# Changes in Level of Several Functional Components and ACE-Inhibitory Activity in Developing Soybean Seeds

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**ABSTRACT :** Soybean quality is determined based on protein content, lipid content and fatty acid composition, and several functional components including isoflavones, anthocyanins and functional activity. Because the level of each component changes during seed development, it is necessary to know the concentration of quality-related components in developing seeds. Little is known of the pattern of changes in quality-related components. Seeds from field-grown soybean was harvest from the $R_6$ stage to the $R_8$ stage in 2004. Seed characteristics and the level of nutritional components were examined. Seed moisture content was dropped rapidly after the $R_7$ stage in the tested varieties. Seed growth rate was the highest from the beginning of the $R_6$ stage to the mid-$R_6$ stage. Chlorophyll content was decreased rapidly in pods and seeds. However, seed growth period from the $R_6$ to $R_8$ was 35 days. The crude protein content was increased dramatically between 63 DAF and 70 DAF and then increased slightly. The pattern of isoflavone accumulation was nearly similar to that of seed weight increase. From the late $R_6$ stage to the $R_7$, the accumulation rate was higher as compared to other stages. The angiotensin inhibitory activity was increased according to seed development from 63 ($R_6$) to 84 DAF ($R_8$). The difference of inhibitory activity in heated soybean powder, however, was not great among stages. The inhibitory activity was affected by heating treatment. The most effective heating time was 10 min. Excessive heating longer than 30 min resulted in a lowered inhibitory activity of soybean on ACE.

*Keywords*: seed development, anthocyanins, isoflavones, ACE-inhibitory activity, soybean

S oybean seed has a number of nutritional constituents including a large proportion of protein and lipid and relatively small amount of carbohydrates and several functional constituents like isoflavones, soyasaponins, anthocyanins and others (Badger et al., 2002; Kim, 2002; Holt, 1997). In addition, recently, one of noticeable nutraceutical concerns to soybean is inhibitory activity on angiotensin converting enzyme that is related to high blood pressure.

The concentration of each constituent of seed changes during seed development. Therefore, it is important to know the time at which the concentration of a target chemical is the highest. Because the conventional harvesting is synchronized with the maximal accumulation of major storage component of seed which is used for food, the harvesting time for a certain functional component may be different from the harvesting timing for food production. Due to the increase of needs for functional food, soybean having high level of nutraceutical components should be produced. Among a number of soybean varieties, the soybeans having black seed coat color have known as a medicinal material in Korea so far. Therefore, in this experiment was conducted with a small and black soybean variety, 'Seomoktae'.

Because the nutraceutical effect of soybean is becoming important characteristics as much as yield potential and the levels of other nutritional component, recent researches conducted in Korea are focused on the bioactive components from soybean. Of nutraceutical components, isoflavones is a major component in soybean and have been studied by a number of researchers (Kim et al., 2006; Hoeck et al., 2000). Isoflavones are mainly found as flavonoid forms in soybean seeds and are known for their many chemical activities, such as their antioxidative properties, and their uses as anticancer agents. They are classified into four groups: aglycons, glucosides, malonylglucosides and acetylglucosides. Soybean isoflavones provide potentially beneficial effects for several diseases afflicting human beings, including breast cancer (Holt, 1997). Recently, studies have provided evidences that soybeans might have cancer-preventative properties. In many studies, isoflavones have been shown to have potential benefits for reducing the risk of various cancers (Graber et al., 1992; Messina et al., 1994). Isoflavones concentrations are affected by the environment, the genotype and interaction between these factors. The environment had a greater effect than the genotype (Hoeck et al., 2000; Wang &Murphy, 1994) on the concentration

