STUDIES ON MOLECULAR HETEROGENEITY OF 11S GLOBULINS FROM SOYBEAN AND BROAD BEAN

SHIGERU UTSUMI

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ABBREVIATIONS

AS	acidic subunit
BS	basic subunit
G.	glycinin
L.	legumin
2-ME	2-mercaptoethanol
M.W.	molecular weight
PAGE	polyacrylamide gel electrophoresis
PGEF	polyacrylamide gel electrofocusing
PGGE	polyacrylamide gradient gel electrophoresis
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane

I GENERAL INTRODUCTION

Soybean has a long history as a protein foodstuff in the Orient including our country. Over the centuries a variety of soybean foods, including tofu, shoyu, miso, and kinako, have developed. Besides such traditional utilization, a number of soybean protein products have become established as useful ingredients in the manufacture of processed foods during the past fifteen years. The growing acceptance of these products for use in food manufacture has been prompted by their varied functional properties and good nutritional qualities. The functional properties that make soybean proteins useful in food systems are their abilities of emulsification, gelation, fiber and shred formation and so on [1,2]. It is an important problem to elucidate what kind of chemical and structural properties of soybean proteins are responsible for their excellent functional properties. An understanding of these properties would further the adoption in foods of soybean proteins and other grain legume proteins similar in structure to soybean proteins. However, at present sufficient information is lacking.

The seed storage proteins of legumes including soybean contain two major components, 7S and 11S globulins [3,4], which are stored in subcellular particles called protein bodies [5-7]. Both globulins are composed of plural subunits [8]. Therefore, research on the physical and chemical properties of soybean proteins must be focused on the subunits in order to shed light on the problem described above.

It is generally agreed that 11S globulin is primarily responsible for the texture formations in foods, which are closely related to

thiol:disulfide interchange reaction, because 11S globulin contains much more cysteine residues than 7S globulin in general [8].

Recent years have seen a dramatic development in the elucidation of the structural aspects of 11S globulins from soybean [9-19], broad bean [20-23], pea [23-29], lupin [30,31], peanut [32-37], chickpea [38], pumpkin [39-42], and others [8]. Especially with regard to the 11S globulin from soybean, several structural models of the molecule have been proposed on the basis of observations through electron microscope by Saio et al. [16] and Badley et al. [17]. The model of the latter shown in Fig. I-1 is generally accepted. According to this model and other protein chemical analyses of the subunit structure of the soybean 11S globulin, the constituent components of the 11S globulin are regarded as shown in Fig. I-2. In this figure, A and B refer to acidic subunits and basic subunits, respectively. The intermediary

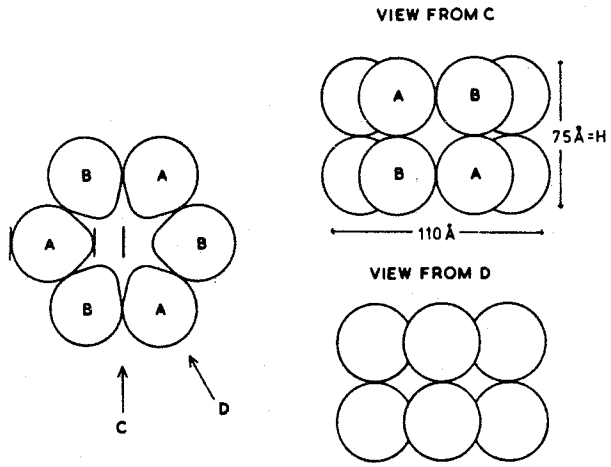
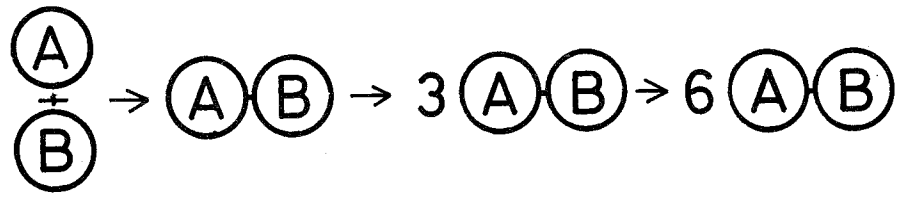


Fig. I-1. Model of subunit arrangement in soybean 11S globulin. Diagram at left is top view of hexagonal rings. Side views from positions C and D are shown at right.



Subunit Intermediary subunit 7S (half-molecule of 11S) 11S

Fig. I-2. Schematic representation of constituents of soybean glycinin.

subunit (AB) is composed of disulfided acidic and basic subunits, which are linked together in specific combinations in 1 : 1 ratio [15]. The half-molecule (3(AB)) consists of three intermediary subunits and has the size of 7S. The 11S component consists of six intermediary subunits; that is, it has the 6(AB) structure. Such a structure of 11S globulin shown in Fig. I-2 is common to 11S-type globulins from pea [26,27], pumpkin [42], sesame [43-45] except peanut whose 11S-type globulin does not have such intermediary subunits as described above [37].

Of various legumes, soybean has the highest protein content, followed by broad bean which is superior to pea, kidney bean and peanut [46]. Broad bean has the advantage that its cultivation time is opposite to that of soybean, which makes it possible to cultivate both soybean and broad bean in the same field in a year.

The present studies are devoted to 11S globulins which are one of the most predominant components of seed storage proteins and closely related to the texture formations in foods. In this thesis the chemical and structural properties of 11S globulins from soybean and broad

bean are investigated from the standpoint of their subunits. Chapters II to V elucidate the occurrence of molecular heterogeneity of 11S globulins from soybean and broad bean in their subunit compositions and molecular species. In Chapters VI and VII, the specificity of subunit interaction in the formation of 11S-structure is investigated and discussed. Chapter VII, moreover, describes the formation of hybrid-11S globulins from the subunits of soybean and broad bean 11S globulins.

II DIFFERENCES IN SUBUNIT COMPOSITIONS OF GLYCININS AMONG SOYBEAN CULTIVARS

II-1 INTRODUCTION

Grain legumes are potential sources of edible vegetable proteins for supplementing dietary needs. It is generally known that the major components of the seed storage proteins are responsible for contributing to the quality of foods made from these seeds, their flour and protein products, particularly the physical and nutritional properties. Soybeans are used not only for various kinds of traditional Japanese foods, but their protein products as well are used commercially as ingredients in food system. However, the lack of basic information about protein components from soybean and other legume seeds has hindered the full and effective utilization of legume seeds and their protein products for food.

The 11S-type globulin of soybean is called glycinin. The structural features of glycinin have been fairly elucidated up to now as described in General Introduction [8-19]. However, the subunit composition of glycinin has not yet elucidated sufficiently. This was investigated by Catsimpoolas et al. [9,11,47], Kitamura et al. [15,48] and Badley et al. [17]. Their results were different among each other, which indicates the differences in subunit compositions of glycinins among soybean cultivars.

In this chapter, the subunit compositions of glycinins from a wide range of cultivars of soybean seeds were analyzed by gel electrophoresis under various conditions. The results obtained here indicate that

the subunit compositions of glycinins were classified into five groups from the standpoint of charges.

II-2 MATERIALS AND METHODS

The samples of soybean seeds (Glycine max) examined and their country of origin are listed in Table II-I. The cultivars were grown at some places in Japan in 1977 where the seeds of each cultivar normally developed and matured: sample Nos. 1 and 2 were grown at Hokkaido Central Agricultural Experimental Station; Nos. 3 to 5 and 15 to 18, Iwate University Agronomy Farm; No. 6, The University of Tokyo Agrobiology Farm; Nos. 7 and 8, Kyushu National Agricultural Experimental Station; and Nos. 9 to 14, Tohoku National Agricultural Experimental Station. Urea and 2-ME purified for biochemical research were obtained from

Table II-I. List of seed samples examined with country of origin.

Country	Cultivar	Sample No.
Japan	Tokachi Nagaha	1
	Shiro Tsuru-no-ko	2
	Rikuu No. 20	3
	Raiden	4
	Goyo Daizu	5
	Sakagami No. 2	6
	Iyo Daizu	7
	Matsuura	8
United States	Hill	9
	Hark	10
	Corsoy	11
	York	12
	Dare	13
	Ford	14
China	Bai-hua-zuo-zi	15
	Tianjin-dachingdou	16
Korea	Kinzu	17
	Huk-tae	18

Nakarai Chemicals. SDS purified for electrophoresis was obtained from Wako Pure Chemical Industries. DEAE-Sephadex A-50 was purchased from Pharmacia Co., Ltd. Ampholine (pH 3.5-10) was purchased from LKB Co. Other chemicals are the highest grade.

Preparation of acetone powder

The seeds of soybean cultivars were soaked overnight in distilled water at 4°C. Cotyledons from which the germ had been removed were homogenized with 15 volumes (w/v) of 63 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-ME and allowed to stand for 1 h at 20°C with gentle stirring. The homogenate was filtered through gauze, then centrifuged at 9000 rpm for 15 min at 0°C. Chilled acetone was added slowly to the supernatant to 60 % at below 0°C. The precipitate was collected by centrifugation at -20°C, washed once with chilled acetone, washed once with diethyl ether, dried, and stored in a desiccator at 4°C until used.

Preparation of purified glycinin

Crude glycinin fraction was prepared from the acetone powder according to the method of Thanh et al. [49]. Chromatographic fractionation of the crude glycinin fraction was performed on a column of DEAE-Sephadex A-50 equilibrated with 0.035 M potassium phosphate buffer (pH 7.6) containing 0.25 M NaCl, 10 mM 2-ME, and 0.02 % NaN_3 at 5°C. Elution was carried out in steps using 0.25 M, 0.275 M, and 0.35 M NaCl. The eluate between 0.275 M and 0.35 M of NaCl (glycinin fraction) was dialyzed against 0.035 M potassium phosphate buffer (pH 7.6) containing 10 mM 2-ME, 0.4 M NaCl and 0.02 % NaN_3 in the cold and then fractionated by centrifugation on a 10 to 30 % (w/v) linear sucrose density gradient

in the same buffer at 34000 rpm at 20°C for 16 h in a Hitachi RPS 40T rotor. After centrifugation, the gradient was divided into 0.4 ml fractions with an ISCO density gradient fractionator. Of the three major peaks which appeared in the gradient, the third one (purified glycinin fraction) was subjected to further analyses.

Fractionation of native subunits of glycinin

The purified glycinin fraction was equilibrated with 0.1 M sodium phosphate buffer (pH 6.3) containing 6 M urea and 0.2 M 2-ME and applied on a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was performed with 2500 ml of linear gradient (0 to 0.5 M) of NaCl in the buffer. All operations of the procedure were performed at 5°C.

Electrophoreses

SDS-PAGE was performed according to the method of Laemmli [50] at room temperature with 10 % polyacrylamide gels in the presence or absence of 2-ME. 50 µg of sample was dialyzed against 62.5 mM Tris-HCl buffer (pH 6.8) containing 1 % SDS (and 0.2 M 2-ME) for 20 h at room temperature and then electrophoresed at first for 30 min at a constant current of 1 mA/gel and then for 2.5 h at 2 mA/gel . Alkaline urea-PAGE was performed with 7.5 % polyacrylamide gels at 4°C according to the method of Davis in 7 M urea in the presence or absence of 2-ME. The gels were pre-run for 1 h at a constant voltage of 200 V in order to remove excess ammonium persulfate [51]. 50 µg of sample was dialyzed against 7 M urea with or without 0.2 M 2-ME for 20 h and then electrophoresed at 4°C for 4 h at a constant voltage of 200 V. After electrophoresis, the gels were stained with 0.5 % amido-black 10B in

20 % acetic acid for 1 h and destained with 7 % acetic acid.

PGEF in the presence of urea and 2-ME was performed in 6 % polyacrylamide gels according to the method of Wrigley [52] with a slight modification. Anode and cathode solutions were 0.02 M phosphoric acid and 1 M NaOH, respectively. Prior to electrophoresis, 50 µg of sample was equilibrated with 8 M urea and 0.2 M 2-ME. After the sample containing 2 % Ampholine was layered on a gel and 50 µl of 54 mM glutamic acid containing 2 % Ampholine and 15 % glycerol was layered on the sample, electrophoresis was carried out for 5.5 h at 4°C with a constant voltage of 180 V. After electrophoresis, the gels were fixed and washed with two changes of 0.35 % perchloric acid and then stained in 0.04 % Coomassie brilliant blue G-250 in 0.35 % perchloric acid.

Densitometric scanning

The destained gels were scanned at 590 nm on a Shimadzu-dual-wavelength chromatoscanner model CS-910.

Protein concentration

Protein was determined by the method of Lowly et al. [53] or by absorbance measurements at 280 nm based on the relationship $E^{1\%}_{1cm}$ (280 nm) = 8.0 [13].

II-3 RESULTS

Fractionation and characterization of native subunit proteins of glycinin

Native subunit proteins of glycinin from var. Tsuru-no-ko were fractionated by chromatography on a DEAE-Sephadex column in the presence of urea and 2-ME as described in MATERIALS AND METHODS. As shown in

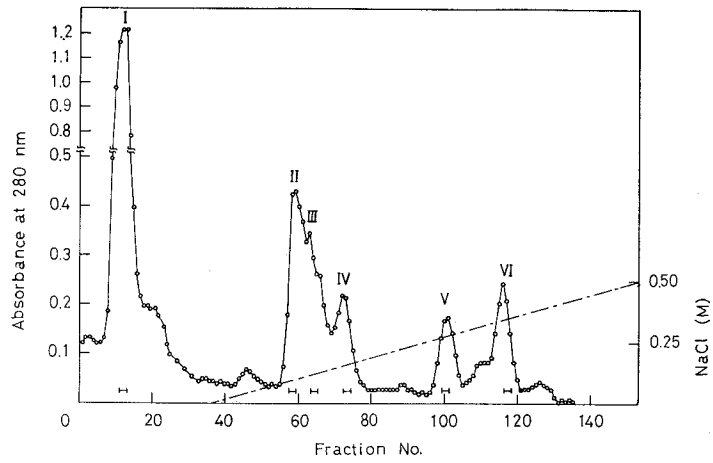


Fig. II-1. Fractionation of native subunit proteins of glycinin by DEAE-Sephadex A-50 column chromatography.
The purified glycinin preparation (197 A_{280} units) was fractionated on a 2.5 x 45 cm column at 5°C as described in MATERIALS AND METHODS. Flow rate was 20 ml/h, and 10 ml was collected in each fraction. Fraction numbers 11-13 (I), 57-59 (II), 63-65 (III), 72-74 (IV), 99-101 (V), and 116-118 (VI) were collected.
—O— , absorbance at 280 nm; — - — , concentration of NaCl.

Fig. II-1, glycinin was separated into six fractions. The first peak flowed through the column and the following five peaks were eluted with a gradient of NaCl. The flowed through fraction and the adsorbed fractions of glycinin were analyzed by gel electrophoresis under different conditions as shown in Fig. II-2. The flowed through fraction was composed of basic subunits with molecular weights of 19000 and 18300 (Fig. II-2-A and B). The adsorbed fractions were the acidic subunits (Fig. II-2-A and C). As shown in Fig. II-2-C, peaks II and III consisted of AS_{1+2} and AS_{2+3}, respectively, and IV, V and VI consisted of AS_4, AS_5 and AS_6, respectively. The molecular weights of AS_{1+2}, AS_{2+3}, AS_4 and

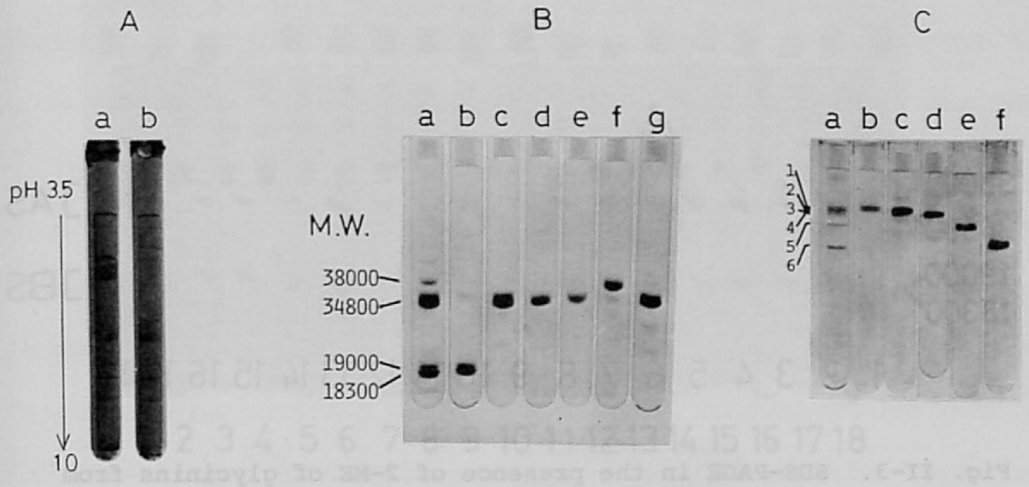


Fig. II-2. Characterization of isolated subunits of glycinin by various electrophoreses.
A, PGEF of the purified glycinin preparation (a) and the flowed through fraction (b). B, SDS-PAGE (in the presence of 2-ME) of the purified glycinin preparation (a) and its subunits (Gels b to g) obtained as described in Fig. II-1. Gel b, flowed through fraction; Gel c, II; Gel d, III; Gel e, IV; Gel f, V; Gel g, VI. C, Alkaline urea-PAGE of the purified glycinin preparation (a) and adsorbed fractions (Gels b to f) obtained as described in Fig. II-1. Gel b, II; Gel c, III; Gel d, IV; Gel e, V; Gel f, VI. Electrophoretic conditions were described in MATERIALS AND METHODS. Migration is from top to bottom.

AS_6 and AS_5 were 34800 and 38000, respectively (Fig. II-2-B). AS_5 seems to correspond to A_4 of Kitamura et al. [15].

Difference of subunit compositions among soybean cultivars

The purified glycinin from various cultivars were analyzed by SDS-PAGE in the presence of 2-ME. As shown in Fig. II-3, all glycinins were composed of three major subunit proteins with molecular weights of 38000, 34800, 19000 and 18300. The larger two subunits and the smaller two were acidic and basic subunits, respectively, as shown in

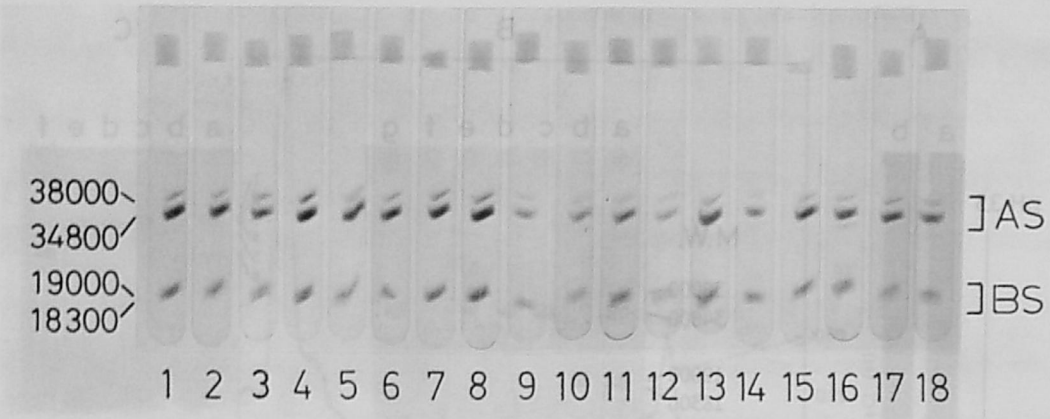


Fig. II-3. SDS-PAGE in the presence of 2-ME of glycinins from samples, no. 1-18, listed in Table II-I.
50 μg of each glycinin was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom.

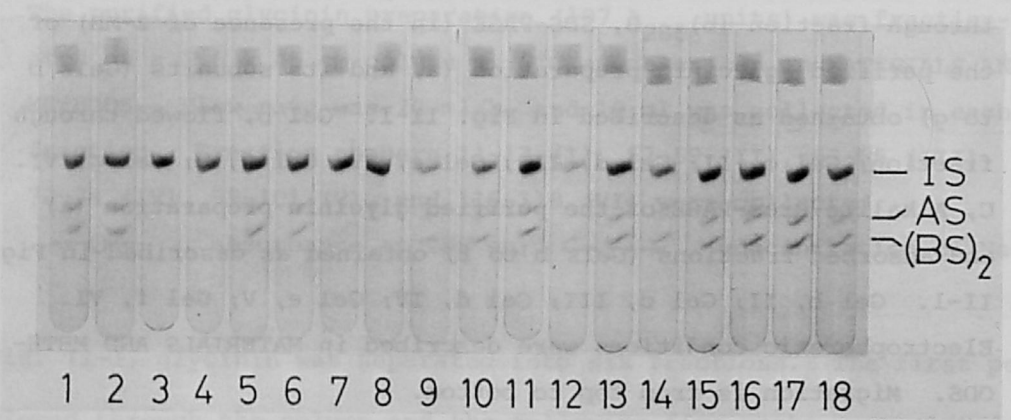


Fig. II-4. SDS-PAGE in the absence of 2-ME of glycinins from the same samples described in Fig. II-3.
50 μg of each glycinin was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom.

Fig. II-2-B. When each glycinin was analyzed by SDS-PAGE in the absence of 2-ME, two band patterns were observed (Fig. II-4). In one, only a band corresponding to the intermediary subunits with a molecular weight of about 50000 appeared, while in the other, two more band:

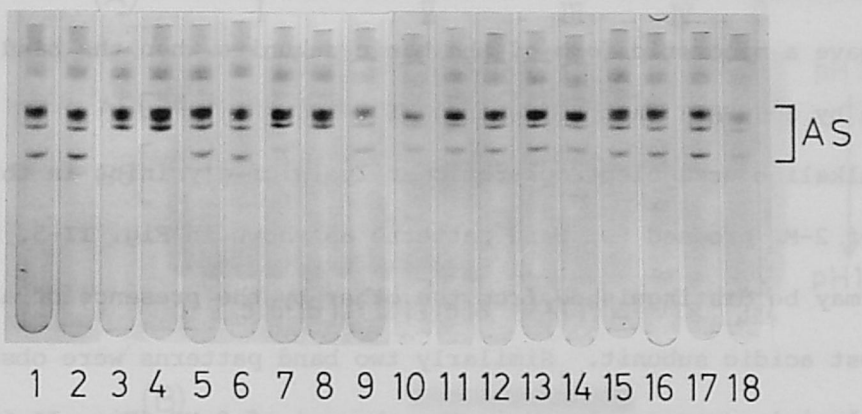


Fig. II-5. Alkaline urea-PAGE in the presence of 2-ME of glycinins from the same samples described in Fig. II-3. 50 μg of each glycinin was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom.

