

Enzymatic hydrolysis on protein and β -glucan content of Sang-yod rice bran hydrolysates and their anti-inflammatory activity on RAW 264.7 cells

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Submission Date: September 1st, 2017, **Acceptance Date:** December 27th, 2017, **Publication Date:** December 31st, 2017

Citation: Phantuwong N., Thongraung C., Yupanqui C.T., Enzymatic hydrolysis on protein and β -glucan content of Sang-yodrice bran hydrolysates and their anti-inflammatory activity on RAW 264.7 cells. *Functional Foods in Health and Disease* 2017; 7(12): 958-971

ABSTRACT

Background: Research focusing on the improvement of the utilization of rice bran is increasing due to its nutritional properties. Several biological activities of rice bran hydrolysates and its constituents have been reported. Sang-yod rice, a local rice variety in Southern Thailand, is a pigmented rice. Furthermore, its bran has high nutritive value and health beneficial components. Accordingly, there is growing interest in transforming this by-product into a functional food ingredient.

Objective: To investigate the effect of enzymatic hydrolysis processes on the digestion of protein and β -glucan and evaluate anti-proinflammatory properties of selected hydrolysates on RAW 264.7 macrophage cells.

Method: Sang-yod rice bran hydrolysates were obtained using a single or co-enzymatic hydrolysis process and sequential hydrolysis process using amyloglucosidase and protease G6. Effects of enzyme concentration (3-5% v/w) and hydrolysis duration (30, 60, and 120 min) on soluble protein and β -glucan contents of obtained rice bran hydrolysates were evaluated. The selected rice bran hydrolysates were evaluated for their cell viability and inhibition against NO and pro-inflammatory cytokines generation on RAW 264.7 mouse macrophage cell lines.

Results: Protein content (0.59-3.37 %) of the rice bran hydrolysates (RBHs) was increased by increasing of enzyme concentration (3-5% v/w) and hydrolysis time (60-120 min). However, the β -glucan content (0.88-4.63%) of RBHs decreased with the increase of those parameters. The RBHs derived by the sequential process using 5% v/w enzyme concentration and 60 min hydrolysis time

gave high protein (3.23%) and high β -glucan (4.02%) contents. The hydrolysates with high amount of protein and/or β -glucan contents demonstrated no cytotoxicity against RAW 264.7 cells at a concentration range of 100-2,000 $\mu\text{g/ml}$. Additionally, they demonstrated NO inhibition and pro-inflammatory inhibition ranges of 49.09-71.63% and 9.37-71.96% respectively. Generation of TNF- α , IL-6, and IL-1 β cytokines was inhibited differently by the selected RBHs.

Conclusion: Pre-digestion of Sang-yod rice bran with amyloglucosidase followed with co-hydrolysis of amyloglucosidase and protease G6 of the sequential hydrolysis process was the most effective process to release β -glucan and protein from the rice bran. The hydrolysate obtained from the process using an enzyme concentration at 5% v/w and 60 min hydrolysis duration of each stage had the highest soluble β -glucan and protein content. Moreover, the process provided the hydrolysates with potential anti-inflammatory properties on nitric oxide inhibition and pro-inflammatory cytokine inhibition on RAW 264.7 macrophage cell lines.

Keywords: Sang-yod rice, Rice bran hydrolysate, β -glucan, Enzymatic hydrolysis, Anti-inflammatory activity

BACKGROUND

Rice bran comprising about 10–15 % by weight of rough rice is a by-product from the rice-milling process [1]. Recently, there has been rising research attention towards increasing its utilization because of its nutritional properties [2]. Rice bran is generally accepted as a rich source of protein and other nutrients. Rice bran's protein contains unique nutritional and hypoallergenic properties compared to proteins from other cereals and legumes [3, 4, 5]. Moreover, a recent study revealed that rice bran hydrolysate is composed with β -glucan as a water-soluble polysaccharide [6]. Many nutrients and bioactive compounds known as antioxidants, including phenolic compounds, are found in pigmented rice and particularly in rice grain. Alternative products from rice have reported that five varieties of pigmented rice from Thailand revealed low to medium glycemic index, and could have potential of being used in diabetic diets [7]. Utilization of ricegrass can be improved in nutritional value and bioactive compounds, in addition to antioxidant activity [8, 9].

