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FULL PAPER

Evaluation of hypoglycaemic potency in tempe with soybean germination process and extended fermentation time

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Abstract

One of the potential hypoglycaemic food from Indonesia is tempe. The use of germinated soybean and the extended fermentation time are potential methods to increase hypoglycaemic activity. In this study, the potential hypoglycaemic activity of tempe due to the use of germinated soybeans as raw material and the extended fermentation time (observation of 48, 72, and 96 hrs) was evaluated. The observations included insulinotropic free amino acids, soluble protein, peptides profile, antioxidants capacity, isoflavones, total phenolic compounds, and the α- glucosidase inhibitor activity. During the extended fermentation time from 48 hrs to 96 hrs, the germinated soy tempe had the highest insulinotropic free amino acids level, while non-germinated soy tempe had the highest antioxidant components (isoflavones, total phenolic compounds, and antioxidants capacity). However, the activity of α-glucosidase inhibitor due to treatments was decreased, suspiciously related to the decrease of the 11.40 kDa peptide, which was dominant in this study. This study showed that the extended fermentation time from 48 to 96 hrs and the use of germinated soybean was able to increase the tempe hypoglycaemic potencies based on the insulinotropic free amino acids and antioxidant component, but not for α-glucosidase activity.

1. Introduction

Diabetes mellitus (DM) is one of degenerative diseases which can increase the severity and mortality risks of other disease, such as cardiovascular and coronavirus disease 2019 (COVID-19) patients (Satria *et al.*, 2020; Gregory *et al.*, 2021). The preventive and curative management of DM must be implemented intensively to reduce those risks. One of the efforts is the provision of hypoglycaemic functional food. Tempe is an Indonesian fermented food product known for its hypoglycaemic activity (Huang *et al.*, 2018). Several compounds in tempe are suspected of its hypoglycaemic activity. Insulinotropic amino acids (lysine, arginine, alanine, leucine, phenylalanine, and isoleucine) are commonly found in tempe (Astawan *et al.*, 2020a). These amino acids could trigger insulin secretion (Van Loon *et al.*, 2003; Kanetro and Setyowati, 2013). Antioxidant compounds and phenolic components were also found, which were dominated by isoflavones (Haron *et al.*, 2009; Surya *et al.*, 2021; Yudiono *et al.*, 2021). In addition, α-glucosidase enzyme inhibitor compounds have also been found (Astawan *et al.*, 2020a). Each compound has roles in pancreatic tissue improvement and inhibition of glucose absorption in the small intestine. The antioxidant and insulinotropic compounds improved the pancreas performance and optimised insulin secretion (Astawan *et al.*, 2015; Lo *et al.*, 2018; Astawan *et al.*, 2020a). In addition, the inhibition of α-glucosidase could reduce glucose absorption, so the blood glucose rise would be controlled (Wresdiyati *et al.*, 2015).

The extended fermentation time in tempe can increase its hypoglycaemic potency. Tempe with extended fermentation time from 48 hrs to 96 hrs experienced a rise in aglycone isoflavone content (Nakajima *et al.*, 2006; Kuligowski *et al.*, 2017), phenolic components (Kuligowski *et al.*, 2017), and antioxidant activity (Chang *et al.*, 2009; Widoyo and Handajani, 2015). In addition, germinated soybean also experienced higher hypoglycaemic potency. The insulinotropic free amino acids (Kanetro and Setyowati, 2013; Kanetro, 2018), phenolic components (Shohag *et*

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al., 2012; Huang *et al.*, 2014; Liu *et al.,* 2022), isoflavones (Shi *et al.*, 2010; Huang *et al.*, 2017), hypoglycaemic bioactive peptides (Gonzalez-Montoya *et al.*, 2018), and antioxidant activity (McCue *et al.*, 2005; Huang *et al.*, 2014) were reported to be increased. The use of germinated soybean as the raw material of tempe also increased antioxidant activity, α-glucosidase inhibitor activity, aglycone isoflavones, total phenolic compounds, and insulinotropic amino acids content (Astawan *et al.*, 2020a). The combination of germinated soybean and extended fermentation time was observed to increase the tempe hypoglycaemic activity. This has not been reported before.

This study aimed to evaluate the tempe hypoglycaemic potential due to a combination of germinated soybean application and extended fermentation time from 48 hrs (normal fermentation time) to 72 hrs and 96 hrs (extended fermentation time). Six samples of tempe from the treatment combinations were observed for the activity of hypoglycaemic potential.

