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Antioxidant activity of a mixture between water-soluble tempeh extract and whey powder that has undergone a Maillard reaction

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The high amino acid content in over fermented tempeh and lactose in whey powder can be combined to perform a Maillard reaction. This strategy can be used to produce functional food products. Like the Maillard reaction products,, melanoidins have been reported to have high antioxidant capacities and own a preferred flavor. The study's general objective was to obtain the optimal processing conditions in a mixture of water-soluble tempeh extract and whey powder with the highest antioxidant capacity. The research was carried out in three stages: (i) selecting the best whey powder concentration, (ii) initial reaction pH, and (iii) obtaining the kinetic parameters of the Maillard reaction with respect to the antioxidant activity of the reacted mixture. The mixture ratio between water-soluble tempeh extract and whey powder of 5.0 % (w/v), reacted at 100 °C and pH 5.0 for 90 min, could increase the antioxidant activity from 67.91 ± 2.62 % to 89.89 ± 0.17 %. That concludes as the optimal conditions for the Maillard reaction between water-soluble tempeh extract and whey powder with an IC50 of 47.82 mg/mL.

1. Introduction

Fermented soybean, otherwise known as tempeh, is a traditional food from Indonesia, which is widely known by the general public. Rhizopus sp. is the dominant microorganism for fermenting soybean during tempeh production (Fibri and Frøst 2019). The macromolecules in soybeans are converted into simpler forms by the extracellular enzymes produced during tempeh's fermentation process. Specifically, the fermentation increases the levels of free amino acids (AA), ammonia, and low molecular weight peptides due to protein degradation by proteases (Aryanta 2000; Liu and Pan 2011). There is no significant difference observed in protein content between soybean and tempeh. However, the dissolved protein content in tempeh increased due to the activity of the proteases (Astuti *et al.*, 2000). Tempeh contains 20-55 % db protein (Kiers *et al.*, 2000; Babji *et al.*, 2010; Bavia *et al.*, 2012; Rusdah *et al.*, 2017). Low molecular weight peptides in tempeh have the potential to be bioactive compounds, such as antioxidative, antimicrobial, antihypertensive, antidiabetic, anticancer, and anti-tumor compounds (Amadou *et al.*, 2017; Sanjukta and

Rai 2016; Tamam *et al*., 2019). Apart from peptides, other bioactive compounds found in tempeh are flavonoids, especially isoflavones that act as antioxidants (Kuligowski *et al*., 2017). The optimal soybean fermentation for producing tempeh is about two to three days. Tempeh is considered to be over fermented when the fermentation is longer than this period. The traditional tempeh sellers normally use the over fermented tempeh as part of animal feeds or possibly discharge it directly to the environment. Considering the high amount of protein in over fermented tempeh, simple aqueous extraction can be used to recover water-soluble protein, which is useful for other purposes (Arianingrum *et al*., 2007).

Whey is a by-product produced from the manufacture of cheese and casein in the dairy industry. About 85% of the total milk used was wasted as a whey by-product from dairy manufacture (Panesar *et al*., 2012). Whey can be purified, pasteurized, and dried to produce whey powder. Whey powder contains about 11 - 14.5% protein, 63 - 75% lactose, and 1 - 1.5% fat (Kadam *et al*., 2018). The composition of the whey product varies depending on the milk source, production method, cheese type, and industry specifications. Lactose is a disaccharide in milk and is the main component in solid whey (Chegini and Taheri 2013). High lactose content in whey powder can be used as a source of sugar to form flavor resulting from caramelization. As a by-product of the cheese-making industry, the utilization of whey processed into food products is still quite low (less than 50%), the rest is disposed of as waste into the environment (Alonso *et al*., 2011; Sitanggang *et al*., 2016). Whey will be beneficial for the cheese industry because it reduces the cost of waste processing and valorizes the by-products.

The Maillard reaction or non-enzymatic browning reaction is a complex reaction between compounds with a carbonyl group (reducing sugars) and compounds containing amine groups (amino acids, peptides, or proteins) (Chawla *et al*., 2007; Hwang *et al*., 2011).

The Maillard reaction is divided into three stages the: initial stage, the intermediate stage, and the final stage (Hodge 1953). The final stage of the Maillard reaction produces a brown pigment known as melanoidins (Liu *et al*., 2008) that have been reported to have functional properties as antioxidants (Wagner *et al*., 2002). Melanoidin compounds, which are polymers with large molecular weights, have fairly large antioxidant capacities. That is due to functional structures in melanoidins such as enol and enamiol, which can act as antioxidants. The corresponding hydroxyl groups can reduce the oxidation process by reducing metals, chelating metals, and scavenging free radicals (Fardiaz *et al*., 2006).

