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RESEARCH ARTICLE

The effect of *Aspergillus oryzae* and *Rhizopus aspergillus* fermentation on daidzein content in edamame (*glycine max*)

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Abstract

Background: Previous studies show that fermentation using *Aspergillus oryzae* and *Rhizopus oligopsorus* increases the aglyconic isoflavone in soybean with the highest content reached on the seventh day. Still, no research has been done on edamame. **Objective:** This study was conducted to validate the method and determine aglyconic isoflavone, daidzein, content in non-fermented and *A. oryzae* and *R. oligopsorus* fermented edamame using TLC densitometry. **Method:** TLC densitometry method used was validated, then used to determine daidzein content in non-fermented edamame, and edamame fermented with the combination of *A. oryzae* and *R. oligopsorus* for four days (D1 - D4). **Results:** The TLC method met all the validation method, except for the selectivity. The daidzein content on non-fermented and fermented edamame (D1 - D4) was $0.0354 \pm 1.49*10^{-3}$, $0.0099 \pm 1.19*10^{-3}$, $0.0118 \pm 0.16*10^{-3}$, $0.0156 \pm 0.65*10^{-3}$, and $0.0618 \pm 10.67*10^{-3}$, respectively. **Conclusion:** The highest daidzein content was reached at the fourth day of fermentation.

Introduction

Edamame or what is known as vegetable soybean is a variant of soybean (Zeipina, Alsina, & Lepse, 2017). Thus, it also exhibits soybean properties. Soybean is one of the phytoestrogen sources safe for menopausal women, including those suffering from breast cancer. Phytoestrogens are substances with oestrogen-like properties originating from plants (Chang, 2009). Oestrogen is a hormone that is responsible for secondary development in women as well as for maintaining their cardiovascular health and bone density. Oestrogen production decreases in menopausal women resulting in hot flushes, cardiovascular diseases, osteoporosis, insomnia, and other inconvenient conditions. Hormone replacement therapy can be an option, but it also increases the possible incidence of cancer (Rossouw *et al.*, 2002).

Isoflavones are compounds that act as the main phytoestrogen in soybeans. Soybean is known to have abundant isoflavone (Chang, 2009), more or less 1.2-4.2 mg/g of dried sample (Wang & Murphy, 1994). Isoflavones are commonly found as glycosidic isoflavone (Teekachunhatean, Hanprasertpong, & Teekachunhatean, 2013), while the aglycone isoflavone is more biologically active than that of the glycosidic ones (Pandit & Patravale, 2011). Aglyconic isoflavone showing estrogenic activity is found in soybean, i.e. daidzein (Picherit *et al.*, 2000), genistein (Santell *et al.*, 1997), and glycitein (Song, Hendrich, & Murphy, 1999).

Glycosidic isoflavone will be converted into aglyconic isoflavone by fermentation (Purwoko, Pawiroharsono, & Gandjar, 2001). Two fungi widely used for food fermentation are *Aspergillus oryzae* (Machida, Yamada, & Gomi, 2008) and *Rhizopus oligosporus* (Purwoko *et al.,* 2001). These fungi produce β-glucosidase that will convert glycosidic isoflavone into aglyconic isoflavone (Machida *et al.*, 2008). Previous studies showed that fermentation using *A. oryzae* and *R. oligosporus* increased the aglyconic isoflavone, with the optimum fermentation time of three days (Praharini *et al.*, 2015; Yunindarwati *et al.*, 2017). However, no research has been done on edamame. This study was conducted to validate the TLC densitometry method and to determine the major aglyconic isoflavone, daidzein, content in non-fermented edamame, and in *A. oryzae* and *R. oligosporus* fermented edamame from Jember using the earlier validated method. A new analysis method must be validated first to prove that its performance parameters are able to overcome specific analysis problems. Validation methods are also carried out to ensure that the analytical methods are accurate, precise, reproducible, and able to analyse individual analytes at a particular range of concentrations (ICH Expert Working Group, 2005).

Methods

Plant material

Edamame (*Glycine max* var. SPM 1) used was obtained from PT. Mitra Tani Dua Tujuh, Jember, Indonesia. Edamame was then soaked in boiling water for five minutes, peeled, and divided into 50 g for sterilisation using autoclave prior fermentation.