2. Materials and methods

2.1 Sample preparation

The sample preparation consisted of soybean germination, tempe production, tempe flour production, defatting of tempe flour, and tempe flour extraction. The soybean germination step referred to the method described by Astawan *et al.* (2020d). The non-GMO (genetically modified organism) soybean (Sb&B Food Inc., Casselton, USA) was sorted and soaked in water for two hrs. After being drained, the soybean was stored for 28 hrs in a dark room and watered for every 3 hrs until a radicle of ±0.5 cm long sprouted.

The tempe production procedure referred to Rumah Tempe Indonesia, Bogor, West Java tempe production procedure (Astawan *et al.*, 2020c). The germinated and non-germinated soybean (soaked for 2 hrs before processing) were boiled, soaked for 18 hrs, peeled off, washed, watered with boiling water, drained, cooled, and air-dried with a blower. The soybean was inoculated with tempe starter (Raprima^{TM}, Bandung, Indonesia) in 1 g/kg of dry soybean, packed in a 20×10 cm perforated plastic, and fermented for 48, 72, and 96 hrs (at 30°C).

The tempe produced was milled following the method described by Astawan *et al.* (2020b). The tempe was sliced, steamed for 10 mins, dried, milled, and sieved in 60-mesh size. The flour was stored in a tightly closed aluminium foil container at 4°C to be used for free amino acids analysis and next preparation.

The defatting method referred to the modified method described by Puteri *et al.* (2018). Tempe flour in hexane with a 1:3 (b/v) ratio was stirred for 1 hr and filtered. This process was carried out two times. The tempe flour was dried and mixed with hexane in a 1:3 (b/v) ratio, then sonicated for 20 mins. The blend was filtered, dried (at 50°C for 2 hrs), and stored in a tightly closed aluminium foil container at 4°C to be used for extraction and analysis of protein molecular weight profiling, soluble proteins, total phenolic content, and antioxidant.

The extraction referred to the method described by Durovic *et al.* (2018). The defatted tempe flour was mixed with methanol: water (4:1) solution with a ratio of 1:5 (b/v), stirred for 3 hrs, rested for 18 hrs, centrifuged (Hermle Z383K, Whingen, Germany) (speed of 3500 rpm at 4°C for 10 mins) and the supernatant was filtered using Whatman no. 1 filter paper. The extract was concentrated using a rotary evaporator (at 50°C and 85 mbar pressure), stored in a bottle covered with aluminium foil, inserted with nitrogen gas, and stored at -5°C until the analysis of α-glucosidase inhibitor activity.

2.2 Analysis of free amino acids, peptides, and soluble protein

2.2.1 Free amino acids profile

The analysis procedure referred to the method described by Waters Corporation (2012). The tempe flour in water (1:50) was filtered with ash-free filter paper and GHP/RC 0.2 µm syringe filter. The filtrate of 500 µL or 40 µL standard solution of the amino acid mixture was added with 40 µL of 2.5 µM/mL AABA (α-aminobutyric acid), followed by distilled water until 1000 µL, and mixed using vortex for 2 mins. The sample was derivatised by mixing 10 µL solution standards or samples with 70 µL of AccQ.Tag Fluor Borate Buffer derivative solution and 20 µL of AccQ. Tag Reagent 2A using a vortex, and then heated at 60°C for 10 mins. After cooling down, 1 µL of the sample was injected into the UHPLC (ultra-high-performance liquid chromatography) (Acquity Arc Bio System, Waters, UK) with AccQ.Tag Ultra C18 (2.1 × 100 cm) (Acquity Arc Bio System, Waters, UK) column at 49°C, gradient pump system with 0.5 mL/min flow rate, PDA detector (λ_{265}), mobile phase A (acetonitrile: formic acid: ammonium formate 10:6:84), B (acetonitrile: formic acid: aquabidest 88:2:10), C (aquabidest), and D (acetonitrile: formic acid 98:2).