Angiotensin-converting enzyme (ACE) plays an important role in the regulation of blood pressure. ACE has been associated with the rennin-angiotensin system, which regulates the peripheral blood pressure. The enzyme can increase

blood pressure by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Inhibition of ACE may therefore exert an antihypertensive effect through a decrease of angiotensin II. Of the bioactive peptides in soybean, angiotensin I–converting enzyme (ACE) inhibitory peptides have been studied due to their potential usage as a functional food additive and natural alternative to ACE inhibitor drugs. Among the bioactive peptides, angiotensin I–converting enzyme (ACE) inhibitory peptides derived from food proteins have attracted particular attention and have been studied the most comprehensively for their ability to prevent hypertension. These peptides could be used as a potent functional food additive and represent a healthier and natural alternative to ACE inhibitor drugs. It has been generally known that the ACE catalyzes the hydrolysis of angiotensin I to angiotensin II, which is a potent vasoconstrictor, and inactivates the vasodepressor compound, bradykinin. ACE-inhibitory peptides have been discovered from soybean protein (Shin et al., 2001; Wu & Ding, 2001). In addition, soybean fermented products have been found that contain antihypertensive peptides (Gibbs et al., 2004). The peptides might be utilized to control blood pressure, especially among people with a high risk of essential hypertension (Yoshii et al., 2001).

## MATERIALS AND METHODS

A soybean variety 'Seomoktae', having black seed coat was used in the experiment. Soybean seeds were sown with $60 \times 20$ cm planting space on May 26 at the Experimental Farm of Gyeongsang National University in 2004. Standard management practices for soybean production were applied. Harvesting date based on the soybean growth stage described by Fehr and Caviness (1977) was determined from the $R_6$ to the $R_8$ stage by 7 days interval. From green bean stage to full maturity, soybean seeds were harvested and dried at low temperature (45°C) to minimize the chemical changes of seed constituents. Dried seeds were ground with cutting mill with 1 mm screen and stored at 4 until use. Heat treatment was carried out with powdered sample at 100 for 1, 5, 10, 30 min in boiling pot after hydration with distilled water for 12 hr. The heat-treated paste was freeze dried and powdered with mortar and pestle.

Crude protein content was examined by micro-kjeldahl method (AOAC, 1980). Conversion factor (6.25) was multiplied to the nitrogen content obtained by micro-kjeldahl method to calculate crude protein content. Reducing sugar content was analyzed by DNS (dinitrosalicylic acid) method (Bernfeld, 1955). Sugar concentration was measured at 570 nm and calculated using standard curve of anhydrous D-glucose. Sucrose content was analyzed with enzymatic method

(Guglielminetti, 1995). Starch content was analyzed by enzymatic method using amyloglucosidase. In brief, use the pellet from the extraction for sugar analysis. Add 1.8 mL of distilled water to the pellet and boiled for 10 min. After cooling to room temperature, 0.1 mL of 1.25 M citrate buffer (pH 4.5) and 0.1 mL of 375~400 units $mL^{-1}$ amyloglucosidase (reconstituted in 0.05 M citrate buffer) was added to sample. Incubate at 55°C for 12 h. Cool to room temperature and centrifuge for 5 min at 3000 rpm. Supernatant was used for sugar analysis by DNS method.

Isoflavones concentration was determined by HPLC (Mitani et al., 2003) after hydrolysis into aglycones. In brief, 50mg of soybean powder was extracted with 95% EtOH for 3 hr at room temperature and the extract was dried in vacuo then resuspened with 3N HCl and heated at 95°C for 30 min. The hydrolysate was neutralized with NaOH and subsequently filtered using 25 μm syringe filter. The 20 μL sample injected into the HPLC system. A Waters NovaPak $C_{18}$ ($250 \times 3.9$ mm, 4 μm) reversed-phase column was used to carry out the separation. UV-absorption spectra of purified isoflavones were analyzed in HPLC equipped with a pump delivery system (LC-10ATVP, Shimadzu, Japan). The mobile phase consisted of acetonitrile (A) and 0.1% acetic acid (B). All elution were carried out at a flow rate of 0.8 mL $min^{-1}$ and at ambient temperature. The following gradient was used to analyze the extracts: 0 min: 23% A and 77% B; 15 min: 90% A and 10% B; 22.5 min: 90% A and 10% B; 25~30 min: 23% A and 77% B. Analytes were monitored at 260 nm.

