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The Enhancement of Isoflavone Aglycones Daidzein and Genistein from Soybean Devon-1 Tempeh through Heat Treatment after 61 Hours of Fermentation

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ABSTRACT

Daidzein and genistein, the main isoflavone aglycone in soybean, are significantly gaining attention due to the numerous health benefits. Devon-1 variety of soybean, found in Indonesia, has the highest total isoflavone content. However, the compound predominantly exists in glycoside form, requiring hydrolysis during tempeh fermentation to obtain bioactive aglycone. Previous investigations indicated that ethanol extract of Devon-1 soybean after 61 hours showed lower daidzein and genistein levels compared to 1 N HCl hydrolyzed soybean. Therefore, this study aimed to enhance daidzein and genistein contents through heat treatment of Devon-1 soybean tempeh after 61 hours of fermentation. The sample was heated using a dry oven at 40, 50, and 60°C for 2 hours and extracted with ultrasonic bath with ethyl acetate solvent and 100% amplitude for 10 mins. Extract was analyzed using RP-HPLC with UV/Vis detector at 265 nm, methanol, and 0.1% acetic acid (47:53) as mobile phase. The results showed a 2-3 times increase in daidzein and genistein levels after heat treatment in all temperatures, particularly at 40°C. This suggested that heat treatment was a viable means to enhance the main isoflavone in tempeh.

Keywords: Daidzein, Genistein, Heat treatment, Isoflavone aglycone, Tempeh

Introduction

Daidzein and genistein are the two main isoflavone aglycones in soybean, attracting significant attention due to the numerous health benefit, including agonistic or antagonistic estrogen, antioxidant, anti-inflammatory, anti-allergic, anti-cancer,[1] and hepatoprotector.[2] In Indonesia, a variety of soybean with the highest level of total isoflavone is Devon-1.[3] However, the amount of genistein in soybean is highly dependent on both plant and pod age. A previous study in several varieties of Indonesian soybean seeds reported that the optimal pod age for high genistein was approximately 80-83 days (medium plant age category). Specifically, the varieties Anjasmoro, Argomulyo, and Devon-1 have contents of 15.996 ± 2.51 µg/g, 14.175 ± 2.03 µg/g, and 13.081 ± 4.18 µg/g, respectively.[4] Anjasmoro and Argomulyo were also found to have a high content of genistein, as reported by Sulistyowati *et al* (2018).[5] The result found that daidzein content in Argomulyo was higher compared to in Anjasmoro but daidzein and genistein of Devon-1 variety were not determined.[5] In 2017, a new soybean variety was launched in Indonesia, namely Detap-1, resulting from crossing both Anjasmoro-1-2 and Anjasmoro with 78 days of pod age.[6] Consequently, this study aimed to investigate the amount of daidzein and genistein after ultrasound-assisted extraction of Detap-1 as a modified soybean variety compared to Argomulyo and Devon-1 for selecting a variety before proceeding with tempeh production.

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Ultrasound-assisted extraction (UAE) method is effective for extracting daidzein and genistein due to the cavitation phenomenon. This method facilitates the release of the cell content into extraction medium by breaking the biological cell walls.[7] Furthermore, the advantages include reduced quantities of solvents, shortened extraction time, and increased isoflavone yield.[8] Regarding the solvent used in UAE method, Charpe and Rathod reported that the presence of water in ethanol solvent could significantly increase the percentage yield of extract compared to 100% ethanol. This phenomenon occurs due to the propensity of water to inflate the cellular substance of the sample, influencing the rise in cell wall permeability to easily break when exposed to ultrasonic.[9]

The compounds of daidzein and genistein in soybean are still in glycoside form, necessitating the breakdown into aglycone through both chemical (acidic and basic reagent) as well as enzymatic hydrolysis processes. Among these methods, acidic hydrolysis is exceptional due to its cost-effectiveness and time efficiency.[10] Enzymatic hydrolysis of soybean occurred through a fermentation process using fungi species of *Rhizopus* sp. found in the tempeh production process.[11] According to Hasanah *et al*, the maximum level of genistein was obtained after 61 hours of fermentation, which was further improved through heat treatment due to continuous β-glucosidase enzyme activity.[12] Therefore, this study aims to evaluate the enhancement of daidzein and genistein after 61 hours of fermentation and the heat treatment process. The effect of soybean varieties, the concentration of ethanol in extraction, and the hydrolysis process on daidzein and genistein levels is also investigated. The study novelty is to investigate different incubation temperatures to isoflavone aglycone content regarding the activity of β-glucosidase enzyme.

