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Effect of Cultivar and Processing on the Hemagglutinin Activity of Soybean

Penelope Felipe, Dai-Eun Sok[1], Ok-Soon Heo[2], Hyoung Chin Kim[3], Won Kee Yoon[3], Hwan Mook Kim[3] and Mee Ree Kim*

Dept. of Food and Nutrition, Chungnam National University, Daejeon 305-764, Korea
[1]College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea
[2]Daejeon Regional Food & Drug Administration, Daejon 302-713, Korea
[3]Bio-Evaluation Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

Abstract Effects of cultivars, cooking, and processing on hemagglutinin activity were evaluated by observing macroscopic hemagglutination using serial twofold dilution of trypsinized human blood type-O or rabbit blood. Hemagglutinin activity was expressed as maximal geometric dilution fold. Agglutination of rabbit blood was more sensitive compared to human blood. Hemagglutinin activities of glyphosate-tolerant soybean, HS2906, and imported conventional soybeans were not statistically different, although significant differences were observed among conventional soybean cultivars cultivated in Korea (286 to 1535 HU/mg protein). Time required to reach fifty percent inhibition of hemagglutinin activity (IT_{50}) value decreased with increasing cooking temperature and pressure. Most effective conventional cooking method to inhibit hemagglutinin activity was pressure-cooking (IT_{50}: 1.36 min). Calculated activation energy based on reaction rate constant was 4.88 kcal. No hemagglutinin activities were detected in processed soybean products such as *tofu*, soybean paste, and soysauce.

Keywords: hemagglutinin, soybean, cultivars, cooking, procedding

Introduction

Soybeans (*Glycine max*) have long been recognized not only as a good source of edible protein but also as a cheap source of protein particularly in developing countries, where animal source is scarce. Unfortunately, utilization of the available proteins is much lower than the calculated amount based on the chemical compositions, because a number of antinutritional components, such as trypsin inhibitors, chymotrypsin inhibitors, and hemagglutinins, reduce the digestion of proteins in animals, particularly monogastrics (1-3). Hemagglutinins are carbohydrate-binding proteins resistant to digestion (4), obstructing the optimal exploitation of nutrients present in foods and decreasing nutritive value of soybeans. Moreover, hemagglutinins have oral toxicity due to their ability to bind to different types of blood (5-6). In addition to their ability to agglutinate the erythrocytes of numerous animal species, they negatively affect nutrient utilization by various mechanisms and inhibit the enzyme activity of the brush border of the enterocytes (7-8). Several animal experiment results showed impaired growth of rats, enlargement of small intestine, and stimulated hypertrophy and hyperplasia of the pancreas (9-10). At high levels, hemagglutinins may induce depletion of body and skeletal muscles, lipid, and glycogen (11-13). Therefore, method of inactivating the antinutritional effect of hemagglutinin in soybeans is an important step for their safe consumption. In Korea, soybean consumption has recently been increasing, while the domestic production of soybeans is on the decreasing trend (14). Most commercial soybeans are imported (15) and used for the production of soybean-based foods such as tofu and soybean paste known to have health-promoting effects (16-18). Genetically modified foods, especially soybeans, have been projected to take part in increasing the amount of food supply. For genetically modified foods that were not developed to have intentionally altered nutritional value, nutritional evaluation should be assessed to insure that there has been no unintentional changes in the levels of key nutrients or antinutrients. In this case, food substitution using products from the genetically engineered food should not adversely affect the health or nutritional status of the consumers. To our knowledge, there have only been few reports concerning hemagglutinin activity of some soybean cultivars cultivated in Korea or imported, including that of the genetically modified ones.

The objectives of this study were to examine the hemagglutinin activities of genetically modified soybean and other local cultivars or processed soybean-based foods, and to evaluate the effect of conventional cooking methods on the hemagglutinin activity of soybeans.

