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Effects of Sulfur Nutritional Forms on Accumulation of Seed Storage Proteins in Soybean(*Glycine max*)

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ABSTRACT

Improvement of seed protein quality might be an essential issue in soybean and would give more profit directly to both farmers and users. This study was carried out to investigate the effects of reduced-S form(s) on seed storage protein components in soybean during seed filling stages. The reduced-S forms during seed fill were sodium thiosulfate, sodium sulfite, sodium sulfide, thioacetate, β-mercaptoethanol, thiourea, thiamine-HCl, L-cysteine, L-cystine, and L-methionine. Seed storage protein concentration did not appear to be affected by any reduced-S forms. However, glycinin and β-conglycinin concentrations seemed to be changed greatly by L-methionine. This resulted in the increase in the 11S/7S ratio(3.58). Among the β-conglycinin, β-subunit was not accumulated at all, α-subunit concentration appeared to be decreased and α' -subunit concentration was not altered in comparison with sulfate control. Also, β-conglycine concentration, especially β-subunit concentration, tended to be decreased with L-cystine treatment, resulting in an increase in the 11S/7S ratio(1.83). The glycinin concentration tended to be increased at the expense of the decrease in the β-conglycinin concentration. Therefore, it is suggested that enhancing soybean protein quality would be achieved by improving metabolic pathways of S assimilation in soybean plants during seed filling period under sulfate-sufficient condition.

Key words: Soybean, Seed Storage Protein, Glycinin, β-Conglycinin, Sulfur Forms, L-Methionine

INTRODUCTION

Soybean (*Glycine max* L.) seeds are major sources of edible plant proteins and the desire for value-added soybean protein has increased continuously for human and animal diets(Kitamura, 1995). Although soybean has the highest seed protein concentration among crop species, the nutritional value of the soybean protein is still below that of animal proteins due to low levels of S-amino acids such as cysteine and methionine(Serretti et al., 1994). Therefore, it is still needed to improve its quality by modifying seed protein components. Soybean protein is composed of seed structural proteins, enzymes, and seed storage proteins (SSP) called globulin. About 70% of the protein is SSP that contains glycinin (11S protein) and β-conglycinin (7S protein)(Derbyshire et al., 1976; Thanh et al., 1975). Glycinin accumulates about 60% of SSP and β-conglycinin does the remaining 40% although there are some variations among cultivars with different genetic backgrounds(Koshiyama, 1983; Nielsen et al., 1994). Glycinin that is composed of five subunits has S-amino acids of about 3.5% which is the standard level for humans(Fukushima, 1991). According to homology of amino acid sequences, glycinin is divided into two groups : Group I ($A_{1a}B_2$, $A_{1b}B_{1b}$, A_2B_{1a}) and group II (A_3B_4, $A_5A_4B_3$)(Nielsen et al., 1994). However, β-conglycinin that is composed of three subunits (α' [76kDa], α [72kDa], β[53kDa]) has S-amino acids lower than 1%(Derbyshire et al., 1976; Harada et al., 1989; Sebastiani et al., 1990). Especially, β-subunit contains only one cysteine and no methionine in about 470 amino acids(Coates et al., 1985). Thus, higher β-conglycinin concentration in soybean seeds may result in lower S-amino acids concentration. If β-

conglycinin can be reduced or removed from soybean seed without decreasing seed protein concentration, the soybean seed will have the best desirable quality for diets as well as industrial uses (Fukushima, 1991; Kitamura, 1995).

