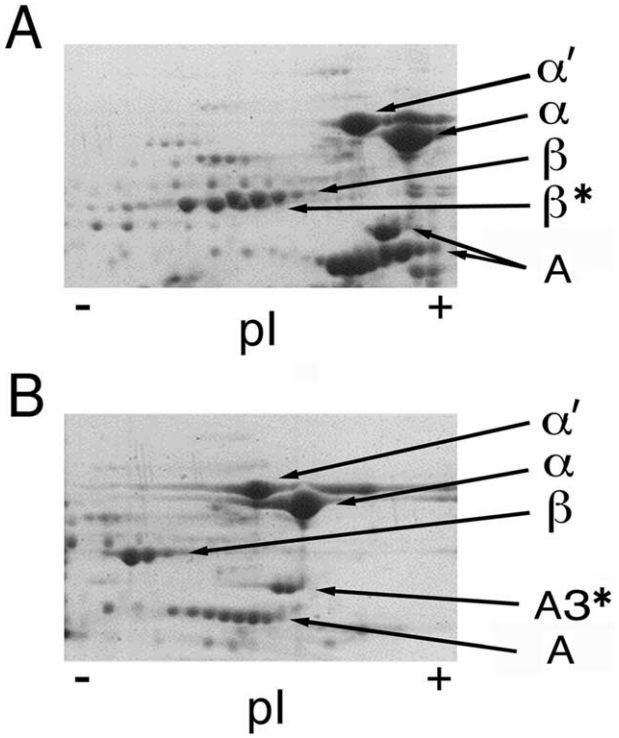

Fig. 2. 2D electrophoresis patterns of proteins from unique soybean cultivars. Gels were stained with coomassie brilliant blue R-250. α′, α and β indicate subunits of β-conglycinin. A indicates acidic polypeptides of glycinin. Panel A, 'Gotenba Zairai'; panel B, 'Zairaishu 51-1-2′.

extracts of soybean seeds ('Gotenba Zairai' and 'Zairaishu 51-1-2′) by using the ConA-HRP method. All spots derived from β* were stained similarly to those of the β subunit (Fig. 3), but the two spots derived from A3* were not stained similarly to those of the A3 polypeptide (Fig. 3). The result indicated that the differences in electrophoretic mobilities between the β subunit and β* and between the A3 polypeptide and A3* are not due to the presence or absence of N-linked glycans.

2.3. Purification of β* and β homo and heterotrimer

To characterize the protein chemical properties of β*, we attempted to purify β* and β homo and heterotrimers from the seeds of 'Gotenba Zairai'. It is known that β homotrimer exists in a glycinin-rich fraction, although most of the molecular species of β-conglycinin are fractionated in a β-conglycinin-rich fraction (Sykers and Gayler, 1981). Therefore, we attempted to purify β* and β homo and heterotrimers from a glycinin-rich fraction by ammonium sulfate fractionation and Mono Q column chromatography. Four major peaks corresponding to the homo and heterotrimers composed of β* and/or the β subunit judged by the SDS-PAGE profile were detected (Fig. 4). In this paper, the homotrimers of β* and the β subunit were designated β*3 and β3, respectively, and the heterotrimers composed of two β* and one β and one β* and two β were designated β*2β1 and β*1β2, respectively.

2.4. Glycosidase treatment

To confirm that the difference of the electrophoretic mobilities of polypeptides between the β* and the β subunit were not due to the presence and absence of N-linked glycan, we deglycosylated the purified β*3 and β3 by PNGase F. Although treatment of PNGase F resulted in the increase of their mobilities (Fig. 5, compare lanes 1and 2 with lanes 3 and 4), it did not affect the difference in the mobility (lanes 3 and 4). Therefore, this result together with that of the glycan detection by ConA-HRP (Fig. 3) clearly indicates that N-linked glycans have no effect on the difference of the electrophoretic mobility between the β* and the β subunit.

