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Production of milk analogues from rice bran protein hydrolysate using the subcritical water technique

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Abstract

Subcritical water treatment is a useful technique for the extraction of active compounds from biomass materials due to its short processing time, low cost, and environmental sustainability. Defatted rice bran is a by-product of rice bran oil, which still contains protein. In this study, rice bran protein was extracted using subcritical water treatment at temperatures of 120, 140, 160, and 180 °C and reaction times of 30, 45, and 60 minutes to determine the effects of following parameters on the production of milk analogues. At higher temperatures, the protein content and emulsifying activity index (EAI) increased, and the nitrogen solubility index (NSI) ranged from 87-100%. The emulsifying stability index (ESI) reduced when the reaction time was increased, while the molecular weight of the protein was less than 50 kDa. These temperatures and reaction times were shown to have direct impacts on both protein extraction and the molecular size of rice bran protein, which significantly increase the production of milk analogues. The results showed that milk analogues appeared to be more viscous than cow's milk, as evidenced by the shear thinning. In sensory evaluation, the color, texture, taste, and acceptability of cow's milk scored higher in comparison to milk analogues. All of the sensory parameters analyzed, except odor, were significantly different ($P \leq 0.05$).

Keywords: subcritical water; defatted rice bran; milk analogues.

Practical Application: Nowadays, many people are focusing more on their health, and the basis of having good health partly comes from food—the essence of human life—we cannot survive without it. Rice is considered to be the main agricultural product consumed by Asian people and many other nations. Thailand is ranked in the sixth position for rice production. The rice milling process produced 3.06 million tons of rice germ in 2011. Rice germ can be used in the production of rice milk, which is a chemical-free product containing fat. Rice milk is rich in nutrients such as iron, fiber, minerals, B1, B2, and Vitamin-E, and can help protect against allergies, acne, and also includes the antioxidant anthocyanin. As an environment-friendly technique, subcritical water extraction is utilized for decomposition and the extraction of protein within the rice germ to produce milk analogues.

1 Introduction

Nowadays, many people focus more on their health, and the basis of good health partly comes from food—the essence of human life—we cannot survive without it. Currently, manufacturers offer many types of beverages, such as milk, made from beans or animal products, to satisfy the needs of consumers, although some protein products can cause allergies.

Rice is considered to be the main agricultural product consumed by Asian people and many other nations. Thailand is ranked in the sixth position for rice production; below China, India, Indonesia, Bangladesh, and Vietnam, respectively, with 20.26 million tons in 2012 (Food and Agriculture Organization of the United Nations, 2012). However, for many decades, Thailand has held the top position for the quantity of rice produced.

In the milling process, aside from the milled rice, which accounts for 69.5%, with the by-products consisting of 20% rice husks and 10.5% rice germ. Accordingly, when the quantity of rice germ was deducted from the total amount of rice produced in 2011, it equated to 3.06 million tons. Rice germ contains 11.3-14.9 (g Nx5.95) of protein, while rice only contains 6.3-7.1 (g Nx5.95) and the husk 2.0-2.8 (g Nx5.95) (Shih, 2003).

The price of rice germ is usually around 0.13-0.16 USD per kg, while oil made from rice germ costs 2.87-3.19 USD per liter, meaning that the total value of rice germ can reach 54.27 USD per kg, providing the manufacturer can maximize the production of protein. Protein from rice germ can be utilized in many industries, such as bread production and infant food (Jiamyangyuen et al., 2005). It made up of amino acid (Shih, 2003) and can be used in the production of cereal, grain, protein supplements, drinks, as well as a meat and sausage component (Prakash & Ramaswamy, 1996). Protein from rice germ can also act as an antioxidant and has cancer-prevention characteristics (Kawamura & Muramoto, 1993).

Therefore, using rice germ in the production of rice milk results in fat and chemical-free product. This product provides

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excellent nutrition with iron, fiber, minerals, B1, B2, and Vitamin-E, which can help protect against allergies and acne. It also contains the antioxidant anthocyanin. Ricebased milk is, therefore, very beneficial to the whole body.