Anthocyanins were analyzed in HPLC equipped with a pump delivery system (LC-10ATVP, Shimadzu, Japan). Soybean powder was extracted with methanol containing 1% HCl. After hydrolysis 20 μL of the sample was loaded onto a $C_{18}$ column ($250 \times 3.9$ mm, 4 μm, Novapak, Waters). The mobile phase consisted of $HCOOH-H_2O$ (1:9) (solvent A) and $HCOOH-H_2O-MeOH$ (1:4:5) (solvent B). All elution were carried out at a flow rate of 1.0 mL $min^{-1}$. After injection of the sample, solvent B was increased to 10% in 4 min, increased from 10% to 100% in 4 - 21 min, continued 100% in 21 - 25 min, decreased to 10% in 25 - 26 min and continued 10% in 26 - 29 min. Absorbance was monitored at 530 nm.

Inhibitory activity for angiotensin converting enzyme (ACE) was examined by the method of Cheung & Chshman (1971) with slight modification. Soybean powder was extracted with 20 volumes of methanol at 100 for 2 h. The aqueous extracts were then filtered, concentrated and lyophilized. The lyophilized powder was kept at -20°C until needed. The 0.05 mL of sample containing 0.1 mL of hip-his-leu was preincubated for 5 min at 37°C. The reaction was initiated by adding 0.15 mL of ACE and terminated by

**Table 1.** Growth and development of pods and seeds during grain filling stage in Seomoktae.

| DAF[†] | Dry weight (g) | | Seed moisture content (%) | Chlorophyll content (mg g⁻¹) | |
|---|---|---|---|---|---|
| | Pod | Seed | | Pod shell | Seed |
| 49 ($R_6$) | 0.153±0.005 | 0.050±0.003 | 73.9±0.69 | 11.66±0.12 | 5.68±0.16 |
| 63 | 0.224±0.013 | 0.115±0.009 | 64.2±1.24 | 12.34±0.11 | 3.78±0.05 |
| 70 | 0.247±0.007 | 0.131±0.006 | 61.0±0.70 | 9.65±0.02 | 3.93±0.02 |
| 77 ($R_7$) | 0.248±0.015 | 0.140±0.012 | 54.2±3.18 | 4.59±0.02 | 2.74±0.03 |
| 84 ($R_8$) | 0.201±0.009 | 0.114±0.003 | 26.0±1.75 | 2.11±0.01 | 1.05±0.01 |

[†]Days after flowering

0.25 mL of 0.5 N HCl after 1 hr of incubation. After the addition of 1.5 mL of ethyl acetate, it was centrifuged at 2,800 rpm for 10 min. The 0.5 mL of supernatant was then dried at 140°C for 15 min and dissolved in 3.0 mL of 1 M NaCl. The absorbance at 228 nm was measured to evaluate the degree of inhibition of ACE activity. The inhibitory activity (%) was represented as follows; [(Ec-Es)/(Ec-Eb)] × 100, where Es was the absorbance of the sample solution, Ec was that of the control solution to which the buffer solution was added instead of the sample, and Eb was that of blank solution to which the stop solution of reaction was added in advance.

## RESULTS AND DISCUSSION

Pod dry weight, seed dry weight and seed moisture content during grain filling stage increased until 77 days after flowering (DAF), then decreased (Table 1). On the other hand, the chlorophyll content decreased steadily after the $R_6$ stage. Decrease in chlorophyll content means that physiological activities related to the quantitative growth of reproductive organs (Sinnecker et al., 2005) reaches to maximum level at the $R_7$ stage (physiological maturity) and then decrease persistently until full maturity. At 77 DAF from which no net increase of dry matter accumulation was observed, several changes linked to qualitative characteristics of reproductive organs might be commenced. Seed size was increased dramatically between 49 DAF and 63 DAF. During this period, translocation rate of photoassimilates might be the highest. Chlorophyll content was positively related to seed moisture content. Because chlorophyll and moisture are essential components for conducting physiological processing, both components were decreased as the growth stage was approached to physiological maturity. As the chlorophyll content began to decrease drastically, seed dry matter was accumulated rapidly. Chlorophyll content was steadily decreased after the $R_6$ stage in seeds, however, the highest level of chlorophyll content was observed at 63 DAF after initiation of the $R_6$ stage in pods. In addition, the
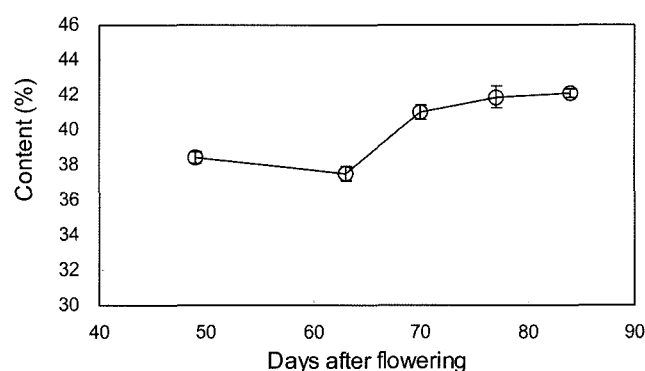