Based on the existing characteristics, the combination of water-soluble protein obtained from over fermented tempeh and whey powder can produce functional food products. The high amino acid (AA) content in water-soluble tempeh extract and lactose in whey powder can be used as substrates to form a derivative food product with a high antioxidant capacity and preferred flavor through the Maillard reaction. This can be used to valorize the by-products and a basis for developing functional food products in the form of beverages. Hence, this study aimed to optimize the Maillard reaction conditions between water-soluble tempeh extract and whey powder.

2. Materials and Methods

2.1. Materials

The over fermented tempeh (4-d fermentation) was obtained from the Indonesian Tempe Cooperative (KOPTI), Bogor, West Java, Indonesia. Whey powder was a gift from Fonterra, Indonesia. Sodium hydroxide (NaOH), methanol (CH_3OH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals were analytical grade.

2.2. Preparation of water-soluble tempeh extract

4-d tempeh was cut (3.0 x 0.5 x 0.5 cm) and placed in a cabinet dryer at 70 oC for seven hours. Size reduction was performed by pin disc mill, and the tempeh flour was sieved using 80 Tyler mesh. The preparation of water-soluble tempeh extract was conducted by dispersing tempeh flour in distilled water with a tempeh flour-to-water ratio of 1:5 (w/w). The extraction was performed at 30 oC, with an agitation of 500 rpm for two hours. The slurry was centrifuged at 3000 rpm for 15 min, and the supernatant was recovered (Christianti 2019).

2.3. Maillard reaction

The preparation of the reacting volume was carried

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out on a 100-mL basis. The controls consisted of water-soluble tempeh extract (C), whey powder at a concentration of 2.0, 5.0, and 10.0 % (w/v). The unreacted mixtures, such as C11, C12, and C13, were the mixtures of water-soluble tempeh extract with whey powder at a concentration of 2.0, 5.0, and 10.0 % (w/v), respectively. The selection of the best concentration of whey powder was made by reacting the mixtures at 100 ° C, initial pH 7 for one hour, and shaking at 200 rpm. The liquid fraction of the sample was obtained by centrifugation at 300 rpm, followed by 0.45 µm membrane filtration. The recovered liquid was analyzed for antioxidant activity, browning intensity, and lightness value. The reacted mixtures between water-soluble tempeh extract with whey powder at a concentration of 2.0, 5.0, and 10.0 % (w/v) were denoted as R11, R12, and R13.

The best concentration of whey powder was used to investigate the best initial reaction pH. The six pH values were pH 3.0, 4.0, 5.0, 6.0,7.0, and 8.0. The procedures to recover reacted mixture for the analyses were the same as mentioned above.

The kinetics of the Maillard reaction based on the changes in antioxidant capacity was carried out by reacting the mixture of water-soluble tempeh with whey powder at the best whey powder concentration and initial reaction pH. The Maillard reaction was performed at four levels of temperature (70, 80, 90, 100 oC) for three hours, in which the sampling was conducted every 30 min. The rate constant k, a parameter that indicates how fast a reaction can proceed in each heating temperature, was evaluated following zeroth (0th), first (1^{st}), and second (2^{nd}) reaction order, as following (eqs. (1), (2), and (3)):

0^{th} reaction order: $$A = A_0 + kt \quad (1)$$

1^{st} reaction order: $$\ln A = \ln A_0 + kt \quad (2)$$

2^{nd} reaction order: $$\frac{1}{A} = \frac{1}{A_0} - kt \quad (3)$$

where A_0 and A: initial and final antioxidant capacity (mg AEAC/L), respectively, and *t*: reaction time (h). The selection of the appropriate reaction order for describing the rate constant was based on the coefficient of determination (R^2) obtained from the linear equations above. The calculation of the energy activation for the Maillard reaction was following Arrhenius equation (eq. 4):

$$\ln k = \ln k_0 - \frac{Ea}{R}\frac{1}{T} \quad (4)$$

where k_0: a constant, *Ea*: activation energy (kJ/mol), R: gas constant (8.31x 10^{-3} kJ/K mol) and *T*: temperature (^{o}K).

2.4. Antioxidant activity

The antioxidant capacity was analyzed according to Brand-William *et al.* (1995) and Sitanggang *et al.* (2020a) with modifications. A total of 1.0 mL sample was added with 3.0 mL of DPPH solution in methanol (120 µM) in a screw-cap test tube. The mixture was vortexed for 15 s and incubated in a dark room for 30 min. The absorbance was monitored at a wavelength of 515 nm with a UV-VIS spectrophotometer (Thermo Scientific Genesys-150, USA). 1.0 mL of distilled water was used to substitute the sample for the control. The antioxidant activity was expressed as percent inhibition or radical scavenging activity (eq. 5):

$$Inhibition(\%) = \frac{(A_c - A_s)}{Ac} x100\% \quad (5)$$

where A_c and A_s: absorbance of the control and sample, respectively (-). The ascorbic acid standard curve was used to calculate antioxidant capacity. The capacity was expressed as ascorbic acid equivalent antioxidant capacity (mg AEAC/L). In addition to this, the concentration of the recovered supernatant (mg/mL) from the best reacting conditions, which can inhibit the oxidation process by 50 %, or the half-maximal inhibitory concentration (IC_{50}), was also measured.

