International Journal of Food Science and Nutrition
ISSN: 2455-4898, Impact Factor: RJIF 5.14
www.foodsciencejournal.com
Volume 1; Issue 5; September 2016; Page No. 22-27

Time and temperature dependence of antioxidant activity from soybean seeds (*Glycine max L. Merr*.) during germination

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Abstract
The influence of germination temperature and time on the antioxidant activity of soybean seeds was investigated. The MTĐ 760 soybean cultivar was germinated in dark condition at 22, 25, 28ºC and ambient temperature for 0, 12, 23, 36, 48, 60 and 72 hours. The total phenolic content (TPC), total flavonoid content (TFC), vitamin C and α-tocopherol contents as well as antioxidant activity (AA) assayed by DPPH radical-scavenging activity in terms of IC50 of germinated soybean were determined. These values increased with the increase of germination time for all applied germination temperature and they tend to reach the maximum values after a period of 60 hours. Germination temperature also influenced these parameters and generally at 25ºC they reached the highest values. The TPC, TFC, vitamin C and IC50 value of soybean seed germinated at 25ºC for 60 hours were respectively 8.11 ± 0.03 mgGAE/g (DW); 6.45 ± 0.09 mgQE/g (DW); 12.80 ± 0.02 mg/g (DW) and 5.49 ± 0.03 mg/ml. The maximum content of α-tocopherol after 72 hours of germination was 0.247±0.004mg/g (DW). Correlation studies indicated significant negative correlation between the values of IC50 with TPC (r = –0.968).

Keywords: Soybeans, germination, temperature, total phenolic content, IC50 value

1. Introduction

The soybean (*Glycine max L. Merrill)* belongs to the family Leguminosae and have been extensively consumed worldwide as important source of dietary protein and oil [1]. Soybean contains about 20% oil that includes high proportion of essential fatty acids for humans such as linoleic and linolenic acids. Soybean contains about 40% high–quality protein that includes all the essential amino acids, most of which are present in amounts that closely match those required by humans [2]. In addition, soybeans are preferred due to many bioactive compounds such as the phenolic compounds, isoflavones, soya saponins, tocopherols, etc. Phenolic compounds are potential candidates as selection criterion for antioxidant activity in soybeans [3]. Soy isoflavones are interested due to the potential in lowering cholesterol levels, preventing prostate and breast cancers, osteoporosis, cardiovascular disease as well as relieving menopausal symptoms [4, 5]. However, soybeans and soy products get limited acceptance by consumers due to an unpleasant bean flavour during processing [6], the bitter taste of soybean peptides [7] and the presence of antinutritional factors such as trypsin inhibitors, oligosaccharides and phytic acid [8]. Germination is one of the most common and effective processes to overcome these disadvantages of soybean seed [9, 10]. During germination, the amount of the antinutritive materials (trypsin inhibitor, phytic acid, oligosaccharides) as well as the beany flavour decreases [11] and it also improves the bioavailability of the various minerals, vitamins and phytochemical properties (natural antioxidants) which are great significance from both health and nutritional point of view [12]. Germination is considered one of the best methods to be utilized in the improvement of nutritional profile of the seed grains [13]. This process is influenced by some external factors such as temperature, humidity, lightness condition, etc [12, 14]. Temperature plays a major role in determining the periodicity of seed germination [15]. Germination occurs within a defined temperature range and will not occur out of range of these limits. The germination reaches maximum in the least amount of time at the ideal temperature [16] or optimum temperature, at which allows for the most efficient combination of the percentage and speed of germination [17]. However, the influences of germination temperature on antioxidant compounds in soybean seeds have not been published. The objective of this research was to investigate the influence of temperature on the changes in total phenolic content, total flavonoid content, vitamin C, α-tocopherol and antioxidant activity of soybean seeds during germination. Beside, the relationship between antioxidant activity and the contents of total polyphenols was evaluated.

2. Materials and methods

2.1 Soybean and germination process

Soybeans (*Glycine max L.*, MTĐ 760 variety) were supplied from Department of Agricultural Genetic, College of Agricultural and Applied Biology, Cantho University.