2.2.2 Soluble protein

The analysis referred to the method described by Bradford (1976). Approximately 1 g of defatted tempe flour (Ws) in Tris-HCl buffer (0.05 M, pH 12) (Vs) with 1:10 (b/v) ratio was mixed for 2 mins using a vortex, and

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then centrifuged (3000 rpm, 45 mins, 4°C). The supernatant or BSA standard (0 – 1000 µg/mL) was added by 150 µL of Bradford solution (0.1 g Coomassie Brilliant Blue G-250 dye, 50 mL 95% ethanol, 100 mL 85% phosphoric acid in 1 L water) in a dark condition. The samples were incubated for 5 mins while shaking, then their absorbance (λ_{595}) was read. The soluble proteins concentration from the standard curve was expressed in mg/g tempe dw with the following formula.

$$\text{Soluble proteins (mg/g tempe dw)} = \frac{\text{Protein concentration (µg/mL)} \times \text{Vs} \times 100}{\text{Ws} \times 1000 \times (100 - \text{tempe water content (ww)})}$$

2.2.3 Peptide content (estimation)

The estimated peptide content was determined by calculating the difference between the soluble protein content and total free amino acids. The value was reported as a unit of mg/100 g tempe dw.

2.2.4 Peptide profile

The peptide profiling via SDS-PAGE was referred to the method described by Laemmli (1970). The supernatant of the sample (prepared similarly with soluble protein analysis) with concentration 1.6 mg/mL mixed with sample buffer with a ratio of 1:4. Then, the mixture was heated in boiling water for 10 mins and inserted into gel electrophoresis well with a volume of 20 µL. The electrophoresis was activated with a 20 mA electric current and voltage of 70 V and ended when the sample distance was 1 cm from the gel base. Finally, the sample was dyed using silver dye.

2.3 Analysis of antioxidant components

2.3.1 Total phenolic compounds

The analysis was performed under the method described by Chen *et al.* (2016). Approximately 1 g of defatted tempe flour in 10 mL 80% methanol was vortexed for 1 min and centrifuged (3000 rpm, 45 mins, 4°C). A 0.1 mL of supernatant or gallic acid (50 – 250 µg/mL) was mixed with 2 mL of 2% Na_2CO_3 for 3 mins, then added with 0.1 mL of 50% Folin-Ciocalteu reagent, rested for 30 mins, and measured for its absorbance (λ_{750}) using spectrophotometer. The total phenolic content was determined from the gallic acid standard curve.

2.3.2 Aglycone isoflavones (daidzein and genistein)

The aglycone isoflavones observation referred to the method described by Astawan *et al.* (2020a). Approximately 2 g of defatted tempe flour (Ws) was added with 30 mL of 1N HCl: acetonitrile (1:4) solution mix (Vs), stirred for 2 h and filtered using Whatman no. 1 filter paper. The filtrate was diluted ten-fold with methanol: ammonium acetate 1 µM (6:4) as the mobile phase. Then, the sample and the standards (daidzein and genistein with each concentration series of 0.5 – 50 µg/mL) were filtered using 0.22 µm syringe filter nylon membrane, and 20 µL of the solution was pipetted into the HPLC (high-performance liquid chromatography) instrument (Agilent 1200 Series, Agilent Technologies, USA) with isocratic pump system with a flow rate of 0.5 mL/min, in C-18 5 µm (15 cm × i.d. 4.6 mm) column at 25°C, and detected with multiwavelength detector (λ_{265}). The concentration of isoflavones in the sample was determined from the standard curve and converted into a unit of mg isoflavones/100 g tempe dw with formula as followed:

$$\text{Isoflavones (mg /100 g tempe dw)} = \frac{\text{µg/mL isoflavones} \times \text{Vs (mL)} \times \text{FP} \times 100}{\text{Ws (g)} \times 1000\ \text{µg/mg} \times (100 - \text{tempe water content (\%bb)})} \times 100$$

2.3.3 Antioxidant capacity DPPH inhibition method

The DPPH inhibition analysis was carried out according to the method described by Hashim *et al.* (2018). Ascorbic acid (25 – 200 µg/mL) or 0.2 mL of supernatant sample (extracted using a similar method of antioxidant analysis) were mixed with 3.8 mL of DPPH solution (0.1 mM in methanol), incubated in a dark condition for 30 mins, and measured for its absorbance (As) at λ_{517}. A blank sample filled with 80% methanol solution (Ab). The percentage of inhibition value was calculated with the equation as follows. The antioxidant capacity value unit was reported as mg AEAC (ascorbic acid equivalent antioxidant capacity)/100 g tempe dw.