Materials and Methods

Materials Commercially available 11 soybean cultivars cultivated in Korea (2003), and 2 imported cultivars were analyzed (Table 1). HS2906 is a glyphosate-tolerant soybean, which contains the CP4 EPSPS gene, while the rest are conventional soybeans. Soybean seeds of uniform size, absolutely sound, and undamaged were used for analysis. Processed soybean products were purchased from a local market in Daejon, Korea (Table 3).

*Corresponding author: Tel: 82-42-821-6837; Fax: 82-42-821-8887
E-mail: mrkim@cnu.ac.kr
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Table 1. Characteristics of soybean cultivars for hemagglutinin activity assay

Cultivars	Characteristics	Crop year	Origin
HS2906	Glyphosate-tolerant (white shelled)	2003	USA
WS82	Conventional (")	"	USA
Hwangum kong	Conventional (")	"	NewGene Co., Ltd., Korea
Pungsan kong	" (")	"	"
Ouyu kong	" (")	"	"
Sinpaldal kong	" (")	"	RDA[1], Korea
Hwangum kong	" (")	"	"
Daewon kong	" (")	"	"
Taekwang kong	" (")	"	"
Pungsan kong	" (")	"	"
Somyung kong	" (")	"	"
Yakkong	" (black shelled)	"	NongHyup, Korea"

[1]RDA: rural development administration.

Table 2. Hemagglutinin activity (HU/mg protein) of different cultivars of soybeans using rabbit blood

Cultivars	HU/mg protein
HS2906	1232.01±77.49[b]
WS82	1240.78±70.67[b]
Hwangum kong	288.64±10.55[e]
Pungsan kong	464.40±55.24[d]
Ouyu kong	343.65±33.18[e]
Sinpaldal kong	501.11±20.29[d]
Hwangum kong	1159.80±35.61[bc]
Daewon kong	337.03±18.79[e]
Taekwang kong	1182.23±34.42[bc]
Pungsan kong	1214.27±65.40[b]
Somyung kong	1535.53±65.61[a]
Yakkong	1105.09±31.55[c]

[a]Any two means in same column followed by the same superscripts are not significantly different (p<0.05) by Duncan's multiple range test.

Soaking All soybean samples were immersed in distilled water (5°C) for 6 hr and cooked using the following methods:

Ordinary cooking Soybeans were cooked in water (seed:water, 1:5) at 60, 80, and 100°C for 5, 15, 30, 45, 60, and 120 min.

Pressure cooking Soybeans were autoclaved in water (seed:water, 1:5) at pressure higher than or equal to 1 kgf/ m^2 for 1, 2, 3, and 5 min.

Microwave cooking Soybeans were cooked in a microwave (MR-202, LG Co., Ltd., Korea) at 2,450 MHz in water (seed:water, 1:5) at medium power for 0.5, 1, 2, 3, and 5 min.

Sample extraction Five hundred milligrams of commercially available soybean product or homogenized cooked soybean sample was extracted with 10 mL of 10 mM phosphate buffered saline (PBS, pH 7.4) for 2 hr. The suspension was centrifuged for 20 min at 1,500×g, 4°C. The resulting supernatant was used for assay.

Blood preparation Human type-O erythrocyte or specific pathogen-free rabbit erythrocytes from New Zealand white rabbit was anticoagulated with sodium citrate and centrifuged at 1,500×g, 4°C, to remove all soluble blood constituents. The suspended erythrocytes were then washed twice with PBS and incubated with 100 μL/ mL trypsin (Sigma-Aldrich Co., St. Louis, MO, USA) for 1 hr. Trypsin inhibitor (Sigma-Aldrich Co.) was then added to the blood suspension, and the optical density was adjusted at 620 nm to 0.8-0.9 (approximately 0.15-0.20% v/v) using PBS.

