Concentrations of Water-Soluble Nitrogen and Amino Acids as Criteria for Discriminating Vegetable-type and Grain-type Soybean Cultivars

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Summary

Soybean (*Glycine max* (L.) Merr.) cultivars occur in two types, the vegetable-type (edamame) and the grain-type cultivars. This study was carried out to examine the characteristics of accumulation of water-soluble nitrogen and amino acids in immature and mature seeds from these two types of soybean cultivars. Ten vegetable-type and thirteen grain-type cultivars were grown in an upland field of Yamagata University. The immature seeds were sampled 30 to 40 days after flowering. Concentrations of the water-soluble nitrogen measured in 1991 were highly correlated with amino acid concentrations measured in 1992. The immature seeds of the vegetable-type cultivars contained higher concentrations of seven free amino acids (asparagine, alanine, glutamic acid, arginine, serine, histidine and valine) than those of the grain-type cultivars. Based on concentrations of these amino acids in immature seeds, we correctly discriminated the vegetable-type and grain-type cultivars, indicating that differences in amino acid concentrations in the immature seeds were sufficient to distinguish the two types of soybean cultivars. Furthermore, the concentration of water-soluble nitrogen may be a simple measure that represents concentrations of free amino acids, and can thus be used as one of the selection indices in breeding programs of the vegetable-type cultivars.

Key Words: *Glycine max*, vegetable-type soybean, water-soluble nitrogen concentration, amino acid composition.

Introduction

Two types of soybean cultivars with different utilization characteristics are found in Asian countries. One of these is the vegetable-type (edamame) cultivars (Lumpkin *et al.*, 1993). Another is the grain-type cultivars that are utilized for making soy sauce, soybean paste, soy curd, fermented soybeans (Taira, *et al.*, 1979 a and b), and feed grains, as well as products derived from these foods.

The protein content of soybean seeds of the grain-type cultivars is more than 40 % of the dry weight (Fehr, 1987; Kitamura, 1991). Thus, it has been questioned whether an increase in protein concentration was required in soybean breeding programs (Fehr, 1987;

Kitamura, 1991). In contrast to the few studies on quantitative improvement of soybean seed proteins, studies on the qualitative improvement of soybean seed proteins have been conducted extensively, including studies designed to increase glycinin (11S subunit) content with a corresponding decrease in β -conglycinin (7S subunit) content (Saio *et al.*, 1973; Orf and Hymowitz, 1978; Ogawa *et al.*, 1989).

Furthermore, studies on the oil quality of grain-type cultivars have also been conducted extensively. These studies have included exploitation of genotypes that lack or contain less lipoxygenase isozymes in order to reduce the incidence of rancidity (Hildebrand and Hymowitz, 1981; Wilson *et al.*, 1981; Yabuuchi *et al.*, 1982; Kitamura 1984; Hajika *et al.*, 1991).

The immature seeds of the vegetable-type cultivars are harvested during the period 30 to 40 days after flowering, and are eaten as a vegetable after boiling for two min. Many vegetable-type cultivars are landraces, and have been selected and conserved by farmers for many decades in Japan (Aoba, 1956) although breeding programs for vegetable-type soybeans have attempted to improve disease- and insect-resistances, maturity, quality, yield, and so on (Shanmugasundaram *et al.*, 1991; Takahashi, 1991). The immature seeds of the grain-type cultivars can be eaten following the same cooking procedures used for the vegetable type cultivars. However, the latter immature seeds have a better flavor and sweetness as compared with the immature seeds from the grain-type cultivars.

In order to better understand the differences in protein quality between vegetable-type and grain-type soybean cultivars, and to assist in the development of breeding programs, the present study examined the accumulation characteristics of water-soluble nitrogen and free amino acids in immature and mature seeds from the vegetable-type and grain-type soybean cultivars.

Materials and Methods

Experiment 1

In 1991, 10 vegetable-type and 13 grain-type cultivars were selected for this study on the basis of water absorption characteristics in a previous study (Akazawa and Sasahara, 1990). Their growth characteristics are shown in Table 1. In Japan, these vegetable-type and grain-type cultivars are mainly cultivated in the Tohoku and Hokkaido areas (38° to 43° N lat.), but some grain-

Received July 12, 1996. Accepted December 11, 1996.

type cultivars with late maturities are cultivated in the Kanto area (35 ° to 38 ° N lat.).