Soybeans were cleaned and rinsed with cleaned water before being soaked for 12 hours to reach the equilibrium moisture content at ambient temperature (30 ± 2ºC). The soaked beans were drained, rinsed and placed in a germination chamber in dark condition. Watering automatically the seeds was set up two minutes for every 4 hours with cleaned water. The germination process was carried out at the temperatures setting as 22, 25, 28 and ambient temperature for 0, 12, 24, 36, 48, 60 and 72 hours. Germinated soybeans were freeze dried for analyzing the TPC, TFC, vitamin C, vitamin E (α–tocopherol) and IC50 value. The extraction procedure for analysis of the antioxidant compounds followed the study results of Duong *et al.* (2015) [18], the α-tocopherol content was detected in soybean oil.

2.2 Determination of the TPC, TFC, vitamin C and Antioxidant capacity (IC50)

The TPC was estimated by Folin-Ciocalteu method [19]. The TPC of samples was expressed as milligrams garlic acid equivalents per gram of dry matter (mg GAE/g).

The TFC was determined by the Dowd method with slight modification [20] and using a standard curve of quercetin. Thus, the results were expressed as milligrams of quercetin equivalents (QE) per gram of dry matter sample (mg QE/g).

Ascorbic acid (vitamin C) content was determined by redox titration with iodine [21].

Vitamin E (α–tocopherol) content was determined by Emmerie-Emmerie Engel reaction which is based on the reduction by tocopherol of ferric to ferrous ions which is then form a red complex with α,α-dipyridyl that can reading at optimum wave length of 520nm [22].

Antioxidant activity of the phytochemicals extracted from soybean was assessed by measuring their radical scavenging activity that was measured by the bleaching of the purple-coloured methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses stable DPPH radical as a reagent. The DPPH radical scavenging activity was evaluated from the difference in peak area decrease of the DPPH radical detected at 517nm between a blank and a sample [23]. Percentage of radical scavenging activity was plotted against the corresponding concentration of the extract (μg/ml) to obtain IC50 value in mg/ml. IC50 is defined as the amount of antioxidant material required to scavenge 50% of free radical in the assay system. The IC50 values are inversely proportional to the antioxidant activity.

2.3 Statistical analysis

All evaluations of total phenolic content, total flavonoid content, antioxidant activity were performed thrice. The data were submitted to analysis of variance (ANOVA) by Portable Statgraphics Centurion 15.2.11.0 and were expressed as mean values and standard deviation. The regression graphs were plotted by Microsoft Excel 2007.

3. Results & Discussion

3.1 Effect of soaking process on antioxidant activity of soybean seeds

Table 1 showed the changes in chemical compositions and antioxidant compounds of soybean seeds after 12 hours of soaking. The TPC, TFC and vitamin C in soybean seeds decreased significantly after soaking leading to increase in IC50 value and they are displayed in Table 1. TPC and TFC reduced 21 and 12.8% respectively. This phenomenon of decreasing TPC and TFC during soaking has also been observed by Xu and Chang (2008) [24] for other seed legumes when soaked at ambient temperature for 24 hours, such as green pea (4.9–11.5%), yellow pea (2.2–11.6%), chickpea (2.8–9.0 %) and lentil (9.5–37.8%), depending on the hydration degree. Aharon *et al.* (2011) [25] found the significant reduction of 30% to 40% TPC in desi chickpeas after soaking at room temperature for 22 hours. Seasonal changes in these parameters after soaking due to the water-soluble phenolics and vitamin C leaching into the soaking water [24, 25]. Xu and Chang (2008) [24] have proved their explanation by identifying the levels of TPC in soaking water. They found that the TPC in soaking water for green pea, yellow pea, chickpea and lentil were respectively 0.1, 0.09, 0.15 and 1.55 mg gallic acid equivalents/g at 100% hydration. The difference in the reduction of TPC and TFC from different grains during soaking depend on distribution and content of phenolic compounds in the seed coat and cotyledon [24], the degree of hydration of soaking and the soaking water temperature [25].

The α-tocopherol content increased significantly after 12 hours soaking (29.8%). This result may be due to the leaching of other water soluble compounds causing the increasing in proportion of α-tocopherol in sample. Sindhu and Sumathi (2015) [26] also found that after soaking chickpea seeds, the α-tocopherol increased from 0.37±0.02 to 0.43±0.03mg/100g. However, Afify *et al*. (2012) [27] noticed that α-tocopherol content in raw sorghum ranged from 1.74 to 5.25 mg/kg and after soaking α-tocopherol content was significantly reduced and ranged from 1.50 to 4.25mg/kg.