The most common method for the production of rice protein is alkaline hydrolysis, followed by acid precipitation. This method is simple and easily available (Jiamyangyuen et al., 2005). Unfortunately, the high pH conditions can lead to undesirable results, including protein modification, which decreases the native value and formation of the toxic compound e.g. lysinoalanine (Cheftel et al., 1985). Therefore, methods such as subcritical water extraction have been studied to overcome these problems. Recent research has been carried out on subcritical water for the decomposition and extraction of various compounds (Wiboonsirikul et al., 2007; Herrero et al., 2006). Subcritical water extraction is a new and powerful technique, as well as being environmentally friendly. It is performed using hot water at a temperature of between 100 and 374 °C and under high pressure to maintain its liquid state (critical point of water, 22.4 MPa and 374 °C) (Baig et al., 2013; Asl & Khajenoori, 2013; Goto et al., 2004). Based on recent research, it has been shown that subcritical water extraction is cleaner, faster, and cheaper than conventional extraction methods (Asl & Khajenoori, 2013) As the subcritical water extraction utilizes water, it gives benefits on both low-cost and environmental acceptable solvent (Wang et al., 2008).

Due to its innovative character, it is necessary to study rice bran beverage. This study aims to study methods for extracting protein from defatted rice bran, especially subcritical water extraction. Moreover, sensory evaluation and shelf-life of milk analogues are investigated in this study.

2 Materials and methods

In this study, the experiments were set in a laboratory and divided into three parts. First is the study time (30, 45, and 60 min) and temperature (120, 140, 160, and 180 °C) of the extraction of protein hydrolysis from defatted rice bran by subcritical water treatment. Second is functional protein hydrolysis from defatted rice bran. The third is the development of milk analogues from rice bran protein hydrolysis and the rheology, shelf-life, and sensory evaluation of milk analogues.

2.1 Materials

Defatted rice bran was obtained from Kasisuru Co., Ltd. (Nonthaburi, Thailand).

2.2 Subcritical water hydrolysis

Subcritical water extraction was performed using a stainless steel vessel resistant to high pressure and temperature. A sample consisting of 100 g of bran and 500 mL of distilled water was placed in the vessel. The extraction was performed at 120, 140, 160, and 180 °C for 30, 45, and 60 min at constant pressure 20 MPa. After the desired conditions were achieved, the vessel was immediately removed from the oven and cooled in an ice bath to reach room temperature. The bran mixture was centrifuged at 12,000 rpm for 20 min. The soluble products from hydrolysis were freeze-dried at -40 °C and stored in a desiccator until use, normally withing 24 hrs.

2.3 General properties of rice bran

The properties investigated in this study consist of emulsifying activity (EA), emulsion stability (ES), protein content, and nitrogen solubility index (NSI). The EA and ES were determined following the study by Pearce & Kinsella (1978) using a grey triggerfish (*Balistes capriscus*) protein sample dispersion of 1.0% (w/v) with distilled water. Soybean oil (10 mL) and protein (30 mL) samples were homogenized in a mechanical homogenizer at 22,000 rpm for two minutes at room temperature to produce the emulsion. The 50 µL portions of emulsion were pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1% SDS. The absorbance of emulsions was measured at 500 nm with a UV-VIS spectrophotometer. Therefore, the absorbance measured immediately after emulsion formation was expressed as the EA and ES of a protein. The EA and ES values acquired from the experiments are investigated with Equation 1 and 2, respectively.

$$\text{Emulsifying activity index}\left(\text{m}^2/\text{g}\right)=\frac{2\times 2.203\times \text{A}_{500}}{0.5\times \text{P}} \quad (1)$$

$$\text{Emulsifying stability index}\left(\text{min}\right)=\frac{\text{A}_0\times \Delta\text{T}}{\Delta\text{A}} \quad (2)$$

Where A_{500} is the absorbance of emulsion at 500 nm, P is the protein content, A_0 is the absorbance of the emulsion at 500 nm after homogenization, A10 is the absorbance of the emulsion at 500 nm after homogenization for 10 min, ΔT is 10 min, and ΔA is A_0-A_{10}.

The protein content of the bran extract was assessed by modifying the Lowry-Folin assay (Lowry et al., 1951). Standard aqueous solutions of bovine serum albumin (BSA) were used for the preparation of the calibration curve. An alkaline solution (5 mL) was mixed to the protein samples or the standard BSA solution. After standing for 15 min at room temperature, 25 °C, the diluted Folin-Ciocalteu reagent (1:1 w/v) was quickly added and mixed immediately. The mixture was stored for 30 min before measuring the absorbance at 750 nm using the UV-VIS spectrophotometer and deionized water.