Fermentation

Sterile edamame was fermented using a combination of A. oryzae and R. oligosporus for four days (D1 - D4), since the following day, the fifth day, the fermented edamame has already rotten. The fungi were grown in potato dextrose agar at 30 ^{0} C for one day (A. oryzae) and three days (R. oligosporus) (Jayanti, Wuryanti, & Taslimah, 2013). The fermentation used 5 ml of 106/ml A. oryzae in combination with 5 ml of 106/ml of R. oligopsorus. At the end of the fourth day, the fermented edamame was sliced and dried using an oven at 60 ^{0} C for 30 hours, then ground, and sieved. The day 0 - fermentation was used as control and considered as non-fermented edamame.

Extraction

The ground-sieved fermented edamame was then extracted using the method previously described (Hutabarat, Greenfield, & Mulholland, 2000) with slight modification. The non-fermented edamame was also extracted as a comparison. The powder was soxhleted using n-hexane (1:5) for three hours (Miao, Qi, & Zhao, 2005), followed by air-drying. Then, it was extracted by ultrasonication using 70% ethanol (1:6) for one hour and centrifugated at 2,600 rpm for ten minutes. The extraction was done in triplicate. The filtrate was then evaporated using a rotary evaporator until the thick extract was obtained.

Validation method of analysis

TLC densitometry (Camag 3) was done using silica gel 60 F_{254} as the stationary phase and a mixture of n-hexane, ethyl acetate, acetic acid (2:5:0.15) as the mobile phase, concentration test 120 mg/ml with methanol p.a as solvent, was detected at 273 nm (Yunindarwati *et al.*, 2017). The qualification and quantification of aglyconic isoflavone were calculated using daidzein (Sigma Aldrich 16587), standard. The method validation was performed to confirm the suitability of the proposed analytical method for its intended use, including linearity, LOD and LOQ, selectivity, precision, and accuracy (ICH Expert Working Group, 2005).

Determination of daidzein content in A. oryzae and R. oligosporus fermented edamame using TLC densitometry

Standard solutions used were in the concentration of 10, 20, 50, 70, 90, and 100 µg/mL. The sample solution concentration was 80 µg/mL. The bottling was carried out using capillary pipes with a standard volume and a sample of 6 µL. Specific stains were observed under a 254 nm UV lamp and then scanned using a densitometer.

Statistical analysis

The daidzein content was analysed using Kruskal Wallis and followed by post hoc Mann Whitney with *p* level 95%.

Results

Characteristics of fermented edamame

Fermented edamame using a combination of *A. oryzae* and *R. oligosporus* characteristics are presented in Figure 1. Mycelium was observed to grow at the first day of fermentation (D1), while the browning started at day four (D4) (Dwinanto, 2011).

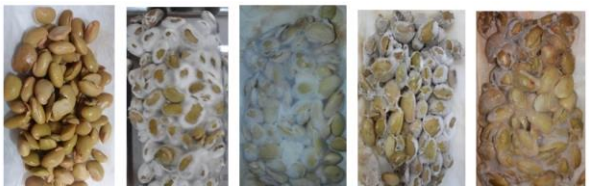


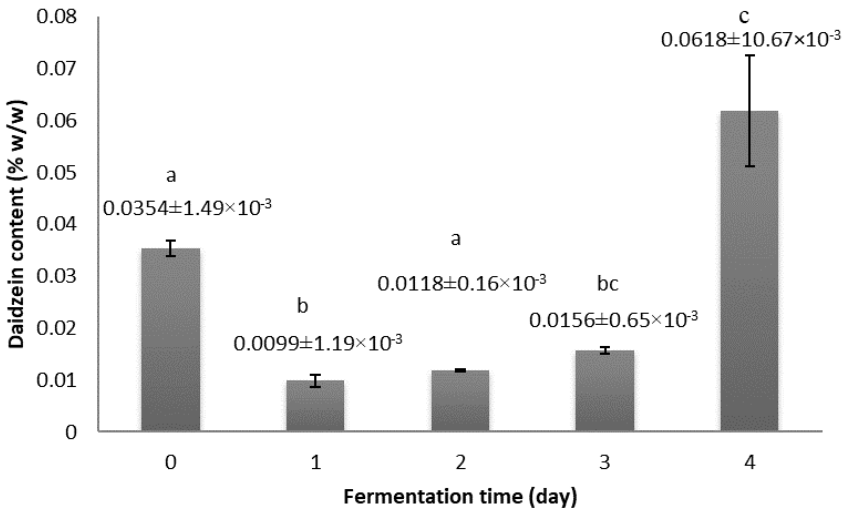
Figure 1: **Morphological characteristics of fermented edamame from zero to four days of fermentation (D1 - D4)**