Hydrolysates from rice brans have been found to exhibit several biological activities including anti-oxidant and anti-metabolic syndrome activities [10]. They are able to lower the risk of hypercholesterol, coronary heart disease, and cancer formation [11]. Furthermore, they exert anti-inflammatory activity, anti-diabetes [12], and support the growth of beneficial intestinal micro flora [13].

Sang-yod rice is a pigmented rice and an indigenous Southern Thai rice cultivar [14]. There are bioactive compounds especially in anthocyanin, which is presented in the bran layer. Sang-yod rice bran hydrolysate or extract has been discovered to exhibit apparent antioxidant activity and anti-inflammatory activity [15, 16]. Therefore, hydrolysates have the potential to be developed into bioactive ingredients for functional foods and nutraceutical products.

The objectives of the present study were to investigate the effect of enzyme and hydrolysis conditions on the release of β -glucan and protein from Sang-yod rice bran and to ascertain anti-proinflammatory properties of selected hydrolysates on RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Materials:

Sang-yod rice bran (SYRB) (*Oryza sativa* L.) was obtained from a small rice mill located in Phatthalung province in southern Thailand. The SYRB was sieved with a 40-mesh screen. A commercial Protease G6 (alkali serine protease) was purchased from Siam Victory Chemicals Co.,

Ltd (Thailand). Amyloglucosidase from *Aspergillus niger* (A7095) was purchased from Sigma-Aldrich Co. Ltd.

Preparation of Sang-yod rice bran hydrolysates:

The fine SYRB was suspended 5 times in distilled water and adjusted to pH 8.0 with 1 N NaOH. The alkaline suspension was sonicated at 55 °C and at the frequency and power of 20 kHz and 750 watts respectively for 1 hour. The treated suspension was subjected to a single- or two-stage enzymatic hydrolysis at pH 8.0 and 55°C. In the single hydrolysis process, either amyloglucosidase (A) or protease G6 (P) at 3, 4, or 5% v/w of SYRB was used. The hydrolysis process of amyloglucosidase at 3, 4, or 5% v/w was named as 3A, 4A, or 5A respectively. This notion was also applied for the process using protease G6 which is named 3P, 4P, or 5P. In another case these two enzymes at equal concentrations, 3, 4, or 5% v/w of SYRB, were combined and used in the single digestion. The process would be named as a co-hydrolysis process. In the two-stage enzymatic hydrolysis, digestion was started by using amyloglucosidase and afterwards an identical concentration of protease G6 was added into the mixture to co-hydrolyze the suspension. The process would be named as a sequential hydrolysis process.

The hydrolysis duration for each stage was either 30, 60, or 120 min. Thus, the hydrolysis duration of the sequential hydrolysis process would last for 60, 120, or 240 min respectively. The processes were terminated by raising the suspension to 95 °C for 10 min. Non-soluble residue was packed by centrifugation at 10,000 g for 30 min at 4°C. The supernatants or Sang-yod rice bran hydrolysates (RBHs) were then separated and freeze-dried.

Determination of total protein content and total β-glucan contents:

Total protein content of the RBHs was determined by the Kjeldahl method [17]. Total β-glucan content of the RBHs was determined by using a Megazyme assay kit [18].