$$\%inhibition = \frac{A_b - A_s}{A_b} \times 100$$

2.4 Activity of α-glucosidase inhibitor

The analysis was carried out based on the method described by Sancheti and Seo (2009). A 10 µL of the sample (6.25 – 200 mg/mL in DMSO) and blank sample for each sample was added with 25 µL of 5 µM pNPG (in 0.01 M phosphate buffer pH 7.0) and 25 µL of the α-glucosidase enzyme (0.1 U/mL in 0.01 M buffer phosphate pH 7.0). After incubated (37°C, 30 mins), the sample was added by 100 µL of 0.2 M Na_2CO_3 and measured for its absorbance (λ_{410}) using a microplate spectrophotometer (Epoch, BioTek, Vermont, US). There were samples (Asc) and blank samples (Abc) which were not added with α-glucosidase as a correction factor. The calculation of inhibition percentage used calculation below and reported as IC_{50} value.

$$\%inhibition = \frac{(A_b - A_{bc}) - (A_s - A_{sc})}{A_b - A_{bc}} \times 100$$

2.5 Data analysis

The data from this study were analysed using SPSS 12 data processing software to observe treatment effects on the sample. The statistical analysis performed was multivariate ANOVA (analysis of variance) with the

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Duncan test at a 5% significance level.

3. Results and discussion

3.1 Insulinotropic free amino acids profiles, peptides, and protein of tempe

3.1.1 Insulinotropic free amino acid

The use of germinated soybean and extended fermentation time increased the total free amino acids of tempe than normal tempe 48NG (Table 1). Tempe 96NG achieved the highest value based on the total free amino acids (Figure 1). At the same time, 96G tempe showed the highest values in total insulinotropic (lysine, arginine, alanine, leucine, and phenylalanine) free amino acids and its ratio to non-insulinotropic amino acids. The domination of germinated soybean in both parameters were expressed at a fermentation time of 96 hrs. In the 96G tempe, the total insulinotropic free amino acids were higher than the total non-insulinotropic counterpart with an above 1 ratio value.

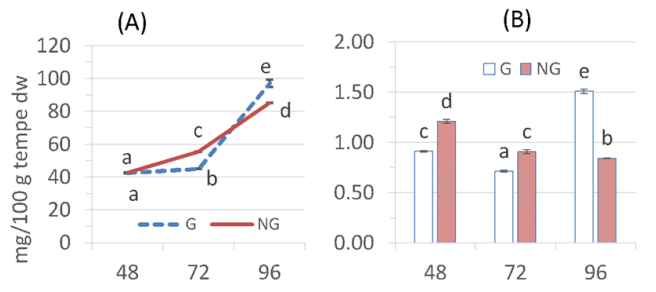

Figure 1. Total insulinotropic free amino acids (A) and the ratio of insulinotropic and non-insulinotropic free amino acids (B) of tempe. Bars and lines with different notations are statistically significantly different ($p<0.05$). 48, 72, 96: fermentation time (hours), G: using germinated soybean, NG: using non-germinated soybean.

The rise of tempe free amino acids, including insulinotropic free amino acids, from non-germinated soybean after being fermented for more than 48 hrs has been reported in several studies (Stillings and Hackler, 1965; Handoyo and Morita, 2006; Ali *et al.*, 2016; Utami *et al.*, 2016). The germinated soybean also experienced an increase in insulinotropic free amino acids (Kanetro and Setyowati, 2013; Kanetro, 2018). Its application as a raw material of fermented food, soymilk yoghurt, also improved the insulinotropic free amino acids (Yang and Li, 2010). This increase in tempe was caused by proteolysis during the fermentation process and soybean germination (Baumann and Bisping, 1995; Sanjukta and Rai, 2016; Kadar *et al.*, 2018; Tuan *et al.*, 2018). The results were similar to this study. The effects of the germination process in tempe insulinotropic free amino acids were varied and different in each addition of fermentation time. The increase in phenylalanine and arginine due to the germination in tempe fermented for 96 hrs increased the total of insulinotropic free amino acids.

3.1.2 Soluble protein and peptides

The extended fermentation time up to 96 hrs increased the soluble protein in non-germinated soy tempe, while the germinated soy tempe only experienced the increase until 72 hrs fermentation time (Figure 2A). The germination of soybean did not increase the soluble protein tempe in 48 to 96 hrs fermentation time. The concentration of peptides in non-germinated soy tempe was higher compared to germinated soy tempe at a