Seeds were sown in black thin vinyl pots (10 cm in diameter $\times$ 12 cm in depth) that were filled with an upland soil without application of chemical fertilizer on May 10. In order to ensure uniform growth, seedlings were grown in a greenhouse nursery bed for 14 days, and those with one single foliate leaf were transplanted to an upland field of Yamagata University farm on May 24.

A chemical fertilizer containing 60 g N, 180 g P₂O₅, and 240 g K₂O per kg was applied at the rate of 15 kg/ha at plowing on May 14. Two seedlings were transplanted per hill. The hills were spaced 10 to 45 cm apart depending on the plant height of the cultivars. All rows were 80 cm wide. These planting densities were adopted according to standard cultivation procedures in the Shonai plain.

A plot of a single cultivar consisted of 6 rows $\times$ 10 hills. Sixty-nine adjacent plots were set up in a field. Thirty plots were used for the vegetable-type cultivars (3 replications for each of 10 cultivars) and 39 plots were used for the grain-type cultivars (3 replications for each of 13 cultivars). The locations of all 69 of the plots were randomly chosen.

One hundred pods at the middle positions of the culm were sampled from the central 6 hills of the middle 2 rows excluding the border of 2 rows and 2 hills in each plot. Sampling of the immature seeds occurred 30 to 40 days after flowering depending on when a suitable softness was reached for use as a vegetable. This was determined by chewing the raw immature seeds and comparing them to commercially harvested seeds. After removal of the pods, seeds that were pooled from each plot were freeze-dried using an EYELA FD-80 type Freeze Drier (Hitachi-Koki Co., Ltd., Ibaraki, Japan), and then pulverized to 150 μ m flour using a Cyclone Sample Mill (UD Co., Boulder, CO, USA).

One gram of seed flour was dispersed with 25 ml of distilled water and shaken at 20 °C for 1 hr. The milky precipitates formed by the addition of 10 ml of 5 g/l MgSO₄ at 75 °C were removed by filtration through Toyo-Roshi No. 2 filter paper (Toyo-Roshi, Co., Ltd., Tokyo, Japan). Ten ml of the filtrate was used to determine the water-soluble nitrogen concentration, using a Kjeltac System 1026 (Nippon General Co., Ltd., Tokyo, Japan) in 3 replications from 3 plants of each cultivar (3 $\times$ 3 = 9 samples per cultivar).

Experiment 2

In 1992, the same 10 vegetable-type and 13 grain-type cultivars were cultivated as in 1991. The procedures of management of seedlings and transplanting, the time that the pods were sampled, and the level of fertilizer application were also the same as in 1991. Seed flour and its filtrate were also prepared according to the procedures in 1991. The filtrate was vacuum-dried, and then dissolved in 2 ml of a trifluoroacetic acid (13.094

mM/l):acetonitrile solution that was mixed at a 1:1 volume ratio. One ml of the extract was cleaned by passing through a SEP-PAK plus C18 cartridge according to the manual (Millipore Co., MA, USA). Then, 1 ml of trifluoroacetic acid (13.094 mM/l) and 1 ml of trifluoroacetic acid (13.094 mM/l): acetonitrile solution (1:1 volume ratio) were passed through the cartridge to elute the hydrophilic and hydrophobic free amino acids, respectively. The total amino acids were dissolved in 20 μ l of an ethanol:H₂O: triethylamine solution (2:1:1 volume ratio), and freeze-dried. Amino acids were labeled with phenylisothiocyanate (PITC) by the addition of 20 μ l of ethanol:H₂O:triethylamine:PITC (7:1:1:1 volume ratio) to the dried sample for 20 min at room temperature. The PITC-labeled amino acids, called PTC-amino acids, were dissolved in 100 μ l of a solution of 0.05 M Na₂HPO₄ and 953.7 mM/l acetonitrile at pH 5.8, and passed through a columngard-LCR4 column (Nihon Millipore Co., Ltd., Tokyo, Japan) for purification. The PTC-amino acids were then analyzed using a PROTEIN-PAK 300 column (7.8 mm in diameter $\times$ 30 cm in length) (Millipore Co., MA, USA) and ultraviolet detector at 254 nm in a PICO $\cdot$ TAGTM system (660E High Performance Liquid Chromatography) (Waters Nihon Millipore Co., Ltd., Tokyo, Japan) in 3 replications from 3 plots of each cultivar.