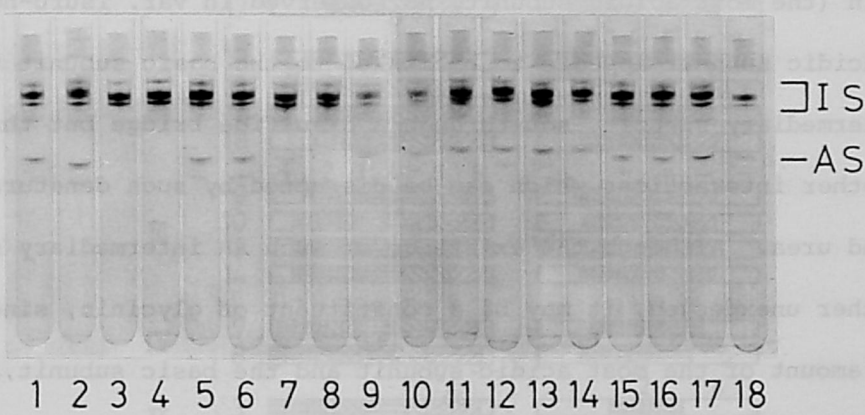


Fig. II-6. Alkaline urea-PAGE in the absence of 2-ME of glycinins from the same samples described in Fig. II-3. 50 μg of each glycinin was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom.

appeared besides the intermediary subunit band. One of the extra bands corresponded to the acidic subunit with a molecular weight of 34800, and

the other band was a dimeric form of the basic subunits, since the band gave a monomeric form of the basic subunits when the band was analyzed by SDS-PAGE in the presence of 2-ME.

Alkaline urea electrophoretic analysis of glycinins in the presence of 2-ME produed two band patterns as shown in Fig. II-5. One group may be distinguished from the other by the presence of a band of the most acidic subunit. Similarly two band patterns were observed when glycinins were analyzed in the absence of 2-ME (Fig. II-6). One group had only the intermediary subunit band, while the other group had an additional band corresponding to the most acidic subunit.

These results indicate that glycinins of various cultivars could be classified into two groups, one which contained an extra subunit protein (the most acidic subunit, AS_6 observed in var. Tsuru-no-ko). This acidic subunit appears to be linked to the basic subunit to form an intermediary subunit, not through a disulfide bridge but through some other interactions which can be disrupted by such denaturants as SDS and urea. Although the existence of such an intermediary subunit is rather unexpected, it may be a constituent of glycinin, since the total amount of the most acidic subunit and the basic subunit, both of which constitute the intermediary subunit, is comparable to the other subunits (Figs. II-4, 5 and 6). Recently, Moreira et al. reported the existence of an acidic subunit with a molecular weight of around 10000 in glycinins from CX635-1-1-1 [19], and so did Kitamura et al. from Norin No. 1 and Amsoy [54]. However, the author did not observe such an acidic subunit in the SDS electrophoretic analysis under the conditions used here.

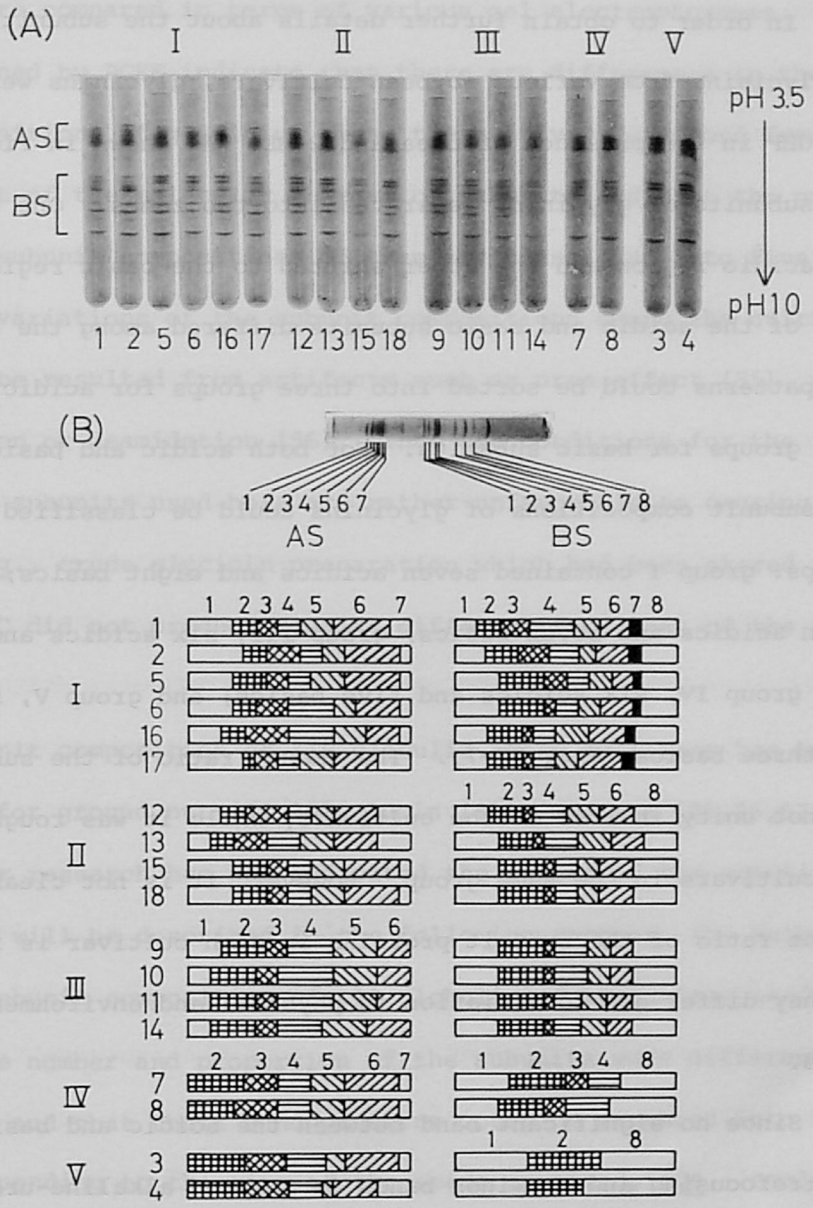


Fig. II-7. PGEF of glycinins from the same samples as described in Fig. II-3.

100 μg of each glycinin was electrophoresed as described in MATERIALS AND METHODS. A, PGEF patterns. B, The relative proportion of the acidic and the basic subunits from each cultivar was calculated from densitometric scanning of the gels and is represented schematically.

In order to obtain further details about the subunit compositions of glycinins from various soybean cultivars, glycinins were analyzed by PGEF in the presence of urea and 2-ME. As shown in Fig. II-7-A, the subunits of glycinins separated into two zones. One shifted to the acidic region and the other shifted to the basic region. The numbers of the acidic and basic subunits differed among the cultivars. The patterns could be sorted into three groups for acidic subunits and four groups for basic subunits. For both acidic and basic subunits the subunit compositions of glycinins could be classified into five groups: group I contained seven acidics and eight basics; group II, seven acidics and seven basics; group III, six acidics and seven basics; group IV, six acidics and five basics; and group V, six acidics and three basics (Fig. II-7). The amount ratio of the subunit proteins was not unity in most of the cultivars, while it was roughly similar for cultivars in the same group. However, it is not clear whether the amount ratio of the subunit proteins in each cultivar is invariant or if they differ due to variation in physical and environmental conditions.

Since no significant band between the acidic and basic regions in electrofocusing and no minor bands in SDS and alkaline-urea electrophoreses were present, the purities of the glycinin preparations used here seems to be sufficient to clarify their subunit composition in detail.

II-4 DISCUSSION

The subunit compositions of glycinins from 18 kinds of cultivars

of soybean were compared in terms of various gel electrophoreses. The results obtained by PGEF indicate that there are differences in the subunit compositions of glycinins among the cultivars examined from the standpoint of the molecular charges of subunits and that the patterns of the subunit compositions of them are classified into five groups. The variations of the subunit compositions among the cultivars may not be resulted from artifacts such as urea effect [55], proteolytic action or deamidation [56], since the conditions for the isolation of the subunits used here are rather unfavorable to causing such artifacts, e.g., crude glycinin preparation which had been stored for a month at 5°C did not produce results different from that of the fresh one.

The subunit composition of 11S globulin among cultivars has been investigated for ground nut [32,33], lupin [31], and pea [25,26,57]. However, their research has not indicated the number of the constituent subunits. As will be described in the following chapter, the author studied the subunit composition of 11S globulin of broad bean seeds and found that the number and proportion of the subunits were different for the cultivars and that their band patterns could be arranged into three groups corresponding to the size of the seeds examined, i.e., small-, medium- and large-size groups. Moreover, recently, Davies has reported that the subunit compositions of 11S globulins of pea cultivars are related to seed phenotype, round and wrinkle [58]. However, the band patterns of glycinin could not be grouped according to the size and other characteristics of the seeds unlike the cases of broad bean and pea 11S globulins. Since the present study on soybean cultivars

is not exhaustive, other types of subunit composition of glycinins may exist in soybean cultivars different from those described above. At any rate, these observations (Fig. II-7) support those of others as to the diversity of subunit composition and also suggest the heterogeneity of glycinin molecular species, as has already been postulated for peanut arachin [32,33,59] and pea 11S globulin [25], because more than six subunits were observed as the acidic subunits or the basic subunits in groups I, II, III and IV, and the amount ratio of subunit proteins was not unity among the acidic subunits and among the basic subunits.

The differences in the subunit compositions of glutenins among wheat cultivars and their relation to the bread-making quality of the flours have been studied by several people [60-64]. Although differences in the subunit compositions of glutenins were observed in cultivars, no significant correlation with bread-making quality could be made. However, Payne et al. have recently found that the presence of a subunit of glutenin, whose molecular weight is about 145000, correlated with bread-making quality [65]. On the other hand, it has been reported that the quality of tofu (one of the Japanese traditional foods made from soybeans) differs according to the cultivars used [66]. Saio et al. found the proportion of 7S and 11S globulins to be responsible for the differences in the physical properties of tofu-gel among soybean cultivars [67]. Thus, it seems likely that the subunit composition of glycinin is related to the physical properties of foods made from soybeans or their isolated proteins. From such a viewpoint it is important to study the relationship between the subunit compositions of glycinins and the exhibition of their functional properties such as

gelation, emulsification, and so forth in the food system.

Moreira et al. have recently reported that the sulfur amino acids in glycinin from soybean cultivar CX635-1-1-1 are not distributed evenly among the various subunits, of which six acidic and four basic subunits have been distinguished [19]. Their observation seems to have significance in efforts to improve soybean quality genetically with respect to sulfur amino acid level. As described above, the author could distinguish many more subunits than they did; e.g., seven acidic and eight basic subunits of glycinin existed in group I. Therefore, analyses of the amino acid composition of those subunits as well as among the cultivars may provide further information about the uneven distribution of the sulfur amino acids in glycinins among the subunits for the purpose of soybean breeding.

III DIFFERENCES IN SUBUNIT COMPOSITIONS OF LEGUMINS AMONG BROAD BEAN CULTIVARS

III-1 INTRODUCTION

The 11S-type globulin of broad bean is called legumin. This has been purified by several people [20-22,68,69]. The subunit structure of legumin has been investigated by Bailey and Boulter [21] and Wright and Boulter [22]. The former reported that legumin consisted of three kinds of subunits with molecular weights of 56000, 42000 and 23000 from the SDS-PAGE analysis by the method of Shapiro et al. [70]. The latter identified five distinct subunits, of which two were acidic with a molecular weight of 37000, and three basic with molecular weights of 23800, 20900 and 20100. Thus, the subunit composition has not yet been elucidated sufficiently.

On the other hand, differences of subunit compositions of 11S-type globulins among cultivars have been found in ground nut [32,33], lupin [31] and pea [25,26] as well as soybean as described in the preceding chapter.

In order to elucidate the diversity of subunit composition among different seeds, the subunit compositions of legumins from various cultivars of broad bean planted in Japan were investigated. The results obtained indicate that the subunit patterns can be classified into three groups corresponding to size of the seeds examined, i.e., small-, medium- and large-size groups from the standpoint of molecular charges.

III-2 MATERIALS AND METHODS

Six cultivars of broad bean (Vicia faba L.) seeds planted in Japan were obtained from Takii Seed Co., Ltd. The seeds of these cultivars differ in size, that is, the small seeds of Sanuki-Nagasaya (S.N.), Sanuki-Nagasaya-Wase (S.W.) and Gifu-Wase (G.W.), the medium seeds of Kumamoto-Churyu (K.C.) and the large seeds of Otafuku (O.) and Issun (I.). Average weights per 100 seeds of the three groups were 90, 115, and 230 g, respectively. Other materials were as described in the preceding chapter.

Preparation of crude extract

Bean meal (10 g) was homogenized with 100 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl for 3 h at 4°C, filtered through gauze and then centrifuged at 20000 x g for 30 min. The supernatant was brought to 75 % saturation with ammonium sulfate and stirred for 1 h, and the precipitate was collected by centrifugation at 20000 x g for 30 min. The precipitate was suspended in 15 ml of 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 M NaCl and 0.02 % NaN_3 (standard buffer) and dialyzed against the standard buffer. The dialyzate was centrifuged at 30000 x g for 30 min. This supernatant was used as crude extract.

Sucrose density gradient centrifugation

Aliquot of the crude extract was fractionated by centrifugation on 10 to 30 % (w/v) linear sucrose density gradient in the standard buffer at 36000 rpm and 20°C for 16 h in a Hitachi RPS 40T rotor as described in the preceding chapter.

Amino acid analysis

Amino acid compositions of legumins prepared from various cultivars by sucrose density gradient centrifugation were determined by using a Hitachi KLA-5 amino acid analyzer according to the method of Spackman et al. [71]. About 0.5 mg protein was precipitated by adding an equal volume of 10 % trichloroacetic acid and the precipitate was hydrolyzed with 0.5 ml of 6 N HCl in a sealed, evacuated tube for 20 and 70 h at 110°C. Threonine and serine values were corrected by extrapolation back to zero time. For valine and isoleucine the 70 h values were used. Cysteine was determined by the performic acid oxidation method [72]. Tryptophan was determined spectrophotometrically according to the method of Goodwin and Morton [73].

Analytical Ultracentrifugation

Determination of S value was performed in a Hitachi ultracentrifuge UCA 1A with a double sector cell and a Schlieren optical system at 60000, 51200 and 34450 rpm for 2S, 7S and 11S and 15S components, respectively, at 20°C. The concentration of the sample was 9, 9, 9 and 7 mg protein/ml for 2S, 7S, 11S and 15S components, respectively. $S_{20,w}$ for each component was calculated from each S values obtained. $S^0_{20,w}$ of 11S component was determined by extrapolating the values at 0.9, 0.5, 0.375 and 0.25 % of protein concentrations back to zero concentration. Molecular weights of 11S and 15S components were determined by sedimentation equilibrium method of Yphantis [74] using a six channel cell at 10490 and 6520 rpm for 11S and 15S, respectively, at 20°C. The samples were dialyzed exhaustively against the standard buffer containing 0.02 % NaN_3 prior to centrifugal analysis. Values of the

partial specific volume used for determination of molecular weights and/or S values of 2S, 7S and 11S and 15 S components were 0.75 ml/g, 0.73 ml/g [75] and 0.72 ml/g which was calculated [76] from the amino acid composition of 11S component (Table III-II), respectively.

Fractionation of native subunits of legumin

In order to separate the constituent subunits, DEAE-Sephadex A-50 column chromatography in the presence of 6 M urea and 2-ME was performed using 1.5 x 14 cm column at 4°C. 18 mg of legumin of var. Sanuki-Nagasaya was applied on the column and eluted with a 480 ml of linear gradient (0 to 0.5 M) of NaCl as described in the preceding chapter. Eluates were collected in 6 ml fractions and the absorbance at 280 nm was measured.

Electrophoreses

PAGE was performed in 6.5 % polyacrylamide gels at 4°C according to the method of Davis [77]. The procedures of SDS-PAGE and PGEF were the same as described in the preceding chapter. The destained gels were scanned at 590 nm as described in the preceding chapter.

Protein concentration

Protein was determined by the method of Lowry et al. [53] or by absorbance measurements at 280 nm based on the relationship $E_{1cm}^{1\%}$ (280 nm) = 8.5, which was calculated from comparison of the value obtained by absorbance measurement at 280 nm with that obtained by microkjeldahl method.

III-3 RESULTS

General properties of legumin and other protein components

Each crude protein fraction prepared from the seeds of various cultivars was fractionated by centrifugation on sucrose density gradient as described in MATERIALS AND METHODS. The centrifugation pattern of the protein fraction from Sanuki-Nagasaya is shown in Fig. III-1-A. Similar patterns were also obtained in the cases of other cultivars. Broad bean seed storage proteins were fractionated into four components, 2S, 7S, 11S and 15S. There was no large difference in the contents of 11S components among the six cultivars of broad bean seeds (Table III-I). The underlined part of each component in Fig. III-1-A was collected and then recentrifuged under the same condition. The

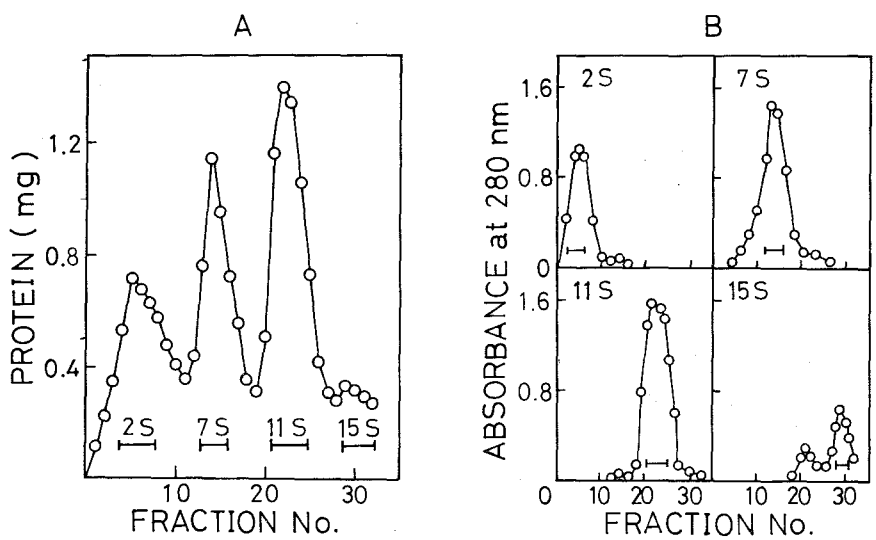


Fig. III-1. Fractionation of broad bean proteins by sucrose density gradient centrifugation.
A, 0.4 ml (26 mg of protein) of the crude extract was fractionated by centrifugation as described in MATERIALS AND METHODS.
B, underlined part of each peak in (A) was collected and centrifuged again under the same condition as in (A).

Table III-I. Proportions of four protein components in each extract from six cultivars.

Cultivars	Components of seed storage protein			
	2S	7S	11S	15S
	(%*)			
Sanuki-Nagasaya	24.4	28.6	40.2	6.8
Sanuki-Nagasaya-Wase	24.6	28.3	41.4	5.8
Gifu-Wase	23.4	30.5	39.2	6.7
Kumamoto-Churyu	24.3	29.5	40.4	5.9
Issun	27.3	29.4	38.6	4.6
Otafuku	29.0	28.9	36.8	5.3

* The proportions of the four protein components were estimated from the area of each peak in Fig. III-1.

profiles of the second centrifugal fractionations are shown in Fig. III-1-B. The underlined part of each component in Fig. III-1-B was pooled and subjected to characterization of each component without further purification as follows.

Each component fractionated by the recentrifugation was analyzed by PAGE. As shown in Fig. III-2, electrophoretic patterns of legumins and the other components from various cultivars were similar to each other.

Each component was analyzed by ultracentrifugation in order to examine the purity of the components and estimate the size of the components. All components were homogeneous in their Schlieren patterns. $S_{20,w}$ of each component (2S, 7S, 11S and 15S) was 1.64, 6.71, 11.3 and 17.2, respectively. $S^{0}_{20,w}$ and a molecular weight of 11S component

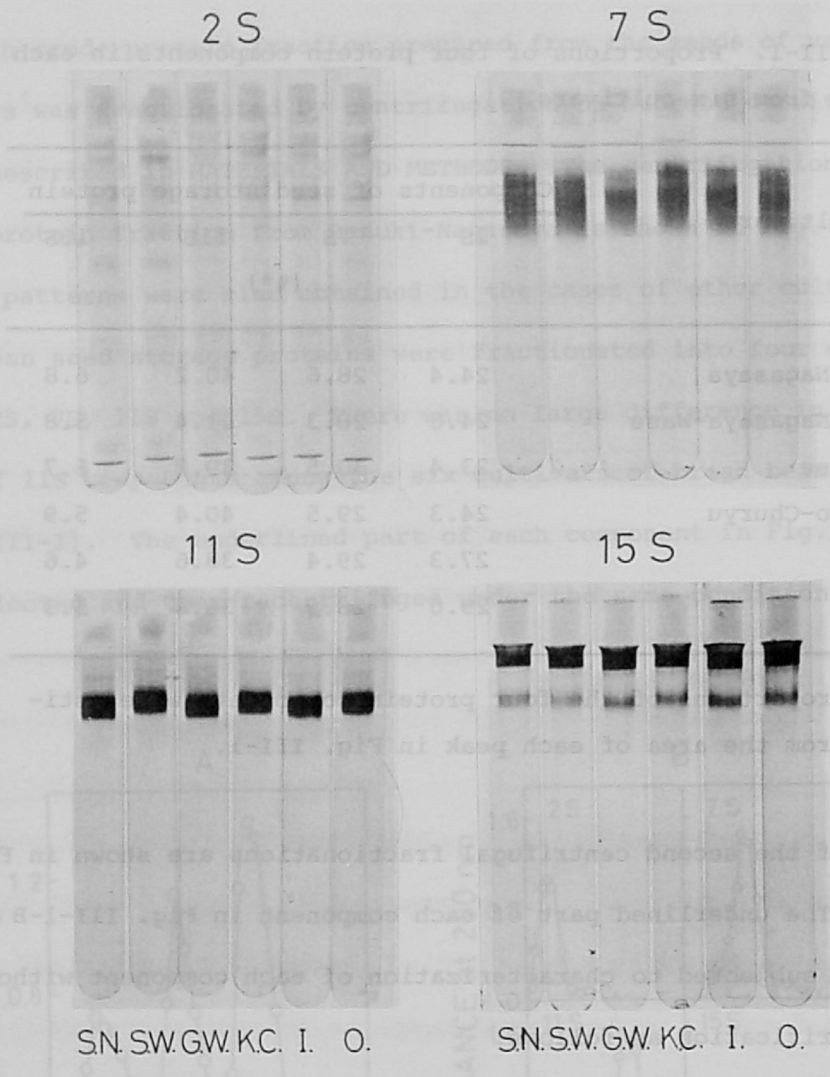


Fig. III-2. PAGE of each component from six cultivars. 50 μg of each component obtained from the centrifugation shown in Fig. III-1 was electrophoresed for 2 h at a constant current of 3 mA/gel at 4°C. S.N., S.W., G.W., K.C., I. and O. are broad bean cultivars as described in MATERIALS AND METHODS. Migration is from top to bottom.

were 12.3 and 319000, respectively. The molecular weight of 15S component was calculated as 599000, which is close to twice of the molecular weight of 11S component.