β-conglycinin subunits were known to accumulate differently according to sulfur nutritional condition. Gayler and Sykes(1981; 1985) reported increased accumulation of β-conglycinin and decreased quantity of glycinin in sulfate-deficient soybean plants. Among the β-conglycinin, the β-subunit increased with sulfate deficiency most dramatically and accemulated to a 3-fold level as compared with normal conditions without altering seed protein concentration. When developing soybean seeds were *in vitro* cultured in the medium containing L-methionine, β-subunit did not accumulate and α and α' subunits was not affected(Holowach et al., 1984a; 1984b; Thompson et al, 1984; Thompson & Creason, 1990). It was suggested that the inhibition of the β-subunit accumulation might be controlled by L-methionine at the transcriptional level, and that the regulation might be reversible because synthesis of the β-subunit began when the seeds moved to non-methionine medium(Creason et al., 1984; 1985). Infusion study(Grabau et al., 1986) also showed positive relationship between inhibition of β-subunit and L-methionine infusion into stem. Paek et al.(1997) reported that substitution of urea (the reduced form of N) for nitrate (the oxidized form of N) under sulfate-sufficient condition during seed fill resulted in increases in N assimilation and protein concentration of about 5%. However, this increase in the protein concentration(Ohtake et al., 1994) resulted from an increase in β-subunit that can be observed under sulfate-deficiency and there was no difference in glycinin concentration between ammonia and nitrate(Gayler & Sykes, 1981). It was also indicated that N-deficient stress resulted in a decrease in seed protein and subunit concentrations especially in β-subunit concentration(Ohtake et al., 1994).

A most sulfur form that exist about 98-99% in soil is sulfate, the oxidized S form. Sulfur as a plant nutrition is mostly uptaken as sulfate through an active transport system of root and 1-2% sulfur is uptaken as S-amino acids and secondary S-metabolites by root or as sulfur gases by leaf(Anderson, 1990). Sulfate uptaken by root is reduced by ATP, metabolites and other catalysts in S assimilation procedure and the reduced S is incorporated for synthesis of methionine, a final product of S assimilation. Five percent increase in protein concentration of soybean seed was observed during seed fill when N nutritional form was changed from the oxidized form, nitrate, to a reduced form, ammonia(Paek et al., 1997). This result suggests that S-amino acids concentration in seed may be affected if a reduced form of S is substituted for an oxidized form, sulfate. Therfore, this study was conducted to invertigate the effects of substitution of several reduced S forms for an oxidized-S form, sulfate, during seed fill on seed protein and subunit concentrations in soybean during seed filling period by hydroponic culture.

MATERIALS & METHODS

1. Plant Materials and Hydroponic Solutions

The study was carried out from February 15 to July 31 in the Agronomy greenhouse at Iowa State University, USA. Kenwood, one of the best soybean variety cultivars in Iowa, was grown in a hydroponic medium as originally developed by Imsande & Ralston(Imsande & Ralston, 1981). Hydroponic system enabled us to change nutritients according to plant growth stages. Some modification was made according to S forms used in hydroponic solutions to see the effects (Table 1). Until R4.5(Fehr & Caviness, 1977), 5mM KNO_3 and 0.4mM Na_2SO_4 were maintained to make sulfate-sufficient condition during vegetative growth stage of soybean plants, which is lower than 16 in a ratio of N/S, as suggested by Agrawal & Mishra(1994). Hydroponic solution containing nitrate was buffered to about pH 5.6-5.7 with 2mM MES. Temperature in the greenhouse was maintained at near 26/18℃(day/night) and supplemental lightening of $200\mu Em^{-2}s^{-1}$ was provided at the greenhouse and was extended to daylength of 16 hours.

After R4.5, KNO_3(oxidized-N, 5mM) was substituted for urea(reduced-N, 2.5mM) to maximize N nutrition during seed fill as described(Paek et al., 1997). Na_2SO_4(oxidized-

Table 1. Chemical composition of the stock solutions used in hydroponic culture. All chemicals were obtained from Sigma.

Stocks	Chemical composition	Grams per liter	Dilution used
1	KNO_3	202.25	1 to 400
2	urea (NH_4CONH_4)	60.6	1 to 400
3	$Na_2SO_4^+$	56.8	1 to 1000
4	MES[a]	97.6	1 to 250
5	KCl	176.8	1 to 800
	K_2HPO_4	13.9	
6	$CaCl_2 \cdot 2H_2O$	312.5	1 to 1000
7	$MgCl_2 \cdot 6H_2O$	366.0	1 to 1000
8	$FeCl_3$	18.0	1 to 2000
	DTPA[b]	10.9	
9	$MnCl_2 \cdot 4H_2O$	1.16	1 to 4000
	$CuSO_4 \cdot 5H_2O$	0.8	
	$Na_2MoO_4 \cdot 2H_2O$	0.62	
	H_3BO_3	1.12	
	$ZnSO_4 \cdot 7H_2O$	0.8	
	$CoSO_4 \cdot 7H_2O$	0.19	
	$NiCl_2 \cdot 6H_2O$	0.011	

a : MES: 2-[N-morpholino]ethanesulfonic acid

b : DTPA: Diethylenetriaminepentaacetic acid

+ : Reduced forms of S were substitued for $Na_2SO_4^+$ after R4.5.