Results

The plant heights of the vegetable-type cultivars tended to be lower than those of the grain-type cultivars. The number and weight of pods of the vegetable-type cultivars also tended to be lower than those of the grain-type cultivars (Table 1). Concentrations of the water-soluble nitrogen that were measured in 1991 were positively and significantly correlated with the concentrations of the amino acids that were measured in 1992, especially in the immature seeds. Similar trends were observed in the mature seeds except for glutamic acid (GLU) and valine (VAL), although the correlation coefficients were lower in the immature seeds (Table 2).

Concentrations of asparagine (ASN), alanine (ALA), and GLU were significantly higher than those of arginine (ARG), serine (SER), histidine (HIS) and VAL in the immature seeds of both the vegetable-type and grain-type cultivars. Furthermore, concentrations of ASN and ALA tended to be higher than those of other amino acids in the mature seeds of the vegetable-type cultivars (Fig. 1).

Concentrations of ASN, ALA and GLU in the immature seeds were high compared with those in the mature seeds of both the vegetable-type and grain-type cultivars (Figs. 2 to 4). Among these three amino acids, the concentration of ASN in a vegetable-type cultivar, 'Higan-ao' (No. 1) was lower than the concentrations of ASN in the grain-type cultivars. On the other hand, the concentration of GLU in a grain-type cultivar, 'Hitorimusume' (No. 23) was as high as the concentrations of

Table 1. Growth characteristics of the vegetable-type and grain-type cultivars used in the experiments in 1991

Cultivar	Onset of flowering	Harveting time	Plant height (cm)	Number of pods (/plant)	Weight of pods (g/plant)
	Date				
Vegetable-type (edamame)					
1. Higan-ao	Jul. 24	Sep. 17	94.3±3.4	105.7± 4.9	201.4±20.8
2. Shonai 1	Jun. 30	Aug. 12	42.6±0.8	24.7± 3.0	45.0± 1.8
3. Wase-shirayama-dadacha	Jul. 4	Aug. 12	52.3±0.9	30.0± 3.0	52.0± 4.2
4. Shirayama-dadacha	Jul. 16	Aug. 19	60.6±1.6	32.0± 5.1	61.0± 3.2
5. Denko-okuhara	Jun. 20	Jul. 29	32.0±1.5	18.3± 0.7	31.4± 0.7
6. Ezo-nishiki	Jun. 20	Jul. 29	28.7±1.6	24.7± 3.0	47.2± 4.3
7. Sapporo-midori	Jun. 18	Jul. 29	19.9±0.4	20.3± 0.9	39.2± 1.3
8. Okuhara-wase (HS 1)	Jun. 18	Jul. 29	27.2±0.8	27.3± 5.2	53.0± 6.2
9. Shinsetsu-midori	Jun. 20	Aug. 1	28.3±0.4	16.0± 0.6	38.2± 1.5
10. Nakate-hikari-guro	Jun. 28	Aug. 12	47.6±1.0	38.7± 4.7	80.1± 3.3
Grain-type					
11. Isuzu	Jun. 26	Aug. 8	45.1±1.6	32.7± 0.9	51.1± 3.2
12. Karikachi	Jun. 28	Aug. 8	53.8±2.1	41.7± 4.4	80.8± 4.8
13. Hime-yutaka	Jun. 26	Aug. 4	46.1±1.0	34.7± 3.2	61.3± 3.0
14. Norin 1	Jul. 18	Aug. 22	54.9±2.2	104.7±23.8	69.4±10.1
15. Ibaraki 7	Jul. 16	Aug. 22	77.6±2.1	66.7±12.8	80.9± 8.7
16. Dewa-musume	Jul. 16	Aug. 22	124.9±3.4	83.7± 7.0	107.8± 5.5
17. Chamame	Jul. 24	Sep. 9	83.0±1.9	117.0±16.5	213.1±22.1
18. Tanba-guro	Jul. 24	Sep. 13	96.7±1.4	79.3±11.6	144.8±16.5
19. Ootama	Jul. 24	Sep. 27	127.5±2.2	87.3±11.3	138.0±12.9
20. Sekai-ichi	Jul. 28	Sep. 24	85.3±3.3	40.7± 5.2	108.4± 7.4
21. Ooshoku-aki-daizu	Aug. 11	Dec. 8	101.2±2.9	291.3±14.3	452.8±14.1
22. Tsurunoko	Jun. 28	Aug. 12	34.7±1.3	28.7± 2.9	63.4± 3.3
23. Hitori-musume	Aug. 3	Dec. 8	87.8±0.7	169.7±14.1	464.0±22.6

Values shown are mean±SE.