Table 1. The profile of free amino acids in tempe

Amino acids (mg/100 g)	48G	48NG	72G	72NG	96G	96NG
L-leucine*	3.55±0.07^{a}	4.16±0.06^{b}	4.28±0.07^{b}	5.29±0.04^{c}	10.66±0.02^{d}	15.37±0.18^{e}
L-alanine*	8.02±0.01^{c}	7.21±0.03^{b}	7.01±0.01^{a}	10.38±0.01^{d}	14.65±0.02^{e}	15.93±0.11^{f}
L-isoleucine*	7.52±0.05^{a}	8.38±0.00^{ab}	8.81±0.02^{ab}	10.23±0.02^{bc}	11.28±2.33^{c}	17.63±0.01^{d}
Glycine	6.66±0.13^{a}	t (<6.32)	t (<5.28)	6.99±0.01^{b}	9.13±0.06^{c}	10.41±0.14^{d}
L-valine	6.06±0.02^{a}	6.75±0.09^{b}	7.82±0.01^{c}	11.93±0.10^{d}	20.39±0.04^{f}	18.72±0.01^{e}
L-phenylalanine*	nd (<0.44)	nd (<0.55)	nd (<0.46)	nd (<0.53)	30.58±0.10^{b}	14.81±0.11^{a}
L-tyrosine	7.13±0.14^{b}	6.84±0.03^{a}	30.90±0.05^{f}	8.79±0.01^{c}	11.47±0.07^{d}	15.13±0.03^{e}
L-lysine*	8.41±0.06^{a}	9.69±0.00^{b}	12.62±0.05^{c}	14.88±0.14^{d}	17.44±0.03^{e}	21.51±0.01^{f}
L-arginine*	15.12±0.07^{d}	13.34±0.25^{b}	12.43±0.15^{a}	14.71±0.16^{c}	12.65±0.02^{a}	t (<0.70)
L-histidine	6.78±0.01^{a}	8.55±0.05^{b}	10.45±0.02^{d}	9.76±0.16^{c}	12.33±0.21^{e}	18.26±0.11^{f}
L-aspartate	t (<4.24)	nd (<1.89)	nd (<1.57)	t (<5.11)	t (<4.48)	nd (<1.82)
L-glutamate	9.23±0.02^{b}	nd (<6.20)	5.19±0.01^{a}	10.46±0.01^{c}	t (<10.96)	28.93±0.17^{d}
L-threonine	5.44±0.04^{c}	5.91±0.02^{d}	4.28±0.04^{b}	5.93±0.01^{d}	4.09±0.05^{a}	nd (<1.09)
L-proline	7.18±0.07^{a}	8.60±0.21^{d}	6.70±0.14^{b}	9.00±0.03^{e}	8.04±0.06^{c}	11.48±0.02^{f}
Total free amino acids	91.1±0.12^{b}	79.43±0.49^{a}	110.50±0.28^{c}	118.33±0.02^{d}	162.72±2.26^{e}	188.18±0.44^{f}

Values are presented as mean±SD, n = 2. Values with different superscripts within the same row are statistically significantly different with One-Way ANOVA test ($p<0.05$). 48, 72, 96: fermentation time (hours), G: using germinated soybean, NG: using non-germinated soybean, t: trace (concentration below limit of quantification and above limit of detection), nd: not detected (concentration below limit of detection). *insulinotropic amino acids.

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longer fermentation time (Figure 2B). The domination of non-germinated soy tempe compared to germinated soy tempe in soluble protein and peptides results were in line with the total free amino acids result (Table 1).