2.5. Browning intensity

The browning intensity (A_{420}) of the sample was evaluated by measuring the absorbance of the recovered supernatant using a UV-VIS spectrophotometer (Thermo Scientific Genesys-150, USA) at 420 nm (Karseno *et al.*, 2018).

2.6. Lightness value

Colour analysis was performed by chromameter (Minolta CR 300, Osaka, Japan) in triplicate. The values of

L (lightness value), *a* (red-green), and *b* (yellow-blue) for each sample were measured. Prior to measurement, the instrument was calibrated with a standard white plate (CIE L *: +97.30, CIE a *: −0.09, CIE b *: +1.94).

2.7. Amino acid analysis

The analysis of amino acids was performed by a commercial laboratory, PT. Saraswanti Indo Genetech (18-5-17/MU SMM-SIG, UPLC), where the method has also been reported elsewhere (Sitanggang *et al*., 2020b).

2.8. Statistical analysis

Each data point presented was an average of three replicates with a standard deviation. The analysis of variance (ANOVA) was performed using SPSS software (IBM, USA). One-Way ANOVA was used select the best concentration of whey powder and initial reaction pH. Two-way ANOVA was used in a completely randomized factorial design for choosing the best combination between reaction time and temperature. For both analyses, a confidence level of 95% was considered.

3. Results and Discussion

Selecting optimal whey powder concentration

As seen in Figure 1a, water-soluble tempeh extract and whey powder at different concentrations (2.0, 5.0, and 10.0 %) exhibited antioxidative properties. The water-soluble tempeh extract could inhibit free radicals with a percentage of 54.59 ± 2.95 %. The ability of water-soluble tempeh flour to inhibit free radicals has been reported in a range of 30 to 40 % (Puteri *et al*., 2017). The higher inhibition value within this study was due to a higher tempeh flour-to-water ratio compared to that of Puteri *et al*. (2017). The high antioxidant activity in tempeh is due to the presence of flavone derivatives and low molecular weight peptides that are produced during fermentation (Chang *et al*., 2009). In addition, water-soluble tempeh extract had the highest browning intensity corresponding to the lowest lightness value (Figure 1b, 1c, and 1d). Whey powder also shows the antioxidant property, and the increase in concentration is proportional to the increases in the antioxidant activity and the browning intensity (Figure 1a and 1b). Whey powder contains antioxidant compounds such as lactoferrin and lactoperoxidase (Bylund 2013). It can be considered a natural antioxidant (Ashous *et al*., 2013), thus, used as an ingredient for producing functional foods.

Water-soluble tempeh extract can be used as a source of amino groups, while whey powder contains reducing sugars. The use of heat onto the mixture between water-soluble tempeh extract and whey powder can facilitate the Maillard reaction. The Maillard reaction is a typical non-enzymatic browning, a reaction between the carbonyl group of the reducing sugar and the amino group from amino acids or proteins, which forms the final compound, melanoidin. The reaction between the polar fraction of water-soluble tempeh extract and whey powder is homogeneous and produces water-soluble Maillard reaction products that exhibit antioxidant activity.

After the heating process (where the Maillard reaction took place), there was an increase in antioxidant activity and browning intensity of the mixture at whey powder of 2.0 and 5.0 % (w/v) (Figure 2a and 2b). The increase in antioxidant activity reached saturation as the inhibitions of the free radicals were relatively similar between mixtures at 5.0 and 10.0 % (w/v) whey powder. The application of heat (100 oC for one hour) could degrade the antioxidative isoflavones, such as genistein and daidzein (Ungar *et al*., 2003; Kuligowski *et al*., 2017). Herein, the enhancement of antioxidant activity for the reacted mixtures at 2.0 and 5.0 % (w/v) whey powder might be attributed to the formation of melanoidins. As a consequence, the brown colour was developed, followed by the reduction of lightness value (Figure 2c and 2d). The antioxidant activity of the reacted mixture at a whey powder of 10.0 % (w/v) was lower than that of the unreacted mixture despite having a higher browning intensity. A significant increase in total solid for the reacted mixture with 10.0 % (w/v) whey powder might require a higher heat content to establish a completed Maillard reaction. Besides having a Maillard reaction, the formation of brown colour in the reacted mixture with 10.0 % (w/v) whey powder could also be due to caramelization. According to Ajandouz *et al*. (2008), caramelization, a reaction where the sugars are heated at high temperatures, especially at the alkaline pH range, also contributes