Validation method of analysis

The linearity (r) of daidzein analysis was 0.9991, with Vx0 of 3.068% and Xp of 55.675. The LOD was 20.2623 ng and the LOQ was 60.787 ng. The separation of daidzein from two other isoflavone aglycone compounds (glycitein and genistein) showed poor resolution. The Rs value of daidzein-glycitein was 0.128, while the Rs value of daidzein-genistein was 0.198. The repeatability precision resulted in $4.4860\pm14x10^{-4}$ %, and the intermediate precision was $3.1810\pm87x10^{-4}$ %. The accuracy was $92.6210\pm16x10^{-1}$ %.

Determination of daidzein content in A. oryzae and R. oligosporus fermented edamame

The daidzein content on non-fermented edamame and fermented edamame (D1 - D4) was $0.0354\pm1.49*10^{-3}$,$0.0099\pm1.19*10^{-3}$, $0.0118\pm0.16*10^{-3}$,$0.0156\pm0.65*10^{-3}$, and $0.0618\pm10.67*10^{-3}$, respectively (Figure 2).



The data was shown as mean ± SD (n = 3). The different annotation shows significant differences (Kruskal-Wallis, $p < 0.05$)

Figure 2: Daidzein content in non-fermented edamame and *A. oryzae and R. oligosporus* fermented edamame

Discussion

In this study, a validation method had been carried out. The values of *r*, Vx0, and Xp obtained have met the linearity requirements, that was the value of Vx0 <5%, Xp was smaller than the concentration of the smallest analyte used, and the *r* value of table 0.88 with a confidence level of 99%. The selectivity did not meet the minimal standard of *Rs* > 1.5 (ICH Expert Working Group, 2005). This failure to meet the requirement of selectivity was most probably due to the similarity in the chemical structures of genistein, daidzein, and glycitein, resulting in similarities in chemical and physical properties of these compounds (Choi *et al.*, 2008). In this study, the precision acceptance limit used was 5.3% (ICH Expert Working Group, 2005). Based on the data, the repeatability precision and intermediate precision did not exceed the acceptable limit. Therefore, it can be concluded that the results obtained were precise. The requirements for accepting the accuracy tests for analyte levels of more than 0.001% (percent recovery values) were in the range of 90-107% (ICH Expert Working Group, 2005). The authors can say that the method of analysis for daidzein content determination in the non-fermented and *A. oryzae and R. oligosporus* fermented edamame have met the requirements for validation, except for selectivity. The lower the resolution value for selectivity, the higher the probability to result in an incorrect compound measurement, since the peak of the intended compound may be spiked with the other nearby compound. However, the lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s) (ICH Expert Working Group, 2005). Therefore, this method can be used to

calculate the isoflavone aglycone content in the non-fermented and fermented edamame.

The daidzein content, however, showed insignificant difference after the fermentation for three days, but increasing significantly at the fourth day. The highest daidzein content was that at the fourth day of fermentation. The converting process of glycosidic isoflavone into aglyconic one was driven by the β-glucosidase activity produced by *A. oryzae* and *R. oligosporus*. However, it still cannot be explained why the daidzein level decreased on the first day of fermentation and then increased significantly on the fourth day. The rate of *A. oryzae* and *R. oligosporus* is diverse in processing the conversion (Kameda *et al*., 2018). Moreover, edamame is a raw soybean harvested earlier than that of the other soybean variants (BPPP Lembang, 2015). Thus, it would have different level of secondary metabolite content, including the isoflavone (Teekachunhatean *et al*., 2013). Hence, further studies need to be conducted on the utilisation of this fermented edamame on its oestrogenic activity compared to the non-fermented one, for which either of these could be used as initial data.

Conclusion

The method of analysis for daidzein content determination in non-fermented edamame and in *A. oryzae* and *R. oligosporus* fermented edamame were valid, but not selective. The highest daidzein content was obtained from the fourth day of fermentation.

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