2.5. Thermal stability

The thermal stability of the homo and heterotrimers composed of β* and/or the β subunit are shown in Fig. 6. β3 purified from the seeds of 'Gotenba Zairai' exhibited a T_m value (89.7 °C) similar to that of the

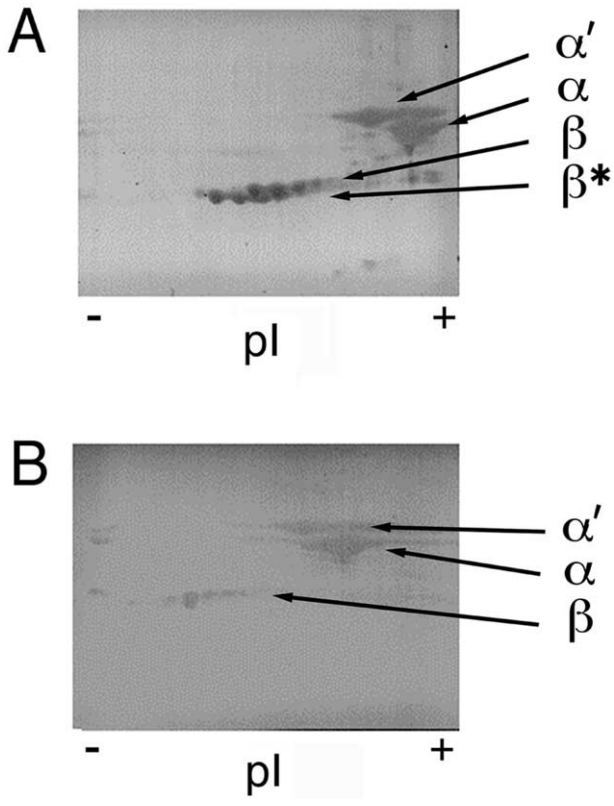

Fig. 3. Detection of glycoproteins in extracts from unique soybean cultivars on 2D electrophoresis by a method using ConA-HRP. α′, α and β indicate subunits of β-conglycinin. Panel A, 'Gotenba Zairai'; panel B, 'Zairaishu 51-1-2′.

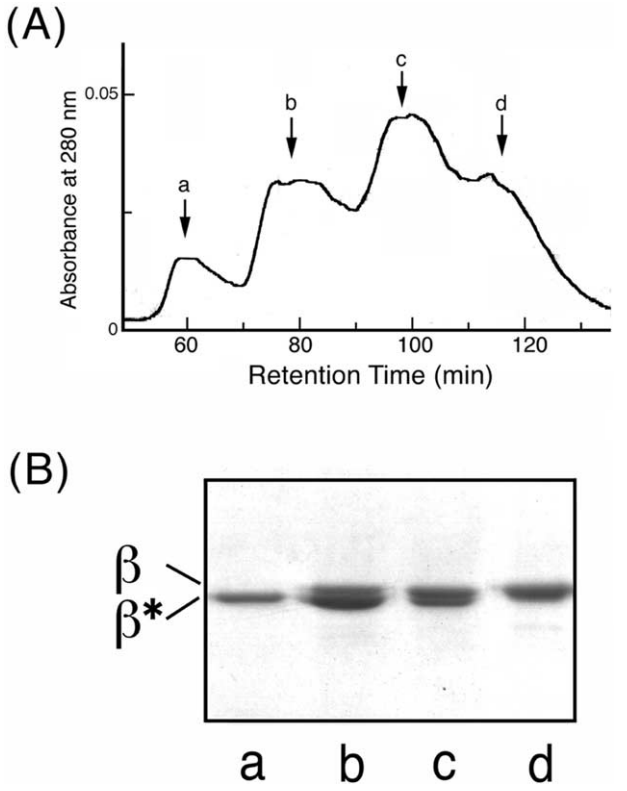

Fig. 4. Purification of molecular species composed of the β subunit and/or β*. (A) Pattern of MonoQ column chromatography. a-d indicate peaks. (B) SDS-PAGE profiles of peaks a-d. Peak a, β*3; peak b, β*2β1; peak c, β*1β2; peak d, β3.

recombinant β3 (90.7 °C) whose cDNA was prepared from the developing seeds of 'Wasesuzunari' (Maruyama et al., 1998), with a small shoulder at a lower temperature than that of the major peak. On the other hand, β2β*1, β1β*2 and β*3 gave a major peak at 89.7 °C with a minor peak at 83.7 °C, and the minor peak of β*3 was significantly larger than those of β2β*1 and β1β*2. In the previous studies, the homotrimers composed of one kind of subunit of β-conglycinin gave single peaks without shoulders under the same condition as here (Maruyama et al., 1998, 2002). Therefore, β* and the β subunits ('Gotenba Zairai') probably contain several molecular species having different thermal stability.