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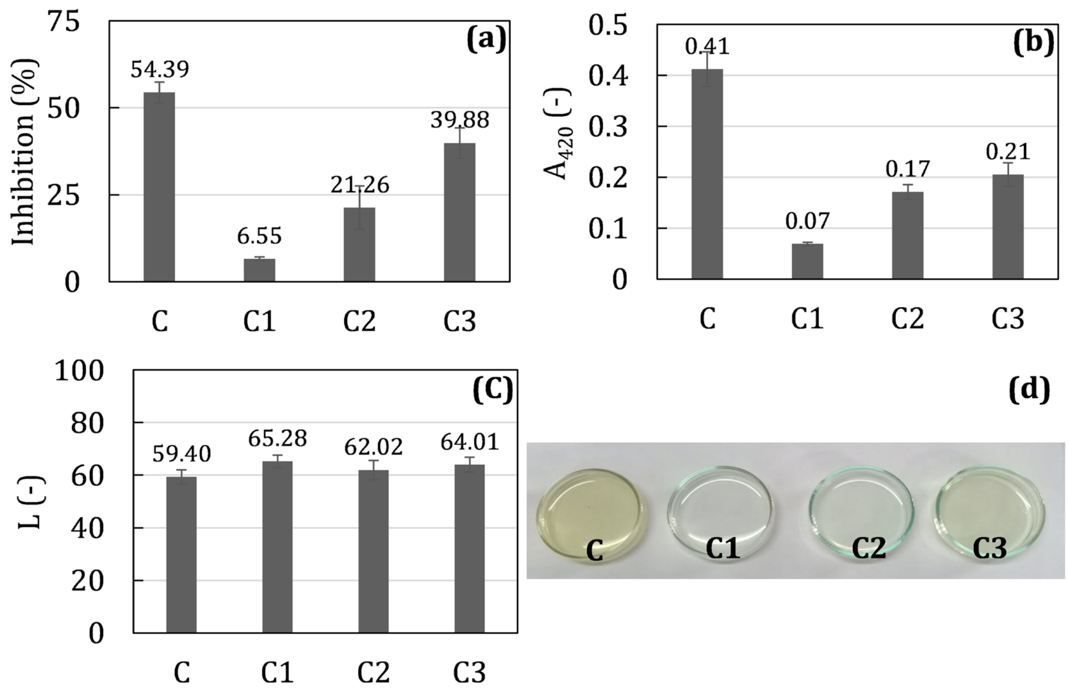

Figure 1. The antioxidant activity (a), browning intensity A_{420} (b), lightness value L (c), and the appearance (d) of water-soluble tempeh extract and whey powder control (C = water-soluble tempeh extract , C1, C2 and C3 = whey powder at 2.0, 5.0, and 10.0 % (w/v)).