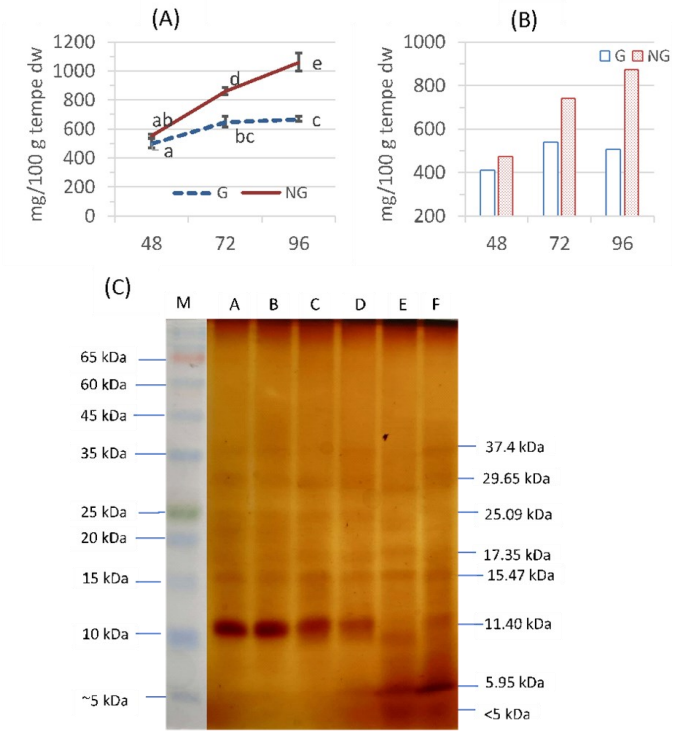

Figure 2. Soluble protein (A), the estimated concentration of peptides according to the difference of soluble protein and total free amino acids value (B), and peptides profile from SDS-PAGE analysis (C) of tempe. Bars and lines with different notations are statistically significantly different ($p<0.05$). 48, 72, 96: fermentation time (hours), G: using germinated soybean, NG: using non-germinated soybean, M: marker protein, A: 48G, B: 48NG, C: 72G, D: 72NG, E: 96G, F: 96 NG. The value of peptide concentration is the difference of means without repetition.

Based on the SDS-PAGE result, the extended fermentation time affected the presence of dominant peptides in tempe (Figure 2C). Peptides with a molecular weight of 11.40 kDa were predominant in 48 hrs fermented tempe, reduced in 72 hrs tempe, and had the least value in 96 hrs fermentation time. Meanwhile, a peptide with a molecular weight of 5.95 kDa was predominant in 96 hrs fermentation time. The effect of soybean germination was significant in 96 hrs of fermented tempe. The 96NG tempe had more of 5.95 kDa peptides compared to the 96G tempe.

Previous studies reported that the dominant peptides molecular weight in non-germinated soy tempe were 37.2, 16.8, and 10.6 kDa (Sitanggang *et al.*, 2020), while germinated soy tempe had a molecular weight of 14.2 kDa (Puteri *et al.*, 2018). The different kinds of soybean caused the differences in molecular weights in predominant peptides. The soybean used in the previous studies were local Grobogan (Indonesian) variety soybean (Puteri *et al.*, 2018) and imported American GMO soybean (Sitanggang *et al.*, 2020), while this study used USA non-GMO soybean.

The different types of soybean are related to the different gene inside. Gene modification basically alters gene expression and also the expressed protein. Astawan *et al.* (2020b) reported that the protein content of local Grobogan was the highest, followed by non-GMO soybean and GMO soybean, respectively. This suggests a possibility of different peptide profile among those soybeans (Nishizawa *et al.* 2008).

The active hypoglycaemic peptides were previously reported with molecular weight less than 5 kDa (Sitanggang *et al.*, 2020) and 3 kDa (Tamam *et al.*, 2019). In this study, the peptides with similar molecular weight were dominantly found in 96 hrs of fermented tempe, specifically in non-germinated soy tempe.

The 96NG tempe had the highest potency of hypoglycaemic peptides based on the SDS-PAGE result, along with soluble proteins and peptides concentration. The better results in non-germinated soybean with longer fermentation time was not following the initial hypothesis, which is the germinated soy tempe was better than non-germinated soy tempe. The exact mechanism for this remains unknown. The increase of soluble proteins, smaller peptides, and free amino acids was fundamentally caused by the activity of proteolytic enzymes during the germination and fermentation process (Sanjukta and Rai, 2016; Tuan *et al.*, 2018; Kadar *et al.*, 2020).

Based on the results, the hydrolysis in non-germinated soybean, which was fermented for 72 and 96 hrs, was higher than the germinated soybean. It caused by the higher initial protein content found in the non-germinated soy tempe than to the germinated soybean. The protein in germinated soybean was mostly hydrolysed during the germination process. As a result, the initial protein content declined, and the value continued to decline during a longer fermentation time (Kim *et al.*, 2011). This was also related to the molecular protease hydrolysis mechanism from the soybean during the germination process and resulted from the mould during the fermentation process. Furthermore, a detailed and comprehensive study in protein and tempe amino acid profiles caused by fermentation time and germination factors needs to be done.

3.2 Antioxidant components

The extended fermentation time from 48 to 96 hrs was shown to increase the antioxidant capacity, total phenolic compounds, and aglycone isoflavones content

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(Figure 3). The use of germinated soybean during extended fermentation time did not increase the antioxidant component in tempe. In addition, the germinated soybean also reduced the total phenolic compounds on 72 hrs fermented tempe and aglycone isoflavones on 72 and 96 hrs fermented tempe. The non-germinated soy tempe with 96 hrs fermentation time was the tempe with the highest antioxidant components in all three parameters.