2.6. Cloning of β* and A3*B4cDNAs

We isolated the cDNA of the β subunit or β* of 'Gotenba Zairai' by RT-PCR and sequenced five clones (Gmβ/β*1–5). Two to six nucleotides in Gmβ/β*1–5 were different from the sequence of the β subunit ('Wasesuzunari') reported previously (Maruyama et al., 1998). We aligned the deduced amino acid sequences of Gmβ/β*1–5 with that of the β subunit ('Wasesuzunari') (Fig. 7). We could not detect deletions, which might account for the difference of the electrophoretic mobility, in all the sequences compared with that of the β subunit ('Wasesuzunari'). Phe13 in the β subunit ('Wasesuzunari') was substituted for Leu in Gmβ/β*2, 3 and 5. This substitution is observed in the β subunit of the mutant variety lacking α and α′ subunits (Maruyama et al., 2001). Glu177 and Glu178 in the β subunit ('Wasesuzunari') were replaced by Gln and Gly, respectively, in both Gmβ/β* 4 and 5. Tyr93 and Arg222 in the β subunit ('Wasesuzunari') were replaced by His in Gmβ/β*2 and 3, respectively. These replacements affect isoelectric points (pI of Wasesuzunari calculated from an amino acid sequence, 5.59; that of Gmβ/β*2, 5.67; that of Gmβ/β*3, 5.56; those of Gmβ/β*4 and 5, 5.83). Therefore, in addition to posttranslational modifications (Ladin et al., 1987), the primary structure may be responsible for the charge heterogeneity of the β subunit and β* on 2D electrophoresis (Fig. 2). Cysteine residues were detected in Gmβ/β*1 and 4, although all the sequences of the β subunit reported so far do not contain cysteine residues. Therefore, this is a first report that the β subunit contains cysteine residues. The possibility that PCR causes the mutations at these positions is very low, because the enzyme used for PCR in this experiment has a relatively high fidelity.

On the other hand, we sequenced the regions of A3* in three clones of cDNA ('Zairaishu 51-1-2′) for A3*B4. The fact that we could not detect the band of the A3 polypeptide on SDS-PAGE of the extracts from the seeds ('Zairaishu 51-1-2′) indicates that the sequences

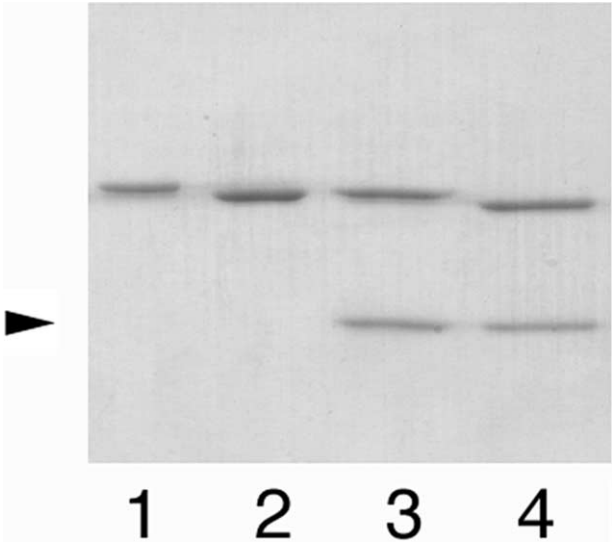

Fig. 5. Effect of PNGase F on the mobility of the β subunit and β* on SDS-PAGE. SDS-PAGE of β3 (lanes 1 and 3) and β*3 (lanes 2 and 4) without (lanes 1 and 2) and with (lanes 3 and 4) PNGase F treatment. Arrowhead indicates the position of PNGase F.