Table 2. Correlation coefficients between concentrations of water-soluble nitrogen in 1991 and amino acids in 1992 in immature and mature soybean seeds from 10 vegetable-type and 13 grain-type cultivars

	ASN	ALA	GLU	Amino acid ARG (mM/kg-dry wt.)	SER	HIS	VAL
Water-soluble nitrogen (g/kg-dry wt.)	0.968*** 0.626**	0.836*** 0.617**	0.673*** 0.139	0.864*** 0.584**	0.764*** 0.525*	0.893*** 0.533**	0.823*** 0.257

*, **, and *** : Significant at 0.05, 0.01 and 0.001 probability levels, respectively.

Upper numerals indicate the correlation coefficients in immature seeds. Lower numerals indicate the correlation coefficients in mature seeds.

GLU in the vegetable-type cultivars. However, the concentrations of ALA in the immature seeds clearly differed from those in the mature seeds.

Based on the concentrations of a combination of amino acids in immature seeds in Fig. 1, ASN, ALA and GLU, which exhibited the highest concentrations, were assigned to the A Group, and the other amino acids ARG, SER, HIS and VAL were assigned to the B Group. Using these two groups and the A plus B Group, the 10 vegetable-type cultivars were correctly discriminated from the 13 grain-type cultivars (Table 3). The corresponding discriminant functions were as follows:
 $Z_1 = -0.013 \times \text{ASN} - 0.408 \times \text{ALA} - 0.136 \times \text{GLU} + 5.135$, $Z_2 = -0.783 \times \text{ARG} - 0.908 \times \text{SER} - 0.280 \times$

$\text{HIS} + 0.312 \times \text{VAL} + 5.421$, and $Z_3 = -0.066 \times \text{ASN} - 0.482 \times \text{ALA} + 0.011 \times \text{GLU} + 0.773 \times \text{ARG} - 1.069 \times \text{SER} - 0.019 \times \text{HIS} + 0.751 \times \text{VAL} + 5.531$, respectively. Two cultivars 'Higan-ao' and 'Hitori-musume' were also correctly discriminated into each cultivar group. However, the concentrations of the same combinations of amino acids in mature seeds as in immature seeds could not discriminate the two types of soybean cultivars (data not shown).

Discussion

The present results showed that the cultivars studied varied in the concentrations of seven amino acids. These

Table 3. Discriminant analyses based on concentrations of amino acids in immature seeds of the vegetable-type and grain-type soybean cultivars grown in 1991

Cultivar	Combination of amino acids		
	ASN+ALA+GLU (A)	ARG+SER+HIS+VAL (B)	(A) Group+ (B) Group
Discriminant score			
Vegetable-type (edamame)			
1. Higan-ao	-1.598	-2.816	-2.972
2. Shonai 1	-4.814	-3.433	-4.285
3. Wase-shirayama-dadacha	-5.059	-2.579	-5.472
4. Shirayama-dadacha	-3.567	-3.091	-4.739
5. Denko-okuhara	-3.671	-3.211	-4.870
6. Ezo-nishiki	-4.048	-3.433	-4.430
7. Sapporo-midori	-4.292	-3.491	-4.680
8. Okuhara-wase (HS 1)	-3.749	-2.806	-4.144
9. Shinsetsu-midori	-3.256	-3.254	-3.979
10. Nakate-hikari-guro	-5.518	-3.926	-5.481
Discriminant boundary score	-0.457	-0.371	-0.520
Grain-type			
11. Isuzu	2.179	0.236	2.580
12. Karikachi	1.668	1.220	1.228
13. Hime-yutaka	4.239	2.530	4.777
14. Norin 1	3.184	3.145	3.566
15. Ibaraki 7	2.851	2.541	3.274
16. Dewa-musume	4.493	4.875	5.421
17. Chamame	2.112	2.615	2.407
18. Tanba-guro	4.051	3.619	4.516
19. Ootama	2.107	1.035	2.287
20. Sekai-ichi	2.846	2.822	4.007
21. Ooshoku-aki-daizu	3.713	3.622	4.259
22. Tsurunoko	3.622	2.643	3.753
23. Hitori-musume	2.484	1.092	2.437