Table III-II. Amino acid compositions of legumins from six cultivars.

Amino acids	Broad bean cultivars*					
	S.N.	S.W.	G.W.	K.C.	I.	O.
	(g/100 g of legumin)					
Lys	5.52	4.72	4.38	5.46	7.10	5.06
His	2.27	2.71	2.64	2.90	2.76	3.03
Arg	10.00	10.06	10.01	10.02	9.35	10.84
Asp	11.38	11.17	11.51	11.12	10.03	11.18
Thr[a]	3.10	3.07	3.17	3.03	3.08	3.18
Ser[a]	5.16	5.26	5.64	5.34	6.37	5.46
Glu	20.75	22.14	22.09	21.91	19.72	19.96
Pro	4.38	4.39	4.15	4.90	4.67	4.72
Gly	3.87	3.68	3.69	3.35	4.08	3.58
Ala	3.31	3.37	3.35	3.16	3.30	3.29
Cys[b]	0.75	0.69	0.74	0.69	0.70	0.71
Val[c]	4.11	3.96	4.23	4.03	4.38	4.59
Met	0.56	0.49	0.51	0.50	0.52	0.56
Ile[c]	4.69	4.43	4.42	4.35	4.05	4.63
Leu	7.67	7.33	7.39	7.24	6.68	7.59
Tyr	3.21	3.48	3.39	3.54	3.70	3.65
Phe	3.76	3.62	4.35	3.55	3.61	3.21
Try[d]	1.22	0.98	1.03	1.02	0.81	1.21

a Extrapolated to zero time.

b Determined as cysteic acid.

c 70 h only.

d Determined spectrophotometrically.

* Abbreviations described in MATERIALS AND METHODS were used.

Amino acid composition of each legumin prepared by the recentrifugation is shown in Table III-II. There was no large difference in

the composition among the six cultivars except that the amounts of lysine and serine were slightly high in Issun. This result essentially coincided with the result reported by Bailey and Boulter [21] except that the amount of glutamic acid was slightly higher and those of proline, cysteine and tyrosine were lower in this result. The amino acid composition of broad bean was similar with that of soybean seed [78] except that the amounts of cysteine and methionine were slightly lower and that of arginine was higher in broad bean.

Fractionation and characterization of native subunits of legumin from var. Sanuki Nagasaya

In order to characterize the subunits of legumin of broad bean, the constituent subunits were fractionated by DEAE-Sephadex A-50 column chromatography in the presence of urea and 2-ME. As shown in Fig. III-3, legumin was separated into four fractions. The first peak flowed through the column and the following three peaks were eluted with a gradient of NaCl. The amounts of protein determined by absorbance at 280 nm of the flowed through fraction and the adsorbed fractions were approximately equal. The flowed through fraction and the adsorbed fractions were analyzed by PGEF and SDS-PAGE (Fig. III-4). The flowed through fraction was composed of basic subunits with molecular weights of 23000, 20500 and 19000. The adsorbed fractions were the acidic subunits. Peaks II, III and IV coincided with the subunits with molecular weights of 36000, 36000 and 49000 and 51000, respectively. The densitometric patterns of the gels showed that the amount ratios of the acidic subunits (bands 1, 2 and 3) and the basic subunits (bands 4, 5 and 6) were approx. 1 : 1 : 10 and 3 : 2 : 1, respectively.

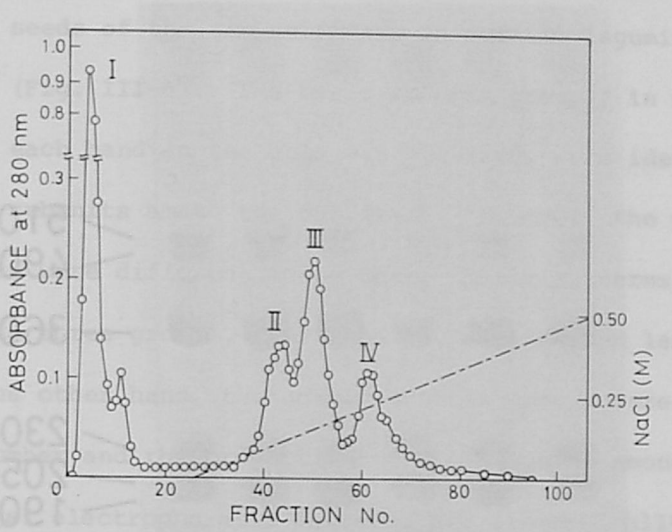


Fig. III-3. Fractionation of native subunit proteins of legumin by DEAE-Sephadex A-50 column chromatography.
11S component obtained as shown in Fig. III-1-B was fractionated as described in MATERIALS AND METHODS. The flow rate was 15 ml/h.
—o— , absorbance at 280 nm; —-— , concentration of NaCl.

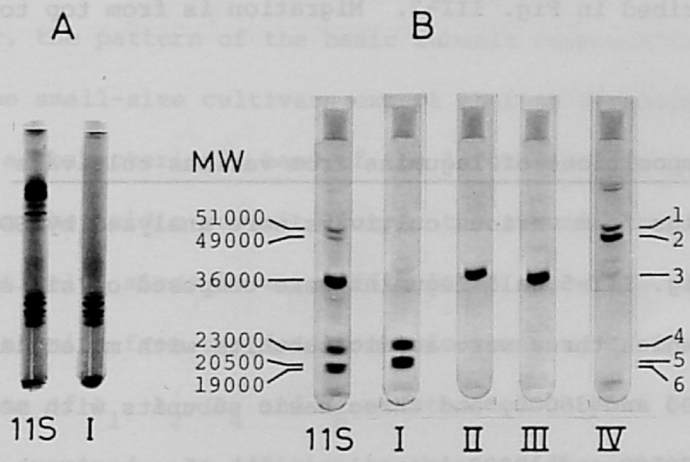


Fig. III-4. Characterization of isolated subunits of legumin by PGEF (A) and SDS-PAGE (B).
Electrophoreses were performed as described in MATERIALS AND METHODS. Migration is from top to bottom. I-IV correspond to those in Fig. III-3.

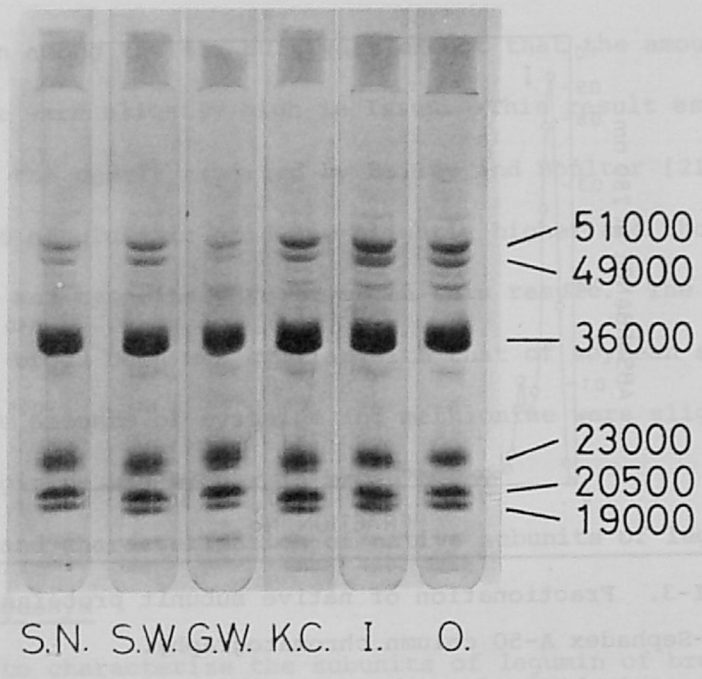


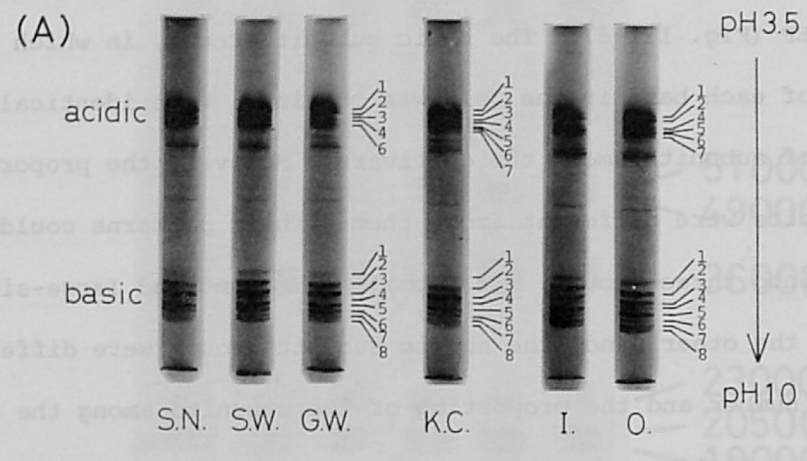
Fig. III-5. SDS-PAGE of legumins from six cultivars.
100 μg of each legumin from six cultivars was electrophoresed as described in MATERIALS AND METHODS. Gel symbols are the same as described in Fig. III-2. Migration is from top to bottom.

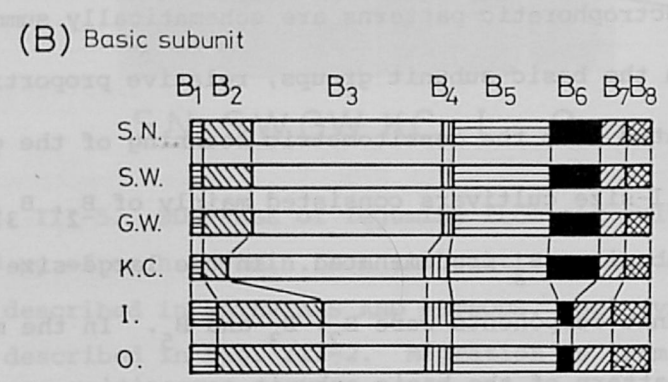
Subunit compositions of legumins from various cultivars

Legumins from various cultivars were analyzed by SDS-PAGE. As shown in Fig. III-5, all legumins were composed of six subunit proteins, of which three were acidic subunits with molecular weights of 51000, 49000 and 36000, and three basic subunits with molecular weights of 23000, 20500 and 19000 in analogy with the result shown in Fig. III-4-B. The densitometric patterns of the gels showed that the amount ratios of the acidic subunits and the basic subunits were similar among the cultivars.

In order to investigate further the subunit compositions of leg-

mins from the seeds of the six cultivars in detail, legumins were analyzed by PGEF (Fig. III-6). The basic subunit groups, in which good separation of each band in the gels was obtained, were identical in the number of subunits among the cultivars. However, the proportions of the subunits were different among them. Their patterns could be classified into three groups, i.e., small-, medium- and large-size groups. On the other hand, the acidic subunit groups were different both in the number and the proportion of the subunits among the cultivars. These gel electrophoretic patterns are schematically summarized in Fig. III-6-B. In the basic subunit groups, relative proportion of subunits was calculated from the densitometric scanning of the gels. Legumins of the small-size cultivars consisted mainly of B_2, B_3, B_5 and B_6. Of these subunits, B_3 predominated. In the large-size cultivars, the major subunit components were B_2, B_3 and B_5. In the medium-size cultivar, the pattern of the basic subunit composition was similar to that of the small-size cultivars except that it contains much more B_4, which is a characteristic band of the large-size group. On the other hand, in the acidic subunit groups, the subunit compositions were shown qualitatively, since it was difficult to calculate relative proportions of subunits from the densitometric scanning of the gels. The acidic subunits of A_1, A_2, A_4 and A_6 existed commonly in legumins from all cultivars examined. In addition to these acidic subunits, A_3, A_5 and A_7 and A_3, A_5 and A_7 existed in legumins of small-, large- and medium-size cultivars, respectively. Thus, the number of the acidic subunits was different among the cultivars, i.e., five in the small-size, six in the large-size and seven in the medium-size cultivars.





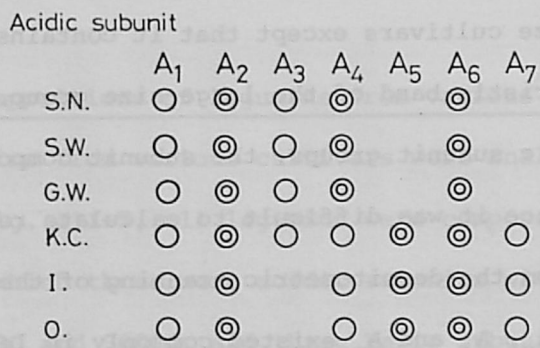


Fig. III-6. PGEF of legumins from six cultivars.
100 μg of each legumin was electrophoresed as described in MATERIALS AND METHODS. A, PGEF patterns. B, Schematic representation of subunit compositions. The relative proportions of the basic subunits were calculated from densitometric scanning of the gels.

Fig. III-7. SDS-PAGE in the absence of 2-ME of legumins from six cultivars.
100 μg of each legumin was electrophoresed as described in MATERIALS AND METHODS. Gel symbols are the same as described in Fig. III-2. Migration is from top to bottom.

The pattern of the acidic subunit composition of the medium-size cultivar seemed to be rather similar to that of the large-size cultivars. As a whole subunit composition, the medium-size cultivar had both characteristics of the small- and large-size cultivars.

Intermediary subunit compositions of legumins from various cultivars

In order to investigate the intermediary subunits in which acidic subunits linked with basic subunits through disulfide bridges [15] in legumins from the seeds of various cultivars, legumins were analyzed by SDS-PAGE in the absence of 2-ME. As shown in Fig. III-7, three major bands with molecular weights of 61700, 59800 and 48000 which did

not coincide in position with the subunit components (Fig. III-5) newly appeared in all legumins. The number, proportion and molecular sizes of the intermediary subunits of legumins were similar among the cultivars.

III-4 DISCUSSION

The subunit compositions of legumins from various cultivars of broad bean seeds were compared in terms of gel electrophoreses. The results described in this chapter indicate that there are differences in the subunit compositions of legumins among the cultivars examined from the standpoint of the molecular charges of subunits (Fig. III-6) but not from that of molecular sizes (Fig. III-5 and 7). The variations of the subunit compositions among the cultivars may not be resulted from artifacts such as urea effect [55], deamidation [56] or proteolytic modification, on the basis of the following observations; (1) the isolation of subunits of legumin was performed at acidic pH (pH 6.3) and at low temperature (4°C), (2) there was no change in the electrophoretic patterns of the subunits of legumins even if the extracts prepared using 50 mM potassium phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 7.6) each contained 1 M NaCl were incubated for 5 days at 20°C; (3) the patterns of the subunit compositions were constant and reproducible for each cultivars. Therefore, the variations among the cultivars seem to be substantial characteristics for them.

The patterns of the subunit compositions of the cultivars can be classified into three groups, that is, small-, medium- and large-size groups. The medium-size cultivars, Kumamoto-Churyu, has the character-

istics of both small- and large-size cultivars (see Fig. III-6). Recently, it has been reported that there are differences in the subunit compositions of legumin-like proteins of some kinds of legumes among their cultivars [25,26,30-33]. In the case of Pisum sativum, Thomson et al. [25,79] observed an additive inheritance of subunit components in the crosses between cultivars. Therefore, the medium-size cultivar should be regarded as a situation intermediate between the small- and the large-size cultivars, with partial representation of gene products, that is partial additive inheritance. This regard may support the conjecture that a small-size cultivar might be introduced from China into Japan for the first time and then a large-size cultivar, Otafuku, might be done.

The presence of the electrophoretic variants of the subunit proteins of legumin revealed in this chapter suggests that there may be heterogeneity of molecular species of legumin in broad bean, if legumin is anticipated to be also composed of A_6B_6, which has been reported in legumin-like proteins of Glycin max [9,15,17], Avena sativa [80], Sesamum indicum [43], Pisum sativum [26,27], Arachis hypogaea [37] and the others [8], because the amount ratio of the acidic subunits with molecular weights of 51000, 49000 and 36000 and the presence of more than six kinds of subunits are in conflict with the A_6B_6 structure.

IV HETEROGENEITY OF MOLECULAR SPECIES OF SOYBEAN GLYCININ

IV-1 INTRODUCTION

The structural properties of glycinin have been well investigated [9-19]: several structural models of glycinin have been proposed [16, 17], and NH_2-terminal amino acid sequences of glycinin subunits have recently been presented [18,19]. In these studies, glycinin was considered as a homogeneous protein with respect to molecular species. Thus, glycinin has been regarded as a single molecular species with an inherent subunit composition. However, as discussed in Chapter II, the results relating to the subunit compositions of glycinins from various cultivars suggest the heterogeneity of glycinin molecular species.

In the present study, the heterogeneity of glycinin molecular species from various soybean cultivars was investigated. The results obtained demonstrate that glycinin exhibits the heterogeneity of molecular species from the standpoint of molecular size.

IV-2 MATERIALS AND METHODS

Polyacrylamide gradient gel (4-30 %) was purchased from Pharmacia Co., Ltd. The glycinin rich fractions and purified glycinins from various cultivars were prepared as described in Chapter II except glycinin from var. Tsuru-no-ko. Other materials were the same as described in the previous chapters.

DEAE-Sephadex A-50 column chromatography of glycinin rich fraction from var. Tsuru-no-ko

The glycinin rich fraction from var. Tsuru-no-ko prepared by the

method of Thanh et al. [49] were applied to a DEAE-Sephadex A-50 column equilibrated with 0.035 M potassium phosphate buffer (pH 7.6) containing 0.25 M NaCl, 10 mM 2-ME and 0.02 % NaN_3 at 5°C. Elution was performed with a linear gradient (0.25 to 0.45 M) of NaCl.

Electrophoreses

SDS-PAGE was performed by the method of Laemmli[50] as described in Chapter II. PGGE was performed by using 8 x 7 cm slab gradient gel, 4-30 % (w/v) polyacrylamide in concentration, with Pharmacia Electrophoresis Apparatus GE-4. Electrode buffer was Tris-glycine buffer (50 mM Tris, 384 mM glycine, pH 8.3). Equilibrium run was carried out for 1 h at a constant voltage of 70 V. After the samples were applied on the gel, electrophoresis was carried out for 30 min at a constant voltage of 70 V and for 72 h at a constant voltage of 100 V at 4°C. The molecular weights of glycinin molecular species were estimated by comparing their mobilities with those of markers of known molecular weights. Molecular weight markers used were thyroglobulin (669000), ferritin (460000), catalase (240000) and bovine serum albumin (67000).

IV-3 RESULTS AND DISCUSSION

Heterogeneity of glycinin from soybean var. Tsuru-no-ko

Glycinin-rich fractions obtained by the method of Thanh et al. [49] were applied to a DEAE-Sephadex A-50 column. The chromatogram of glycinin from Tsuru-no-ko is shown in Fig. IV-1. The flowed through fraction may be derived from the 7S component which contaminated the glycinin-rich fraction (see Fig. IV-3, Gel 2). Glycinin eluted as a single peak which was comparatively sharper than that of legumin from

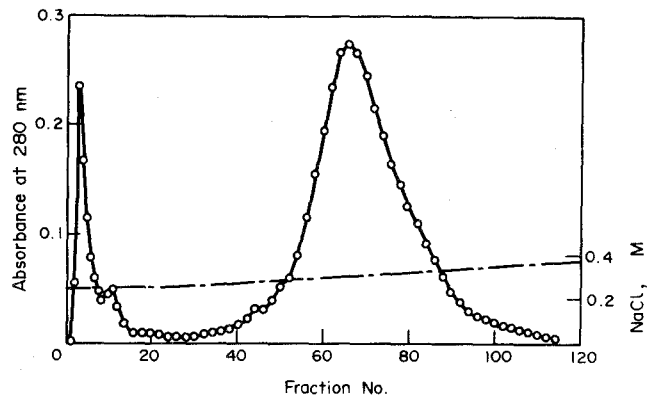


Fig. IV-1. DEAE-Sephadex A-50 column chromatography of glycinin-rich fraction from Tsuru-no-ko.
Glycinin (60 mg) was equilibrated with 35 mM potassium phosphate buffer (pH 7.6) containing 0.25 M NaCl, 10 mM 2-ME and 0.02 % NaN_3 and then applied on the column (1.5 x 17 cm) which had been equilibrated with the buffer at 4°C. The column was washed with the buffer. Development: 600 ml buffer, 0.25 to 0.45 M NaCl; flow rate, 20 ml/h; fraction volume, 5ml. —O— , absorbance at 280 nm; — - — , concentration of NaCl.

broad bean (see Chapter V, Fig. V-1).