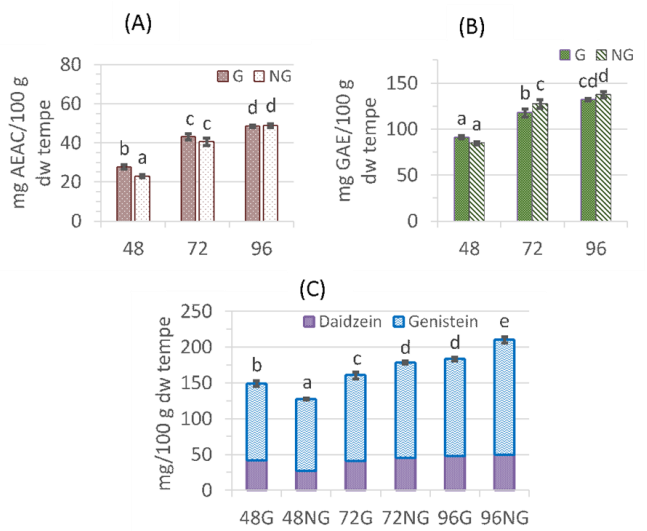

Figure 3. Antioxidant capacity (A), total phenolic compounds (B), and aglycone isoflavone concentration (C) in tempe. Bars and lines with different notations are statistically significantly different ($p<0.05$, $n = 4$). 48, 72, 96: fermentation time (hours), G: using germinated soybean, NG: using non-germinated soybean, AEAC: Ascorbic acid equivalent antioxidant capacity, GAE: gallic acid equivalent.

The increase in total phenolic compounds in extended fermentation time was caused by the carbohydrate-hydrolysing enzymes secreted from the microbes, which resulted in the breakdown of the phenolic-carbohydrate bond (Adetuyi and Ibrahim, 2014; Bei *et al.*, 2018). The increase of aglycone isoflavones resulted from the extended fermentation process was caused by the activity of β-glucosidase enzyme from the tempe mould and lactic acid bacteria, which converted isoflavone glycones into aglycones (Murakami *et al.*, 1984; Otieno and Shah, 2007; Nurdini *et al.*, 2015; Lo *et al.*, 2018). The increase in aglycone isoflavones and phenolic compounds which formed during the extended fermentation time affected the increase of tempe antioxidant capacity.

Short-chained peptides in tempe also had antioxidant properties, such as peptides with molecular weight less than 5 kDa (Sitanggang *et al.*, 2020) and 3 kDa (Tamam *et al.*, 2019). In this study, the presence of peptides below 5 kDa (Figure 2) was directly proportional with the antioxidant capacity increase in 96 hrs of fermented tempe. This information supported the hypothesis that short-chained peptides possessed antioxidant properties. In addition, other compounds such as tocopherol (vitamin E), β-carotene (pro-vitamin A), and ascorbic acid (vitamin C) (Astawan *et al.*, 2017; Astawan, *et al.*, 2020a) had roles in tempe antioxidant capacity, which were not observed in this study.

The cause of lower aglycone isoflavones content in the 72 hrs and 96 hrs fermentation time of germinated soy tempe compared to non-germinated soy tempe has not been determined before. This result was relevant to a previous study with a longer fermentation time, 120 hrs (Puspitasari *et al.*, 2020). The increase of aglycone isoflavones in non-germinated tempe was in accordance with the soluble protein (Figure 2A) and free amino acids results (Table 1). Wu and Muir (2010) reported that the protease enzyme secreted by mould and lactic acid bacteria during the soybean fermentation was associated with the β-glucosidase enzyme. The result of the protein hydrolysis process with protease was in line with the increase of aglycone isoflavones content. Similar results were also found in the soymilk fermentation study (Hati *et al.*, 2017). This strengthened the notion that enzyme activity resulting from microbes in the extended fermentation time was higher in non-germinated soybean in than the germinated soybean. However, this needs to be proven through a more comprehensive study.

3.3 Activity of α-glucosidase inhibitor

The combination of germinated soybean and extended fermentation time reduced the α-glucosidase inhibitor activity (Figure 4). This was shown by increasing the IC_{50} value (concentration required to inhibit 50% enzyme activity) of tempe extracts. The highest inhibitor activity was found in the 48 hrs fermented tempe. The use of germinated soybean did not affect the inhibitor activity and even reduced the activity in fermentation time of 96 hrs.