Materials and Methods

Preparation of soybean extract

A total of three varieties of soybean, namely Detap-1, Devon-1, and Argomulyo, were purchased in August, 2022, from Various Nuts

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Agricultural Instruments Standardization Agency (BSIP Aneka Kacang) Malang, East Java, Indonesia (-8.04733, 112.62534) with voucher number 190 for Detap-1, 034 for Devon-1, and 021 for Argomulyo. Soybean was ground to a fine powder using a blender (Philips HR 2116) and stored in sealed plastic bags at 4°C for use.
Ethanol soybean extract was prepared by extracting 5 g powdered sample in 50 mL ethanol 96% (Merck, technical grade) using an ultrasonic bath (WUC-D06H, DAIHAN Scientific Co., Ltd., Korea) with 100% amplitude for 10 mins. The solute mixture was filtered and then evaporated using a rotavapor (R-215, BUCHI Labortechnik AG, Switzerland) at 50°C, under reduced pressure to obtain the dried extract. Furthermore, the effect of ethanol concentration was evaluated on the selected soybean extract with the highest extract yield.

Acid hydrolysis
Acid hydrolysis procedure of soybean followed Lee and Choung (2011) method with modification.[13] Subsequently, 1 g powdered soybean was extracted with ethanol 96%, added with 1 mL methanol, and 1 mL in 1 N HCl (Merck, pro analysis grade). The mixture was heated in the oven at 100°C for 2 hours and extracted by ethyl acetate (1:2), which was evaporated to obtain a dry extract.

Enzymatic hydrolysis (fermentation)
Enzymatic hydrolysis was treated through the preparation of tempeh as soybean fermentation food using a standard method from the National Standardization Agency of Indonesia (BSN).[14] Soybean from the selected variety was sorted, washed, and boiled for approximately 30 mins, followed by soaking overnight to obtain acid condition. The next day, soybean was dehulled, washed using water, steamed, and added with mold *Rhizopus oligosporus* (RAPRIMA, Indonesia) after soybean was cooled and dried. Subsequently, the molded soybean was wrapped in plastic bags and fermented for approximately 61 hours at room temperature to obtain soybean tempeh. Extract obtained was cut, dried for 24 hours at room temperature, and ground into powder. Extraction of tempeh powder was carried out with ethanol 96% using an ultrasonic bath. The solute mixture was filtered and evaporated using a rotavapor at 50°C under reduced pressure to obtain the dried extract.

Heat treatment
The 61-hour soybean tempeh was heated at 40°C, 50°C, and 60°C for 2 hours using an oven for the pre-treatment and dried at 50°C for 24 hours before extraction process using ethyl acetate solvent (Merck, technical grade). All extraction mixtures were filtered and evaporated using a rotavapor at 50°C under reduced pressure to obtain the dried extract.

Preparation of standard solutions
Daidzein and genistein purchased from ChemFaces, China (≥ 98% purity) were dissolved into solvent HPLC grade methanol (Merck). The stock solution of 100 ppm standards was prepared by dissolving 1 mg of standard powder in 10 mL methanol. Subsequently, the working solutions were prepared in 10, 20, 40, 80, and 100 ppm concentrations for standard daidzein and 10, 14, 20, 30, and 40 ppm for genistein. All working solutions were filtered using a 0.2 µm syringe filter (Sartorius Minisart), followed by injection of 20 µL filtered solutions into HPLC system.

HPLC instrumentation
The analysis was performed using RP-HPLC (LC-20AD, Shimadzu Corporation, Japan) with conditions as follows: column of LiChrospher® 100 C-18 (125 mm x 4 mm, 5 µm), the mixture of methanol and 0.1% acetic acid (47:53) were used as mobile phase and delivered isocratically with flow rate at 1.0 mL/min. The analytes were detected by UV/Vis at 265 nm.

HPLC analysis of daidzein and genistein in samples
The samples from all treatments were weighed and dissolved in methanol with the ratio of 500 mg samples in 3 mL methanol and 900 mg in 20 mL for ethanol extract 96% and 50%, respectively. Furthermore, there were 30 mg samples in 1 mL methanol for acid hydrolysis treatment, 1000 mg in 60 mL for enzymatic hydrolysis treatment, and 400 mg in 100 mL for the heat treatment. All solutions were filtered through a 0.2 µm syringe filter (Sartorius Minisart) and 20 µL filtered solutions were injected into HPLC system. Daidzein and genistein concentrations were calculated as mg of isoflavone per 100 g samples.