Table 1: Changes in antioxidant activity of soybean seeds after soaking

	Soybean seeds	Soaked soybean seeds
TPC (mgGAE/g)	$3.04^{a}\pm0.01$	$2.40^{b}\pm0.09$
TFC (mgQE/g)	$2.11^{a}\pm0.03$	$1.84^{b}\pm0.02$
Vitamin C (mg/g)	$6.51^{a}\pm0.12$	$4.87^{b}\pm0.20$
IC50 (mg/ml)	$9.19^{b}\pm0.01$	$10.46^{a}\pm0.16$
α–tocopherol (mg/g)	$0.067^{b}\pm0.001$	$0.087^{a}\pm0.002$

(Mean ± SD, The values showing different superscripts within a row are significantly different at p = 0.05)

Recently, several studies demonstrated that there is a high correlation between the total phenolic content and antioxidant activity [28]. For this result, wherein a decrease in the total phenolic content along with a decrease in antioxidant activity was noted. In this study, the decreasing in antioxidant activity expressed through the increasing in IC50 value (13.8%) that was shown in Table 1. Besides finding TPC losses during soaking of seeds mentioned above, Xu and Chang (2008) [24] also provides the reducing in DPPH free radical scavenging capacity from these seeds, i.e. green pea (17.6%), yellow pea (13.6%), chickpea (10.5%) and lentil (9.4%).

3.2 Time–temperature dependence of antioxidant compounds and antioxidant activity from soybean seeds during germination process

Phenolic compounds are secondary metabolites that possess high antioxidant. Flavonoids are belong to phenolic compounds and they contain is flavones that are typical antioxidant in soybean and soy products. Other compound known as antioxidant that present in soybean is vitamin C and vitamin E. All of these compounds contribute into the antioxidant activity of soybean. The changes of them during germination of soybean seeds at different temperature were determined and displayed in Figure 1, Figure 2, Figure 3 and Figure 4. Addition, the change in antioxidant activity that expressed as IC50 value was also studied and shown in Figure 5.

The results showed the increasing in TPC during germination for all applied germination temperature and it tend to reach the maximum values for 60 hours of germination, after that (72 hours of germination) it slowed down. In addition, the TPC showed significant higher in germinated soybean compared to soybean seeds. According to Cevallos-Casals and Cisneros-Zevallos (2010) [29], germination process generally increases the bioactive compounds including phenolics. Beside, several studies reported that the level of phenolic compounds increase in time dependent during germination, for example, Fouad and Rehab (2015) [30] found that phenolics content in increased from 1341.13 mg gallic acid/100 g DW in raw lentil seeds to maximum value of 1630.20 mg gallic acid/100 g DW at the fifth day of germination and decreasing to1510.10 mg gallic acid /100 g DW in sample germinated for 6 days. Another study, Kou and Zhou (2016) [31] determined the changes of TPC during the germination process of soybean supplied by the Midaocailai Company (Jilin, China) and found that the TPC of soybean was significant higher from the second day and reached a peak on the fourth day (6.67 mg GAE/g), which was almost 1.51 fold of the seeds, then reduced at the fifth and sixth days. The increasing in TPC during several beginning days of germination could be due to the biosynthesis and bioaccumulation of phenolic compounds as a defensive mechanism to survive under environmental stresses [32], and the reducing of TPC after that might be due to mobilization of stored phenolics by the activation of enzymes such as polyphenol oxidase during germination [33].

Flavonoids belong to phenolic group, so the tendency of TFC change during germination was similar to the change of TPC. As germination days progressed, the TFC were gradually increased and reached the highest level for soybean seeds germinated for 60 hours at all applied temperatures. This result was in agreement with those of earlier studies of Fouad and Rehab (2015) [30] as well as Kou and Zhou (2016) [31] on the seed germination induced accumulation of flavonoid compounds in germinated seeds.