**Fig. 1.** Changes of seed crude protein over seed developmental stages. Crude protein content was calculated multiplying the nitrogen factor of 6.25.

chlorophyll content in pod shell decreased slowly until 70 DAF. Because seed weight showed the highest value at 77 DAF, it was deduced that the seed weight attributed partly to the translocation of photoassimilates produced in pods.

The crude protein content was increased dramatically between 63 DAF and 70 DAF and then increased slightly in seeds (Fig. 1). The content did not increase after the $R_7$ stage at which seed dry weight also did not increase. The crude protein content during seed development nearly coincided in the pattern with quantitative increase in seed size and weight. This result means that the protein accumulation in seed ceased when the seed growth stopped. The seed storage protein that constitutes a large portion of seed is formed with assimilated carbon and nitrogen compounds translocated from vegetative part as sugars and ureids (Schubert, 1986). Therefore, import of raw materials for producing protein may be dependent on the sink strength that is higher at 77 DAF based on the seed size in this experiment. The crude protein contents at the $R_6$, $R_7$ and $R_8$ stage were about 38.1, 41.9 and 42.1%, respectively, showing no great differences between late reproductive stage. Because the protein content was calculated based on the nitrogen content, there can be some discrepancy in content between kjeldahl-based protein and actual protein content. Nevertheless, because the higher
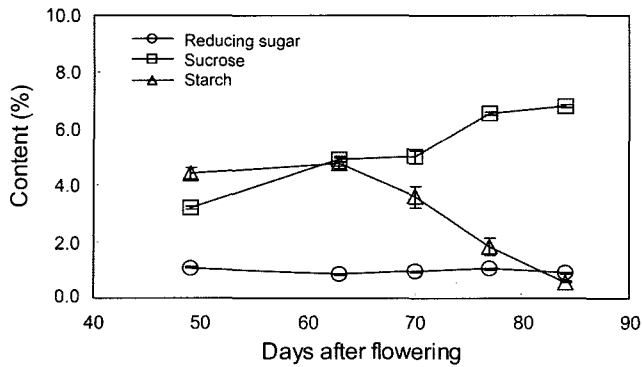
**Fig. 2.** Changes of soluble sugars, sucrose and strach content in developing soybean seeds.

**Fig. 3.** Changes of isoflavone concentrations in developing soybean seed. Isoflavones were determined after hydrolysis into aglycones.

**Fig. 4.** Changes of anthocyanin concentrations in developing soybean seed. Del, Cya, Pela, Peo and Mal are aglycones of anthocyanins and indicate Delphinidin, cyanidin, pelargonidin, peonidin and malvidin, respectively.

content of crude protein means the higher content of nitrogen compounds in seeds, it was concluded that the inflow amount of N compounds into seeds was great at the late $R_6$ stage. From the $R_7$ stage, the level of crude protein content was slightly increased. This result may be due to the ceasing of inflow of nitrogen compounds into seeds at this period.

Carbohydrates contents during seed development were presented in Fig. 2. During seed development, reducing sugar content was nearly stable. However, sucrose was continuously increased showing similar pattern with crude protein. This result may due to the role of reducing sugar that is not participated directly into the biochemical process to producing other constituents in developing seed through translocation into seeds. Because sucrose is a prevalent carbohydrate in soybean seed (Jeong & Lee, 2003), the maximum level was attained at the $R_7$ stage as shown in protein content. It has been known that soybean has a small amount of starch. Starch is, however, a major transporting assimilates to sink organs during translocation. The level of starch was lowered from 4.4% at the $R_6$ stage to 0.6% at the $R_8$ stage. The highest value of starch content was observed at 63 DAF. Seed growth rate of soybean is dependent on assimilate availability (Munier-Jolain et al., 1998). Therefore, the amount of translocated assimilates was great at this time and then decreased rapidly. There was negative relationship between the level of seed major components, crude protein and sucrose, and starch content after 63 DAF. This result implies that chemical reconstitution of unloaded assimilates might be occurred actively at this time.