For nitrogen solubility, protein dispersion (1%) was achieved with distilled water, centrifuged (1,000 g min^{-1}), and analyzed for protein content in the supernatant following the Lowry method (Lowry et al., 1951) using BSA as the standard. Protein solubility was calculated as grams of soluble protein per 100 g of the sample. Each sample was measured in triplicate. The outcome of experiments can be compared with the Equation 3.

$$\text{NSI}(\%)=\frac{\text{Total protein content in the supernatant}}{\text{Total protein content in the sample}}\times 100\% \quad (3)$$

2.4 Gel permeation chromatography

Molecular weight distributions of rice bran protein were determined by gel permeation chromatography (GPC) performed with TSKgel® G4000SWXL column (7.8 i.d. x 30 cm, TOSOH,

Tokyo, Japan) at 25 °C. The mobile phase consisted of 50 mM sodium phosphate (pH 7.2), 50 mM sodium sulfate, and a flow rate of 0.7 mL/min. Protein samples (5 mg/0.5 mL mobile phase), centrifugation at 10,000 g for 20 min, and filtration through a 0.45 µm nylon filter membrane. The elution was monitored at 280 nm. The protein standard mixture (Sigma-Aldrich, USA), containing thyroglobulin bovine (670 kDa), γ- globulins (150 kDa) albumin chicken (44 kDa), and ribonuclease (13 kDa) were used as molecular weight standards for calibration.

2.5 Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical electrophoresis unit according to a method proposed by Laemmli (1970), using 12% Mini-PROTEAN® TGXTM Precast protein gel, 10-well, 50 µl (BIO-RAD, CA, USA). A sample buffer was used for non-reducing SDS-PAGE. The protein samples (15 mg) were dissolved in a 300 µL sample buffer, and then heated in boiling water for 5 min and centrifuged at 10,000 g for 10 min before electrophoresis. Protein brans were stained with Bio-Safe™ Coomassie G- 250 stain.

2.6 Processing of milk analogues

The process shown in Figure 1 produces 100 mL of milk analogues, with 2.5 g of rice bran protein diluted with hot water to obtain a total soluble solid of 10%. Added to this soluble solid beverage were 2.5 g of rice bran oil, 1 g of lecithin, and 4 g of maltodextrin. The mixture was homogenized at 1,000 bars, with five cycles of microfluidizer. The proposed milk analogue was then produced.

2.7 Rheology

Steady shear viscometry was performed in triplicate at 25 °C using a concentric gap (0.053 mm), with an available parallel plate (60 mm in diameter) and a measuring system in a modular advanced rheometer system, HAAKE MARS 60 Thermo Fisher Scientific (Germany). After 10 s of equilibrium, shear stress was measured at 30 logarithmically spaced shear rates ranging from 0.1-100 s^{-1}. Each sample was measured in triplicate.

2.8 Sensory analysis

A panel of 30 experts investigated the descriptive sensory analysis. The number of panelist is acceptable for this preliminary research. The treated milk samples were refrigerated, randomly coded, and served at 15 °C in aliquots of 10 mL. A comparison between milk analogues and cow's milk was carried out using product-specific color, odor, texture, taste, and overall acceptability on a nine-point hedonic scale. The lowest and highest scores were 1 and 9, respectively.

2.9 Shelf-life

The selected quality parameters of the sample, namely, pH, appearance, conductivity, and particle size using the dynamic light scattering technique, were measured after processing and throughout the four-weeks shelf-life period under storage at room temperature.

2.10 Statistical analysis

Data were presented as mean ± SEM. Comparison among the groups was performed using one-way ANOVA followed by the Duncan's Multiple Range Test (MRT). Data were considered to be statistically significant when $P \leq 0.05$.