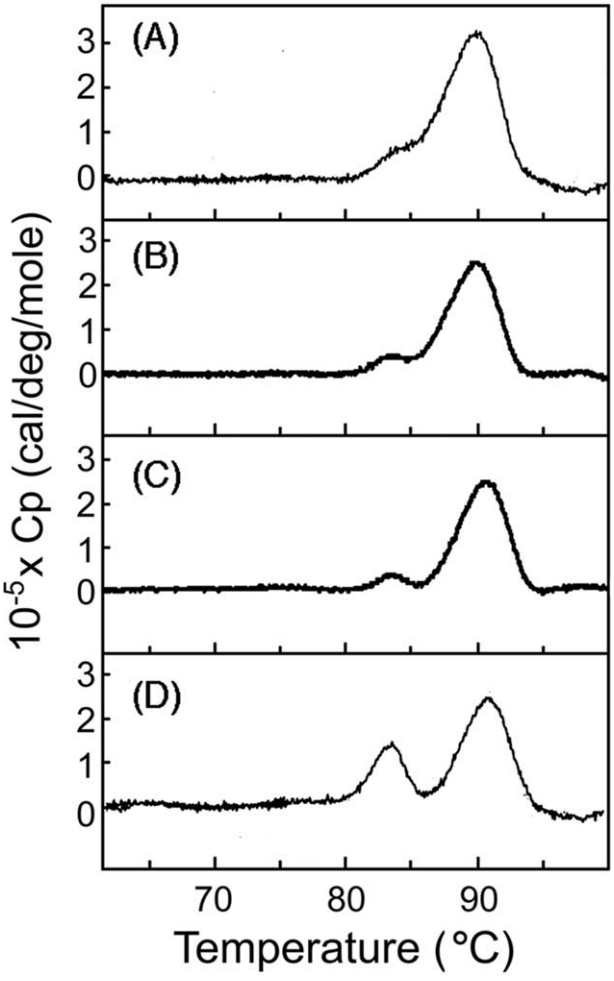

Fig. 6. DSC scans of the homo and heterotrimers composed of the β subunit and/or β*. Panel A, β3; panel B, β2β*1; panel C, β1β*2; panel D, β*3.

determined here were derived from that of A3* in A3*B4. Compared with that of the A3 polypeptide in A3B4 ('Wasesuzunari', Accession numbers AB049440), we could not detect insertions except a difference in only one position (Arg22→Leu) in all clones. Further, we confirmed that this change exists in soybean genome by sequencing of genomic PCR product. It is known that the A3 polypeptide ('Wasesuzunari') migrates normally on SDS-PAGE. Therefore, it is considered that the replacement of a single amino acid affects the electrophoretic mobility of A3*.

2.7. TOF mass spectrometry and C-terminal amino acid sequencing

Molecular masses of the β subunit and β* determined by TOF mass spectrometry were very similar to each other (β, 48,120 Da; β*, 48,147 Da) (data not shown). This is consistent with the result that we could not detect deletions in all the sequences of the β subunit or β* ('Gotenba Zairai') compared with that of the common one as described above. However, these values (β, 48,120 Da; β*, 48,147 Da) were smaller than the expected values (49,400-49,802 Da) of the β subunit and β* which were calculated from their molecular masses (47,842-47,920 Da) predicted from the amino acid sequences and those (1,558 to 1,882 Da) of the glycans of β-conglycinin previously reported (Yamauchi et al., 1975; Yamauchi and Yamagishi, 1979). On the other hand, the molecular mass (47,972 Da) of the recombinant β having no N-linked glycans determined by TOF mass spectrometry was almost identical to that (47,973 Da) predicted from the deduced amino acid sequence, indicating that we could measure masses of the samples correctly. We suggest that the C-terminal peptides of the β subunit and β* may be processed, because the N-terminal amino acid sequences of the β subunit and β* ('Gotenba Zairai') were identical to that of the common one. Therefore, we examined the C-terminal amino acid sequences of the β subunit and β* by a C-terminal amino acid sequencer. Although we applied sufficient amounts of the samples to the sequencer, clear peaks were not detected for both the samples. The C-terminus of both the β subunit and β* may be proline residues resulting from C-terminal peptide cleavage since C-terminal proline residues cannot be analyzed by C-terminal amino acid sequencer.