The fractions from the column were analyzed by PGGE. The electrophoretic patterns are shown in Fig. IV-2. Two zones were separated in all samples, the faster and slower groups of bands being monomers (half-molecules of glycinin) and dimers (glycinin), respectively. The monomer bands may be derived from the dissociation of the glycinin molecule during the electrophoresis, because the glycinin molecule has been shown to undergo reversible dissociation to a 7S monomer [81], although it is unclear whether it is intact or not. In fact, a similar monomer band was observed on PAGE by Kitamura et al. [82]. The unfractionated glycinin gave three bands with apparent molecular weights of

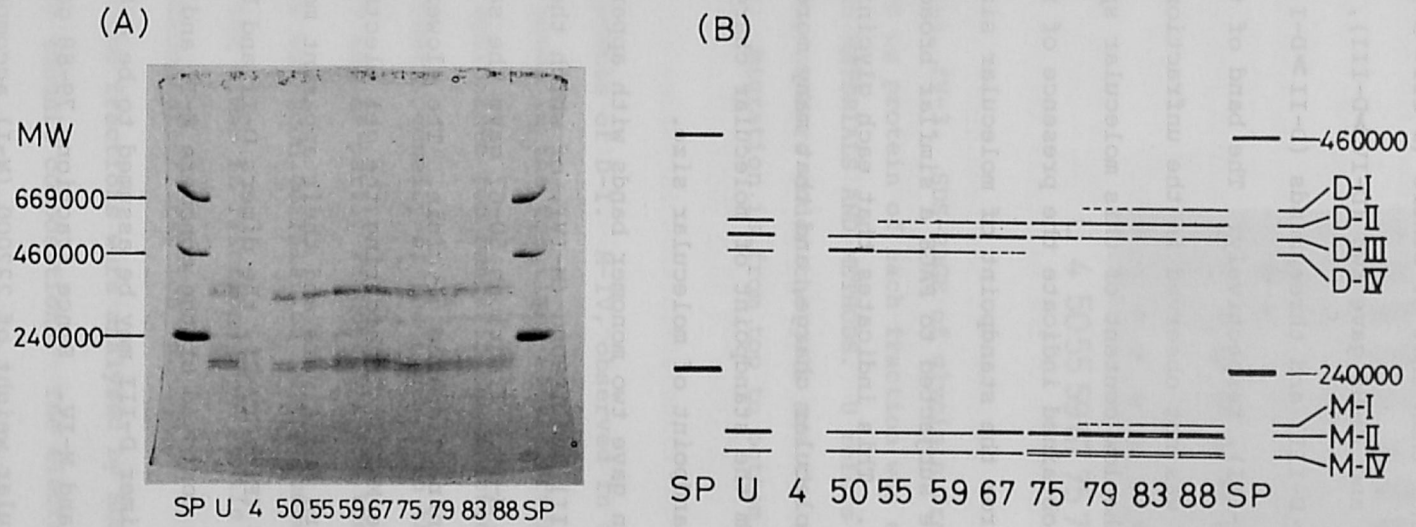


Fig. IV-2. PGGE of glycinin fraction from a DEAE-Sephadex A-50 column.
A, 20 μg protein of each fraction equilibrated with 35 mM potassium phosphate buffer (pH 7.6) containing 0.2 M NaCl, 10 mM 2-ME and 0.02 % NaN_3 was applied on the gel as described in MATERIALS AND METHODS. SP and U refer to standard proteins (thyroglobulin, 669000; ferritin, 460000; catalase, 240000) and the unfractionated sample, respectively. The numbers under the gel are the fraction numbers of DEAE-Sephadex A-50 column chromatography. Migration is from top to bottom. B, Diagramatic representation of A.

360000 (D-II), 345000 (D-III) and 340000 (D-IV), of which the proportion of the middle band was higher than that of others (D-III > D-II, D-IV). Fractions 50, 59-67, 75 and 79-88 gave two (D-IV > D-III), three (D-III > D-II, D-IV), two (D-II = D-III) and three bands (D-II > D-I (molecular weight of 375000), D-III), respectively. The band of the largest molecular species (D-I) was not observed in the unfractionated glycinin. This may be due to the low content of this molecular species in glycinin. The results thus obtained indicate the presence of four molecular species of glycinin from the standpoint of molecular size. However, when the fractions were subjected to PAGE a similar broad band was observed in every fractions. This indicates that each glycinin molecular species has similar molecular charge, and that many more molecular species may exist from the standpoint of molecular charge than those observed from the standpoint of molecular size.

The unfractionated glycinin gave two monomer bands with apparent molecular weights of 211000 (M-II) and 199000 (M-IV), of which the proportion of M-II was higher than M-IV. Fractions 50-67 gave the same monomer bands as those of the unfractionated glycinin. The slower eluting fractions contained more M-II. Considering the gel electrophoretic patterns of the monomers and dimers and their apparent molecular weights in fractions 50-67 (Fig. IV-2), the dimers D-II and D-IV may be assumed to be homodimers composed of the monomers M-II and M-IV, respectively. Similarly, the dimer D-III may be assumed to be a heterodimer made up of monomers M-II and M-IV. Since fractions 79-88 gave another monomer band with a molecular weight of 222000 (M-I) accompanying the appearance of D-I, it may be assumed that M-I is one of the monomer

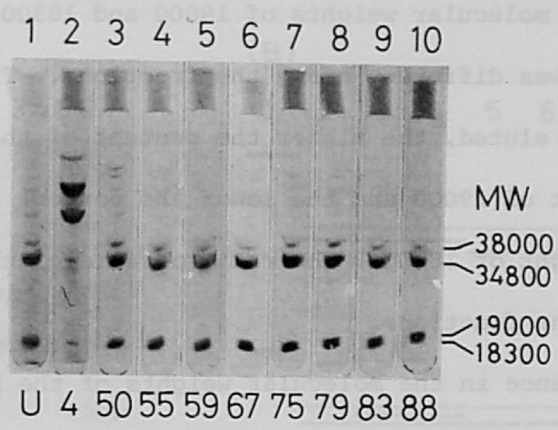


Fig. IV-3. SDS-PAGE of glycinin fractions.
30 μg protein of each fraction was electrophoresed as described in MATERIALS AND METHODS. U refers to the unfractionated sample. The numbers under the gels are the same as described in Fig. IV-2. Migration is from top to bottom.

components of D-I. M-IV, observed in fractions 50-67, was split into two bands in the fractions eluted after fraction 75. Similarly M-II was split into two bands in the fractions after fraction 79. This microheterogeneity of the monomer in the fractions after fraction 75 or 79 may be partly responsible for the heterogeneity of the dimer. There was good correlation in the number of bands between the monomer and the dimer in fractions 55-67, while this was not the case in the fractions after fraction 75.

The fractions were analyzed by SDS-PAGE in order to elucidate their subunit compositions. The electrophoretic patterns are shown in Fig. IV-3. All the glycinin fractions gave four bands, of which two were acidic with molecular weights of 38000 and 34800, and another two

were basic with molecular weights of 19000 and 18300. The ratio of basic subunits was different among the fractions. Thus, the more slowly glycinin eluted, the higher the content of the subunit with a molecular weight of 19000 and the lower the content of the subunit with a molecular weight of 18300. The ratio of acidic subunits was almost similar among the fractions.

The difference in the molecular weights of the basic subunits does not seem to account for the differences in the molecular weights of the dimers and those of the monomers. However, it has been shown that the acidic subunit with a molecular weight of 34800 and the basic subunits are heterogeneous from the standpoint of their molecular charges [9,15, 19,48,54], and that glycinin has intermediary subunits (AB), disulfide-bonded acidic (A) and basic (B) subunits, and is composed of 6(AB) [15, 17]. Therefore, there may be some kinds of intermediary subunits composed of subunits which are identical in their molecular weights but are different in their molecular charges. Thus, the number and the position of disulfide bonds contributing to the formation of intermediary subunits may be different. These differences should effect the compactness of their structure, which in its turn may influence the apparent molecular weight of each molecular species of glycinin, which was observed on PGGE.

From analysis of the subunit composition of glycinin fractions eluted from the column by PGEF in the presence of urea and 2-ME, the author found differences in the subunit compositions from the standpoint of their charges among the fractions (data not shown).

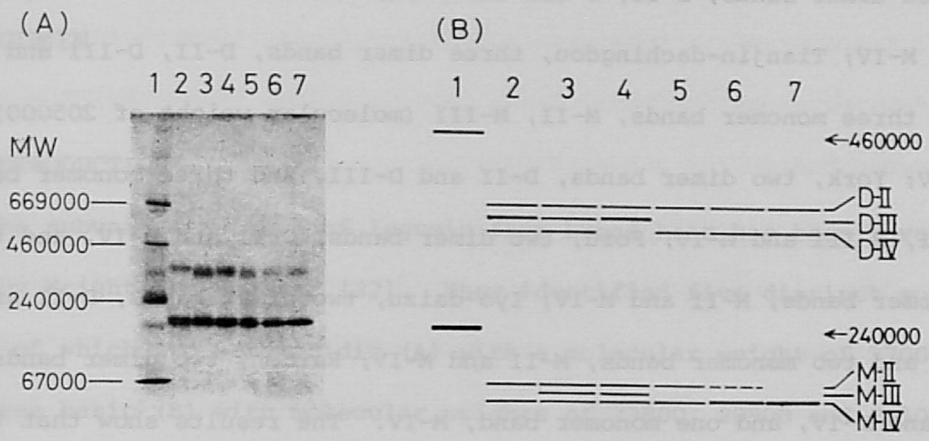


Fig. IV-4. PGGE of glycinins from various soybean cultivars. A, 20 μg protein of each cultivar was electrophoresed as described in Fig. IV-2. Migration is from top to bottom. Lane 1, standard proteins (thyroglobulin, ferritin, catalase and bovine serum albumin, 67000); Lane 2, Shiro-tsuru-no-ko; Lane 3, Tianjin-dachingdou; Lane 4, York; Lane 5, Ford; Lane 6, Iyo-daizu; Lane 7, Raiden. B, Diagramatic representation of A.

Heterogeneity of glycinin in other cultivars

To elucidate whether each glycinin from a wide range of soybean cultivars exhibits the heterogeneity of molecular species, glycinins from various cultivars, i.e., Shiro-tsuru-no-ko, Tianjin-dachingdou, York, Ford, Iyo-daizu and Raiden, were analyzed by PGGE. As shown in Chapter II, glycinins of these cultivars are different in their subunit compositions. Thus, Shiro-tsuru-no-ko and Tianjin-dachingdou are composed of 7 acidics and 8 basics; York, 7 acidics and 7 basics; Ford, 6 acidics and 7 basics; Iyo-daizu, 6 acidics and 5 basics; Raiden, 6 acidics and 3 basics from the standpoint of the molecular charges of subunit. As shown in Fig. IV-4, glycinin from Shiro-tsuru-no-ko gave

three dimer bands, D-II, D-III and D-IV, and two monomer bands, M-II and M-IV; Tianjin-dachingdou, three dimer bands, D-II, D-III and D-IV, and three monomer bands, M-II, M-III (molecular weight of 205000) and M-IV; York, two dimer bands, D-II and D-III, and three monomer bands, M-II, M-III and M-IV; Ford, two dimer bands, D-II and D-IV, and two monomer bands, M-II and M-IV; Iyo-daizu, two dimer bands, D-II and D-IV, and two monomer bands, M-II and M-IV; Raiden, two dimer bands, D-II and D-IV, and one monomer band, M-IV. The results show that the numbers of glycinin molecular species are different among cultivars. The extent of the heterogeneity of glycinins seems to correlate with the diversity of the subunit compositions of glycinins.

V HETEROGENEITY OF MOLECULAR SPECIES OF BROAD BEAN LEGUMIN

V-1 INTRODUCTION

The subunit structure of legumin from broad bean has been investigated by Wright and Boulter [22]. They identified five distinct subunits, of which two were acidic (A) with a molecular weight of 37000, and three basic (B) with molecular weights of 23800, 20900 and 20100 in var. Triple White. They proposed that legumin of Triple White was composed of A_6B_6. However, they did not consider the heterogeneity of legumin molecular species. On the other hand, the author observed other two acidic subunits with molecular weights of 51000 and 49000 in legumins from various cultivars, and the ratio of the amounts of three acidic subunits with molecular weights of 51000, 49000 and 36000 was approx. 1 : 1 : 10 as shown in Chapter III. Moreover, legumins from various cultivars contained eight kinds of basic subunits from the standpoint of their molecular charges as shown in Chapter III. These may indicate the heterogeneity of legumin molecular species.

In view of the evidence described above, legumins from various cultivars were fractionated and their subunit compositions were investigated in order to elucidate the heterogeneity of legumin from broad bean. The results obtained indicate the heterogeneity of legumin molecular species. The possible molecular species of legumin which are common to all cultivars examined are presented.

V-2 MATERIALS AND METHODS

Seeds of Vicia faba vars. Sanuki-Nagasaya, Kumamoto-Churyu and Otafuku were obtained from Takii Seed Co., Ltd. Other materials were as described in the other chapters.

Preparation of purified legumin

Crude extract of each cultivar of broad bean seeds was prepared by homogenizing bean meal with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl and was fractionated into four fractions, called 2S, 7S, 11S and 15S fractions, by sucrose density gradient centrifugation as described in Chapter III.

DEAE-Sephadex A-50 column chromatography

DEAE-Sephadex A-50 column chromatography was performed using 1.5 x 20 cm column equilibrated with 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 M NaCl and 0.02 % NaN_3 at 4°C. Legumins from three cultivars prepared by sucrose density gradient centrifugation were equilibrated with the buffer and then applied on the columns. The column was washed with the buffer. Elution was performed by 500 ml of the buffer containing NaCl in linear concentration of 0.2 M to 0.35 M.

Electrophoreses

PAGE, SDS-PAGE and PGGE were performed as described in the other chapters.

Analysis of subunit compositions of legumin molecular species

The analysis of subunit compositions of legumin molecular species was carried out by combining two disc electrophoresis systems with PAGE and SDS-PAGE as described by Kanda et al. [83] with a slight modification. 20 μg protein dialyzed against 50 mM potassium phosphate buffer

(pH 7.6) containing 0.2 M NaCl at 4°C was electrophoresed on polyacrylamide gel using 0.6 x 10 cm glass tube according to the method of Davis [77] as described in Chapter III. After electrophoresis, the gel was stained with 0.5 % amidoblack 10B in 20 % acetic acid for 5 min and destained with 7 % acetic acid. The protein bands were cut off and rinsed with 63.5 mM Tris-HCl buffer (pH 6.8) containing 1 % SDS, 0.2 M 2-ME and 30 % sucrose for 30 min at room temperature and another 30 min at 50°C with a change of the buffer. The isolated gel slices were put on the top of the SDS-polyacrylamide gel in a 0.6 x 10 cm glass tube and a trace amount of tracking dye (0.02 % bromophenol blue in the SDS-buffer) was layered on the gel slices. SDS-PAGE was performed according to the procedure of Laemmli [50] as described in Chapter II.

The destained gels were scanned at 590 nm as described in Chater II.

Analysis of subunit compositions of legumin intermediary subunits

At first, legumin (300 μg) was electrophoresed on SDS-polyacrylamide gel in the absence of 2-ME using 1 x 10 cm glass tube. After electrophoresis, the gel was stained and destained, and the protein band was cut off and electrophoresed in the presence of 2-ME as described above.

V-3 RESULTS

DEAE-Sephadex A-50 column chromatography

Legumin from three cultivars of broad bean were prepared by sucrose density gradient centrifugation and then were applied on a DEAE-Sephadex A-50 column. The chromatograms are shown in Fig. V-1. The

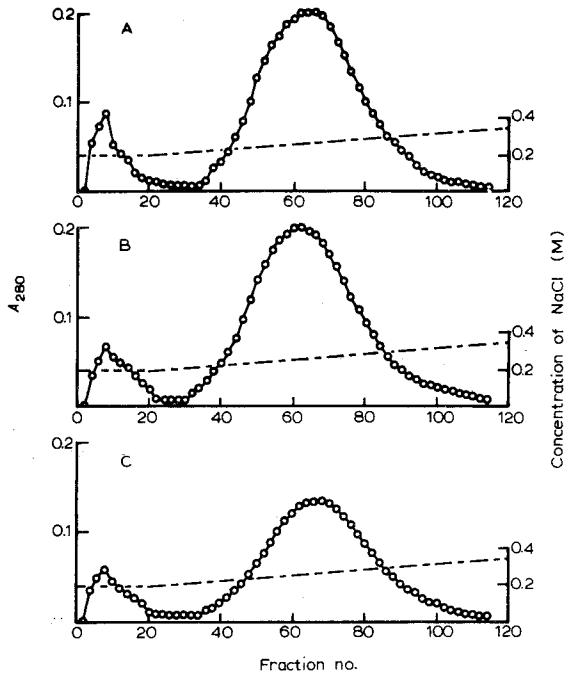


Fig. V-1. DEAE-Sephadex A-50 column chromatography of legumins from three cultivars.
55, 55 and 45 mg proteins of 11S fractions prepared by sucrose density gradient centrifugation were applied on the column for Sanuki-Nagasaya (A), Kumamoto-Churyu (B) and Otafuku (C), respectively, and then chromatographed as described in MATERIALS AND METHODS. The flow rate was 18 ml/h. Fraction volume was 6 ml. —○— : absorbance at 280 nm, — - — : concentration of NaCl.

flow through fraction of each cultivar may be derived from the 7S component which contaminated the legumin fractions of the sucrose density gradient centrifugation (Figs. V-3-8, 4-8 and 5-8). Legumin of each cultivar was eluted as a single peak. However, it was a broad peak in the range of fractions 40 to 100 (approx.). The fractions from the

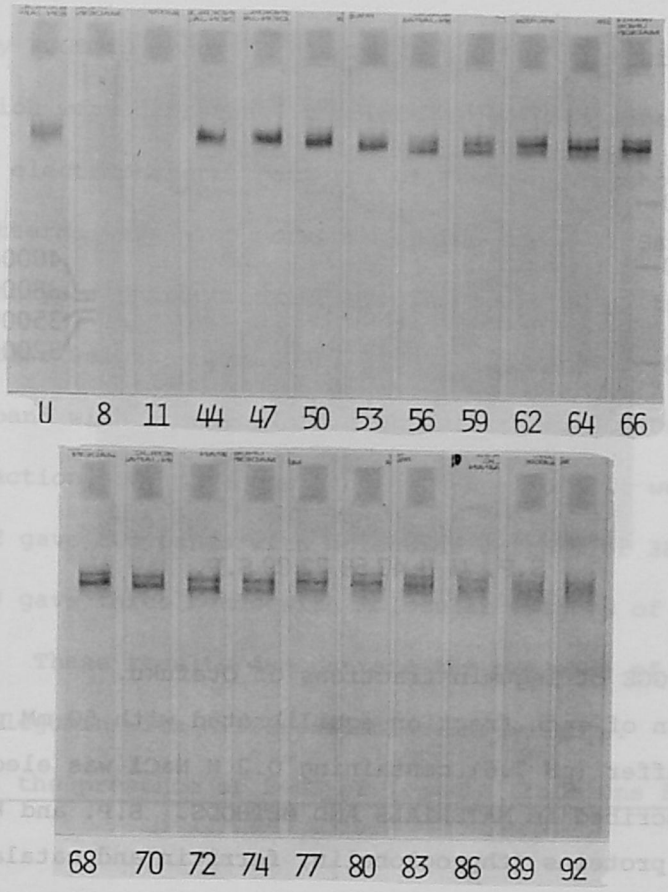


Fig. V-2. PAGE of legumin fractions of Otafuku.
20 µg protein of each fraction was dialyzed against 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 M NaCl at 4°C and then electrophoresed as described in MATERIALS AND METHODS. U refers to the unfractionated sample. The numbers under the gels are the fraction numbers of DEAE-Sephadex A-50 column chromatography. Migration is from top to bottom.

columns of three cultivars were analyzed by various gel electrophoreses.

PAGE of legumin fractions from three cultivars

The legumin fractions eluted from the DEAE-Sephadex A-50 column of three cultivars were analyzed by PAGE. The electrophoretic pat-

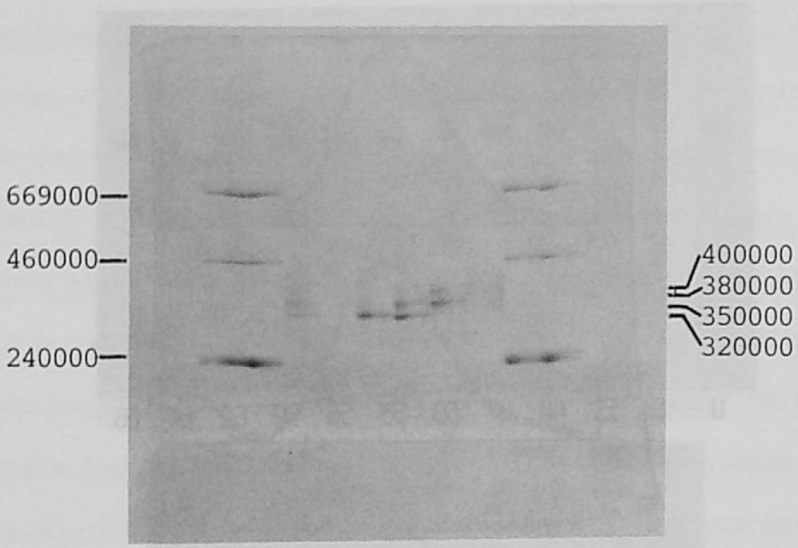


Fig. V-3. PGGE of legumin fractions of Otafuku.
10 μg protein of each fraction equilibrated with 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 M NaCl was electrophoresed as described in MATERIALS AND METHODS. S.P. and U refer to standard proteins (thyroglobulin, ferritin and catalase) and the unfractionated sample, respectively. The numbers under the gels are the fraction numbers of DEAE-Sephadex A-50 column chromatography. Migration is from top to bottom.

terns of Otafuku are shown in Fig. V-2. Similar patterns were also obtained in the cases of Sanuki-Nagasaya and Kumamoto-Churyu. Fractions 8 and 11 of the flow through fraction gave very pale bands. The more quickly eluting fractions of legumin, e.g., fractions 44 and 47, gave a single band, while those eluting more slowly gave the more bands.

PGGE of legumin fractions from three cultivars

The legumin fractions from the column of three cultivars were

analyzed by PGGE in order to elucidate whether molecular species of legumin which were different in molecular weights were present in broad bean. The electrophoretic patterns of Otafuku are shown in Fig. V-3. Similar patterns were also obtained in the cases of Sanuki-Nagasaya and Kumamoto-Churyu. Unfractionated sample consisted of three components with molecular weights of 380000, 350000 and 320000. Fraction 8 gave a vary pale band with a molecular weight of around 200000. The faster eluting fraction, 47, gave one band with a molecular weight of 320000. Fraction 72 gave two bands with molecular weights of 380000 and 350000. Fraction 89 gave three bands with molecular weights of 400000, 380000 and 350000. These results demonstrate the presence of four molecular species of legumin with different molecular weights.