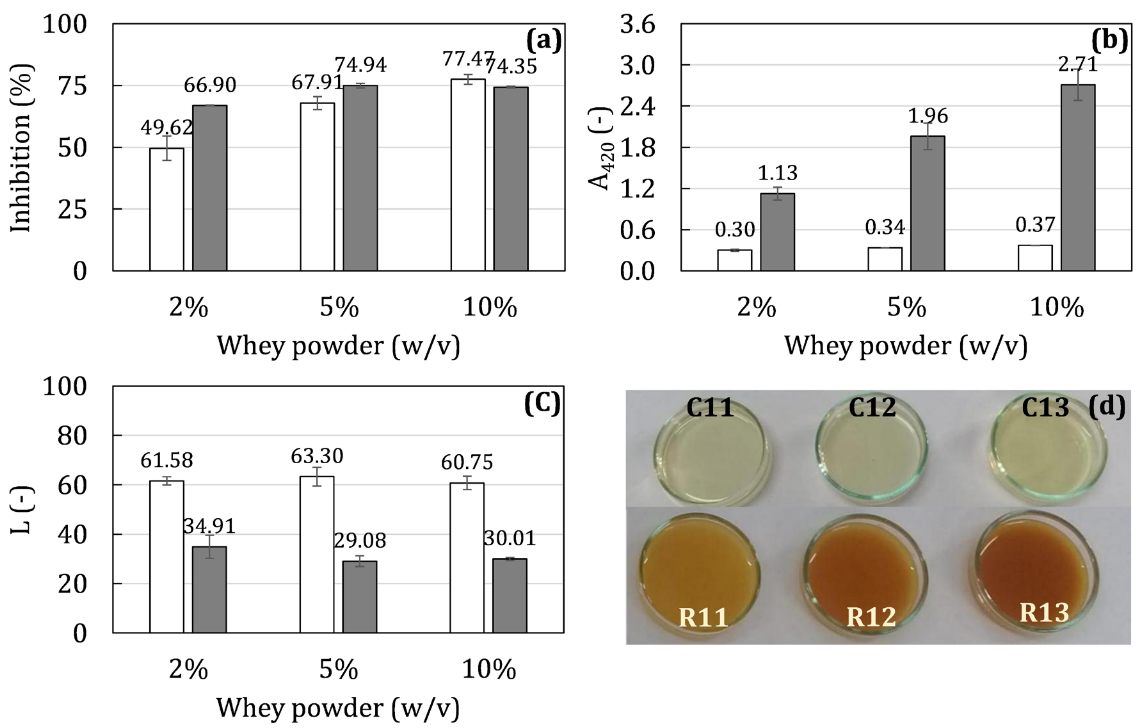

Figure 2. The antioxidant activity (a), browning intensity A_{420} (b), lightness value L (c), and the appearance (d) of a mixture between water-soluble tempeh extract and whey powder. White and grey bars represent unreacted and reacted mixtures, respectively; C11, C12, and C13 were unreacted mixtures between water-soluble tempeh extract and 2.0, 5.0, and 10.0 % (w/v) whey powder, respectively, whereas R11, R12, and R13 were the corresponding reacted mixtures (after the heating process).

to the overall non-enzymatic browning reaction. The antioxidant activity of the reacted mixture with 5.0 % (w/v) whey powder was significantly different compared with 2.0 % (w/v) whey powder. However, this value was not significantly different from that of reacted mixture with 10 % (w/v) whey powder, at a 95 % confidence level. In this study, whey powder at 5.0 % (w/v) was selected as the optimal concentration as the source of reducing sugars.

Selecting initial reaction pH

The initial pH of the mixture is a substantial reacting condition for a successful Maillard reaction. Melanoidin is formed at acidic or alkaline conditions due to the degradation of the Amadori compounds, followed by other chemical reactions such as dehydration, cyclization, and condensation. Within this study, six levels of mixture pH were utilized, namely pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. As shown in Figure 3a, reacted mixture at pH 5.0 obtained the highest antioxidant activity with a value of 89.31 ± 0.15 %. The initial pH impacts the overall Maillard reaction as there was a significant difference in antioxidant activity amongst treatments.

The initial pH of the reaction affects the solubility of proteins, peptides, and amino acids contained in the water-soluble tempeh extract. The solubility of proteins at a pH around the isoelectric point (pI) will decrease due to increased protein interactions (positively and negatively charged protein molecules). Water-soluble tempeh flour contains a high level of essential amino acids, such as leucine and lysine (Cahyani 2020). Lysine is alkaline and has an isoelectric point around pH 9.74 (Nagai and Carta 2004; Zeng *et al.*, 2011). Higher protein solubility in tempeh extract, especially at pH 5.0, caused a higher possibility for more amino acids and proteins to react with reducing sugars during the Maillard reaction. Lysine itself has a considerable influence on the Maillard reaction. Kwak and Lim (2004) reported that lysine is the most reactive amino acid to facilitate the Maillard reaction with various types of sugar compared to 11 other amino acids (aspartate, glutamate, alanine, leucine, isoleucine, valine, proline, serine, cysteine, phenylalanine, and arginine). The high lysine reactivity is due to two amine groups, namely α-amino and ε-amino. The acidic conditions (lower pH values) can also function as a catalyst for the hydrolysis of the glycosidic bonds in lactose. As monosaccharides, both glucose and galactose have higher reducing power than lactose. Herein, reducing lactose at acidic conditions is preferable for the Maillard reaction.