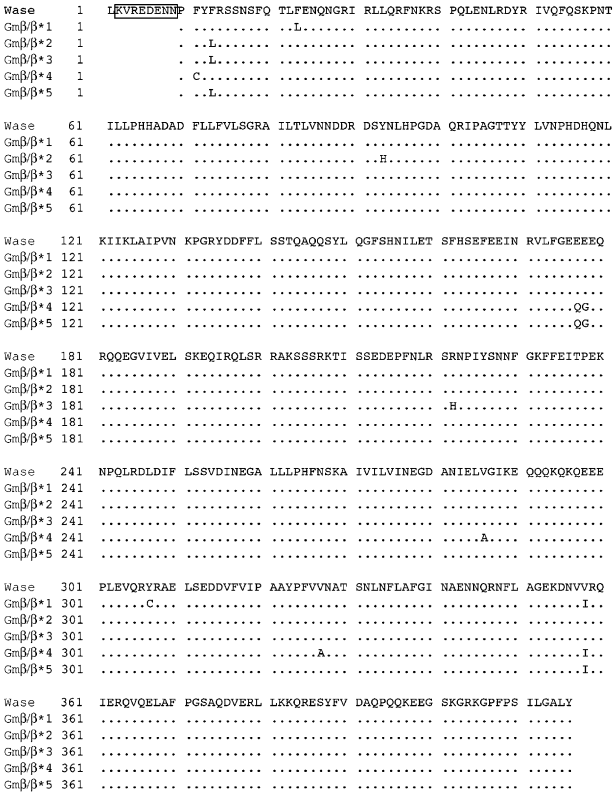

Fig. 7. Alignment of the deduced amino acid sequences of β* or the β subunit of 'Gotenba Zairai' with that of the β subunit of 'Wasesuzunari'. Sequences of the β subunit of 'Wasesuzunari' and five clones of β* or the β subunit of 'Gotenba Zairai' are indicated by Wase and Gmβ/β*1–5, respectively. The sequences of Gmβ/β*1–5 identical to that of 'Wasesuzunari' are indicated by dots and the region for the degenerated primer is surrounded by a box.

3. Discussion

3.1. Electrophoretic variants of soybean seed storage proteins

We determined the sequences of the regions of A3* in three clones of A3*B4 and revealed that these sequences were completely identical to that of the A3 polypeptide previously reported except that Arg22 was substituted for Leu. Furthermore, we could not detect deletions in the sequences of the β subunit or β* ('Gotenba Zairai') compared with the common one. Pacheco et al. (1984) indicated that urea alters the relative electrophoretic mobility of several soybean subunits on SDS-PAGE. From this observation, Davies et al. (1985) pointed out that structural changes that have effects on the electophoretic mobility need not result from large alterations in the primary structure but may arise from a change as small as one amino acid substitution, despite no experimental data. Furthermore, although the molecular mass (65,411 Da) of the recombinant α′ subunit of β-conglycinin containing potent anti-hypertensive peptide sequences is slightly larger than that (65,140 Da) of the wild type, it shows a slightly faster electrophoretic mobility relative to the wild type on SDS-PAGE (Matoba et al., 2001). These reports together with our results indicate that the substitution of a single or a few amino acids of soybean storage proteins change the electrophoretic mobility.

Coates et al. (1985) analyzed the β′ subunit by CNBr cleavage whose electrophoretic mobility is higher

than that of the β subunit and indicated that it contains at least two methionine residues. However, the amino acid sequences of Gmβ/β*1–5 did not contain the methionine residues at all. Further, the N-terminus of the β′ subunit is registant to Edman degradation (Coates et al., 1985), although that of β* was sensitive. These results indicate that β* is different from the β′ subunit. Therefore, there is still a possibility that the β′ subunit contains deletions which affect the electrophoretic mobility compared with the common one.