S, 0.4mM) was substituted for one of the followings during seed fill: 1) 0.2mM sodium thiosulfate, 2) 0.4mM sodium sulfite, 3) 0.4mM sodium sulfide, 4) 0.4mM thioacetate, 5) 0.4mM β-mercaptoethanol, 6) 0.4mM thiourea, 7) 0.4mM thiamine-HCl, 8) 0.4mM L-cysteine, 9) 0.2mM L-cystine, 10) 0.4mM L-methionine. A buffering agent, 2mM MES pH 6.7, was used for urea-containing hydroponic solution after R4.5. The hydroponic solutions were replaced twice weekly until the harvest(R8).

2. Sampling and Analysis Methods

A completely random design(CRD) was used with 4 replications. Soybean seeds were harvested from each plant and oven-dried at 65℃ for 48h. Seed yield (SD, g/plant) and 100-seed weight (SW, g) were measured. To measure seed protein, seed coat was removed before grinding in a mill. Seed powder was oven-dried at 65℃ for 24h additionally. A 0.2g sample of each powder was assayed twice for N concentration by semimicro-Kjeldahl method(Bremner & Breitenbeck, 1983). Percentage of N averaged over two independent samples was measured and if deviation between the two samples was higher than 5%, percentage of N was measured again. Protein concentration was measured by percentage of N×6.25. LSD test was done to see a difference between treatments and control.

3. Analysis of Seed Storage Protein Concentration

A 10 seed sample was randomly selected from each plant. A 20mg of each sample was defatted with cold-hexane four times and soluble proteins were extracted after adding 1.0ml of protein extraction buffer as described(Paek et al., 1997). Soybean seed storage protein (about 10.0mg per lane) was separated by 10-15% gradient SDS-PAGE and all the procedures for staining and densitometric analysis were followed as described(Paek et al., 1997).

RESULTS & DISCUSSION

1. Effects on Seed Yield, Seed Weight and Protein Concentration

During seed filling stages, substitution of reduced forms for sulfate did not appear to affect seed protein concentration(Table 2). Pack et al.(1997) suggested that seed protein concentration tended to increase when ammonia (reduced N) was substituted for nitrate (oxidized N) during seed fill, which appeared to be due to an increase in efficiency of N assimlation. Also, it was suggested that there may exist a positive co-regulatory mechanism between N and S assimilation during the vegetative stage(Bunold & Suter, 1984). Therefore, there seem to be independent relationship between regulatory mechanisms of N and S during seed filling stages.

Thiourea and sodium sulfite as S nutrition was harmful for the plant when added into hydroponic solution. When thiourea was added in hydroponic solution, plant growth and development stopped and both leaves and stems were bleached severely and senescenced about two weeks earlier, resulting in a severe decrease in both seed yield and 100-seed weight(Table 2). All plants treated with sodium sulfite were dead because of its unknown toxicity. L-methionine appeared to decrease seed yield, increase 100-seed weight, and produce the biggest seeds whose coats were cracked perhaps due to slower seed development and longer seed filling period. These seeds were able to be harvested about one month later. These unusual phenomena may be due to an increased level of SAM and consequently an abnormal increase of ethylene by free L-methionine inside the plant(Yang & Hoftman, 1984). The other S-amino acid forms showed no difference in plant growth, seed yield, and 100-seed weight compared to sulfate control (Table 2).