Isoflavone content increased persistently until the physiological maturity (Fig. 3). The pattern of isoflavone accumulation was nearly similar to that of seed weight increase. From the late $R_6$ stage to the $R_7$, the accumulation rate was higher as compared to other stages. In special, genistein, the most prevalent form of isoflavone in Seomoktae, was accumulated rapidly in this period. Glycitein was the most preva-
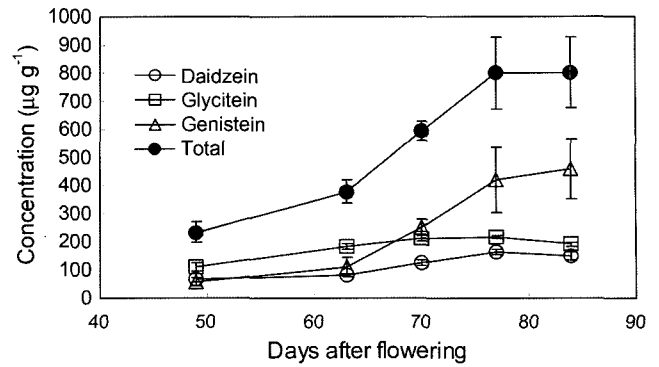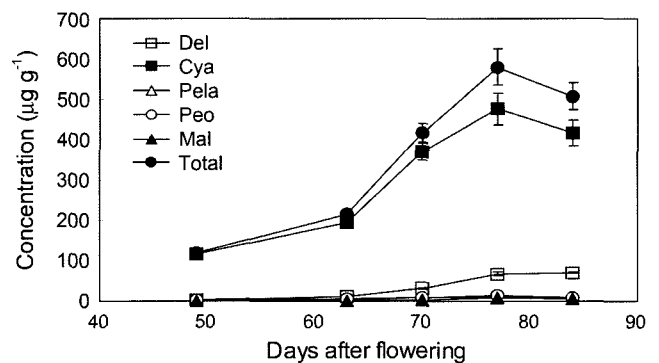
lent isoflavone species until the late $R_6$ stage. Many previous reports showed that glycitein is not a prevalent isoflavone in soybean (Kim et al., 2006; Hoeck et al. 2000; Yi et al., 1997). This isoflavone species, however, showed higher value in relative amount among isoflavone species until 63 DAF. Because chemical reconstitution for storage components started at this time as mentioned above, it was assumed that the chemical reconstitution for producing genistein was more active than other isoflavone species. The contents of daidzein, glycitein and genistein were 150, 192 and 459 μg $g^{-1}$, respectively, at the $R_8$ stage. The isoflavone species which showed relatively stable level was glycitein. Daidzein was persistently increase from the late $R_6$ stage until $R_7$ stage and then slightly lowered at full maturity.

Anthocyanin content also showed tendency with that of total isoflavone content (Fig. 4). In this experiment, the content of each anthocyanin species was determined after hydrolysis to aglycone. The level of cyanidin was the highest in content during all stage among 5 types of aglycones.

Pelargonidin, peonidin and malvidin were detected at trace level less than 10 μg g$^{-1}$ until 70 DAF. Delphinidin showed the second highest level in content from the late R$_6$ stage. Cyanidin and delphinidin, major anthocyanin aglycones, was accumulated rapidly from 63 DAF. This result was similar with that of other seed major constituents such as proteins, sucrose and isoflavones. The increase was stopped at the beginning of the R$_7$ stage and the content was decreased until the R$_8$ stage. In general, total anthocyanins content was determined by the level of cyanidin content which increased until 77 DAF and then decrease slightly at the R$_8$ stage. This result suggests that *de novo* synthesis of anthocyanin commenced after the seed filling was completed at the R$_7$ stage. The content of total anthocyanin was 579 and 507 μg g$^{-1}$ at the R$_7$ and R$_8$ stage, respectively. The seed weight at these stages was 0.140 and 0.114 g, respectively. Based on the seed weight, anthocyanin content was 81.1 and 58.8 μg per seed. Therefore, it was suggested that the optimal harvest timing is the R$_7$ stage if the Seomoktae is grown for producing anthocyanin.