Fig. 1. Contributions of seven amino acids in immature and mature seeds from the vegetable-type and grain-type cultivars in 1991. Vertical bars indicate standard error. **Fig. 2.** Scatter diagram of asparagine concentrations in immature and mature seeds from vegetable-type and grain-type cultivars in 1992. Numerals on the symbols indicate the cultivar number (see Table 1).
◆: Vegetable-type cultivar.
◇: Grain-type cultivar.

Fig. 3. Scatter diagram of alanine concentrations in immature and mature seeds from vegetable-type and grain-type cultivars in 1992. Numerals on the symbols indicate the cultivar number (see Table 1).
 ■: Vegetable-type cultivar.
 □: Grain-type cultivar.

differences may be due to different rates of protein synthesis, including to some extent the delay in protein synthesis in the immature vegetable-type seeds. Furthermore, the higher accumulation rates of free amino acids in the immature seeds may be related to the higher rate of water absorption in the ripened seeds (Akazawa and Sasahara, 1990).

The amino acids that showed higher accumulation rates in the immature vegetable-type seeds represent four of the five amino acid groups that were identified on the basis of their carbon-skeleton structure (Sakurai *et al.*, 1992). These groups are the glutamic acid group including GLU and ARG, the aspartic acid group including ASP and ASN, the pyruvic acid group including ALA, and the serine group including SER. The fifth group, the aromatic amino acid group, was not represented in the present study. ASN, GLU and ASP are the main carrier agents of translocation through phloem (Hoching, 1982; Jeschke *et al.*, 1985; Chino *et al.*, 1987).

ARG, which belongs to the glutamic acid group, is one of the main amino acids that is stored in seeds (Chou and Splittstoesser, 1972). Thus, the higher accumulation rate of amino acids in the immature vegetable-type seeds may be attributed to translocation from the amino acid synthetic sites in the leaves, and/or the delay in protein synthesis in the immature vegetable-type seeds. Furthermore, the higher accumulation rate of ASN, ALA, and GLU may be related to the higher eating quality of the immature vegetable-type seeds because

Fig. 4. Scatter diagram of glutamic acid concentrations in immature and mature seeds from vegetable-type and grain-type cultivars in 1992. Numerals on the symbols indicate the cultivar number (see Table 1).
 ●: Vegetable-type cultivar.
 ○: Grain-type cultivar.

these amino acids are closely associated with sweetness and taste.

In Japan, many vegetable-type cultivars are landraces. This may indicate that the cultivars with the higher accumulation rates of the special amino acids mentioned above were empirically and preferentially selected by farmers. This, in turn, may indicate that these vegetable-type cultivars belong to a different 'ecotype' that differs from the grain-type cultivars. This hypothesis appears to be supported by the discriminant analyses based on concentration differences of the seven amino acids.

Among the seven amino acids, ALA and GLU in the A Group, ARG and SER in the B Group, and ALA, ARG, and SER in the A plus B Group were the higher contribution agents as seen in the coefficients in the discriminant functions.

Furthermore, it is important that concentrations of the water-soluble nitrogen were highly correlated with the amino acid concentrations. Measurement of amino acids is laborious, time-consuming and expensive, but measurement of water-soluble nitrogen is easy and rapid. Therefore, measurement of water-soluble nitrogen may be a simple measure that represents the concentrations of amino acids. Water-soluble nitrogen may thus be used as one of the simple selection indices for breeding programs of the vegetable-type cultivars in which a large number of progenies should be examined.

Acknowledgements

The authors thank Mrs. N. Satoh, Laboratory of Plant Breeding, Faculty of Agriculture, Yamagata University, for her valuable assistance with the calculations used in this study. Thanks are due to the staff of the University farm for their excellent management of the cultivation.

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