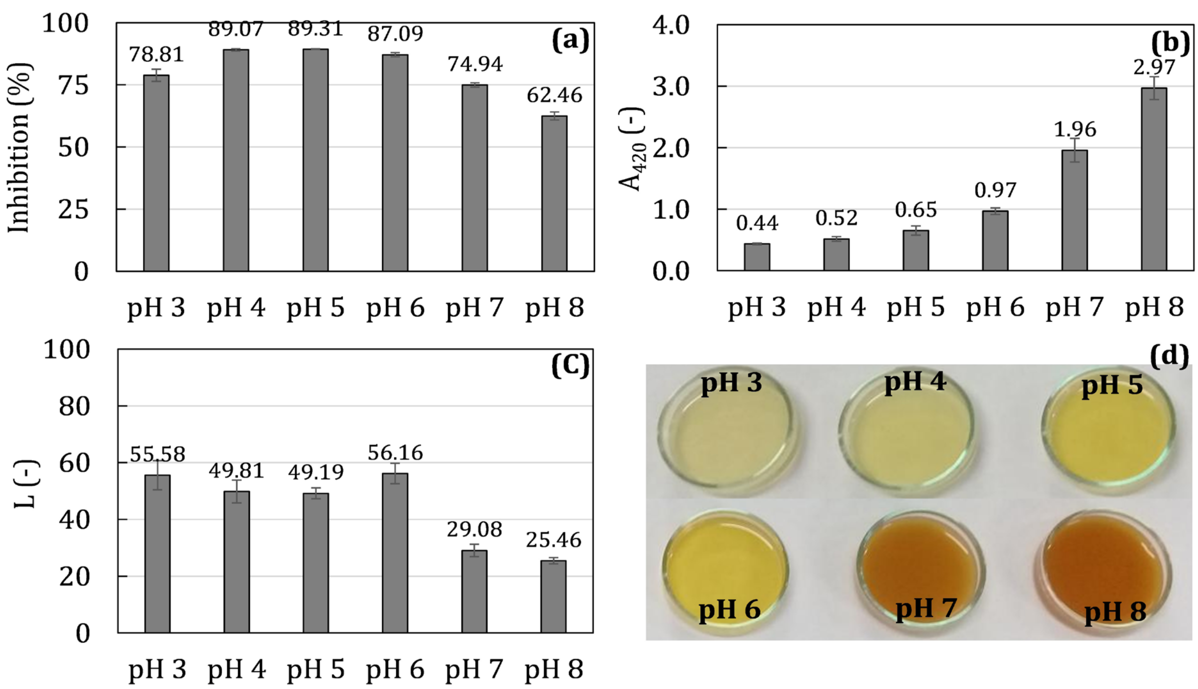

Figure 3. The antioxidant activity (a), browning intensity A_{420} (b), lightness value L (c), and the appearance (d) of reacted mixtures between water-soluble tempeh extract and whey powder at different initial pH values.

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The increases in browning intensity fitted relatively well with the reductions of lightness value (Figure 3b and 3c). However, the increases in browning intensity did not correspond well with the levels of antioxidant activity amongst treatments (Figure 3a and 3b). In this study, the caramelization reaction also contributed to the brown color in the reacted mixtures, especially at high temperatures and high pH values (Ajandouz *et al*., 2008). In alkaline conditions, the formation of Schiff base compounds was enhanced, thus, facilitating a higher rate of the Maillard reaction. Both caramelization and Maillard reactions simultaneously occurred (Wang *et al*., 2013). Martins *et al*. (2000) reported that the formation of hydroxymethylfurfural (HMF) or furfural compounds from Amadori rearrangement is preferred at low pH values (pH< 7.0), whilethe formation of reduction compounds and fission compounds, such as acetyl, diacetyl, and pyruvaldehyde, are preferred at high pH values (pH> 7.0). Within this study, the antioxidant activity of the reacted mixture at pH 5.0 was not significantly different from that of pH 4.0. However, it was substantially different from pH 3.0, 6.0, 7.0, and 8.0, at a 95% confidence level. It concludes that pH 5.0 was the optimal initial pH for the Maillard reaction between water-soluble extract and 5.0 % (w/v) whey powder concentration.

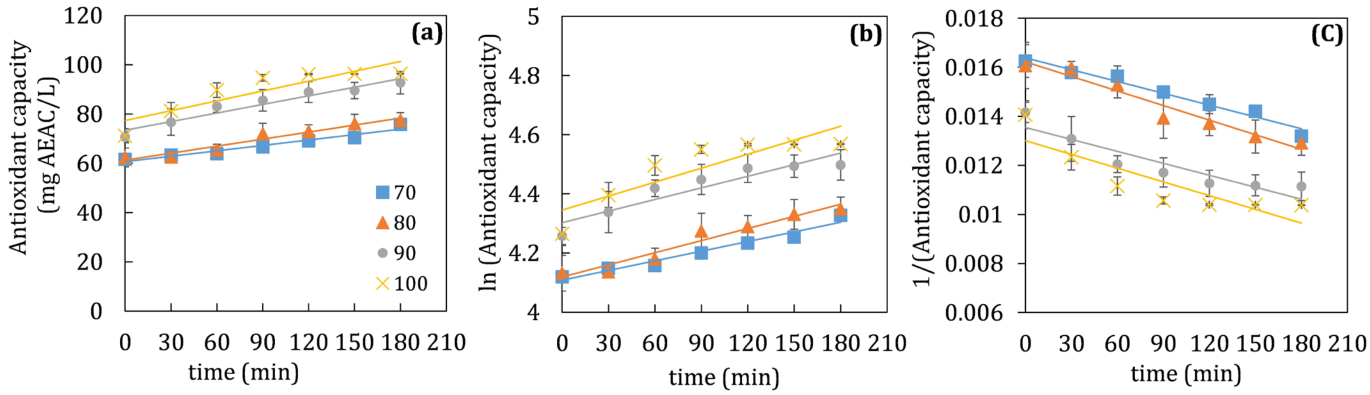


Figure 4. Kinetics of Maillard reaction following zeroth order (a), first-order (b), and second-order (c) of reaction

Table 1. Governing equations and coefficients of determination of the Maillard reactions at different heating temperatures.