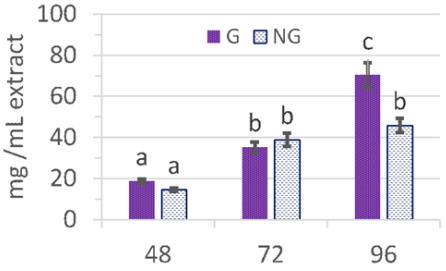

Figure 4. The IC50 value of α-glucosidase inhibitor in tempe. Bars and lines with different notations are statistically significantly different ($p<0.05$, $n = 3$). 48, 72, 96: fermentation time (hours), G: using germinated soybean, NG: using non-germinated soybean.

The increase of α-glucosidase inhibitor activity due to extended fermentation time in fermented soybean product studies had been previously reported, such as in

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douchi (Chen *et al.*, 2007) and okara (Zhu *et al.*, 2013), but there had been no reports in tempe. In the previous studies, germinated soy tempe with 48 hrs fermentation time had higher α-glucosidase inhibitor activity than the non-germinated soybean (Astawan *et al.*, 2020a). The differences were possibly caused by the different soybean types. Local Grobogan (Indonesia) soybean was used in the study, while the soybean used in this study was imported non-GMO soybean from the USA. The different soybean types are related to the different genes, expressed proteins, and peptides with their bioactivity (Nishizawa *et al.,* 2008).

Shorter germination time might result in an insignificant change of α-glucosidase inhibitor activity in tempe, especially in 48 hrs and 72 hrs fermentation time. The increase of α-glucosidase inhibitor activity was reported after four days of germination time (Sun *et al.*, 2018), while the germination time in this study was only 24 hrs.

The result of α-glucosidase inhibitor activity was not in accordance with the hypothesis. Several compounds as inhibitors of α-glucosidase in soybean were phenolic compounds, isoflavones, and peptides (Choi *et al.*, 2010; Ademiluyi and Oboh, 2013; Gonzalez-Montoya *et al.*, 2018). The results of phenolic and aglycone isoflavones compound analysis were not in line with α-glucosidase inhibitor activity. However, the analysis of α-glucosidase inhibitor was in line with the decline of predominant peptides in tempe, which were peptides with a molecular weight of 11.40 kDa (based on SDS-PAGE analysis in Figure 11). According to that result, the decrease of α-glucosidase inhibitor activity was related to the reduction of peptides in tempe.

According to *in vivo* test, this analysis was in accordance with the result of the oral glucose tolerance test (OGTT) performed by Cahyani (2020) and Puspitasari (2020). The studies showed that tempe with two days of fermentation time effectively reduced blood glucose levels compared to 5 and 7 days fermented tempe. Based on that result, a notion was proposed that the effectivity of fresh tempe in reducing blood glucose during OGTT was related to its higher α-glucosidase inhibitor activity found in this study.

The α-glucosidase inhibitor activity was one of several mechanisms related to hypoglycaemic activity in a food material. Aside from the mechanism, other mechanisms were related as well. Therefore, further *in vivo* hypoglycaemic studies need to be done to determine the precise hypoglycaemic mechanism in the body.

Tempe with and without extended fermentation time each had a different mechanism of hypoglycaemic potency. Tempe with normal fermentation time was better in α-glucosidase inhibitor mechanism in the surface of the small intestine. The tempe had roles in DM to control the rise of blood glucose after a meal (postprandial). This tempe was addressed for healthy people who wanted to prevent DM through food products with good sensory quality. Tempe with 96 hrs fermentation time, from both germinated and non-germinated soybean, was better in pancreas function improvement with the help of insulinotropic free amino acids and antioxidant compounds. Tempe with longer fermentation time had the better function in pancreatic tissue improvement and better for people who are going through DM recovery, although it did not meet the sensory quality of fresh tempe.

4. Conclusion

The extended fermentation time from 48 to 96 hrs increased the tempe hypoglycaemic potencies, as indicated by the increased insulinotropic free amino acids and antioxidant components (antioxidant capacity, total phenolic content, and aglycone isoflavones). During this extended fermentation time, the increasing peptide content also described the potency of increasing bioactive peptides. This benefit of extended fermentation time was supported by soybean germination in terms of increasing the insulinotropic free amino acid content in 96 hrs of fermentation time. Along with that superiority, the extended fermentation time and soybean germination were ineffective in increasing the α-glucosidase inhibitor activity. Tempe with 48 hrs of fermentation time and non -germinated soybean had higher α-glucosidase inhibitor activity.

Conflict of interest

The authors declare no conflict of interest.

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