2. Effects on Seed Storage Protein Components

Among all reduced-S sources, L-methionine seemed to increase glycinin concentration very high and appeared to decrease β-conglycinin concentration(Table 2), resulting in a highly significant increase in a 11S/7S ratio(3.58). This seemed to result from a decrease in a α subunit concentration of about 6% compared to sulfate-control. As previously reported(Holowach et al.,

Table 2. Effects of S nutritional forms during seed fill on seed yield(SY), 100 seed weight(SW), seed protein concentration(PC), seed storage protein concentration(SSP), subunit concentrations in SSP and a 11S/7S ratio.

Treatments	SY (g/plant)	SW (g)	PC (%)	SSP (%)	Relative subunit concentration(%) of SSP α	α	β	Ac	Bs	11S	7S	11S/7S ratio
Sulfate(control)	37.4	15.07	42.6	30.4	11.7	15.1	11.3	38.5	23.5	61.9	38.1	1.63
Thiosulfate	39.9	15.99	43.1	32.3	11.5	15.7	12.4	37.5	22.9	60.4	39.6	1.53
Sulfide	41.3	15.50	42.9	31.7	12.0	15.3	11.6	38.1	23.0	61.1	38.9	1.57
Thioacetate	40.7	15.06	41.9	30.3	11.6	15.6	12.3	37.7	22.8	60.5	39.5	1.53
b-Me	41.9	15.55	42.8	31.8	12.1	15.0	11.7	37.9	23.2	61.1	38.9	1.57
Thiourea	14.5*	11.27*	41.9	30.0	11.3	15.6	12.3	37.5	23.3	60.8	39.2	1.55
Thiamine-HCl	36.9	15.88	43.4	32.4	11.9	15.1	12.3	37.7	23.1	60.8	39.2	1.55
L-cysteine	43.5	14..50	41.5	30.3	12.2	15.8	11.6	37.5	23.0	60.5	39.5	1.53
L-cystine	38.6	14.56	42.7	32.0	12.5	14.3	8.5*	40.1	24.5	64.7*	35.3*	1.83*
L-methionine	18.8*	19.81*	42.3	31.2	11.9	9.9*	0.0*	48.3*	29.9*	78.2*	21.8*	3.58*
LSD(0.05)	7.9	2.31	2.8	2.2	1.5	2.1	1.3	2.2	1.7	2.8	2.8	0.14

+ : SSP is composed of 2 major proteins, 11S and 7S, where 11S contains acidic(AC) and basic protein subunits proteins and 7S contains α' , α, and β protein subunits.

* : Significantly different according to LSD(0.05).

1984a; 1984b; Thompson et al., 1984; Thompson & Creason, 1990), β-subunit was not accumulated at all. Their results indicate that supply of L-methionine would increase glycinin cocentration greatly, which suggests that there might be possibility of increasing S-amino acids concentration in a bulk of soybean protein.

L-cystine appeared to accumulate β-subunit concentration of about 8.5% that is less than in sulfate control(11.3%), resulting in a significant increase in a 11S/7S ratio(1.83)(Table 2). However, L-cysteine did not show any variation in subunit concentration(Table 2). There is a doubt whether L-cysteine can be uptaken as an amino acid form in hydroponic condition because it has a highly reactive SH.

Pack et al.(1997) indicated that when ammonia as an N nutritional source was substituted for nitrate during soybean seed fill, seed protein concentration tended to increase due to an increase in β-conglycinin concentration. Among the β-conglycinin, only β-subunit concentration was increased while α' and α subunit concentration were not changed. This increase in β-subunit might result in a decrease in relative abundance of S-amino acids in soybean protein. However, glycinin concentration remained constant.

In view of N and S assimilation for soybean seed protein, the increase in N assimilation resulted in more accumulation in seed protein concentration. The increase in S assimilation with L-methionine resulted in the increase in S-rich glycinin concentration, while the decrease in S assimilation by sulfate-deficiency resulted in an increase in S-poor β-conglycinin concentration, especially S-void β-subunit concentration without altering the concentration of seed protein concentration. With these results, the regulatory mechanism of SSP genes seems to be nutritionally controlled in seed under a given N and S condition. Funthermore, it can be concluded that increasing soybean protein quality would be achieved by improving metabolic pathways of N and S assimilation in soybean plants.

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