3.2. Cleavage at C-terminal region of β subunit

Previously, it was reported that three Asn-carbohydrates ($(GlcNac)_2$ - $(Man)_7$, $(GlcNac)_2$ - $(Man)_8$, $(GlcNac)_2$-$(Man)_9$) were isolated from a pronase digest of β-conglycinin (Yamauchi et al., 1975; Yamauchi and Yamagishi, 1979). The molecular masses of these components are 1,558 to 1,882 Da. The differences in the molecular masses between the glycosylated β purified from the seed ('Gotenba Zairai') and the recombinant β having no glycans (148 Da) as well as between β* ('Gotenba Zairai') and the recombinant β (175 Da) are smaller than those of the carbohydrate moieties (1,558 to 1,882 Da). Further, we could not determine C-terminal sequences of the β subunit and β*, although the C-terminal amino acid predicted from cDNA is not proline residue. These suggest that the C-terminal peptides of the β subunit and β* might be cleaved at Pro407 or Pro409. If the cleavage occurs at either of these residues, the predicted molecular masses of the cleaved β subunit and β* are 48,420-49,067 Da, calculated from the expected values of the β subunit and β* (49,400–49,802 Da) and those of the cleaved peptides (735 and 980 Da). However, these values (48,420–49,067 Da) are slightly different from those (β, 48,120 Da; β*, 48,147 Da) of the β subunit and β* measured in this study. We suppose that parts of the glycans of the β subunit and β* might be removed through the process of purifications.

It is known that many vacuolar proteins such as lectin and 2S albumin have pro regions at C-terminus (Matsuoka and Neuhaus, 1999). Further, it has been reported that phaseolin, the 7S globulin of french bean, also has a pro region at C-terminus (Frigerio et al., 2001). These reports support our idea that the C-terminal peptides of the β subunit and β* may be cleaved.

4. Experimental

4.1. Materials

Soybean seeds (5828 cultivars) were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan.

4.2. SDS-PAGE analysis of proteins from soybean cultivars

Seed meals (50 mg) ground in a mortar with a pestle were vortexed in 500 μl of SDS buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol) followed by centrifugation for 15 min at 20,000×*g*. Supernatants were subjected to SDS-PAGE using 11% polyacrylamide gels according to the procedure of Laemmli (1979).

4.3. Two-dimensional (2D) electrophoresis

Proteins were extracted from soybean seed powders with buffer A (30 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3). Gel strips (IPG ReadyStrip pH 3-7 or pH 4-6 from Bio-Rad) for the first dimensional isoelectric focusing of the extracts were rehydrated overnight at room temperature with 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonaic acid, 0.001% (w/v) bromophenol blue, 14 mM dithiothreitol and the extracts. Isoelectric focusing was performed in Protean IEF cell (Bio-Rad) according to the manufacturer's instructions. After isoelectric focusing, gel strips equilibrated with 375 mM Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) SDS and 20% (v/v) glycerol were subjected to the second dimensional SDS-PAGE according to the procedure of Laemmli (1979).

4.4. Purification of β* and β homo and heterotrimers

Glycinin-rich fractions were prepared from the soybean seeds of 'Gotenba Zairai' by the procedure of Nagano et al. (1992) as described previously (Maruyama et al., 2002). After defatted seed meals (20 g) were stirred with buffer A (240 ml) for 2 h, the supernatants (200 ml) were collected by centrifugation at 4 °C (35,000×*g*, 30 min). We added 196 mg of sodium bisulfite (Wako, Japan) into the supernatant (200 ml) and adjusted to pH 6.4 with HCl. After keeping the mixture at 4 °C overnight, we collected the pellets by centrifugation (35,000×*g*, 30 min) and named the pellet a glycinin-rich fraction. The proteins in the glycinin-rich fraction were fractionated using ammonium sulfate. The precipitate of 70–85% saturation was dissolved in buffer B (35 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% NaN_3) and was dialyzed against buffer B. The dialyzate was subsequently applied to a Mono Q HR 10/10 column (Amersham Pharmacia Biotech) equilibrated with buffer B. The molecular species composed of β* and/or the β subunit were eluted with a linear

gradient from 0.1 to 0.35 M NaCl over a period of 250 min.