Ascorbic acid was recorded an increasing during germination time and were significantly different from each other ($p<0.05$). It reached the maximum level after from 60 to 72 hours of germination period (Figure 3) for all applied temperatures. Different authors have reported that germination causes an increment of vitamin C content in legumes [34, 36, 31]. According to Ahmad and Pathak (2000) [34], vitamin C content of soybean increased by 91.3% after sprouting for 3 days, while the result from Doblado *et al.* (2007) [35] showed that vitamin C was not detected in raw cow pea seeds, as a result of germination, vitamin C content in germinated cowpeas reached up to 25.2 mg/100g d.m after 6 day of germination. When studying on the changes of vitamin C during germination of mung bean and chickpea, Masood *et al.* (2014) [36] concluded that vitamin C contents have not been found in raw mung bean and chickpea, but it appeared 37.0±1.5 and 20.0±0.5 mg/100g in mung bean and chickpea, respectively after 120 hours of germination. According to Davey *et al.* (2000) [37], the difference in level of ascorbic acid biosynthesis during germination might be effected by legume type, maturity, climatic conditions, light conditions, harvesting and grain storage methods. The accumulation of ascorbic acid during seed germination could be due to reactivation of enzyme (L-Galactono- γ-lactone dehydrogenase) involved in the oxidation of L-galactono-1, 4-lactone to ascorbic acid. The activity of this enzyme increased with seed germination [38].

Similar to vitamin C, α-tocopherol contents were found an increasing with germination time and reaching the maximum value after a period of 72 hours (Figure 4). An increase in the α-tocopherol content after germination also has been reported by Suryanti *et al.* (2016) [39] for sorghum, Kou and Zhou (2016) [31] for mung bean, soybean and black bean, Vasantharuba *et al.* (2007) [40] for soybean. In the study of Suryanti *et al.* [39], the highest α-tocopherol contents were obtained at day 4 of germination time. Compared to the imbibed seed, after 4 days of germination, an increase of 4.58 fold for α-tocopherol were observed. According to Vasantharuba *et al.* (2007) [40], the increase in vitamin E content during germination may be due to increased lipoxygenase enzyme activity of seeds.

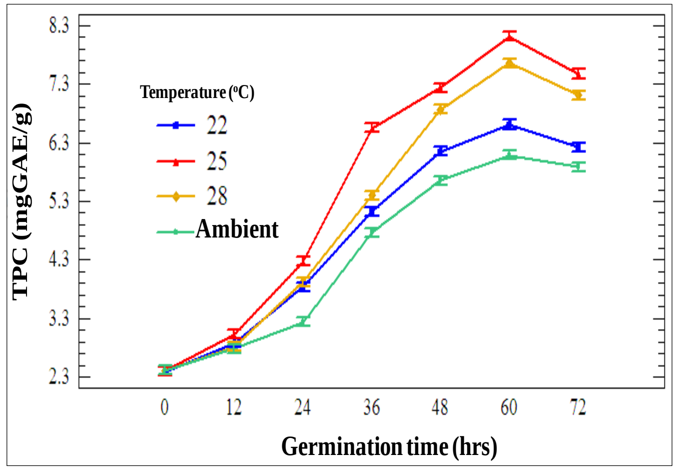

Fig 1: Effect of germination time and temperature on TPC of germinated soybean seeds (Mean and LSD)

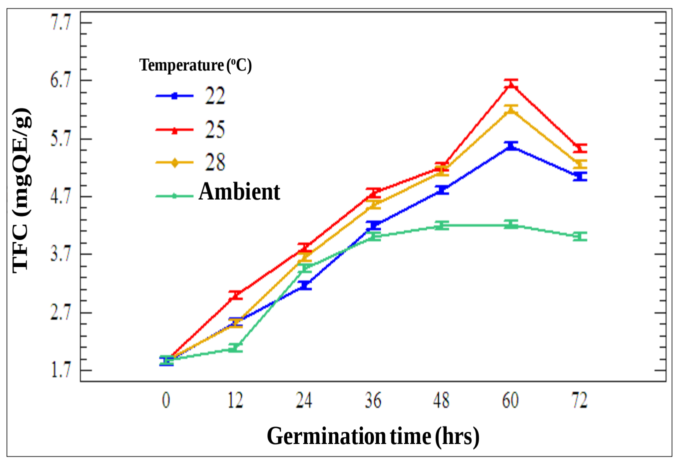

Fig 2: Effect of germination time and temperature on TFC of germinated soybean seeds (Mean and LSD)