Hemagglutination assay Micro assay method for hemagglutinin activity was performed by the method previously described by Lis and Sharon (19). Briefly, 50 μL of twofold serially diluted soybean sample was incubated in a 96-well ELISA plate with 50 μL of rabbit or human type-O blood from a healthy male volunteer. The plate was covered, and the contents were mixed gently but thoroughly and allowed to stand for 2 hr at room temperature. Absence of agglutination and/or blood flow was considered negative. One hemagglutinin unit (HU) was defined as the reciprocal of the highest dilution giving a positive agglutination. After the initial comparison between human type-O and rabbit blood, rabbit blood was utilized in all succeeding assays.

Protein determination Protein Assay was carried out using Bradford method (20).

Statistical analysis Each analysis of the hemagglutinin assay was repeated three times for each cultivar and/or treatment. All data were presented as means ± standard error (SE). All statistical analyses were performed using an SPSS program for window. Statistical assessments were performed using ANOVA for the initial demonstration of significance at p<0.05, followed by post-hoc Duncan's multiple-range test (21). Statistical significance refers to results where p<0.05 was obtained.

Results and Discussion

Sensitivity to human or rabbit erythrocyte Soybean contains hemagglutinins, which are naturally occurring antinutrient factors (22). Hemagglutinin activity was initially measured using human type-O erythrocyte and further compared with erythrocytes from rabbit. Equal amount of blood suspension was incubated with the soybean extract for 2 hr. Rabbit erythrocyte was found to be about 80% more sensitive in detecting the hemagglutinins present in soybeans compared with the human type-O erythrocyte. Kortt (23) found that hemagglutinins in winged bean agglutinated native and trypsinized rabbit blood but not trypsinized human (type O) erythrocytes. Nicolson (24) reported that the extent of agglutination is dependent on the specificity of binding sites, their number, size of the hemagglutinin, as well as the still not well characterized cell properties. Because rabbit blood was found to be more sensitive to hemagglutinin than human type-O, we used rabbit blood in all further assays.

Differences in cultivars The hemagglutinin activities of twelve cultivars including white or black shelled soybeans, as detected by previously reported method of Lis and Sharon (19) using rabbit erythrocytes, are presented in Table 2. The hemagglutinin activities (HU/mg protein) of white-shelled soybeans were significantly different among soybean cultivars cultivated in Korea, ranging from 288.64 to 1,535.53 HU/mg protein. Among cultivars, the hemagglutinin activities of Hwangumkong, Ouyukong, and Daewonkong cultivars cultivated in Korea showed values ranging from 60-426 HU/mg protein (25), while the other nine cultivars showed somewhat higher hemagglutinin activities. Farag (26) reported that hemagglutinin activity of raw soybean is 2,560 HU/mg of sample. The hemagglutinin activity of the glyphosate-tolerant soybean, HS2906, was not significantly different, when compared to that of conventional soybeans, which is consistent with previous reports that there was no significant difference between glyphosate-tolerant soybeans and their non-genetically modified counterparts (27, 28). Furthermore, the hemagglutinin activity of HS2906 soybean was at a similar range as that of the conventional soybean (29, 30). The cultivars of black-shelled soybean were observed to have similar hemagglutinin values with those of the while-shelled ones at 1,105.09 HU/mg protein. According to Lis and Sharon (31) erythrocyte agglutination by hemagglutinin is affected by the molecular properties of hemagglutinin, cell surface properties, and metabolic state of cells. Comparison of hemagglutinin activity among previously reported ones was considered to be difficult due to different cultivars and assay method conditions used (31).

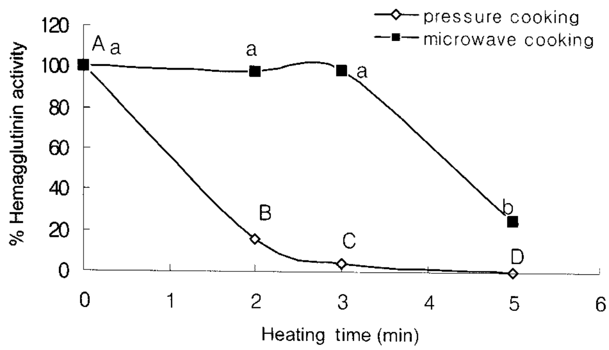

Fig. 1. Effect of heating at 120 with pressure and microwave cooking on the hemagglutinin activity (%) of white soybeans. [a,A]:Values having the same letters in the same treatment or plot are not significantly different at $p<0.05$.