Statistical analysis
Data were expressed as mean ± standard deviation (SD) of triplicate analysis. The differences between daidzein and genistein in three varieties were analyzed using Kruskal-Wallis test coupled with Mann-Whitney. Meanwhile, the variation between both compounds before and after acid hydrolysis treatment in three varieties was analyzed using Wilcoxon. Regarding the differences between daidzein and genistein in all treatments of Devon-1, the analysis was carried out using One-way ANOVA. The values were considered to be significantly different when $P<0.05$ and Minitab® statistical software version 21.3.1 was used for statistical analysis.

Results and Discussion

The percentage yield of extract
Soy isoflavone extraction by ultrasound showed an enhancing yield in several investigations. In this study, ethanol extract of three soybean varieties using UAE method resulted in the percentage yield, as shown in Table 1. The highest percentage yield was obtained from Devon-1 extract, followed by Detap-1, and Argomulyo. These yields were approximately two times higher than Hasanah *et al*,[4] which reported Argomulyo and Devon-1 methanol extract using the reflux method with values of 5.32% and 6.94%, respectively. The variation in results occurred due to the cavitation bubbles phenomenon by ultrasound energy during sonication. Consequently, solvent penetration into cells and the surface area of contact between the solid and liquid phases were enhanced. The cavitation bubble degradation also enhanced mass transfer and cell disruption that could increase the release of plant metabolites from intracellular structures.[15]
Regarding concentrations of ethanol, the existence of water in the aqueous solvent of ethanol increased the dielectric constant of the solvent, which was associated with the absorption of ultrasound in UAE method.[16] However, the increasing water proportion in the mixture of solvents allowed an opposite effect on the percentage yield of extract due to high polarity, which had reached the limit of extraction capability. Singh *et al* reported that the solvent concentration of 65% showed the highest yield of total phenolic content from Potato peels compared to 30% and 100%.[17] In this study, Devon-1 soybean was extracted with ethanol 50% and 96%. The results presented in Table 2 showed that 50% concentration had a higher percentage yield of approximately 1.85 times than the 96% ethanol.

Table 1: Percentage yield (%) of ethanolic extracts of three soybean varieties

Soybean variety	The initial weight of the sample (g)	Weight of extract (g)	Percentage yield (%)
Detap-1	5.00	0.78	15.6
Devon-1	5.00	0.81	16.2
Argomulyo	5.00	0.63	12.6

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Table 2: Percentage yield (%) of soybean extracted in different concentrations of ethanol

Soybean variety	The initial weight of the sample (g)	Weight of extract (g)	Percentage yield (%)
Devon-1	50%	5.00	1.50
	96%	5.00	0.81

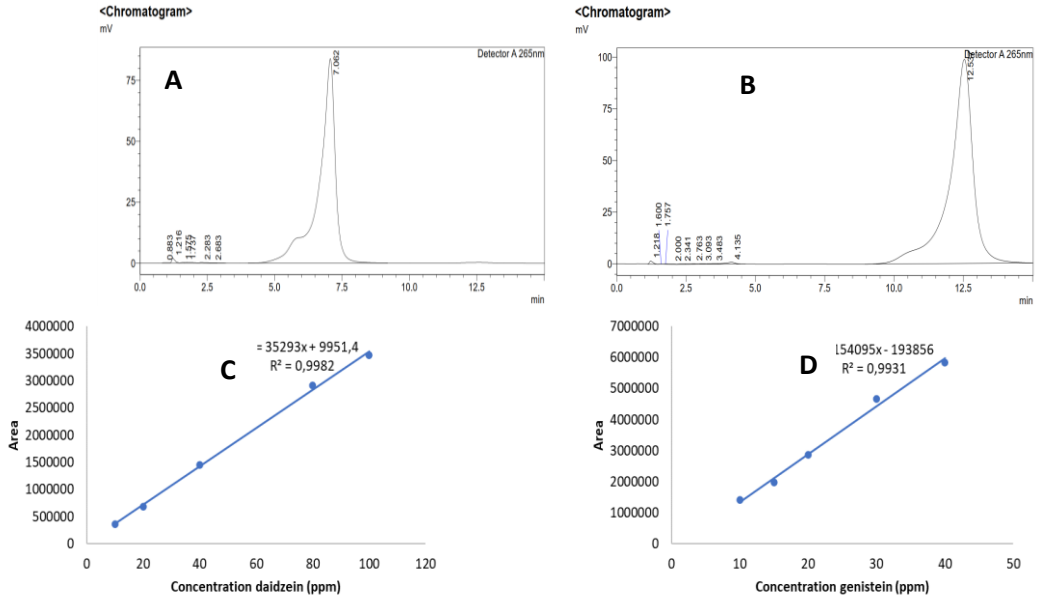

Figure 1: The HPLC chromatogram of standard daidzein (A), genistein (B) and calibration curve of standard daidzein (C) and genistein (D)