SDS-PAGE in the presence of 2-ME of legumin fractions from three cultivars

The legumin fractions eluted from the column of three cultivars were analyzed by SDS-PAGE. The electrophoretic patterns of Otafuku are shown in Fig. V-4. Similar results were also obtained in the cases of Sanuki-Nagasaya and Kumamoto-Churyu. Unfractionated sample consisted of six subunits with molecular weights of 51000, 49000, 36000, 23000, 20500 and 19000, of which the smaller three bands and the larger three bands were assigned to the basic subunits and the acidic subunits, respectively, as shown in Chapter III. The faster eluting fractions of legumin, fractions 44 and 47, consisted of only three components with molecular weights of 36000, 23000 and 20500. On the other hand, the more slowly eluting fractions contained all of the six subunits. Thus the more slowly the legumin eluted, the more plentiful

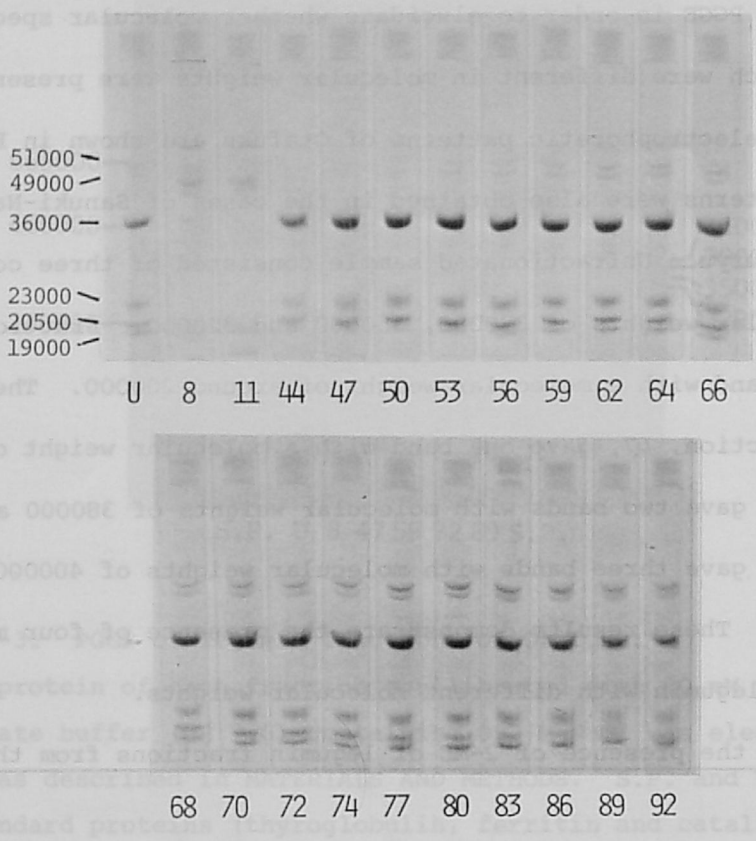


Fig. V-4. SDS-PAGE of legumin fractions of Otafuku.
30 μg protein of each fraction was electrophoresed as described in MATERIALS AND METHODS. U refers to the unfractionated sample. The numbers under the gels are the fraction numbers of DEAE-Sephadex A-50 column chromatography. Migration is from top to bottom.

were the subunits with molecular weights of 51000, 49000 and 19000, while the ratio of the amount of the subunit with a molecular weight of 20500 decreased.

SDS-PAGE in the absence of 2-ME of legumin fractions from three cultivars

The legumin fractions eluted from the column of three cultivars

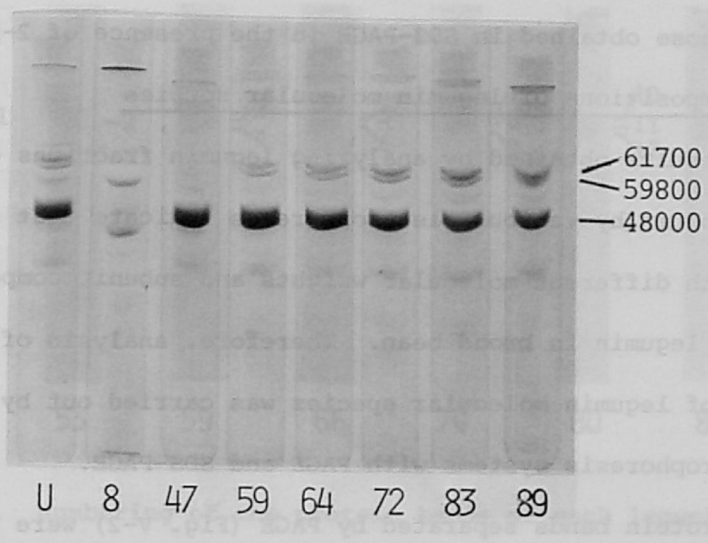


Fig. V-5. SDS-PAGE of legumin fractions of Otafuku in the absence of 2-ME.
30 μg protein of each fraction was electrophoresed in the absence of 2-ME as described in MATERIALS AND METHODS. U refers to the unfractionated sample. The numbers under the gels are the fraction numbers of DEAE-Sephadex A-50 column chromatography. Migration is from top to bottom.

were analyzed by SDS-PAGE in the absence of 2-ME in order to clarify the composition of intermediary subunits. The electrophoretic patterns of Otafuku are shown in Fig. V-5. Similar results were also obtained in the cases of Sanuki-Nagasaya and Kumamoto-Churyu. Unfractionated sample consisted of three intermediary subunits with molecular weights of 61700, 59800 and 48000. The more quickly eluting fraction of legumin, fraction 47, consisted of only one kind of intermediary subunit with a molecular weight of 48000. On the other hand, all of three bands were observed in the slower eluting fractions. Thus the more slowly the legumin eluted, the more plentiful were the intermediary subunits with molecular weights of 61700 and 59800. These results are consist-

ent with those obtained in SDS-PAGE in the presence of 2-ME.

Subunit compositions of legumin molecular species

The results obtained by analyzing legumin fractions of the DEAE-Sephadex column by various electrophoreses indicate that some molecular species with different molecular weights and subunit compositions are present as legumin in broad bean. Therefore, analysis of subunit compositions of legumin molecular species was carried out by combining two disc electrophoresis systems with PAGE and SDS-PAGE.

The protein bands separated by PAGE (Fig. V-2) were numbered according to the order of elution from the column and their electrophoretic mobilities as shown in Fig. V-6. Each band was analyzed by SDS-PAGE from the standpoint of the molecular sizes of the subunits as described in MATERIALS AND METHODS. SDS-PAGE pattern of each protein band is shown in Fig. V-7. The ratio of the amount of each band observed on SDS-polyacrylamide gel was estimated densitometrically and the results are listed in Table V-I.

As shown in Fig. V-6, various bands had the same mobilities on PAGE, e.g., bands 2 and 4, 3, 6 and 9, 5, 8, 11 and 15, and 7, 10 and 14. However, bands 2 and 4, 3, 6 and 9, and 5 and 8 gave similar SDS-PAGE patterns, respectively, while bands 8 (or 5), 11 and 15, and 7, 10 and 14 gave fairly different patterns (Fig. V-7 and Table V-I). This result suggests that there are some molecular species with the same mobilities on PAGE but different subunit compositions in broad bean.

Subunit compositions of intermediary subunits

The intermediary subunits were electrophoresed on SDS-polyacryl-

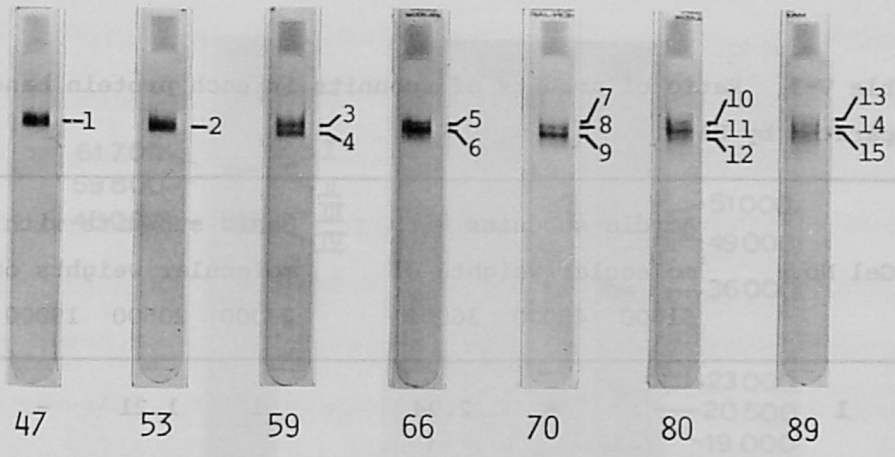


Fig. V-6. Numbering of the protein bands of each legumin fractions from a DEAE-Sephadex A-50 column separated by PAGE. The protein bands separated by PAGE as shown in Fig. V-2 were numbered as described in the text. The numbers under the gels are the fraction numbers of DEAE-Sephadex A-50 column chromatography.

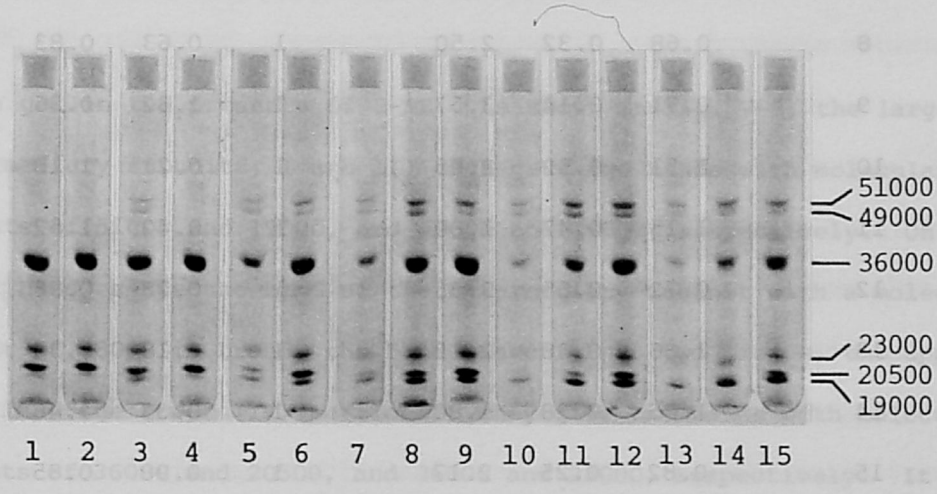


Fig. V-7. SDS-PAGE of the protein bands separated by PAGE. Each stained protein band separated by PAGE (Fig. V-6) was cut off and electrophoresed as described in MATERIALS AND METHODS. The number of gel slices used (two to four) was dependent on the intensity of the stained band. Migration is from top to bottom.

Table V-I. Ratio of amounts of subunits in each protein band separated by PAGE.

Gel No.	Acidic subunits with molecular weights of			Basic subunits with molecular weights of		
	51000	49000	36000	23000	20500	19000
1	-	-	2.94	1	1.21	-
2	-	-	3.24	1	1.59	-
3	0.29	0.18	3.57	1	1.21	0.11
4	-	-	3.15	1	1.89	-
5	0.47	0.35	2.94	1	0.84	0.78
6	0.32	0.17	2.32	1	1.44	0.65
7	0.77	0.62	2.56	1	0.26	1.05
8	0.68	0.32	2.50	1	0.63	0.83
9	0.71	0.14	3.57	1	1.32	0.36
10	1.51	0.52	2.05	1	0.21	1.18
11	1.15	0.47	1.80	1	0.42	1.52
12	0.72	0.55	2.35	1	0.78	0.96
13	1.66	0.38	2.13	1	0.18	1.34
14	1.05	0.28	1.80	1	0.33	1.48
15	0.82	0.25	2.17	1	0.98	0.85

The ratios were estimated densitometrically, taking the basic subunit with a molecular weight of 23000 as unity.

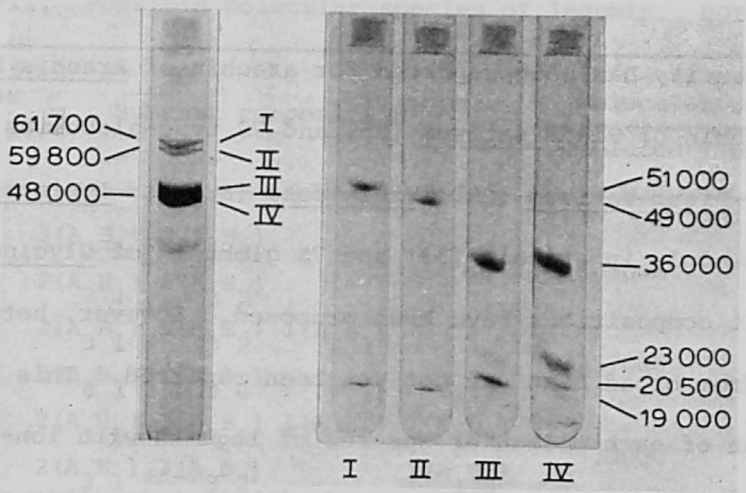


Fig. V-8. Subunit compositions of intermediary subunits. Each protein band (I, II, III and IV) separated by SDS-PAGE in the absence of 2-ME was cut off and electrophoresed in the presence of 2-ME as described in MATERIALS AND METHODS. Migration is from top to bottom.

amide gel in the presence of 2-ME. As shown in Fig. V-8, the larger intermediary subunits, I and II, each gave two bands with molecular weights of 51000 and 19000, and 49000 and 19000, respectively. On the other hand, since the band of the intermediary subunit with a molecular weight of 48000 is broad, the band was cut into two pieces, the upper (III) and the lower (IV) portions. They gave two bands with molecular weights of 36000 and 20500, and 36000 and 23000, respectively. It is of interest that the intermediary subunit composed of 36000 and 20500 migrated slower than that composed of 36000 and 23000. This may be due to the differences in compactness of the structure between these intermediary subunits.

V-4 DISCUSSION

Heterogeneity has been reported for arachin of Arachis hypogaea [32,33], legumin of Pisum sativum [25] and 7S type-globulins of Glycine max [84-87], Pisum sativum [25], Vicia faba [88] and Lupinus angustifolius [31]. Only in arachin [33] and 7S globulin of Glycine max [87] their subunit compositions have been proposed. However, heterogeneity of legumin from broad bean has not yet been reported. This may be due to separation of each molecular species of legumin with ion-exchange chromatography being difficult compared with that in other seeds (Fig. V-1). The heterogeneity of legumin from each cultivar observed here may not be as a result of artifacts such as deamidation [56] or proteolysis, on the basis of the following observations; (1) there was no change in the electrophoretic patterns of the subunits of legumin even if extracts prepared using 50 mM potassium phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 7.6) each containing 1 M NaCl were incubated for 5 days at 20°C; (2) the pattern of heterogeneity was constant and reproducible for each cultivars.

The subunit patterns of legumins from three cultivars were similar among each other from the standpoint of the molecular sizes of the subunits (Figs. V-4 and 5). Therefore, the author attempted to list the possible molecular species of legumin which are common to the three cultivars, constructed from the subunit groups classified according to their sizes. The possible molecular species are shown in Table V-II. Each subunit composition was deduced from the results shown in Table V-I on the basis of the assumption that all the molecular species of legumin are composed of 6(AB), which had been found in glycinin of

Table V-II. Possible molecular species of legumin.

Molecular species	Subunit composition*				Molecular weights	
					Calculated**	Observed***
I - 1	$3(A_3B_1)$	$3(A_3B_2)$			345000	320000
I - 2	$2(A_3B_1)$	$4(A_3B_2)$				
II - 1	$2(A_3B_1)$	$3(A_3B_2)$	$1(A_2B_3)$		357000	350000
II - 2	$2(A_3B_1)$	$3(A_3B_2)$		$1(A_1B_3)$		
III - 1	$2(A_3B_1)$	$2(A_3B_2)$	$1(A_2B_3)$	$1(A_1B_3)$	370000	
III - 2	$2(A_3B_1)$	$2(A_3B_2)$		$2(A_1B_3)$		380000
IV - 1	$2(A_3B_1)$	$1(A_3B_2)$	$2(A_2B_3)$	$1(A_1B_3)$	380000	
IV - 2	$2(A_3B_1)$	$1(A_3B_2)$	$1(A_2B_3)$	$2(A_1B_3)$		
V - 1	$2(A_3B_1)$		$1(A_2B_3)$	$3(A_1B_3)$	396000	400000

* The subunits with molecular weights of 51000, 49000, 36000, 23000, 20500 and 19000 shown in Fig. V-4 were termed A_1, A_2, A_3, B_1, B_2 and B_3, respectively.

** Calculated from the molecular weight of each subunit.

*** Estimated from the results of PGGE (Fig. V-3).

Glycine max [15,17,19], legumin of Pisum sativum [26,27], cucurbitin of Cucurbita maxima [42] and 13S globulin of Sesamum indicum [45], and the following experimental evidences: (1) at least four kinds of molecular species which were different in molecular weights were present as legumin in broad bean (Fig. V-3); (2) the intermediary subunits with molecular weights of 61700, 59800 and 48000 are composed of the acidic subunits with molecular weights of 51000, 49000 and 36000 and the basic subunits with molecular weights of 19000, 19000 and 23000 or 20500, respectively (Fig. V-8). The molecular weight of each molecular species

calculated from a molecular weight of each subunit is also listed in Table V-II, thus five groups of molecular species with different molecular weights are present in broad bean. However, PGGE gave four bands with different molecular weights. This discrepancy may be derived from the closeness of the molecular weights of group III (370000) and group IV (380000), or that they have similar apparent molecular sizes.

As shown in Fig. III-6 of Chapter III, legumins of Sanuki-Nagasaya, Kumamoto-Churyu and Otafuku gave eight bands as basic subunits on PGEF, which were more than those observed on SDS-PAGE. Moreover, the kinds of main bands of basic subunits were different among the three cultivars. Therefore, when the subunit compositions of legumin molecular species are represented by the subunits classified according to their charges, the number of possible molecular species of legumin must be much more than that presented in Table V-II. This suggests that each of nine molecular species presented in Table V-II may have further submolecular species. However, the subunit compositions of them can not be deduced at present.

As described in the preceding chapter, soybean glycinin also exhibits the heterogeneity of molecular species, as well as other 11S-type globulins from ground nut [32,33] and pea [25] and 7S type-globulins from soybean [84-87], pea [25] and lupin [31]. Therefore, heterogeneity of molecular species may be an inherent property of the major storage proteins of legume seeds in general.

VI FORMATION OF PSEUDO-11S GLOBULINS FROM SUBUNITS OF SOYBEAN GLYCININ AND BROAD BEAN LEGUMIN

VI-1 INTRODUCTION

Recent developments in investigations on the structure of the main components of seed storage proteins suggest that 11S globulins are composed of six acidic (A) and six basic (B) subunits. Of various 11S globulins, those from soybean [15,17,19, see Chapter II], broad bean [see Chapter III], pea [26,27], pumpkin [42] and sesame [45] have been shown to have common intermediary subunits, in which the acidic and basic subunits are linked by disulfide bridges in 1 : 1 ratio and to be composed of 6(AB). However, the mechanism of the formation of 11S-structure from subunits or intermediary subunits has not yet been elucidated. Kitamura et al. reported that the 6(AB) structure of soybean glycinin was renatured in renaturation system, where the restoration from denatured and reduced-denatured states were about 70 % and 20 %, respectively [89]. Their results suggest that the step of the formation of disulfide bridges between the acidic and basic subunits is important in the process of the formation of 11S-structure.

The author demonstrated in the preceding chapters that both glycinin and legumin exhibit heterogeneity of their molecular species. This may indicate that specificity of subunit interaction in the formation of 11S-structure is not precise. The similarities of the structure and the amino acid sequences among glycinin acidic subunits, among glycinin basic subunits and among legumin basic subunits were indicated [18,19,90,91]. These may permit the interchange of subunits

among acidic subunits and among basic subunits in the formation of intermediary subunits and 11S-structure.

In the present work the formations of pseudo-glycinin and pseudo-legumin from their isolated native subunit proteins were investigated in order to elucidate the specificity of subunit interaction and subunit assembly mechanism in the formation of 11S-structure and the similarity of the structure among acidic subunits and among basic subunits.

VI-2 MATERIALS AND METHODS

Purified glycinin and legumin were prepared from dry seeds of Glycine max var. Tsuru-no-ko and Vicia faba var. Sanuki-Nagasaya, respectively, by sucrose density gradient centrifugation and DEAE-Sephadex column chromatography as described in the previous chapters. Other materials were as described in the previous chapters.

Preparation of native subunits of glycinin and legumin

The acidic and basic subunits of glycinin and legumin were isolated by DEAE-Sephadex A-50 column chromatography in the presence of urea and 2-ME as described in Chapters II and III. The acidic subunits used in this study were AS_{1-3} (M.W. 34800), AS_4 (M.W. 34800) and AS_5 (M.W. 38000) of glycinin and ASI (M.W. 36000), ASII (M.W. 36000) and ASIII (M.W. 51000 and 49000) of legumin (peaks II, III and IV of Fig. III-3, respectively). The basic subunits of glycinin and legumin were the flow-through fractions, which contained all the basic subunits.

Reconstitution system from isolated native subunits

The isolated acidic subunit (300 μg) was mixed with the basic sub-

units (740 μg) and then left to stand for 1 h at room temperature. The mixtures (2 ml) of glycinin subunits and legumin subunits were dialyzed without stirring against 200 ml of 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 0.02 % NaN_3 and 40 % (v/v) glycerol and 50 mM potassium phosphate buffer containing 0.2 M NaCl, 0.02 % NaN_3 and 40 % (v/v) glycerol, respectively, at 25°C for 24 h and another 24 h with a change of the buffer, and then dialyzed against the above buffer without glycerol for 3 h at 4°C. After dialysis, the sample was centrifuged for 15 min at 2000 rpm to remove the unreacted free basic subunits as a precipitate, because the basic subunits are insoluble in the buffer without denaturant such as urea and SDS. The supernatant was centrifuged on a 12 ml of 10 to 30 % (w/v) linear sucrose density gradient in the above buffer without glycerol as described in Chapter II. After centrifugation, the gradient was divided into 0.4 ml fractions and measured at 280 nm simultaneously using an ISCO density gradient fractionator.