Table 3. Hemagglutinin activity (HU/mg protein) of commercially available processed soybean products using rabbit blood

Soy Products	Company, Location	HU/mg protein
Tofu	Donghwa Foods Co., Busan	ND[1]
Tofu	Dusol Co., Chungnamyesan	ND
Soybean paste	Haechandel Co., Daejon	ND
Soybean paste	Jinmi Foods, Co., Ltd., Daejon	ND
Soysauce	Sampyo Foods Co., Ichon	ND
Soysauce	Taesang Co., Ltd., Chonan	ND

[1]ND:Not detected.

Differences in processed soybean products In Korea, typically processed soybean products such as *tofu*, soybean paste, soysauce, soybean sprout, and soy milk are commonly consumed. The hemagglutinin activities of processed soybean products of Korea are shown in Table 2. *Tofu* showed no hemagglutinin activity in two Korean commercial products. This might be attributed to the processing procedure, which consisted of soymilk preparation and coagulation of proteins with a salt such as calcium sulfate, followed by cooling. Rizzi (32) demonstrated that the procedures of soymilk preparation inactivated most hemagglutinin activity. In addition, hemagglutinin activity was not detected in soybean-based fermented foods such as soybean paste and soysauce (Table 3). Soybean paste is prepared by fermenting soaked and steamed soybeans, and soysauce is a product of enzymatic or acidic hydrolysis of a mixture of fermented soybeans. During processing such as heating and fermentation, hemagglutinin activity in these fermented soybean products could be destroyed. Although hemagglutinin activity in processed food varies depending on the techniques employed, the soybean cultivars, and assay conditions such as moisture, pH, time, and temperature used (33), typical oriental processed soybean foods such as *tofu* or soybean paste were reported to be generally low in antinutrients (34).

Koreans generally consume soybean sprouts after boiling. The hemagglutinin activity of raw soybean sprout was 105.14 HU/mg protein (Table 4), lower than that of raw soybean seed. During germination the hemagglutinin in raw seed may be metabolized via an unknown biochemical pathway. Rizzi (32) confirmed that hemagglutinin activity of soybean sprout rapidly decreased during germination. El-Adawy (35) reported that the hemagglutinin activity of faba beans decreased by about 80% after 3 days of germination. Moreover, after 5 min boiling of the germinated soybean seeds at 100°C, no hemagglutinin activity was detected, indicating that hemagglutinin present in soybean sprout is heat-labile.

Effect of cooking on the hemagglutinin activity Various conventional cooking methods such as moist heating, pressure, and microwave cooking have been utilized to alter the deleterious effects of antinutritional factors naturally present in soybeans, thereby increasing the nutritional quality of soybeans.

Hemagglutinin activity decreased according to time and

Table 4. Hemagglutinin activity (HU/mg protein) activities of soybean sprouts[1] during boiling at 100°C

Boiling time (min)	HU/mg protein
0	105.14±3.35
3	6.80±0.03
5	ND[2)]
10	ND
15	ND

[1]Soybean sprouts were purchased from Pulmuone Co., Cheongju.
[2]ND:Not detected.

temperature, indicating that heat treatment reduces the antinutrient content (Table 5). When hemagglutinins were subjected to heat denaturation, the decreasing rate of hemagglutinin activity varied according to temperature and time of exposure.