Daidzein and genistein in extract and acid hydrolysis

As presented in Figure 1, the standard of daidzein and genistein showed a peak in 7.0 mins and 12.5 mins retention time, respectively. The calibration curve of both compounds showed good correlation coefficients and linearity in the range of concentration studied. Calibration curves can be expressed by a regression equation, as shown in Figure 1, showing the correlation coefficients and range of concentrations evaluated.

The content of daidzein and genistein in extract and acid hydrolysis of three soybean varieties were quantified according to the comparison of sample and standard peaks. Based on the results shown in Tables 3 and 4, daidzein content in Detap-1 extract showed the highest compared to others, but it was not significantly different. Meanwhile, this variety had the lowest genistein content in extract. Furthermore, daidzein and genistein in acid hydrolysis of Devon-1 showed the highest level ($p<0.05$) compared to Detap-1 and Argomulyo. Daidzein in acid hydrolysis of Detap-1 was significantly lower than Argomulyo, while genistein content had no substantial difference.

Daidzein content obtained was higher than Sulistyowati *et al*,[5] which reported 29.68 mg/100 g and 18.69 mg/100 g in methanol extract of Argomulyo and Anjasmoro, respectively. Detap-1 in this study was modified from crossing both Anjasmoro-1-2 and Anjasmoro with 78 days of pod age. Based on the result, this modification affected the increase of daidzein content in ethanol extract compared to Argomulyo, while genistein content remained lower in other studies.[4,5] The genistein content in Detap-1 was higher than Argomulyo, which differed from Anjasmoro in other studies. In the highest genistein content in Devon-1, Krisnawati reported the highest level of isoflavone in Indonesia.[3]

Effect of acid hydrolysis in daidzein and genistein of soybean

Acid hydrolysis increased the content of daidzein and genistein as isoflavone aglycone in soybean, as reported by Horning *et al*.[18] Initially found in low concentrations, these compounds were dominant after acid hydrolysis, with higher significant concentrations in all varieties.[18] Similarly, this study showed that daidzein and genistein content significantly differed in both group varieties and after acid hydrolysis. The increasing length of growth time of soybean seed affected isoflavone aglycone due to the high phenolic metabolites during the maturation of crops and natural plants. Detap-1 had the lowest pod age (78 days) compared to Devon-1 and Argomulyo. Regarding an increase of daidzein and genistein in all varieties after acid hydrolysis, the results could be explained by converting glycoside isoflavone into aglycone through HCl hydrolysis, which almost transformed glycoside and increased the total content of isoflavone aglycone at 1.3 mg/g.[19]

According to group varieties, daidzein and genistein content in Devon-1 were the highest significantly different compared to Detap-1 and Argomulyo presented in Table 4. Although daidzein in Detap-1 was significantly lower than Argomulyo, there was no statistical difference in genistein content. Regarding acid hydrolysis treatment, a significant increase was observed in daidzein and genistein in all varieties based on the Wilcoxon test ($p<0.05$), as shown in Figure 2.

Effect of enzymatic hydrolysis and heat treatment in daidzein and genistein of soybean

The fermentation process in soybean tempeh production is also known as enzymatic hydrolysis due to an enzyme, β-glucosidase, which is produced by *Rhizopus oligosporus* during the process. This enzyme could produce isoflavone aglycone resulting from the hydrolysis of isoflavone glycosides.[20] The effect of heat treatment on the enhancement of isoflavone had been reported by Hasanah *et al*, showing increased genistein from tempeh after boiled, steamed, or fried treatment. This suggested that the continuous hydrolysis process of tempeh through the activation of β-glucosidase enzyme by heat.[12] Further activation of β-glucosidase in tempeh through heat treatment after fermentation significantly increased both daidzein and genistein levels compared to HCl hydrolysis soybean extract and unheated tempeh extract. During fermentation, a mold of *Rhizopus oligosphorus* produced β-glucosidase enzyme, which hydrolyzed the glycosides to form aglycone.[21]