3 Results and discussion

3.1 Composition of defatted rice bran

The defatted rice bran used in this study was analyzed using near-infrared (NIR) spectroscopy, with the compositions shown

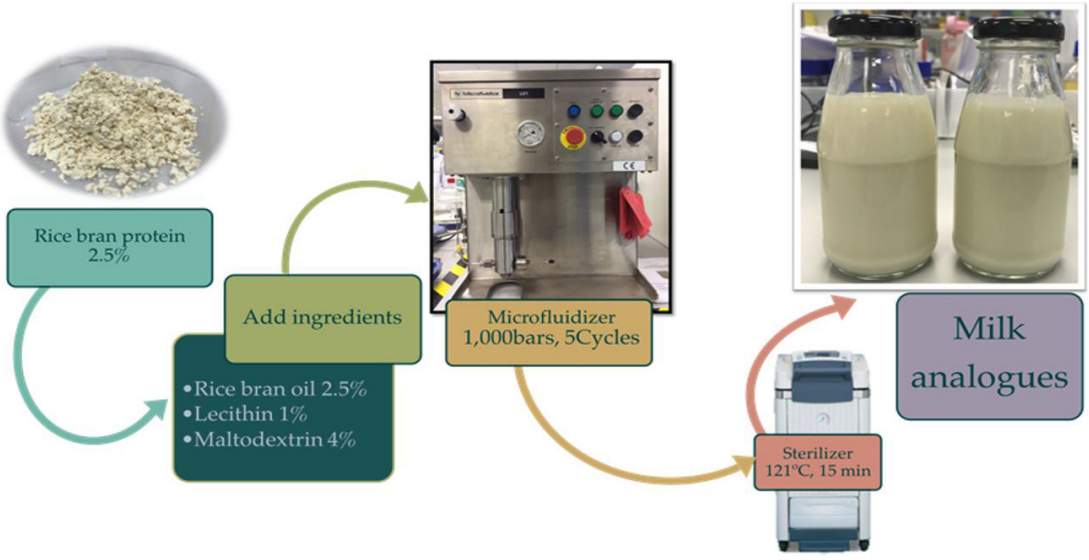

Figure 1. Milk analogues process.

in Table 1. The protein content of defatted rice bran was found to be 16%, fiber 11%, oil content 2%, moisture 12%, but with 12% impurity. The considerable protein content makes it an interesting material for protein extraction.

3.2 Experimental results of an investigation into rice bran properties

The protein content of defatted rice bran extracted using subcritical water hydrolysis is shown in Figure 2A. The results show that the protein content increases as the temperature rises (Sereewatthanawut et al., 2008). The highest protein content is shown at 180 °C, 60 min (30.31 ± 0.57%). The solubility of proteins is a critical factor in the acceptability of beverages, additives, and fortifiers (Chen et al., 2017; Zhang et al., 2015) . Rice bran protein has a nitrogen solubility index (NSI) ranging from 86-100% (Figure 2B).

Table 1. Proximate composition of defatted rice bran from supplier.

Analysis	Specification	Results
Protein (%)	15.00 (Min)	16.83
Fiber (%)	13.00 (Max)	10.63
Oil content (%)	2.50 (Max)	2.27
Impurity (%)	12.00 (Max)	12.00
Moisture (%)	12.50 (Max)	12.40

Source: Kasisuri Co., Ltd. (2016).

The solubility of rice bran protein can increase according to time and temperature. Due to the presence of an extensive hydrogen-bonded structure, water is the most polar solvent with a high dielectric constant (80 at 25 °C) at room temperature and atmospheric pressure. The high levels of H^+ and OH^- (ion concentrations may have higher orders of magnitude than in ambient water) at subcritical conditions mean that many acid or base-catalyzed reactions are accelerated, such as degradation, biomass hydrolysis, and the dehydration of carbohydrates (Teo et al., 2010). When the temperature reaches 160 °C, the solubility decreases in value due to the start of aggregation (Zhang et al., 2012). The increased protein solubility could be due to smaller protein size because of the higher in-contact surface area between the protein and water (Jambrak et al., 2009). High nitrogen solubility is required for protein concentrates used as functional ingredients in many foods (Chandi & Sogi, 2007). The EA and ES of rice bran protein hydrolysis are shown in Figures 2C, 2D.