Electrophoreses

SDS-PAGE and PGGE were performed as described in the previous chapters.

VI-3 RESULTS

VI-3-1 Soybean glycinin

Fractionation of reconstituted products from glycinin subunits by sucrose density gradient centrifugation

In order to investigate the extent of reconstitution of 11S-size complexes from the isolated native subunits, the reconstituted products

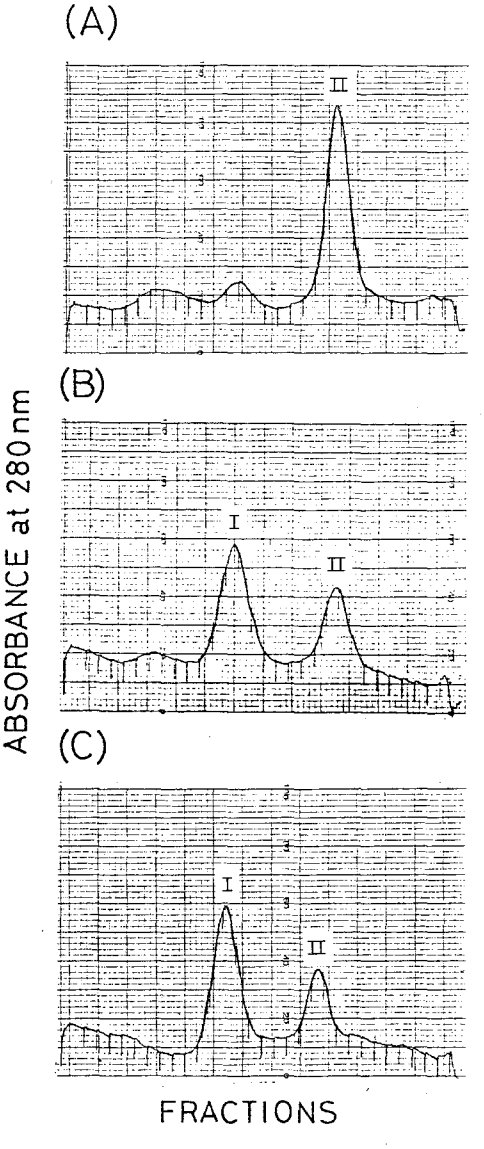


Fig. VI-1. Sucrose density gradient centrifugation of reconstituted products from glycinin subunits.
The isolated acidic and basic subunits were combined and reconstituted in combinations of BS with AS_{1-3} (A), AS_4 (B) and AS_5 (C), and then the reconstituted products were fractionated by sucrose density gradient centrifugation as described in MATERIALS AND METHODS. Sedimentation is from left to right. Absorbance range is 0 to 0.2.

from combinations of one each of glycinin acidic subunits (G-AS) and glycinin basic subunits (G-BS), i.e., AS_{1-3} and BS, AS_4 and BS, and AS_5 and BS (the molar ratio is approx. 1 : 4 in each combination), were fractionated by sucrose density gradient centrifugation (Fig. VI-1). In the absorbance patterns, peaks I and II correspond to 7S (half-molecule of the 11S component) and 11S components, respectively. As shown in Fig. VI-1-A, the formation of 11S component from the combination of AS_{1-3} and BS was about 90 %. In the cases of the combinations of AS_4 and BS, and AS_5 and BS, it was lower (40 to 30 %) and the areas of the 7S components were larger than that of the other combination (Fig. VI-1-B and C). In any of the cases both free acidic subunits and free reconstituted intermediary subunits were scarcely observed. These results suggest that pseudo-glycinins which are different from the native glycinin with respect to the subunit composition can be formed from the isolated constituent subunits of glycinin.

PGGE of reconstituted pseudo-glycinins

In order to examine the molecular size of each pseudo-glycinin from the combinations described above, the 11S component fractions obtained from the sucrose density gradient centrifugation were analyzed by PGGE. As shown in Fig. VI-2, the pseudo-glycinin from the combination of AS_{1-3} and BS gave a main band with a molecular weight of approx. 340000 and a minor band with a molecular weight of about 70000 (Fig. VI-2, Lane 3). The pseudo-glycinin from the combination of AS_4 and BS gave a band with a molecular weight of about 60000, and that from the combination of AS_5 and BS gave a main band with a molecualr weight of approx. 200000 and a minor band with a molecular weight of about 370000 (Fig. VI-2, Lanes

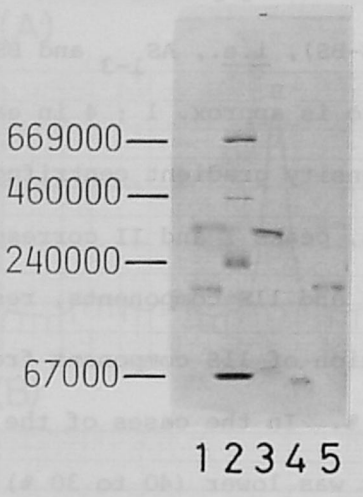


Fig. VI-2. PGGE of reconstituted pseudo-glycinins.
0.02 A_{280} unit of each pseudo-glycinin obtained from the sucrose density gradient centrifugation was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom.
Lane 1, native glycinin; Lane 2, standard proteins (thyroglobulin, ferritin, catalase, bovine serum albumin); Lane 3, AS_{1-3} + BS; Lane 4, AS_4 + BS; Lane 5, AS_5 + BS.

4 and 5). These may be due to dissociation of the pseudo-glycinins to their small components during the electrophoresis. The 7S components formed from these two combinations also gave bands with molecular weights of approx. 60000 and 200000, respectively (data not shown). The native glycinin gave bands corresponding to the 11S-size, the 7S-size and the intermediary subunits (Fig. VI-2, Lane 1). Thus, the dissociation behaviors during the electrophoresis of the pseudo-glycinins formed from the above combinations were different from that of the native glycinin.

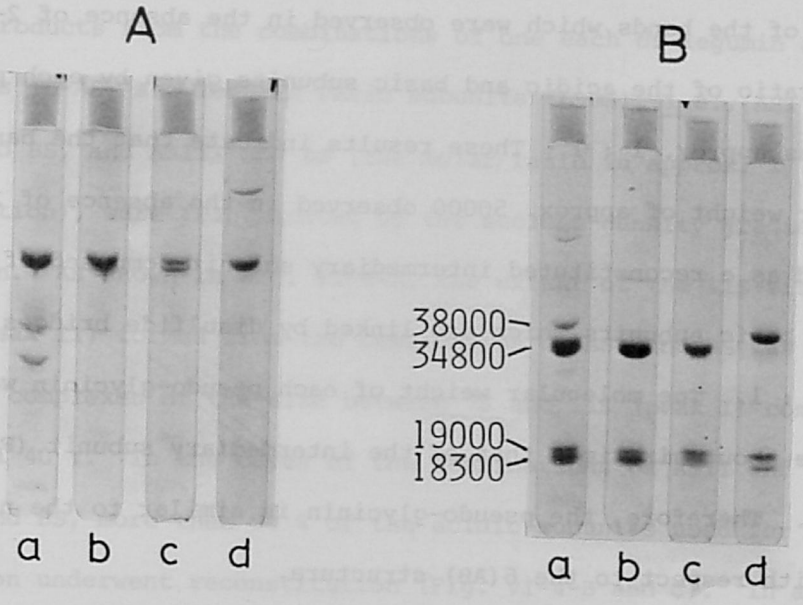


Fig. VI-3. SDS-PAGE of reconstituted pseudo-glycinins. 0.03 A_{280} unit of each pseudo-glycinin obtained from the sucrose density gradient centrifugation was electrophoresed in the absence (A) and presence (B) of 2-ME as described in MATERIALS AND METHODS. Migration is from top to bottom. Gel a, native glycinin; Gel b, AS_{1-3} + BS; Gel c, AS_4 + BS; Gel d, AS_5 + BS.

SDS-PAGE of reconstituted pseudo-glycinins

The pseudo-glycinin from each combination obtained from the sucrose density gradient centrifugation were analyzed by SDS-PAGE in the absence (Fig. VI-3-A) and presence (Fig. VI-3-B) of 2-ME. In the absence of 2-ME (Fig. VI-3-A), all the pseudo-glycinins gave bands with molecular weights of approx. 50000. In any case the bands corresponding to free acidic and basic subunits were not detected. On the other hand, in the presence of 2-ME (Fig. VI-3-B), all the pseudo-glycinins gave bands corresponding to the acidic and basic subunits with the concomitant dis-

appearance of the bands which were observed in the absence of 2-ME. The molar ratio of the acidic and basic subunits given by each pseudo-glycinin was approx. 1 : 1. These results indicate that the band with a molecular weight of approx. 50000 observed in the absence of 2-ME may be regarded as a reconstituted intermediary subunit composed of the acidic and basic subunits which are linked by disulfide bridges in a ratio of 1 : 1. The molecular weight of each pseudo-glycinin was estimated to be about six times that of the intermediary subunit (Figs. VI-2 and 3-A). Therefore, the pseudo-glycinin is similar to the native glycinin with respect to the 6(AB) structure.

A comparison of SDS-PAGE patterns for the pseudo-glycinins with that of the native glycinin indicates that in the formation of an intermediary subunit AS_{1-3} randomly selected both of the basic subunits with molecular weights of 19000 (BS_1) and 18300 (BS_2) as a counterpart (Fig. VI-3-B), while AS_4 and AS_5 exhibited some affinity for BS_1, as is evident from its higher presence in contrast to BS_2 in the pseudo-glycinins in comparison with the situation that exists in the native glycinin (Fig. VI-3-B, Gels c and d). Similar phenomena were observed with respect to 7S components except that of the combination of AS_4 and BS, where AS_4 randomly selected both of the basic subunits.

VI-3-2 Broad bean legumin

Fractionation of reconstituted products from legumin subunits by sucrose density gradient centrifugation

In order to investigate the extent of reconstitution of 11S-size complexes from the isolated native subunits of legumin, the reconsti-

tuted products from the combinations of one each of legumin acidic subunits (L-AS) and legumin basic subunits (L-BS), i.e., ASI and BS, ASII and BS, and ASIII and BS (the molar ratio is approx. 1 : 4 in each combination), were fractionated by the sucrose density gradient centrifugation. As shown in Fig. VI-4-A, the extent of the 11S-size component (peak II) formed from the combination of ASI and BS was about 60 % and the complexes of the size between 7S and 11S (peak I) comprised the residual 40 %. In the cases of the combinations of ASII and BS, and ASIII and BS, more than 90 % of the acidic subunits used for the reconstitution underwent reconstitution (Fig. VI-4-B and C). In any case both free acidic subunits and free reconstituted intermediary subunits were scarcely observed. These results suggest that pseudo-legumins which are different from the native legumin with respect to the subunit composition can be formed from the isolated constituent subunits of legumin.

PGGE of reconstituted pseudo-legumins

11S component fractions obtained from the sucrose density gradient centrifugation were analyzed by PGGE in order to examine the molecular size of each pseudo-legumin reconstituted from the above combinations. As shown in Fig. VI-5, the 11S component fraction from the combination of ASI and BS gave a band with a molecualr weight of 320000 (Fig. VI-5, Lane 3); from the combination of ASII and BS, one band with a molecular weight of 310000 (Fig. VI-5, Lane 4); and from the combination of ASIII and BS, one broad band with a molecular weight of around 430000 and three bands with molecular weights of 260000, 230000 and about 80000 (Fig. VI-5, Lane 5). The native legumin gave three bands cor-

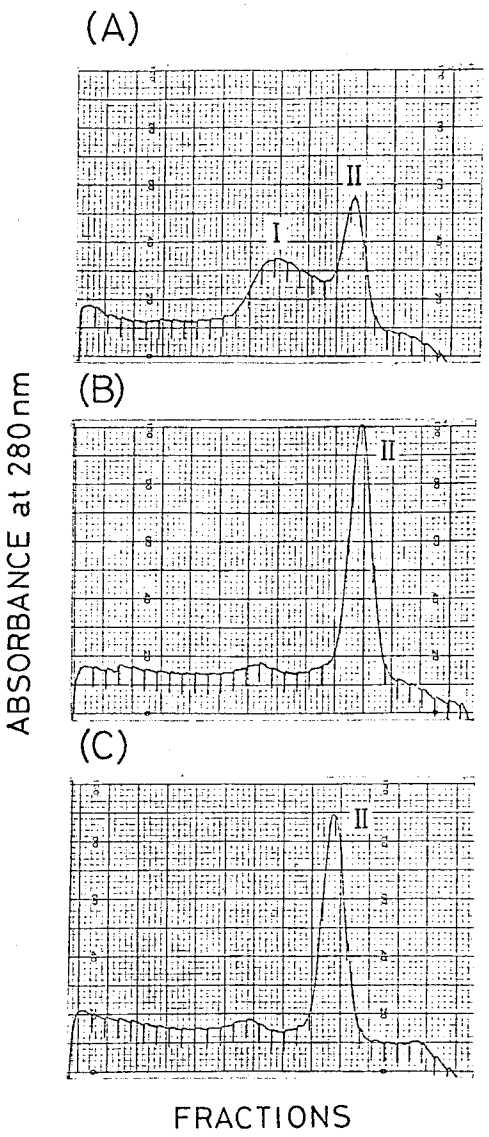


Fig. VI-4. Sucrose density gradient centrifugation of reconstituted products from legumin subunits.
The isolated acidic and basic subunits were combined and reconstituted in combinations of BS with ASI (A), ASII (B) and ASIII (C), and then the reconstituted products were fractionated by sucrose density gradient centrifugation as described in MATERIALS AND METHODS. Sedimentation is from left to right. Absorbance range is 0 to 0.2.

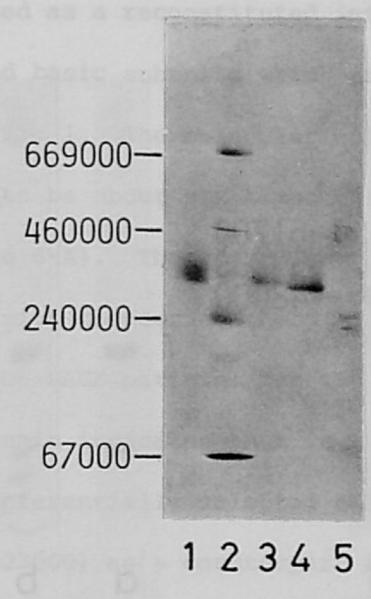


Fig. VI-5. PGGE of reconstituted pseudo-legumins.
0.01 A_{280} unit of each pseudo-legumin obtained from the sucrose density gradient centrifugation was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom. Lane 1, native legumin; Lane 2, standard proteins (thyroglobulin, ferritin, catalase, bovine serum albumin); Lane 3, ASI + BS; Lane 4, ASII + BS; Lane 5, ASIII + BS.

responding to the 11S-size (Fig. VI-5, Lane 1, see Chapter V).

SDS-PAGE of reconstituted pseudo-legumins

The reconstituted pseudo-legumin from each combination obtained from the sucrose density gradient centrifugation was analyzed by SDS-PAGE in the absence (Fig. VI-6-A) and presence (Fig. VI-6-B) of 2-ME. As shown in Fig. VI-6-A, each pseudo-legumin from the combinations of ASI and BS, and ASII and BS (Fig. VI-6-A, Gels a and b) gave only one band with a molecular weight of 48000, and the pseudo-legumin from the

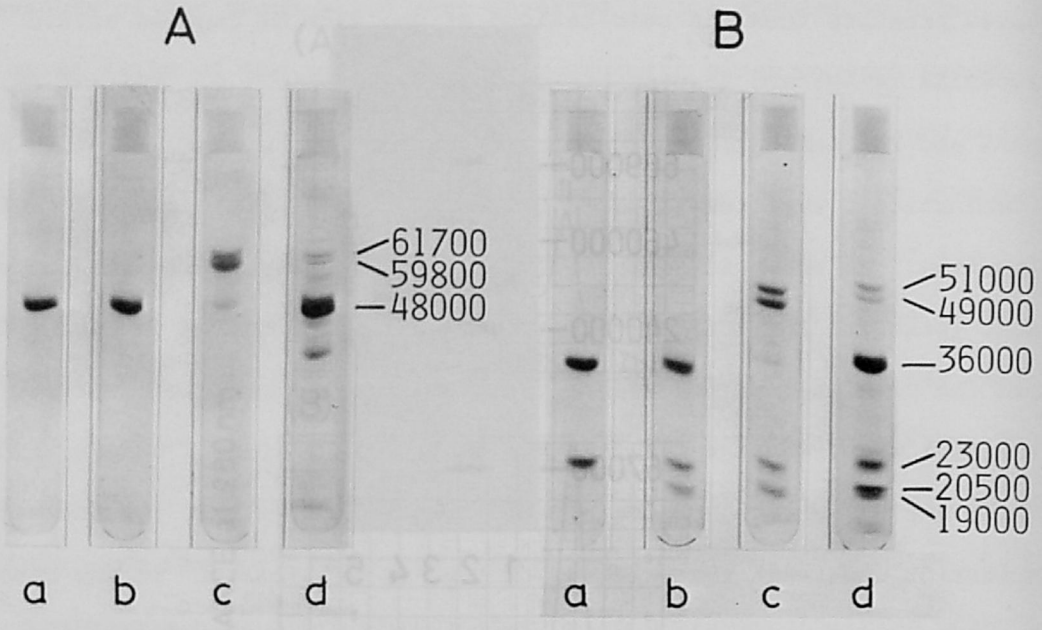


Fig. VI-6. SDS-PAGE of reconstituted pseudo-legumins. 0.03 A_{280} unit of each pseudo-legumin obtained from the sucrose density gradient centrifugation was electrophoresed in the absence (A) and presence (B) of 2-ME as described in MATERIALS AND METHODS. Migration is from top to bottom. Gel a, ASI + BS; Gel b, ASII + BS; Gel c, ASIII + BS; Gel d, native legumin.

combination of ASIII and BS (Fig. VI-6-A, Gel c) gave two bands with molecular weights of 61700 and 59800. In any case the bands corresponding to free acidic and basic subunits were not observed. On the other hand, in the presence of 2-ME (Fig. VI-6-B), the pseudo-legumins from all the combinations gave bands corresponding to the acidic and basic subunits with the concomitant disappearance of the bands which were observed in the absence of 2-ME. The molar ratio of the acidic and basic subunits given by each pseudo-legumin was approx. 1 : 1. These results suggest that the band with a molecular weight of 61700, 59800

or 48000 may be regarded as a reconstituted intermediary subunit composed of the acidic and basic subunits which are linked by disulfide bridges in a ratio of 1 : 1. The molecular weight of each pseudo-legumin was estimated to be about six times that of the intermediary subunit (Figs. VI-5 and 6-A). Therefore, the pseudo-legumin is similar to the native legumin with respect to the 6(AB) structure.

A comparison of SDS-PAGE patterns for the pseudo-legumins with that of the native legumin indicates that in the formation of an intermediary subunit ASI preferentially selected BSI (a basic subunit with a molecular weight of 23000) as a counterpart from among the three kinds of the basic subunits (Fig. VI-6-B, Gel a), while ASII and ASIII did not exhibit such strict specificity for the basic subunits (Fig. VI-6-B, Gels b and c). Peak I from the combination of ASI and BS gave similar results to the pseudo-legumin from the same combination (data not shown).

Effect of amount ratio of acidic and basic subunits on specificity of subunit interaction

In order to clarify the specificities of the acidic subunits for the basic subunits in the reconstitution of the 11S-size component, the effects of the molar ratio of the basic subunits to each acidic subunit in the reconstitution of 11S-structure were investigated. In the combination of ASI and BS, since ASI exhibited a strict specificity for BSI when the molar ratio of AS to BS was approx. 1 : 4, the molar ratio was reduced to 1 : 1 (the molar ratio of ASI to BSI is approx. 1 : 0.35). In the combinations of ASII and BS, and ASIII and BS, since ASII and ASIII did not exhibit any specificity for BS, the molar ratio

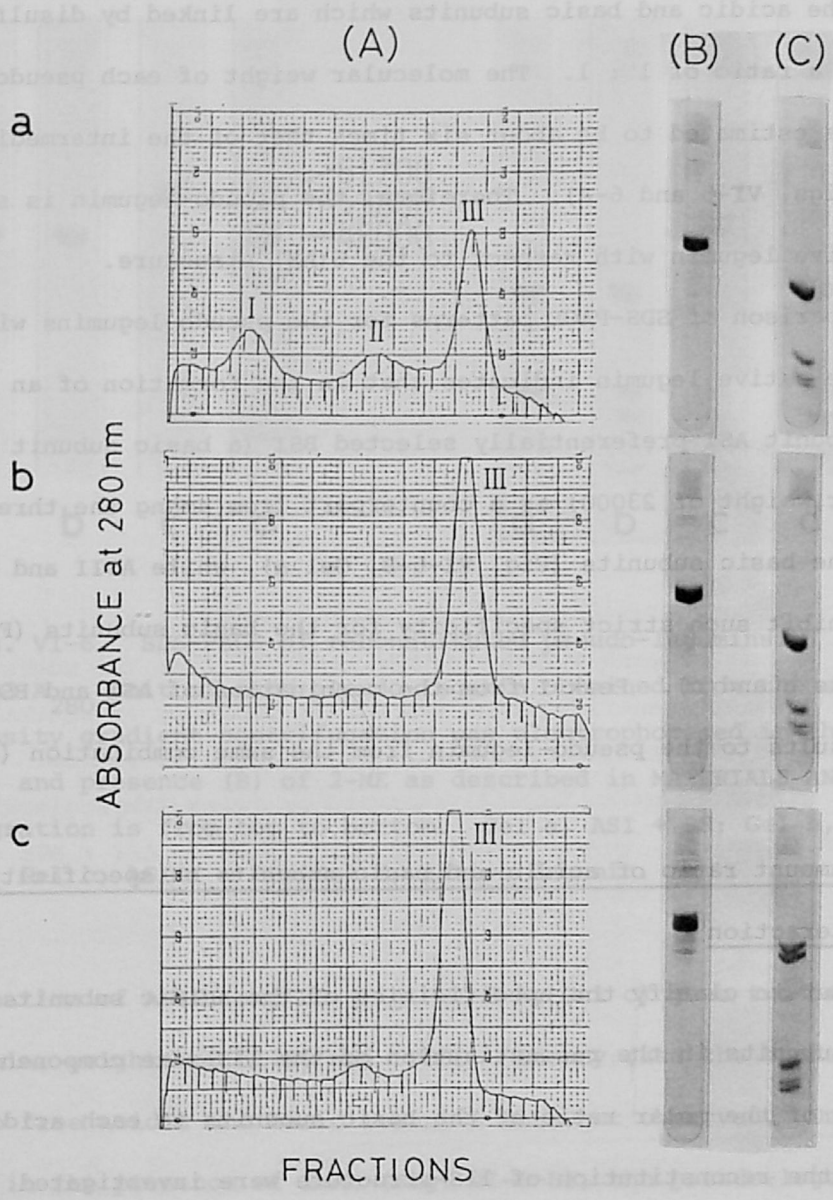


Fig. VI-7. Effect of amount ratio of acidic and basic subunits on specificity of subunit interaction.