A significant reduction in the hemagglutinin activity was observed after 30 min of 60°C heating, whereas at 80 and 100°C, significant changes were observed after heating for only 15 min (p<0.01). Remarkable decreases of 73 and 93% in hemagglutinin activity were observed after only 5 min of cooking at 80 and 100°C, respectively. Initial 15 min of cooking at 80 and 100°C decreased more than 90% of hemagglutinin activity, with a slower breakdown of hemagglutinin activity after 15 min. No hemagglutinin activity was detected after a prolonged heating of 2 hr at 60°C. At 100°C, a 45 min heating was required to entirely eliminate hemagglutinin activity, while at 80°C, 0.5% hemagglutinin activity remained at the same cooking time, further indicating that most of the hemagglutinins are heat-labile. Carlini (36) reported that only 0.76 and 2.80% of hemagglutinin was activities were left after cooking for 1 hr at 96°C for *C. ensiformis* and *C. braziliensis* seeds, respectively. Dhurandhar and Chang (37) reported that only 0.09 and 0.18% hemagglutinin activities were retained in navy and red kidney beans cooked for 1 hr at 82°C, respectively, while 10 min cooking at 100°C was sufficient to inactivate all hemagglutinin activities. In addition, after initial heating of 1 hr at 82 to 94°C, hemagglutinin destruction was slowly accompanied by the gradual destruction of cysteine. The authors concluded that the inactivation of hemagglutinin during cooking could be partly due to the destruction of cystein. The time required to reach 50% inhibition (IT_{50}) of hemagglutinin activity was calculated based on the slope of different heating conditions; IT_{50} values were 30, 5, and 3 min for 60, 80, and 100°C, respectively (Table 6), linearly decreasing as the temperature increased. Moreover, a significant linear relationship was observed between temperature and IT_{50} value (R^2=0.8). The activation energy (Ea), calculated based on the initial 5 min of decreasing reaction rate constant of the three heating temperatures, 60, 80, and 100 °C, was found to be 4.88 kcal/mole. Among the conventional cooking methods, pressure cooking (Fig. 2) was found to be the most effective on the inhibition of hemagglutinin; IT_{50} value was observed only after 1.37 min, and hemagglutinin activity could no longer be detected after only 5 min. Use of microwave oven for cooking has become popular due to its reduction of processing time. Penetration and heating of food by microwaves are instantaneous, while conventional cooking methods transfer thermal energy from product surface towards its center 10 to 20 times slower. Microwave frequencies of 2,450 and 915 MHz are officially recognized in the food industry internationally. Due to higher surface or 'skin' effect, the frequency of 2,450 MHz is commonly employed in microwave ovens (38). However, the effects of this processing are also notably dependent on the techniques and conditions, including time, temperature, moisture content, and pH (37). Figure 1 shows the decrease in hemagglutinin of soybeans cooked at medium heat in a 2,450 MHz microwave oven. Hemagglutinin activity decreased to almost 50% after 4 min. Hernandez-Infante *et al.* (39) reported a 40% decrease in the hemagglutinin activity of soybeans after 5-6 min of microwave cooking, whereas other reports (40) indicated total destruction of antinutrients present in soybeans after 6 min of heating in the microwave oven. Thus, the results of present study indicate pressure cooking is the most effective cooking method to reduce hemagglutinin activity.

Table 5. Effect of heating time at different temperature on the hemagglutinin activity (%) of white soybeans

	Heating temperature		
Heating time (min)	60°C	80°C	100°C
0	100.00[ab]	100.00[a]	100.00[a]
5	104.02[a]	27.77[b]	6.61[b]
15	105.03[a]	7.84[c]	2.14[c]
30	30.83[c]	2.02[d]	0.63[c]
45	9.37[d]	0.56[d]	ND[1]
60	2.53[e]	ND	ND
120	ND	ND	ND

Any two means in same column followed by the same superscripts are not significantly different (p<0.05) by Duncan's multiple range test.
[1]ND: Not detected.

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Table 6. Time (min) required to reach fifty percent inhibition of hemagglutinin activity

Heat processing	IT_{50} (min)
Moist heating, 60°C	30.17
Moist heating, 80°C	5.44
Moist heating, 100°C	3.83
Moist heating, 120°C (with pressure > or = 1kgf/m^2)	1.37
Microwave	4.6

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