For knowing bioactive activity of soybean, the present study was designed to investigate the changes in bioactive potentials of soybean with or without heat treatment. A bioactivity, the inhibitory effect of soybean on angiotensin con-

verting enzyme (ACE), was examined. The inhibitory activity was increased according to seed development. The inhibitory activity on ACE is related to hypertension, a common disease in recent. Considering the dietary intake of soybean product, the inhibitory activity on ACE is an important factor in soybean quality. In this experiment, ACE-inhibitory activity was increased according to seed development (Fig. 5). Seomoktae has been known having medicinal effect in Korea. The result from ACE inhibitory assay reflects a potential of Seomoktae as a nutraceutical food material. The inhibitory activity rapidly increased from 63 DAF and showed the highest activity at the R$_8$ stage. Because the ACE-inhibitory activity is due to the action of specific peptide, it was assumed that the change in substantial activity was coincided with the change in kjeldahl-N level during seed development.

The nutraceutical effect of soybean can be changed by heating during postharvest processing (Trugo *et al.*, 2000). Heating effect on the inhibitory activity of soybean on ACE was examined and the result is presented in Table 2. Heating treatment enhanced the inhibitory activity. The most effective heating time was 10 min. Excessive heating longer than 30 min, however, resulted in a lowered inhibitory activity of soybean on ACE. The inhibitory activity was more enhanced in the samples collected the earlier stages than the R7 stage. This result implies that the overall quality of soybean should be considered for the quality of end-use quality as well as the quality of raw soybean seeds in terms of nutraceutical effect. In most case soybean is heated for producing soybean product like tofu, soy sauce and other fermented products. Therefore, the changes in nutraceutical activity during heating process should be further studied. Many researchers reported the ACE-inhibitory activity of soybean in processed product (Iwamik & Buki, 1986; Kinoshita *et al.*, 1993; Gibbs *et al.*, 2004). In this experiment, the ACE-inhibitory activity of unheated seed was lowered than that of heat-treated powder (data not shown). In addition, there was no close relationship between the level of ACE-inhibitory activity in the raw soybean seed and heated soybean powder. Therefore, the nutraceutical quality of soybean should be
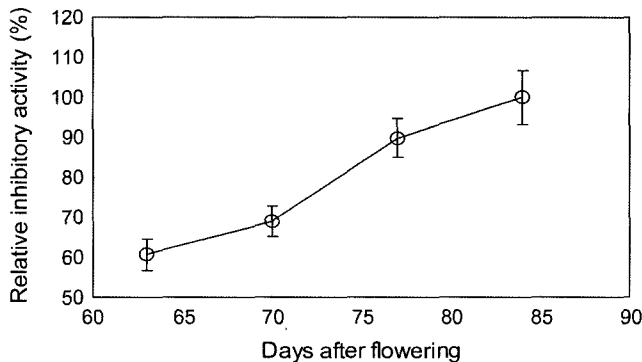


Fig. 5. Changes in inhibitory activity on angiontensin converting enzyme in developing soybean seed. The activity was expressed as relative values compared to the activity at the R$_8$ stage.

Table 2. Effect of growth stage and heat treatment on the inhibtory activity on angiotensin converting enzyme in Seomoktae.

| DAF[†] | Heating time (min) | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 30 |
| 49 | 25.6±1.6 | 32.9±1.5 | 53.9±4.0 | 31.1±1.8 |
| 70 | 39.0±1.2 | 24.9±1.4 | 45.6±3.1 | 30.2±2.0 |
| 84 | 33.6±2.0 | 36.0±1.9 | 36.9±2.7 | 24.6±1.5 |
| Average | 32.3 | 28.9 | 49.8 | 30.7 |

[†]Days after flowering

evaluated considering the changes during post harvest processing because the changes in bioactive compounds during processing is more critical than major constituents like storage proteins, lipids, and carbohydrates in future.

## ACKNOWLEDGEMENT

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