Sucrose density gradient centrifugation (A), SDS-PAGE in the absence (B) and presence (C) of 2-ME were carried out as described in MATERIALS AND METHODS. a, ASI : BS = 1 : 1; b, ASII : BS = 1 : 8.3; c, ASIII : BS = 1 : 8.3.

was increased to 1 : 8.3. The results of the fractionation by the sucrose density gradient centrifugation and SDS-PAGE in the absence and presence of 2-ME are shown in Fig. VI-7. The extent of 11S component (peak III) formed from the combination of ASI and BS (1 : 1) was higher and that of the complex between 7S and 11S (peak II) formed was lower than those in the case of 1 : 4 (Figs. VI-4-A and 7-A-a), and the peak composed of free acidic subunits (peak I) was observed (Fig. VI-7-A-a). In this case, strict specificity of ASI for BSI was lost, that is, ASI formed the intermediary subunits with BSII (a basic subunit with a molecular weight of 20500) and BSIII (a basic subunit with a molecular weight of 19000) in addition to BSI (Fig. VI-7-C-a). When the molar ratios of ASI to BSI were $\geqq$ 1, the specificity of ASI for BSI was not strict (data not shown). On the other hand, the extent of 11S component formed from the combinations of ASII and BS, or ASIII and BS (1 : 8.3), was similar to that in the case of 1 : 4. In these cases ASIII selected BSIII to some extent, but ASII did not (Fig. VI-7-C-b and c).

Heterologous recombinations of isolated subunits with respect to acidic subunits

In order to understand more about the efficiency of reconstitution of 11S-size component and the specificity of interaction between acidic subunit and basic subunit in the reconstitution, the reconstitution reaction was carried out in heterologous recombination of the isolated subunits with respect to the acidic subunit. The reconstituted products in the combinations of ASI, ASII and BS, ASI, ASIII and BS, and ASII, ASIII and BS were fractionated by the sucrose density gradient centrifugation (data not shown). From all the combinations more than

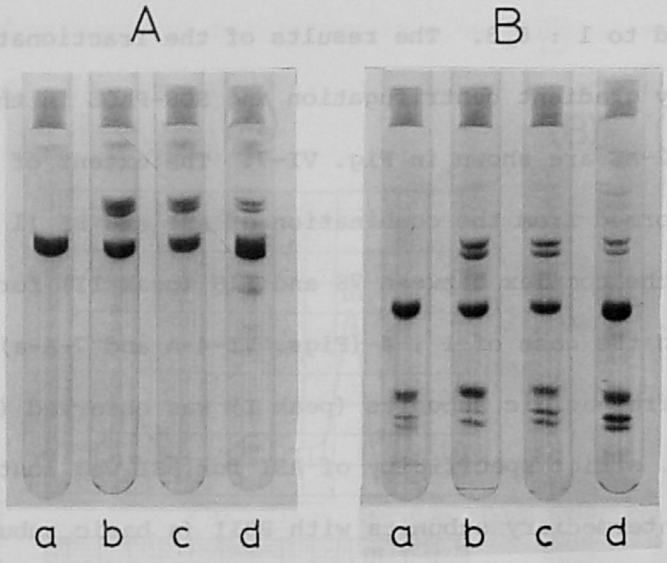


Fig. VI-8. SDS-PAGE of reconstituted pseudo-legumins from heterologous combinations.
Electrophoresis was carried out as described in MATERIALS AND METHODS in the absence (A) and presence (B) of 2-ME. Gel a, ASI + ASII + BS; Gel b, ASI + ASIII + BS; Gel c, ASII + ASIII + BS; Gel d, native legumin.

90 % was 11S component, which was analogous to the results from combinations of ASII and BS, and ASIII and BS (Fig. VI-4-B and C). The 11S component fractions formed from these combinations were analyzed by SDS-PAGE in the absence and presence of 2-ME (Fig. VI-8-A and B). The patterns in the absence of 2-ME (Fig. VI-8-A) indicate that the intermediary subunits were formed in all the above combinations. In the case of the combination of ASI, ASII and BS (Fig. VI-8-B, Gel a), BSIII associated less with the acidic subunits to form intermediary subunits than in the combinations of ASII and BS (Fig. VI-6-B, Gel b) and ASI, ASIII and BS (Fig. VI-8-B, Gel b). In the case of the combination of

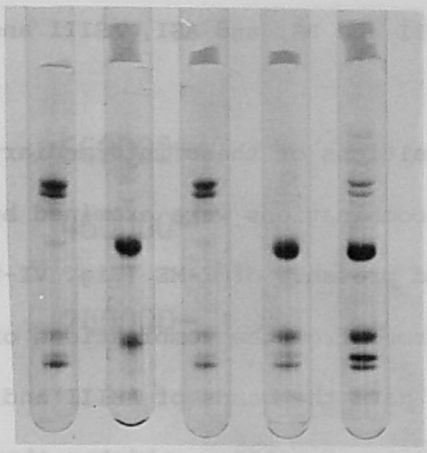


Fig. VI-9. Subunit compositions of intermediary subunits formed from heterologous combinations.
First, 70 µg of pseudo-legumin formed from each heterologous combination was electrophoresed on SDS-polyacrylamide gel in the absence of 2-ME. After electrophoresis, the gel was stained and destained, and the protein band was cut off and electrophoresed in the presence of 2-ME as described in MATERIALS AND METHODS of Chapter V. Migration is from top to bottom. Gels a and c, larger intermediary subunits from the combinations of ASI, ASIII and BS, and ASII, ASIII and BS, respectively; Gels b and d, smaller intermediary subunits from the combinations of ASI, ASIII and BS, and ASII, ASIII and BS, respectively; Gel e, native legumin.

ASI, ASIII and BS (Fig. VI-8-B, Gel b), BSIII associated much more with the acidic subunits to form intermediary subunits than in the combination of ASIII and BS (Fig. VI-6-B, Gel c), and BSII associated less. In the combination of ASII, ASIII and BS (Fig. VI-8-B, Gel c), BSI associated less with the acidic subunits to form intermediary subunits than in the combinations of ASII and BS, ASIII and BS (Fig. VI-6-B,

Gels b and c), ASI, ASII and BS, and ASI, ASIII and BS (Fig. VI-8-B, Gels a and b).

The subunit compositions of these intermediary subunits formed from the heterologous combinations were examined by combining two SDS-PAGE in the absence and presence of 2-ME (Fig. VI-9). The larger intermediary subunits formed from the combinations of ASI, ASIII and BS, and ASII, ASIII and BS gave the bands of ASIII and all the basic subunits, where the intensity of BSIII was higher than those of BSI and BSII (Fig. VI-9, Gels a and c). The smaller intermediary subunits formed from the combination of ASI, ASIII and BS gave the bands of ASI and BSI, and that from the combination of ASII, ASIII and BS gave the bands of ASII and all the basic subunits. Thus, in the heterologous combinations ASI exhibited remarkable specificity for BSI and ASIII exhibited strong affinity for BSIII.

In order to examine the size and the association-dissociation behavior of each reconstituted pseudo-legumin from the above combinations, the 11S component fractions obtained by the sucrose density gradient centrifugation were analyzed by PGGE (Fig. VI-10). The pseudo-legumins from the above combinations gave some bands with molecular weights of 320000-350000 and no band which was derived from dissociation products, even when they contained ASIII (Fig. VI-10, Lanes 4 and 5).

VI-4 DISCUSSION

In this chapter, the author demonstrated the formation of pseudo-glycinins and pseudo-legumins from various combinations of acidic and basic subunits from glycinin and legumin, respectively. All the pseudo-

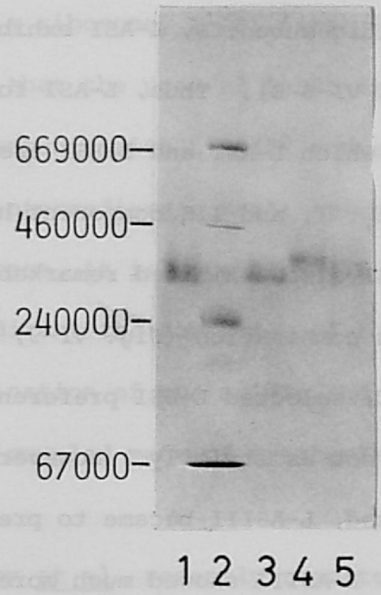


Fig. VI-10. PGGE of reconstituted pseudo-legumins from heterologous combinations.
Electrophoretic conditions are the same as in Fig. VI-5. Lane 1, native legumin; Lane 2, standard proteins; Lane 3, ASI + ASII + BS; Lane 4, ASI + ASIII + BS; Lane 5, ASII + ASIII + BS.

11S globulins were similar to the native glycinin and legumin with respect to the 6(AB) structure, because they have intermediary subunits in which the acidic and basic subunits are linked by disulfide bridges in a ratio of 1 : 1 (Figs. VI-3 and 6) and their molecualr weights were estimated to be six times that of the reconstituted intermediary subunit (Figs. VI-2 and 5). Moreover, hardly any unreacted free acidic subunits in the reconstituted products were found (Figs. VI-1 and 4). These suggest the similarity of the structure among acidic subunits and among basic subunits of each 11S globulin; and that the 6(AB) structure is preferred thermodynamically.

In the formation of pseudo-legumins in homogeneous combinations

with respect to the acidic subunits, L-ASI exhibited remarkable specificity for L-BSI (Fig. VI-6-B). Thus, L-ASI forms the intermediary subunit with L-BSI, in which L-ASI and L-BSI are linked by disulfide bridges in a ratio of 1 : 1, and 11S component having 6(ASI-BSI) structure (Fig. VI-5). L-ASI also exhibited remarkable specificity for L-BSI in the heterologous combination (Fig. VI-9, Gel b). On the other hand, L-ASII and L-ASIII selected L-BSI preferentially in the homogeneous combination, but not as strictly. However, when the molar ratio of BS to AS was increased, L-ASIII became to prefer L-BSIII (Fig. VI-7-C, Gel c). Moreover, L-ASIII showed much more affinity for BSIII in the combinations of L-ASI, L-ASIII and L-BS, and L-ASII, L-ASIII and L-BS than in the combination of L-ASIII and L-BS (Fig. VI-6-B, Gel c, and Fig. VI-9, Gels a and c). Thus, when the amount of the available L-BSIII increased in the reconstitution system, and/or when the amount of other basic subunits decreased due to utilization by L-ASI and/or L-ASII, L-ASIII became to exhibit strict specificity for L-BSIII. Similar phenomena were observed under pH 6.6 to 8.6 and ionic strength 0.05 to 1.0 (data not shown).

As shown in Chapter V, in the native legumin L-ASI and L-ASII form intermediary subunits with L-BSI and/or L-BSII and L-ASIII with L-BSIII. Since L-ASI exhibits remarkable specificity for L-BSI as shown in this chapter, L-ASI may form an intermediary subunit with L-BSI in the native legumin. It may be deduced from these facts and speculation that L-ASII forms an intermediary subunit with L-BSII. If these deductions are valid, together with the evidence presented here, the subunit assembly mechanism of legumin may be presumed to be as follows. After each sub-

unit is synthesized on a ribosome, L-ASI first forms an intermediary subunit with L-BSI, so that the pool of L-BSI is reduced. Then L-ASIII forms an intermediary subunit with L-BSIII and subsequently L-ASII with L-BSII. Finally each intermediary subunit assembles into the 11S-size component. In such a case, it is possible that there are some regulation mechanisms which direct the formation of the intermediary subunit having a specific combination of the acidic and basic subunits, e.g., either a sequential or coupled mode of synthesis of the acidic and basic subunits.

Alternatively, Croy et al. recently reported that in a cell free system, legumin is synthesized as a precursor with a molecular weight of about 60000 consisting of covalently linked acidic and basic subunits from either polyribosomes or mRNAs isolated from developing pea and broad bean seeds [92]. Spencer and Higgins also reported the similar result with regard to pea legumin [93]. If this is also the case for in situ, the combination of AS and BS in the intermediary subunit must be established on the step of translation. This seems to be a reasonable regulatory mechanism for the formation of the intermediary subunit with a particular combination of acidic and basic subunits. The results obtained here that L-ASII and L-ASIII do not exhibit remarkable specificity for L-BS while L-ASI does in in vitro reconstitution system seem to support the idea that the combination in the intermediary subunit is established on the step of translation.

In the formation of pseudo-glycinin, $G\text{-}AS_{1-3}$ randomly selected both of glycinin basic subunits while $G\text{-}AS_4$ and $G\text{-}AS_5$ exhibited some specificity for $G\text{-}BS_1$. These results seem to support the observations

of Croy et al. [92] and Spencer and Higgins [93]. On the other hand, Kitamura et al. reported that each acidic subunit is linked with a basic subunit in a specific combination [15]. However, each acidic subunit could form an intermediary subunit with other basic subunits than the inherent basic subunit, with which each acidic subunit forms an intermediary subunit in the native glycinin. Moreover, these artifactual intermediary subunits were capable of being constituents of the pseudo-glycinins. These results suggest that the specificity of interactions among subunits and among intermediary subunits in the formation of 11S-structure of glycinin is not precise in the reconstitution reaction.

In the combination of L-ASI and L-BS, 11S component having 6(ASI-BSI) structure is formed (Figs. VI-5 and VI-6), but this molecular species is not present in the native legumin as described in Chapter V. Moreover, artifactual intermediary subunits of ASII-BSIII and ASIII-BSI and ASIII-BSII are formed in the combinations of L-ASII and L-BS, and L-ASIII and L-BS, respectively. These are also capable of being constituents of the reconstituted 11S component. These results suggest that the specificity of interactions among subunits and among intermediary subunits in the formation of 11S-structure of legumin is not precise in the reconstitution reaction similar to the case of glycinin. These must be one of the reasons for the occurrence of heterogeneity of glycinin and legumin molecular species. This inference may be also the case for the heterogeneity of the other legumin-type globulins having the 6(AB) structure, e.g., pea legumin [26,27]. In the heterologous recombinations of legumin subunits, some molecular species with

different molecular weights were observed on PGGE (Fig. VI-10). This indicates that their intermediary subunit compositions are different from each other, a conclusion which supports the above discussion.

The pseudo-glycinins from the combinations of G-AS_4 and G-BS, and G-AS_5 and G-BS dissociated to the smaller components on PGGE (Fig. VI-2, Lanes 4 and 5). However, the pseudo-glycinin from the combination of G-AS_{1-3} and G-BS hardly dissocited (Fig. VI-2, Lane 3). These results suggest that G-AS_4 and G-AS_5 are responsible for that glycinin undergo dissociation reaction under lower ionic strength in the normal pH range [81].

The pseudo-legumin from the combination of L-ASIII and L-BS dissociated to the half-molecule and intermediary subunits, but the pseudo-legumins from the combinations of L-ASI and L-BS, L-ASII and L-BS, L-ASI, L-ASIII and L-BS, and L-ASII, L-ASIII and L-BS did not dissociate to the smaller components (Figs. VI-5 and 10). These results suggest that L-ASI and L-ASII are responsible for that legumin does not undergo dissociation reaction with change of ionic strength in the normal pH range [8].

VII FORMATION OF HYBRID-11S GLOBULINS FROM SUBUNITS OF SOYBEAN GLYCININ AND BROAD BEAN LEGUMIN

VII-1 INTRODUCTION

Soybean glycinin and broad bean legumin share many similar structural features including molecular weights [8], N-terminal amino acid sequences of basic subunits [18] and the number and molecular weights of their subunits [8]. They have common intermediary subunits (AB), disulfide-bonded acidic (A) and basic (B) subunits, and are composed of 6(AB). Moreover, as described in the preceding chapter, the specificity of interaction among subunits and among intermediary subunits is not precise in the formation of 11S-structure of both 11S globulins. These informations suggest that some acidic (or basic) subunit of glycinin (or legumin) may be interchanged with that of the other.

The present work was undertaken to investigate the formation of hybrid-11S globulins from different combinations of subunit proteins from glycinin and legumin and thereby gain understanding about the extents of the specificity of subunit interaction and similarities of subunit structures and subunit assembly mechanisms of them.

VII-2 MATERIALS AND METHODS

Purified glycinin and legumin were prepared from dry seeds of Glycine max var. Tsuru-no-ko and Vicia faba var. Sanuki-Nagasaya, respectively, by sucrose density gradient centrifugation and DEAE-Sephadex column chromatography, and their native subunits were isolated by DEAE-Sephadex A-50 column chromatography in the presence of urea and 2-ME

as described in the previous chapters. Other materials were as described in the other chapters.

Formation of hybrid-11S globulins from native subunits of glycinin and legumin

Formation of hybrid-11S globulins was performed according to the reconstitution system described below. The isolated acidic subunit (300 μg) was mixed with the basic subunits (740 μg), and then left to stand for 1 h at room temperature. The mixture (2 ml) was dialyzed without stirring against 200 ml of 0.035 M potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 40 % (v/v) glycerol and 0.02 % NaN_3 at 25°C for 24 h and for another 24 h with a change of the buffer, and then dialyzed against 0.035 M potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl and 0.02 % NaN_3 (standard buffer) at 4°C for 3 h. The sample was then centrifuged on a 12 ml of 10 to 30 % (w/v) linear sucrose density gradient in the standard buffer as described in the preceding chapter.

Electrophoreses

SDS-PAGE and PGGE were performed as described in the other chapters.

VII-3 RESULTS

Fractionation of reconstituted products by sucrose density gradient centrifugation

The reconstituted products from the combinations of G-BS and L-ASI, L-ASII or L-ASIII were fractionated by sucrose density gradient centrifugation (Fig. VII-1). In the absorbance patterns, peaks I and

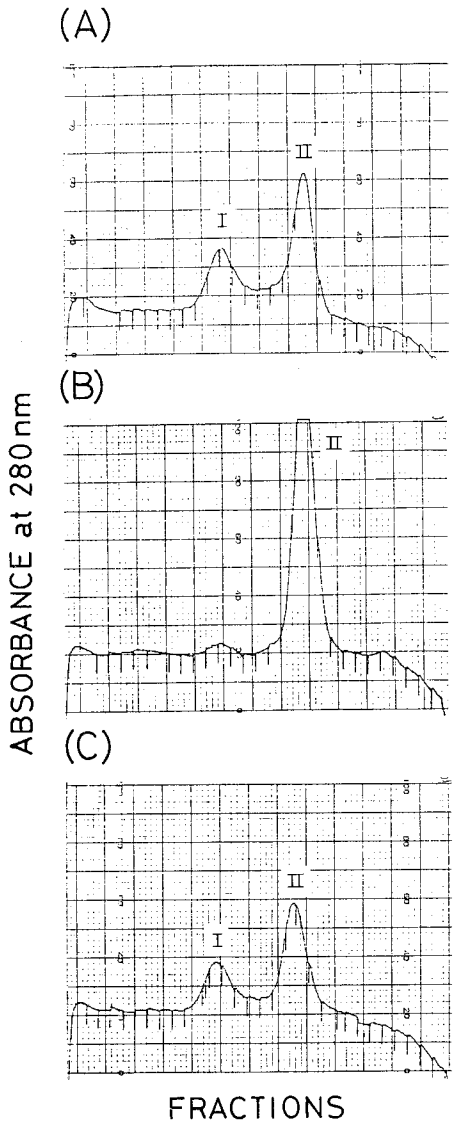


Fig. VII-1. Sucrose density gradient centrifugation of reconstituted products from combinations of L-AS and G-BS.
The isolated acidic and basic subunits were combined and reconstituted in combinations of G-BS with L-ASI (A), L-ASII (B) and L-ASIII (C), and then the reconstituted products were fractionated by sucrose density gradient centrifugation as described in MATERIALS AND METHODS. Sedimentation is from left to right. Absorbance range is 0 to 0.2.

II correspond to 7S (half-molecule of the 11S component) and 11S components, respectively. As shown in Fig. VII-1-A and C, the formation of 11S components from the combinations of L-ASI and G-BS, and L-ASIII and G-BS were 60-70 %. On the other hand, in the case of L-ASII and G-BS (Fig. VII-1-B), more than 90 % of L-ASII was converted. Unreacted acidic subunits or intermediary subunits were scarcely detected in any of the cases (compare with Fig. VII-2-D).

The reconstituted products from the combinations of L-BS and G-AS_{1+2}, G-AS_{2+3}, G-AS_4 or G-AS_5 were fractionated by the sucrose density gradient centrifugation (Fig. VII-2). In the absorbance patterns, peaks II and III correspond to 7S and 11S components, respectively. In contrast to the combinations of L-AS and G-BS, the formation of 11S components by the combinations of G-AS and L-BS was low. This was especially so in the cases of the latter two combinations (Fig. VII-2-C and D). From the combination of G-AS_4 and L-BS, only 7S component was formed. On the other hand, in the case of G-AS_5 and L-BS, the formation of either 7S or 11S component was less than 10 %. Peak I of Fig. VII-2-D contained only unreacted G-AS_5 (see Fig. VII-4-A and B, Gel g), which implies that the reconstitution reaction did not occur in this combination.

These results indicate that the hybrid-11S globulins can be formed from the isolated subunits of glycinin and legumin except in the cases of the combinations of L-BS and G-AS_4 or G-AS_5.