Reaction order	Reaction temp. (°C)	Governing equation	Coef. Determination (R^2)
0^{th} order	70	0.0736x + 60.63	0.9427
	80	0.0949x + 61.297	0.9517
	90	0.1163x + 73.408	0.9333
	100	0.1335x + 77.336	0.7907
1^{st} Order	70	0.0011x + 4.1083	0.9554
	80	0.0014x + 4.1188	0.9496
	90	0.0013x + 4.3023	0.8664
	100	0.0016x + 4.3451	0.7711
2^{nd} order	70	-2E-05x + 0.0164	0.9659
	80	-2E-05x + 0.0162	0.9467
	90	-2E-05x + 0.0135	0.8489
	100	-2E-05x + 0.013	0.7498

Kinetics of maillard reaction based on antioxidant activity changes

The kinetics of the Maillard reaction was performed based on the changes in antioxidant capacity over a certain period. From Figure 4 (a-c) and Table 1, the highest coefficients of determination were obtained by following the zeroth order of the reaction. Reaction order with zero means the magnitude of the reaction rate constant is not affected by the initial concentration of the reactants, and the reaction rate constant increases with the increase in temperature. By employing eq. (4), a correlation between 1/T and ln k was obtained, $y = -2552.7\,x + 4.8552$ (Figure 5). The calculated activation energy Ea was 21.22 kJ/mol K.

This indicates that the minimum energy required to produce compounds exhibiting antioxidant activity was 21.22 kJ/mol K. The reported activation energy of the Maillard reaction ranges from 10-160 kJ/mol (Finot *et al.*, 1990). Ayranci and Dalgic (1990) reported the activation energies of the Maillard reaction of glucose-lysine and lactose-lysine were 153.1 and 162.5 kJ/mol K, respectively. Furthermore, the activation energy of the Maillard reaction between xylose and lysine with ultrasonic assistance was 55.59 kJ/mol K (Yu *et al.*, 2012).

3.4 Selecting the reaction time and temperature

There was a significant interaction between the reacting temperature and time at a confidence level of 95%. In general, the antioxidant capacity of the reacted mixture at 100 °C in each sampling plan was relatively higher than that at other temperatures. At 100 °C, the antioxidant capacity of the reacted mixture at 180 min of reaction was significantly different from 0, 30, and 60 min. However, this value was not significantly different from 90, 120, and 150 min of reaction. Therefore, the best time and temperature for the Maillard reaction between water-soluble tempeh extract and whey powder were 90 min and 100 °C, respectively. By this, the obtained antioxidant capacity was 94.64 ± 1.30 mg AEAC/L.

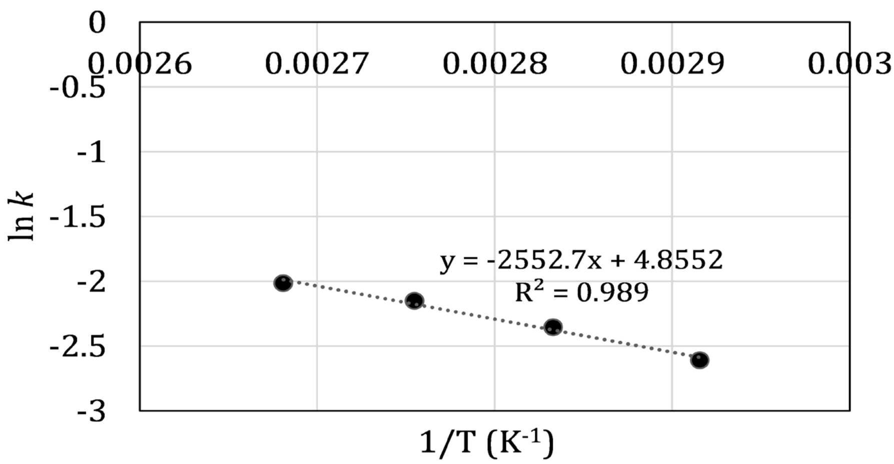
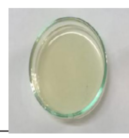


Figure 5. Correlation between 1/T and ln k.

Table 2. Comparison between the unreacted mixture and the optimal reacted mixture.

Mixture	Inhibition (%)	Browning intensity A_{420}	Lightness value L (-)	Appearance
Unreacted	67.91 ± 2.62	0.33 ± 0.003	63.30 ± 3.71	
After reaction (pH 5.0, T = 100 °C, t = 90 min)	89.89 ± 0.17	0.90 ± 0.081	48.28 ± 4.23	