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Furthermore, the heat treatment increased β-glucosidase activities, as reported by Teegarden,[22] confirming the results of this study. Compared to acid hydrolysis, isoflavone aglycone of soybean tempeh after 61 hours of fermentation showed lower levels. Consequently, the heat treatment was selected to increase isoflavone from tempeh. In this study, tempeh was heated at 40°C, 50°C, and 60°C for 2 hours in a dry oven based on the optimum temperature of β-glucosidase activity. The effect of heat treatment on isoflavone levels from tempeh compared to acid (HCl) hydrolysis and tempeh without heat treatment is shown in Figure 3. Regarding the temperature, the levels of daidzein and genistein were highest at 40°C of heating and significantly decreased at 50°C. Meanwhile, the increase of daidzein and genistein was observed at 60°C, but it was not different compared to the 40°C treatment. The decreased level of both compounds after being heated at 50°C was correlated with the stability of β-glucosidase, as reported by Qin *et al*, where enzyme showed low stability at 50°C.[23]

Conclusion

In conclusion, this study showed that UAE method increased the yield of three soybean varieties extract, with Devon-1 extract showing the highest percentage. Extraction of Devon-1 with ethanol 50% showed a higher yield than compared to 96%. Acid hydrolysis showed a significant increase in the level of daidzein and genistein of all varieties compared to soybean without treatment. Regarding varieties, Devon-1 showed the highest level, while enzymatic hydrolysis (fermentation) during tempeh production after 61 hours showed a lower level of daidzein and genistein. The heat treatment after 61 hours of tempeh fermentation, specifically at 40°C significantly increased both compounds compared to unheated tempeh and acid hydrolysis treatment. Consequently, heat treatment at 40°C was suggested to obtain the maximum level of daidzein and genistein from tempeh. The results could be applied for the optimum method to obtain isoflavone aglycone for further nutraceutical formulation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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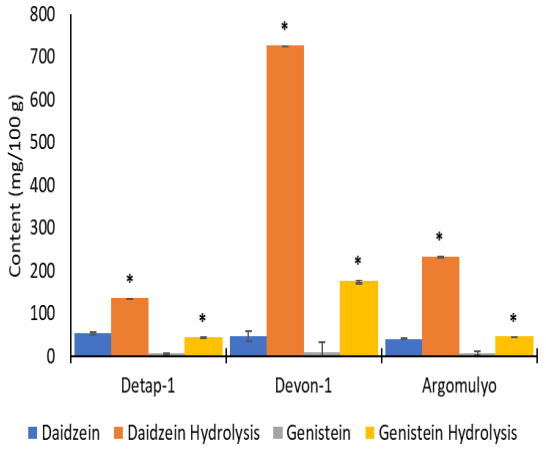

Figure 2: Effect of acid hydrolysis in daidzein and genistein content of three soybean varieties.
(*) means significantly different at p<0.05 before and after treatment.

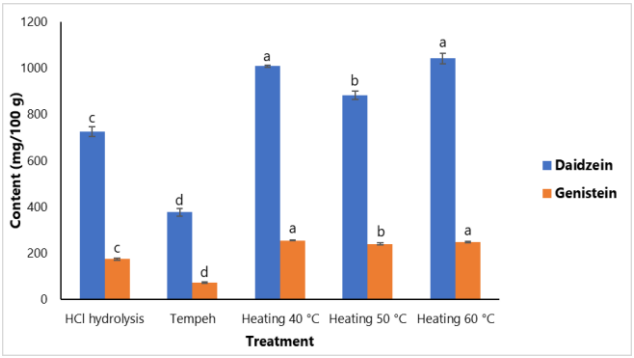

Figure 3: Effect of heat treatment in daidzein and genistein of tempeh compared to HCl hydrolysis
a, b, c = significantly different at p<0.05.

Table 3. Daidzein and genistein content in extracts of the three varieties of soybean

Soybean variety	Daidzein (mg/100 g)	Genistein (mg/100 g)
Detap-1	53.02 ± 3.16	6.05 ± 0.20
Devon-1	45.67 ± 12.07	9.93 ± 1.50
Argomulyo	40.48 ± 1.39	6.55 ± 0.48

Table 4: Daidzein and genistein content after acid hydrolysis of the three varieties of soybean

Soybean variety	Treatment	Daidzein (mg/100 g)	Genistein (mg/100 g)
Detap-1		135.50 ± 0.79^{c}	44.20 ± 0.33
Devon-1	Acid hydrolysis	725.93 ± 22.17^{a}	175.04 ± 3.94^{a}
Argomulyo		232.38 ± 4.77^{b}	46.58 ± 1.02

a, b, c = significantly different at p<0.05.

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