Effect of the Sang-yod rice bran hydrolysates on cell viability:

Cell viability was determined by the MTT colorimetric assay, which was described within S. Wang's study [19]. Briefly, RAW 264.7 cells were seeded in a 96-well plate at a density of 1×10^6 per well. The RBHs were added to the media at appropriate concentrations for cultivation of cells at 37°C for 48 h under humidified air containing 5% CO₂. Following pouring off the culture media, 0.1 mL of 5 mg/mL MTT solution was added to each well. After incubation for 2 h, DMSO was added onto the supernatant-drained cell layer to dissolve the intracellular chromogen. The absorbance of the supernatant was read in a microplate reader at 570 nm. The cytotoxicity as a percentage of cell death was calculated by the formula:

$$[(\text{Abs of sample} - \text{Abs of control})] \times 100$$

Effect of Sang-yod rice bran hydrolysates on NO production by RAW 264.7 cell lines:

NO production by RAW 264.7 cell lines was determined using a method modified by [16]. Briefly, RAW 264.7 cells were seeded in a 96-well plate at a density of 1×10^6 per well and cultured for 2 h. After that the cells were pretreated with SYRB hydrolysates (100, 500, 1000, 1,500, and 2,000 µg/ml) containing 0.5 µg/ml of LPS for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griss reagent (0.1% N-(1-naphthyl) ethylenediamine, 1% sulfanilamide in 5 % phosphoric acid). Their absorbance was measured at 570 nm using a microplate reader. The percent NO inhibition was calculated as:

$$[((\text{Abs control} - \text{Abs blank control}) - (\text{Abs sample} - \text{Abs blank sample})) / (\text{Abs control} - \text{Abs blank control})] \times 100$$

Inhibitory activity of Sang-yod rice bran hydrolysates against pro-inflammatory cytokines:

RAW 264.7 cells were seeded in a 96-well plate at a density of 1×10^6 per well and cultured for 2 h. After LPS media were treated for 6 h., the RBHs were added and incubated for 48 h. The pro-inflammatory cytokine (TNF- α , IL-1 β , and IL-6) concentrations in the supernatant were determined by a quantitative sandwich enzyme-linked immune-sorbent assay (ELISA) using the Murine TNF- α , IL-1 β , and IL-6 ELISA development kit (Peprotech, Rocky Hill, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm by a microplate reader.

Statistical analysis:

All measurements were expressed as mean values \pm standard deviation (SD) from triplicate experiments. Data analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) with ANOVA. A Duncan multiple range test (DMRT) was performed to separate means, and their differences were reported as being significant at the 95% confident level ($p \leq 0.05$).

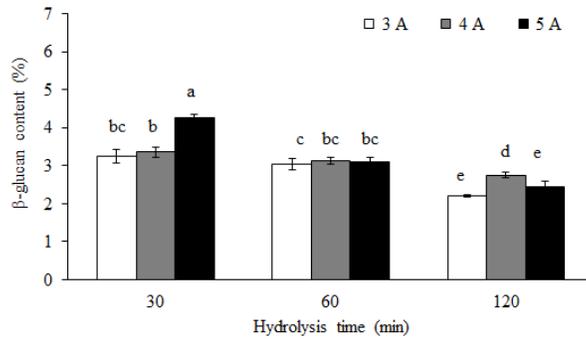
RESULTS AND DISCUSSION**Sang-yod rice bran hydrolysates derived by using a single enzyme hydrolysis:**

Effects of enzymes and hydrolysis conditions on β -glucan and protein contents of the RBHs are shown in Figure 1. The β -glucan content of the RBHs was in the range of 0.73-4.26 % (w/w) (Figure 1A, B). These would represent an extractable glucan range of 0.04-0.21 % g/100g of the rice bran. The highest extractable value was close to the glucan content of Hom Mali 105 (Bureerum) rice bran, 0.22 % w/w [20]. The β -glucan content of the RBHs obtained by using amyloglucosidase hydrolysis was higher than those of the Protease G6-derived RBHs. It was increased through the increasing of amyloglucosidase concentration but decreased through the increasing of hydrolysis time. The highest content (4.26 % w/w) was obtained by using 5 % w/v amyloglucosidase for 30 min (5A-30).