The emulsifying activity index (EAI) increased significantly ($P \leq 0.05$) when the temperature of the extraction was raised. The results in Figure 2C show the EAI stable at 160 and 180 °C, but significantly higher compared to 120 and 140 °C ($P \leq 0.05$). The high temperature affected the molecular size of the protein, which was smaller than at low temperatures. This result is consistent with the finding of Pu et al. (2017) who mentioned that high temperature of subcritical water can increase probability of particle collision, leading to the aggregation and formation of

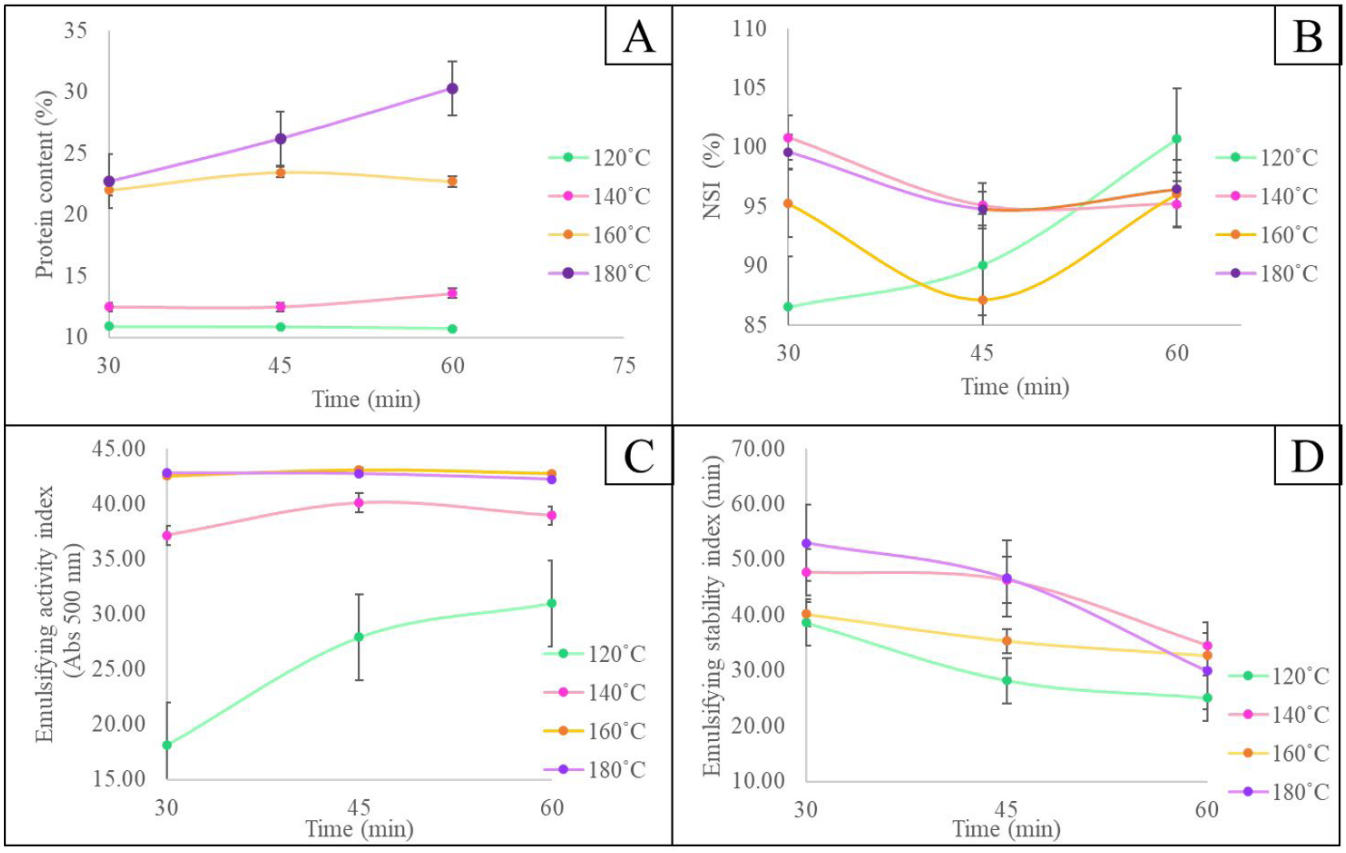

Figure 2. General properties of rice bran: (A) Rice bran protein hydrolysate; (B) Nitrogen solubility index of rice bran protein hydrolysate; (C) Emulsifying activity index (EAI) (Abs 500 nm); and (D) Emulsifying stability index (ESI).

largeparticles. The emulsifying stability index (ESI) is shown in Figure 2D. The reaction time of extracted rice bran is reflected in the ESI values. The ESI reduced when the reaction time increased, which might be due to the smaller molecular size of the protein (Liu et al., 2020).