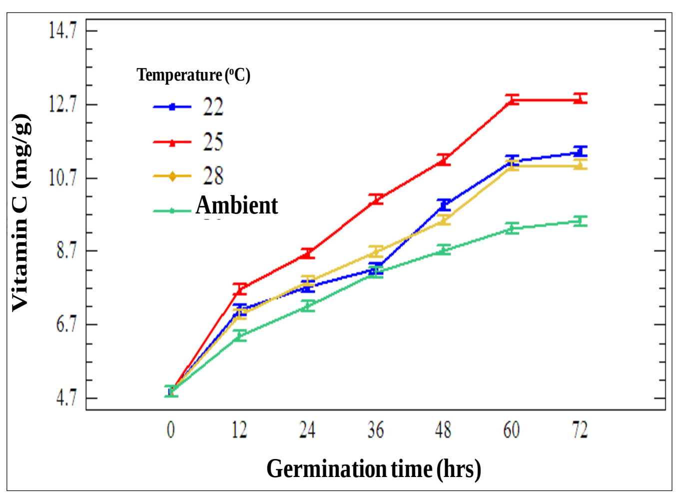

Fig 3: Effect of germination time and temperature on vitamin C content of germinated soybean seeds (Mean and LSD)

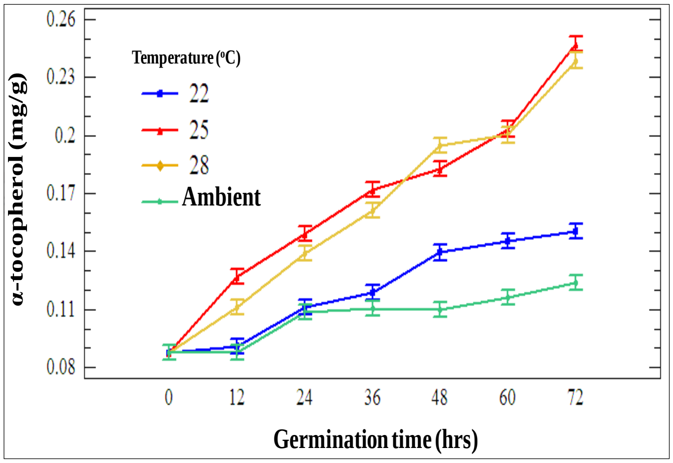

Fig 4: Effect of germination time and temperature on α-tocopherol content of germinated soybean seeds (Mean and LSD)

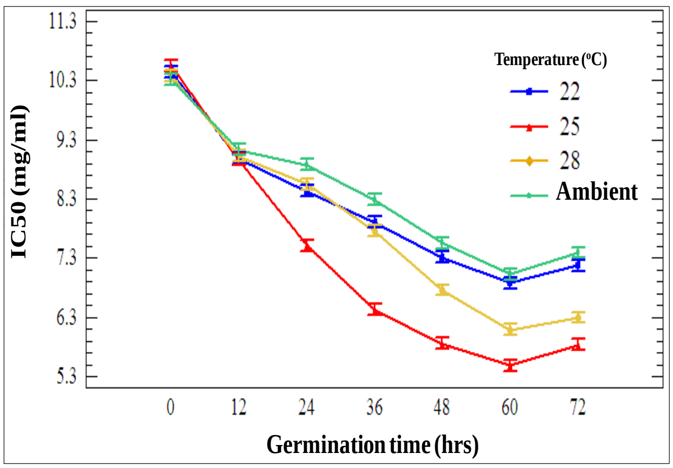

Fig 5: Effect of germination time and temperature on IC50 value of germinated soybean seeds (Mean and LSD)

Germination has also been suggested as a powerful strategy to increase antioxidant activity in seeds [41]. The antioxidant activity of germinated soybean seeds evaluated by IC50 value is reported in Figure 4. It is evident that germinated soybean seeds show a good antioxidant potential. IC50 values of germinated soybean seeds tended to reduced to minimum value after 60 hours for germination for all temperatures applied in the research (Figure 5). The reducing of IC50 value (the increasing in antioxidant activity) resulted from of the biosynthesis of phenolic compounds and vitamin C during germination.