4.5. Amino acid sequence analysis

The proteins in the spots of β* and A3* (~50 pmoles) excised from 2D gels were extracted with the SDS buffer, blotted onto a PVDF membrane by Prosorb cartridge (Applied Biosystems) and subjected to N-terminal amino acid sequencing using a Procise 492 Protein Sequencer (Applied Biosystems). Purified β and β* homotrimers (~10 nmoles) were blotted onto a PVDF membrane by Prosorb cartridge and subjected to C-terminal amino acid sequencing using a Procise C Model 491 (Applied Biosystems) by means of an alkylated thiohydantoin method.

4.6. Detection of glycoprotein

Glycoproteins were detected by using concanavalin A-horseradish peroxidase (ConA-HRP, Seikagaku Kogyo, Japan) as described previously (Kigimoto-Ochiai et al., 1985; Yun et al., 1996). Proteins separated by 2D electrophoresis were transferred electrophoretically to nitrocellulose membranes. The membranes were soaked in a blocking solution (3% (w/v) skim milk in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.05% (v/v) Tween 20) three times for 10 min. After blocking, the membranes were reacted with ConA-HRP (5 μg/ml) diluted with the blocking solution for 1 h. After rinsing the membranes in 15 mM sodium phosphate buffer (pH 6.8) three times for 10 min, proteins on the membranes were reacted with 0.2 mM 3,3′-diaminobenzidine in 0.03% (v/v) hydrogen peroxide.

4.7. Glycosidase treatment

The samples were first denatured by heating at 100 °C for 10 min in 0.5% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and adjusted to N-glycosidase F (PNGase F, New England Biolabs Inc.) digestion condition (50 mM sodium phosphate (pH 7.5), 1% (w/v) NP-40, 0.36% (w/v) SDS and 0.72% (v/v) 2-mercaptoethanol). PNGase F was added to the denatured samples and then the mixtures were incubated for 3 h at 37 °C.

4.8. Differential scanning calorimetry (DSC) measurement

DSC experiments were carried out on a Microcal MC-2 ultrasensitive microcalorimeter (Microcal Inc.). A windows-based software package (Origin) was used for data analysis. All DSC experiments were performed in buffer C (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 10 mM 2 mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% NaN_3). DSC scan rate was 1 °C/min for all experiments.

4.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The β subunit or β* ('Gotenba Zairai') and A3*B4 ('Zairaishu 51-1-2′) cDNAs were isolated by using RT-PCR. Total RNA was isolated from developing soybean seeds according to Shirzadegan et al. (1991). For purification of mRNA from total RNA, mRNA Purification Kit (Amersham Pharmacia Biotech) was used according to the manufacturer's instructions. The β subunit or β* and A3*B4 were amplified using RNA LA PCR Kit (AMV) Ver. 1.1 (Takara). At first, mRNAs were reverse transcribed into cDNAs by the primer1 (CGC GGATCC GGTACC CTGCAG GTCGACTTTTTTTTTTTTTTTT) which is composed of the region complementary to poly(A) and sites of four restriction enzymes indicated by underlines. Next, the degenerate primers (AARGTIMGIGARGAYGARAAYAA for the β subunit or β*; WSIAARTTYAAYGARTGYCA for A3*B4) corresponding to their N-terminal amino acid sequences and the primer2 (CGCGGATCCGGTACCCTGCAGGTCGAC) corresponding to the region of the restriction enzyme sites of primer1 were used. The reactions were performed with 30 cycles at 94 °C for 30 s, 40 °C for 30 s and 72 °C for 4 min. The amplified fragments having the expected sizes were isolated, subcloned into pBluescriptSK(−) (Stratagene), and their sequences were confirmed according to Sanger et al. (1977).

4.10. Time-of-flight (TOF) mass spectrometry

TOF mass spectra were acquired in a Voyager-RP mass spectrometer (Applied Biosystems). Bovine serum albumin (Sigma, >98% purity, fatty acid-free) was used as a calibration standard in determining molecular masses of samples. One μl of each sample (10–30 μM) dissolved in distilled water was mixed with 2°μl of a saturated solution of sinapinic acid in 33.3% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid as a matrix. One μl of the mixture was left to dry on a plate at room temperature and spectra were acquired from dried spots.

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