3.3 Protein electrophoresis

The electrophoresis patterns of rice bran protein extraction (Figure 3) were determined at various times and temperatures, revealing a decrease in the molecular weight.

The bands of extracted protein at various times and temperatures were observed between 10-150 kDa. The study by Hamada et al. (1998) determined the molecular weight range of RBP hydrolysates between 1 and 150 kDa, which is similar to the degree of rice bran protein hydrolysis in this study.

Size exclusion was measured using gel permeation chromatography (GPC) (Figure 4). The results could not be observed since the molecular size of rice bran protein was lower than the standard peaks. The molecular size showed a decreasing trend when the extraction time was increased. It can be confirmed that the molecular size of rice bran protein was smaller than 15 kDa. The experiments observed the molecular size of rice bran protein by using SDS-PAGE and GPC. These methods show the same trend, namely a molecular rice bran protein size of less than 15 kDa.

One can notice the unknown peak at 15 min compared to the standard. However, that peak is eliminated by temperature, and in this work, we focus those peaks after 27 min.

3.4 Rheology

Milk analogues appear to be more viscous and less Newtonian than cow's milk. The flow curves for milk analogues and cow's milk are clearly distinguishable (Figure 5). The apparent viscosities of all samples can increase with the shear rate. This behavior is evidence of shear-thinning properties. This type of behavior is reported for many hydrocolloid solutions due to the formation of aggregated polymers in solutions and their high molecular weight (Saha & Bhattacharya, 2010). At a low shear rate, the aggregates can remain strongly associated but may be easily broken up with a high shear effect.

3.5 Sensory analysis

Table 2 shows the results of the experiment, whereby the color, texture, taste, and overall performance of cow's milk scores are higher than for milk analogues and significantly different ($P \leq 0.05$). However, the odor is not significantly different ($P > 0.05$).

3.6 Shelf-life

The results in Table 3 indicate that the pH value is not significantly different; therefore, the milk analogues are not spoiled. The z-average PDI and zeta potential show no significant difference ($P \leq 0.05$) when kept at room temperature for four weeks. The zeta potential indicated the ability of agglomeration and formation of the system, which consequently associated with the stability of the particles (Beliciu & Moraru, 2011). According to the definition of zepotential reported by Riddick (1968), the values showed in Table 3 are in ranged -16 to -30 mV, mean that it is threshold of delicate dispersion.

4 Conclusions

This study is divided into three parts. Firstly, the extraction of protein hydrolysate from defatted rice bran using subcritical water treatment is performed in various conditions and at different reaction times (30, 45, and 60 min) and temperatures (120, 140, 160, and 180 ˚C). Secondly, the function of protein hydrolysis from defatted rice bran is analyzed. Thirdly, the development of milk analogues from rice bran protein hydrolysis is illustrated.

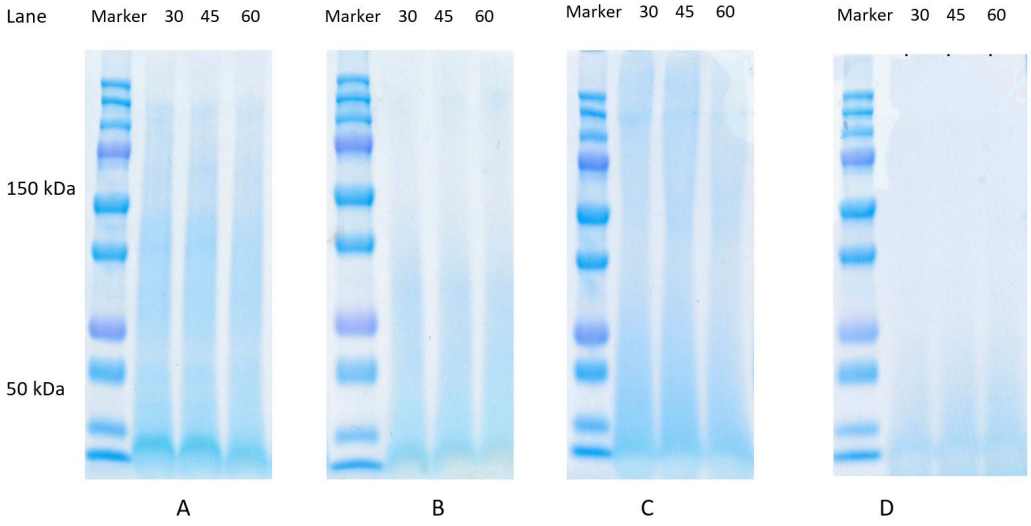

Figure 3. SDS-PAGE of rice bran protein during different conditions: (A) Protein standard mixture 15-600 kDa; (B) RBP 120 °C; (C) RBP 140 °C; (D) RBP 160 °C.