PGGE of reconstituted complexes

In order to examine the size of each hybrid-11S or 7S globulin reconstituted from the isolated subunits of glycinin and legumin, frac-

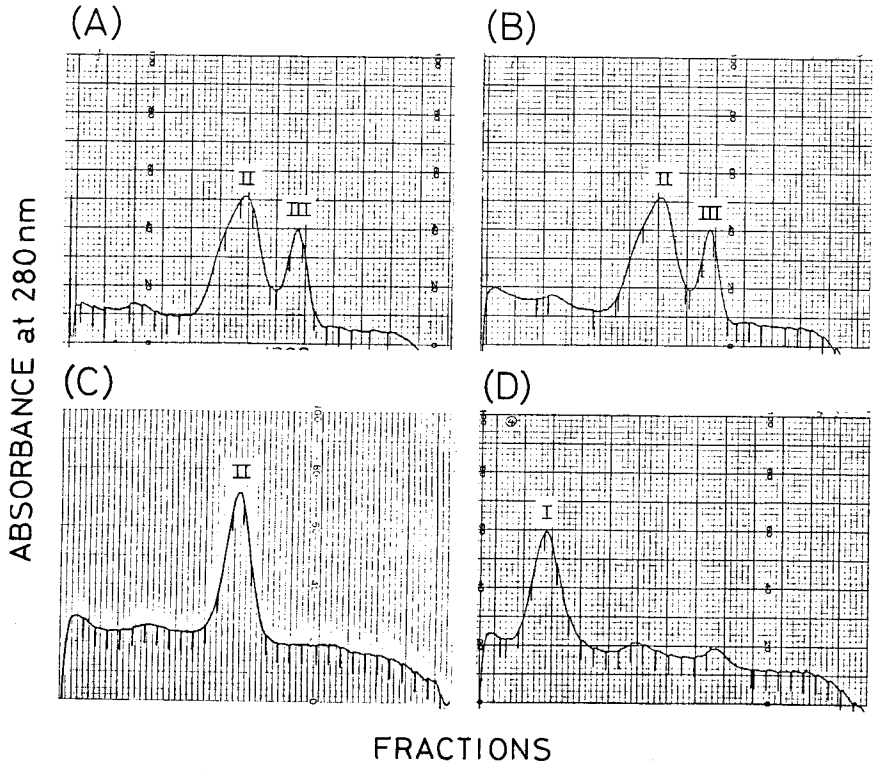


Fig. VII-2. Sucrose density gradient centrifugation of reconstituted products from combinations of G-AS and L-BS.
The isolated acidic and basic subunits were combined and reconstituted in combinations of L-BS with $G\text{-}AS_{1+2}$ (A), $G\text{-}AS_{2+3}$ (B), $G\text{-}AS_{4}$ (C) and $G\text{-}AS_{5}$ (D), and then the reconstituted products were fractionated by sucrose density gradient centrifugation as described in MATERIALS AND METHODS. Sedimentation is from left to right. Absorbance range is 0 to 0.2.

tions of hybrid-11S or 7S globulins obtained by the sucrose density gradient centrifugation were analyzed by PGGE (Fig. VII-3). The electrophoretic patterns of the combinations of L-AS and G-BS, and G-AS and

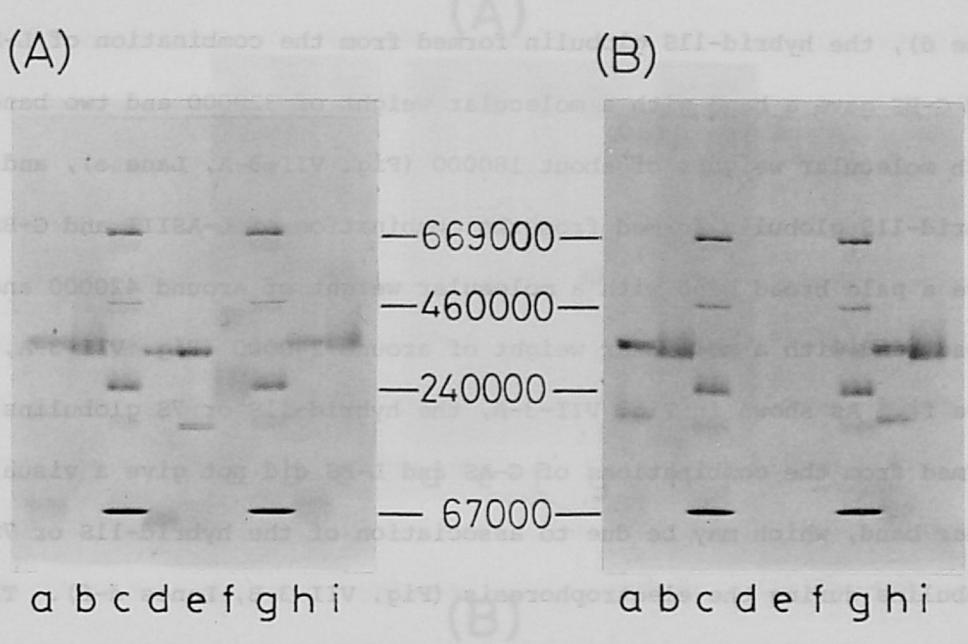


Fig. VII-3. PGGE of reconstituted complexes.
0.01 A_{280} unit of each reconstituted complexes obtained from the sucrose density gradient centrifugation was electrophoresed as described in MATERIALS AND METHODS. Migration is from top to bottom. (A) 11S component fractions from the combinations of L-AS and G-BS. Lanes a and h, native glycinin; Lanes b and i, native legumin; Lanes c and g, standard proteins (thyroglobulin, ferritin, catalase and bovine serum albumin); Lane d, L-ASI + G-BS; Lane e, L-ASII + G-BS; Lane f, L-ASIII + G-BS. (B) 11S or 7S component fractions from the combinations of G-AS and L-BS. Lanes a to c and g to i are the same as in (A). Lane d, 11S component from $G\text{-}AS_{1+2}$ + L-BS; Lane e, 11S component from $G\text{-}AS_{2+3}$ + L-BS; Lane f, 7S component from $G\text{-}AS_{4}$ + L-BS.

L-BS are shown in Fig. VII-3-A and B, respectively. As shown in Fig. VII-3-A, the hybrid-11S globulin formed from the combination of L-ASI and G-BS gave a sharp band with a molecular weight of 325000 and a broad band with a molecular weight of approx. 60000 (Fig. VII-3-A,

Lane d), the hybrid-11S globulin formed from the combination of L-ASII and G-BS gave a band with a molecular weight of 320000 and two bands with molecular weights of about 180000 (Fig. VII-3-A, Lane e), and the hybrid-11S globulin formed from the combination of L-ASIII and G-BS gave a pale broad band with a molecular weight of around 420000 and a broad band with a molecular weight of around 240000 (Fig. VII-3-A, Lane f). As shown in Fig. VII-3-B, the hybrid-11S or 7S globulins formed from the combinations of G-AS and L-BS did not give a visual clear band, which may be due to association of the hybrid-11S or 7S globulins during the electrophoresis (Fig. VII-3-B, Lanes d-f). The associated components may have spread over the gel. In fact, the pale stained region was observed to contain material of a wide range of molecular weights of over 300000. On the other hand, the native glycinin gave bands corresponding to 11S-size, 7S-size and intermediary subunits (Fig. VII-3-A and B, Lanes a and h, see Chapter IV), while the native legumin gave bands corresponding to 11S-size (VII-3-A and B, Lanes b and i, see Chapter V). Thus, the association-dissociation behaviors during the electrophoresis of the hybrid-11S globulins formed from various combinations were different from those of the native 11S globulins.

SDS-PAGE of reconstituted complexes

The hybrid-11S or 7S globulins reconstituted from various combinations were analyzed by SDS-PAGE. The results of SDS-PAGE in the absence and presence of 2-ME are shown in Fig. VII-4-A and B, respectively. In the absence of 2-ME (Fig. VII-4-A), all the hybrid-11S or 7S globulins except that formed from the combination of L-ASIII and

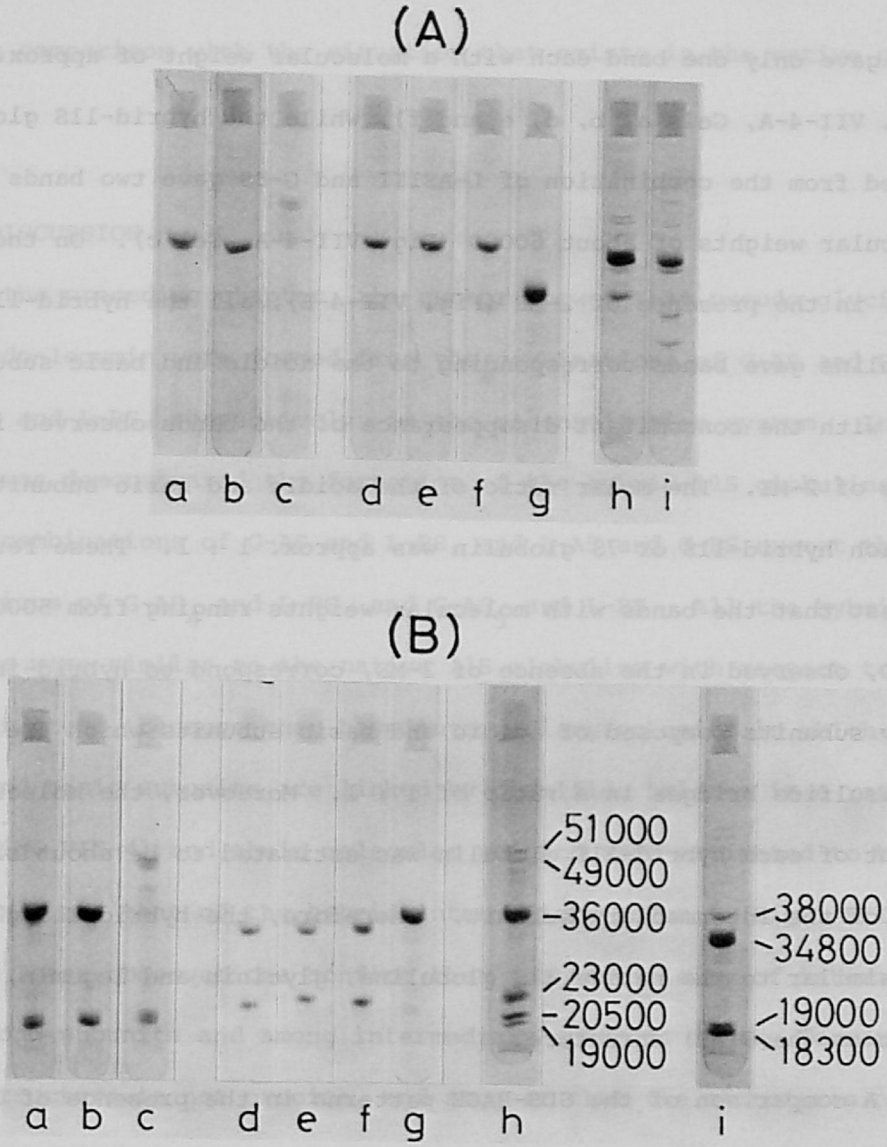


Fig. VII-4. SDS-PAGE of reconstituted complexes.

0.03 A_{280} unit of each reconstituted complexes obtained from the sucrose density gradient centrifugation was electrophoresed in the absence (A) and presence (B) of 2-ME as described in MATERIALS AND METHODS. Migration is from top to bottom. Gels a, b, c, d and e, 11S components from L-ASI + G-BS, L-ASII + G-BS, L-ASIII + G-BS, G-AS_{1+2} + L-BS and G-AS_{2+3} + L-BS, respectively; Gel f, 7S component from G-AS_4 + L-BS; Gel g, peak I from G-AS_5 + L-BS; Gel h, native legumin; Gel i, native glycinin.

G-BS gave only one band each with a molecular weight of approx. 50000 (Fig. VII-4-A, Gels a, b, d, e and f), while the hybrid-11S globulin formed from the combination of L-ASIII and G-BS gave two bands with molecular weights of about 60000 (Fig. VII-4-A, Gel c). On the other hand, in the presence of 2-ME (Fig. VII-4-B), all the hybrid-11S or 7S globulins gave bands corresponding to the acidic and basic subunits used with the concomitant disappearance of the bands observed in the absence of 2-ME. The molar ratio of the acidic and basic subunits given by each hybrid-11S or 7S globulin was approx. 1 : 1. These results suggest that the bands with molecular weights ranging from 50000 to 60000, observed in the absence of 2-ME, correspond to hybrid-intermediary subunits composed of acidic and basic subunits which are linked by disulfide bridges in a ratio of 1 : 1. Moreover, the molecular weight of each hybrid-11S globulin was estimated to be about six times that of the intermediary subunit. Therefore, the hybrid-11S globulins are similar to the native 11S globulins, glycinin and legumin, with respect to the 6(AB) structure.

A comparison of the SDS-PAGE patterns in the presence of 2-ME of the hybrid-11S or 7S globulins with those of the native glycinin and legumin indicates that all the glycinin acidic subunits except $G\text{-}AS_5$ preferentially selected L-BSI (M.W. 23000) in the formation of the hybrid-intermediary subunits and that L-ASI and L-ASII do not exhibit such specificity for the two kinds of glycinin basic subunits. However, L-ASIII seems to exhibit some affinity for $G\text{-}BS_1$ (M.W. 19000) to some extent, as is evident from its higher presence in contrast to the other in the hybrid-11S globulin formed from the combination of L-ASIII and

G-BS, in comparison with the situation that exists in the native glycinin.

VII-4 DISCUSSION

In the preceding chapter, the author showed that pseudo-glycinin and pseudo-legumin were formed from the combinations of G-AS and G-BS, and L-AS and L-BS, respectively, in the reconstitution system. In this chapter was demonstrated the formation of the hybrid-11S globulins from various combinations of G-AS and L-BS, and L-AS and G-BS except the combinations of $G\text{-}AS_4$ and L-BS, and $G\text{-}AS_5$ and L-BS. All the hybrid-11S globulins were similar to the native 11S globulins with respect to the 6(AB) structure, because they have intermediary subunits in which the acidic and basic subunits are linked by disulfide bridges in a ratio of 1 : 1 (Fig. VII-4) and their molecular weights were estimated to be about six times that of the hybrid-intermediary subunit (Figs. VII-3 and 4). These observations confirm that the specificity of interactions among subunits and among intermediary subunits in the formation of 11S-structure is not precise, and suggest that the structures of glycinin and legumin are similar to each other with respect to their intermediary subunits and constituent subunits; that they share common subunit assembly mechanism; and that there may be interchangeabilities of subunits among 11S globulins having the 6(AB) structure.

There is difference in the extent of the formation of the hybrid-11S globulins from isolated subunits among the acidic subunits used, but no difference in the total yield of hybrid-7S and 11S globulins except when $G\text{-}AS_5$ was used (Figs. VII-1 and 2). These results suggest

the occurrence of two types of hybrid-intermediary subunits, i.e., one can assemble into the 11S-size and the other only up to the 7S-size. In the case of the combination of G-AS_4 and L-BS, the reconstituted hybrid-intermediary subunits could assemble only up to the 7S-size. However, at present there is no evidence about the nature of these intermediary subunits.

G-AS_5 could not form an intermediary subunit with L-BS. This suggests that legumin does not contain such a basic subunit as the inherent G-BS with which G-AS_5 forms an intermediary subunit in the native glycinin. In the formation of the hybrid-intermediary subunit, G-AS_{1+2}, G-AS_{2+3} and G-AS_4 preferentially selected L-BSI from among three kinds of L-BS, but in the combination of G-AS_4 and L-BS, 11S component was scarcely formed. On the other hand, L-ASI and L-ASII randomly selected both of G-BS, but L-ASIII exhibited affinity for G-BS_1. These results may indicate that L-BSI is especially similar, with respect to structure, to the inherent G-BS with which G-AS_{1-4} form intermediary subunits in the native glycinin and the similarity of L-BSI to the inherent G-BS with which G-AS_4 forms an intermediary subunit is inferior to the other cases, and that G-AS_1 is similar to the inherent L-BS with which L-ASIII forms an intermediary subunit in the native legumin. These phenomena may shed light on subunit-subunit recognition and assembly mechanisms in the formation of 11S-structure and similarity of 11S-structure among 11S globulins from various seeds, together with the fact that the subunits of glycinin and legumin are similar to each other with respect to their protein chemical properties, e.g., molecular weights, molecular charges and N-terminal amino acids of subunits

[8], and N-terminal amino acid sequence of basic subunits [18].

The association-dissociation behaviors of various hybrid-11S globulins obtained here and pseudo-11S globulins obtained in the preceding chapter on PGGE (Figs. VI-5 and 10 and VII-3) were different from those of the native 11S globulins, glycinin and legumin. Further comparative studies of protein chemical and physical properties of these various hybrid- and pseudo-11S globulins whose subunit compositions are different from those of the native 11S globulins may shed light on such characteristics of glycinin and/or legumin at subunit level, e.g., cryoprecipitation [94], association-dissociation by pH [81,95,96], ionic strength [81,95] and heating [96-100], and functional properties containing gel formation ability [101,102]. Actually the author could elucidate which subunits are responsible for the association-dissociation reaction of glycinin or legumin under low ionic strength as described in the preceding chapter.

VIII SUMMARY

Soybean 11S globulins (glycinins) from the seeds of eighteen cultivars (soybeans from Japan, U.S., China and Korea) were analyzed for their subunit compositions by electrophoreses on polyacrylamide gels under various conditions. Although their subunit compositions could not be distinguished by SDS-PAGE in the presence of 2-ME, five groups were identified according to the differing molecular charges of the subunits: group I consisted of seven acidic subunits and eight basic subunits; group II, seven acidics and seven basics; group III, six acidics and seven basics; group IV, six acidics and five basics; and group V, six acidics and three basics.

Broad bean 11S globulins (legumins) from the seeds of six typical cultivars planted in Japan were also analyzed for their subunit compositions by electrophoreses on polyacrylamide gels under various conditions. Their subunit compositions could not be distinguished by SDS-PAGE but could be classified into three groups according to the size of the seeds from the standpoint of the molecular charges, i.e., small-, medium- and large-size groups.

Glycinin from soybean var. Tsuru-no-ko was fractionated on a DEAE-Sephadex A-50 column and the molecular species were investigated by electrophoretic methods. The results obtained indicate the heterogeneity of glycinin molecular species; four molecular species with molecular weights of 375000, 360000, 345000 and 340000 were detected by PGGE. Glycinins from other cultivars also exhibited a similar heterogeneity and the extent of this heterogeneity seems to correlate with the diver-

sity in the subunit composition.

Legumins from three cultivars (small-, medium- and large-sizes) were fractionated by DEAE-Sephadex A-50 column chromatography and the molecular species were investigated. The results obtained indicate the heterogeneity of legumin molecular species. This heterogeneity was common to the three cultivars from the standpoint of the molecular sizes of the subunits; it was deduced that five groups with nine subspecies were present in each cultivar. Their subunit compositions constructed from the subunit groups classified according to their sizes were presented.

The results described above indicate that molecular heterogeneity may be an inherent property of the major components of seed storage proteins.

The constituent subunits of glycinin and legumin were separated into basic subunits (G-BS: mixture of $G\text{-}BS_1$ and $G\text{-}BS_2$; L-BS: mixture of L-BSI, L-BSII and L-BSIII) and acidic subunits (G-AS: $G\text{-}AS_{1\text{-}3}$, $G\text{-}AS_4$ and $G\text{-}AS_5$; L-AS: L-ASI, L-ASII and L-ASIII). The artificial 11S globulins, pseudo-glycinins and pseudo-legumins with different subunit compositions from the native 11S globulins, were formed in a reconstitution reaction which combined one each of G-AS and G-BS for pseudo-glycinins and one each of L-AS and L-BS for pseudo-legumins. They were similar to the native 11S globulins; they all consisted of reconstituted intermediary subunits which were composed of acidic and basic subunits linked by disulfide bridges in a ratio of 1 : 1 and had the 6(AB) structure. These results suggest a similarity in the structure of the acidic subunits and that of the basic subunits, and that the specificity of the

interactions among subunits and among intermediary subunits in the formation of 11S-structure is not precise. This must be one of the reasons for the occurrence of the heterogeneity of glycinin and legumin molecular species described above.

In the formation of pseudo-legumins L-ASI preferentially selected L-BSI from among the three kinds of L-BS, and L-ASIII exhibited affinity for L-BSIII. Considering all of results, the author presented a presumed subunit assembly mechanism of legumin.

The behaviors of pseudo-glycinin and legumin on PGGE suggest that $G\text{-}AS_4$ and $G\text{-}AS_5$ are responsible for that glycinin undergo dissociation reaction with changes in ionic strength in the normal pH range and that L-ASI and L-ASII for that legumin does not undergo the reaction.

Hybrid-11S globulins were formed from various combinations of L-AS and G-BS, and G-AS and L-BS except for the combination of $G\text{-}AS_4$ and L-BS from which only 7S component was observed and for $G\text{-}AS_5$ and L-BS from which unreacted-free acidic subunit was found. More hybrid-11S globulins were formed from combinations of L-AS and G-BS than from combinations of G-AS and L-BS. The hybrid-11S globulins were similar to the native 11S globulins; they all consisted of six reconstituted hybrid-intermediary subunits which were composed of acidic and basic subunits linked by disulfide bridges in a ratio of 1 : 1. These observations confirm that the specificity of the interactions among subunits and among intermediary subunits in the formation of 11S-structure is not precise, and suggest that the structures of glycinin and legumin are similar to each other and that they share a common assembly mechanism.

In the formation of hybrid-intermediary subunits, G-AS_{1-4} preferentially selected L-BSI, and L-ASI and L-ASII randomly selected both of G-BS, while L-ASIII exhibited affinity for G-BS_1. These results suggest that L-BSI is especially similar, with respect to structure, to the inherent glycinin basic subunits with which G-AS_{1-4} form intermediary subunits in glycinin while G-BS_1 to the inherent legumin basic subunit with which L-ASIII forms an intermediary subunit in legumin.

The association-dissociation behaviors of the hybrid-11S globulins were different from those of the native 11S globulins.

ACKNOWLEDGEMENT

The author wishes to express his sincere gratitude to Dr. Setsuro Matsushita, Professor of Kyoto University, for his kind guidance and continuous encouragement throughout the course of this study.

The author is thankful to Dr. Tomohiko Mori, Associate Professor of Kyoto University, for his continued direction, valuable comments, and critical reading of manuscripts.

Thanks are also due to Dr. Sadaich Takagi, Mr. Hisaji Inaba and Mr. Takashi Nakamura for their helpful collaboration.

The author wishes to express his gratitude to all the members of the Research Institute for Food Science, Kyoto University, for their helpful comments and encouragement.

The author is greatly indebted to Dr. Hideo Chiba, Professor of Kyoto University, and Dr. Ryuzo Sasaki, Associate Professor of Kyoto University, for their direction of the author's graduate studies.

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