The half-maximal inhibitory concentration (IC50) and amino acid composition

The Maillard reaction was considered successful to increase the antioxidant activity of the reacted mixture. This was indicated by the higher antioxidant activity of the optimal reacted mixture as compared to that of the unreacted mixture (Table 2). Additionally, the browning intensity of the reacted mixture was enhanced whilst having a lower lightness value than that of the unreacted mixture. The antioxidant activity of the reacted mixture with optimal reacting conditions was 89.89 ± 0.17 % with an IC_{50} value of 47.82 mg/mL. The calculated IC_{50} value for ascorbic acid (vitamin C) was 0,05 mg/mL. A lower IC50 value indicates stronger antioxidant activity (Salazar-Aranda *et al*., 2011). Hereby, the optimal reacted mixture of water-soluble tempeh extract and whey powder had a weaker antioxidant activity compared to that of ascorbic acid. On the other hand, the obtained antioxidant capacity as mentioned previously was 94.64 ± 1.30 mg AEAC/L. The reported antioxidant capacities of commercial ready-to-drink orange juices and nectars in Brazil were reported between 70-400 and 110-480 mg AEAC/L, respectively (Stella *et al*., 2011). Therefore, the antioxidant capacity of the optimal reacted mixture in this study was comparable to that of Stella *et al*. (2011). Moreover, the dominating amino acids for both water-soluble tempeh extract and optimal reacted mixture were glutamic acid, aspartate, lysine, and leucine (Figure 6). Several amino acids have been reported to have antioxidant properties, such as proline, histidine, tyrosine, cysteine, valine, leucine, phenylalanine, and tryptophan (Sitanggang *et al*., 2020b). These amino acids were also obtained in the optimal reacted mixture.

4. Conclusion

The optimal Maillard reaction conditions between water-soluble tempeh extract and whey powder were 5.0 % (w/v) whey powder, initial reaction pH of 5.0, a reaction time of 90 min, and a temperature of 100 °C. The Maillard reaction was performed with 21.22 kJ/mol activation energy. The Maillard reaction was considered successful as it could enhance the antioxidant activity from 67.91 ± 2.62 % to 89.89 ± 0.17 %. Additionally, the browning intensity of the reacted mixture was enhanced, thus having a lower lightness value than that of the unreacted mixture. The IC50 value of the product was 47.82 mg/mL with an antioxidant capacity of 94.64 ± 1.30 mg AEAC/L. This val-

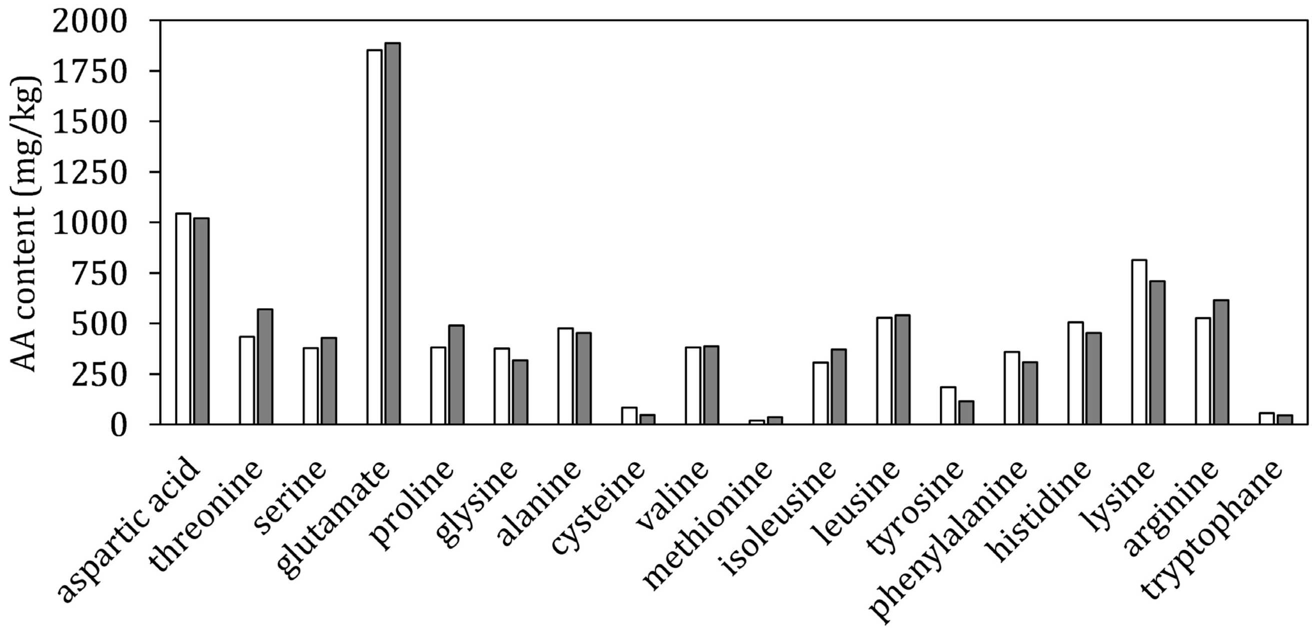


Figure 6. Amino acid (AA) composition of water-soluble tempeh extract (white bar) and optimal reacted mixture (grey bar).

ue was comparable with the antioxidant capacities of commercial ready-to-drink orange juices and nectars reported elsewhere.

Conflict of interest

The authors declare no conflict of interest.

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