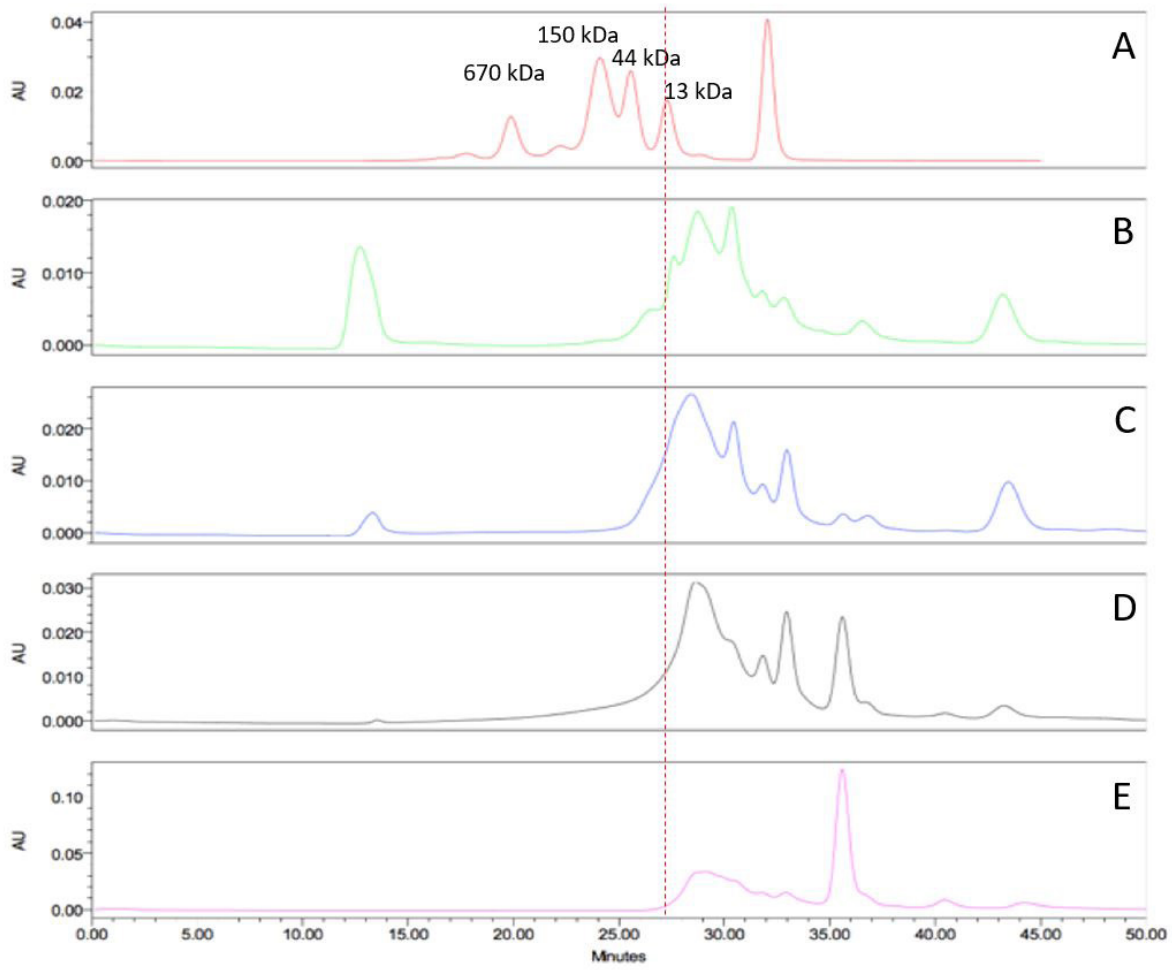

Figure 4. Effect of SCW on the RBP chromatography profile: (A) Protein standard mixture 15-600 kDa; (B) RBP 120 °C; (C) RBP 140 °C; (D) RBP 160 °C; and (E) 180 °C for 30 min.