The results from Figure 1, 2, 3, 4 and 5 clearly indicated that different temperatures used for soybean germination affect the antioxidant compound as well as antioxidant activity and similar to seed germination as observed earlier of Koo *et al.* (2015) [28]. The results showed differential total phenolic content between germinated soybean seeds grown at different temperatures. At the same period of germination, the TPC, TFC, vitamin C and antioxidant activity in the germinated soybean seeds was higher when grown at 25°C than that when grown at 22, 28°C and ambient temperature. In case of α-tocopherol, germination at both 25°C and 28°C enhanced the best level of it. These results are in agreement with those of earlier studies from Koo *et al.* (2015) [28], who proved that the TPC and activities in germinated soybean grown at 25°C were higher than that grown at 20°C. Zhou and Zhang (2012) [42] stated that soybean germination represents optimal quality corresponding to temperature of 25°C.

According to Larcher (2003) [43], higher germination temperatures could potentially alter enzymatic activity and reduce the quantity of amino acids available (via RNA synthesis), thereby modifying metabolic reactions that reduce embryo development and restrict seed germination. However, a reduction in the rate of metabolic reactions could occur at a lower germination temperature (about 20°C), affecting the essential processes that initiate germination that result in the establishment of fewer seedlings and a reduction of biomass (particularly among tropical and sub-tropical species). Thereby reducing the speed and percentage of germination and increasing the average time period required for germination [17]. The study result of Oliveira *et al.* (2013) [17] showed that the optimum temperature for the germination of *Diptychandra aurantiaca* seeds was also at 25°C.

3.3 Correlation of TPC, TFC, vitamin C and IC50 value

Several studies have shown that the increment of antioxidant activity during the germination of mung bean seems to be related with changes in the content of antioxidants, such as polyphenols [31]. To confirm this, the antioxidant activities of soybean seeds and germinated soybean seeds expressed in IC50 value were regression analyzed with TPC of these samples and the correlations were displayed in Figure 6. The antioxidant activities (IC50) was highly correlated to the TPC of germinated soybean (R^2= 0.933, $p< 0.01$. This is consistent with the results previous reported by Gou *et al.* (2012) [44], Kou and Zhou (2016) [31]. In the study on the effect of germination on phytochemicals and antioxidant activity of mung bean sprouts (*Vigna radiata*), the correlation between antioxidant activity with TPC (R^2 = 0.896, $p < 0.05$) were established [44]. Beside, Kou and Zhou (2016) [31] established the high correlation between TPC and antioxidant capacity expressed in ARSC (ABTS radical scavenging capacity) in mung bean with high significance (R^2 = 0.956, $p < 0.01$).

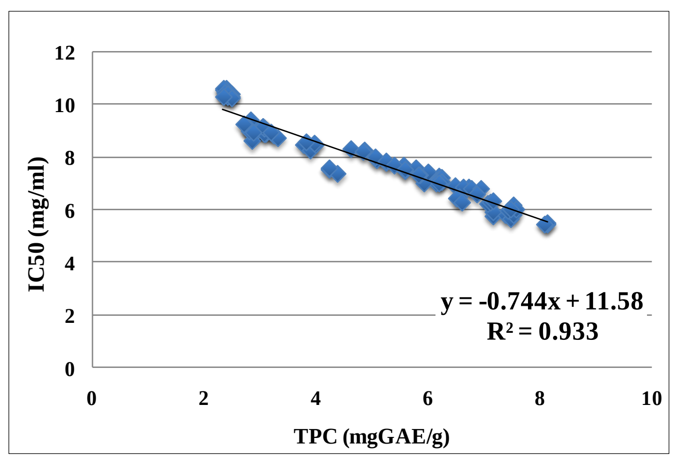

Fig 6: Correlation between IC50 value and TPC of germinated soybean seeds

4. Conclusions

The germination process of soybeans modified their antioxidant compounds. The metabolic changes observed seem to be affected by temperature and time of germination. The results demonstrated that germination for 60 hours at 25°C expressed the greatest enhancement in antioxidant activity. The germination caused a significant increment in total phenolic content, total flavonoid content, ascorbic acid, α-tocopherol and antioxidant activity compared to imbibed soybean seeds. Results suggested that germination is a good way to enhance the antioxidant properties of soybean seeds. The germinated seeds will not only help in the prevention and treatment of various human diseases, but they also be helpful in improving the market of various traditional soybean foods with the development of active components for functional food products.

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