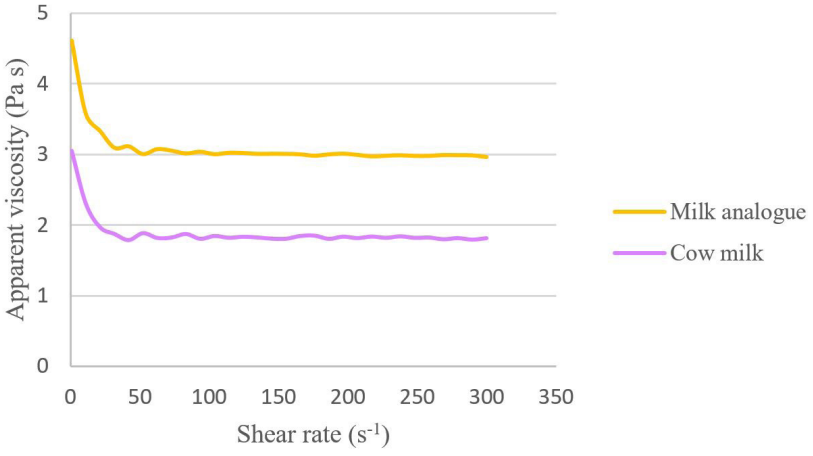

Figure 5. Flow curves (single measurements) for milk analogues and cow's milk.

Table 2. Sensory test for milk analogues and cow's milk.

Example	Color	Odor [ns]	Texture	Taste	Overall
Milk analogues	6.43 ± 1.38[b]	6.5 ± 1.28	6.2 ± 1.65[b]	5.36 ± 1.73[b]	5.93 ± 1.44[b]
Cow's milk	7.33 ± 0.99[a]	6.6 ± 1.54	7.33 ± 0.96[a]	7.36 ± 0.93[a]	7.46 ± 1.07[a]

Different letters in the column indicate significant differences ($P \leq 0.05$) among groups, [ns] No significant difference ($P > 0.05$).

Table 3. Shelf-life of milk analogues and cow's milk.

Week	pH	Z-Average	PdI	Zeta Potential
0	6.12 ± 0.003[b]	141.63 ± 0.59[b]	0.16 ± 0.005[b]	-27.3 ± 2.23[b]
1	6.09 ± 0.006[b]	138.1 ± 0.56[b]	0.17 ± 0.005[b]	-26.4 ± 2.98[ab]
2	6.15 ± 0.003[b]	149.9 ± 0.47[b]	0.17 ± 0.007[b]	-28.4 ± 0.166[b]
3	6.03 ± 0.012[b]	144.3 ± 0.55[b]	0.13 ± 0.001[c]	-20.8 ± 0.81[a]
4	6.08 ± 0.006[b]	145.6 ± 0.61[b]	0.17 ± 0.006[b]	-23.8 ± 0.96[ab]
Cow milk	6.86 ± 0.009[a]	317.4 ± 1.36[a]	0.22 ± 0.002[a]	-28.5 ± 0.87[b]

Different letters in the column indicate significant differences ($P \leq 0.05$) among groups.

Finally, the rheology, shelf-life, and sensory evaluation of milk analogues are analyzed to guarantee quality.

The rice bran protein was prepared using subcritical water hydrolysis. The highest protein content was shown at 180 °C and 60 min (30.31 ± 0.57%). The protein content and EAI increased at high temperatures, while the ESI decreased when the reaction time was reduced. The rice bran protein had an NSI ranging from 86-100%. The reaction time and temperature affected the molecular size of rice bran protein. The molecular weight of the protein was less than 50 kDa. The most suitable reaction time and temperature for protein extraction were 120 °C and 30 min to produce milk analogues. Milk analogues appear to be more viscous than cow's milk, and both show the same shear-thinning behavior trend. In the sensory test, the color, texture, taste, and acceptability of cows milk scored higher than for milk analogues, with significant differences ($P \leq 0.05$). However, the odor was not significantly different ($P > 0.05$). Milk analogues could be stored for at least one month at room temperature. The contribution of this work covers the potential technique to produce milk analouge and the direction to develop. As the research worked closely to sponsor and used their resource, the outcome from this will be further experimented by introduce proportion ingredients, flavor, color to meed the need of sponsors and consumers

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