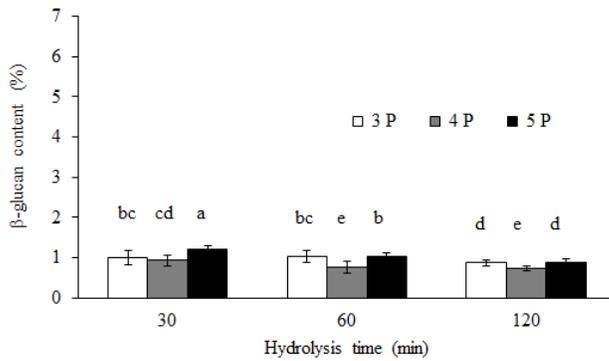
The amyloglucosidase enzyme specifically cleaves the (1-4)- β -linkage next to a (1-3)- β -linkage at the reducing end to yield (1-4)-link-oligosaccharide with one (1-3)-linked glucose unit [21]. Accordingly, its activity likely accounts for the increasing of soluble β -glucan. On the other hand, reduction in soluble β -glucan of the RBHs, especially through the lengthening of hydrolysis time, is likely due to an excess activity of amyloglucosidase specifically against the soluble β -glucan [22]. An unexpected result which was discovered was that a significant amount of β -glucan (0.76-1.20%) was also available in the hydrolysate derived by the proteolytic process. This observation may due to the substantial amount of protein binding with the structural polysaccharides of rice bran. Thus, the digestion of a peptide bond may not only liberate soluble peptide but also release β -glucan, at least partially, as a protein bound β -glucan (Figure 1B).

The protein content of the RBHs was in the range of 0.43-1.16 % (w/w) (Figure 1C, D) by using single a single enzyme hydrolysis. As expected, a high amount of soluble protein was found in the RBHs derived by using protease. The highest content found in the RBH was obtained by using the highest Protease G6 concentration (5% w/v) for 60 min (5P-60). Interestingly, a significant amount of soluble protein (0.35-0.65%) was co-released with β -glucan after the amyloglucosidase treatment. As soluble protein is accounted for a major part of rice bran protein [23], it may thereby readily leach out by the destruction of rice bran through amyloglucosidase activity. Moreover, as protein is also embedded into rice bran matrix, the catalytic activity of amyloglucosidase may also release protein, at least partially, as a protein bound β -glucan.

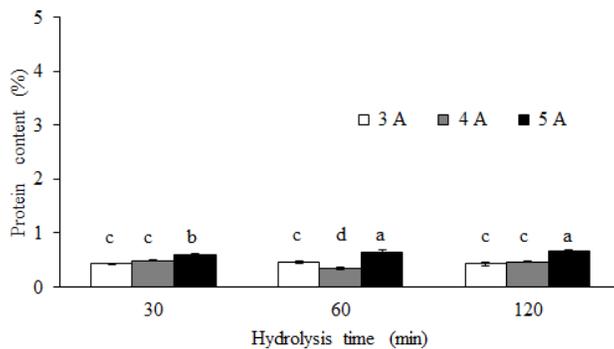
(A)



(B)



(C)



(D)

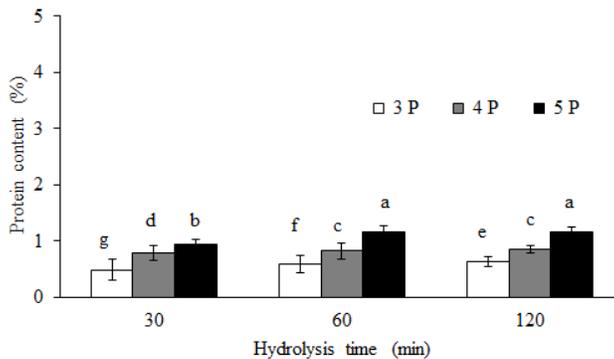


Figure 1. Effect of enzymes and their concentrations and hydrolysis times on β -glucan (A, B) and protein content (C, D) of Sang-yod rice bran hydrolysates. Note: The rice bran hydrolysates were prepared from Sang-yod rice bran using single enzymatic hydrolysis of amyloglucosidase (A) or protease G6 (P) either at concentration of 3, 4, or 5 % v/w, namely 3A, 4A, and 5A or 3P, 4P, and 5P respectively. Values are as mean \pm SD of triplicate determinations. Bars with different alphabets represent significant difference ($p \leq 0.05$).

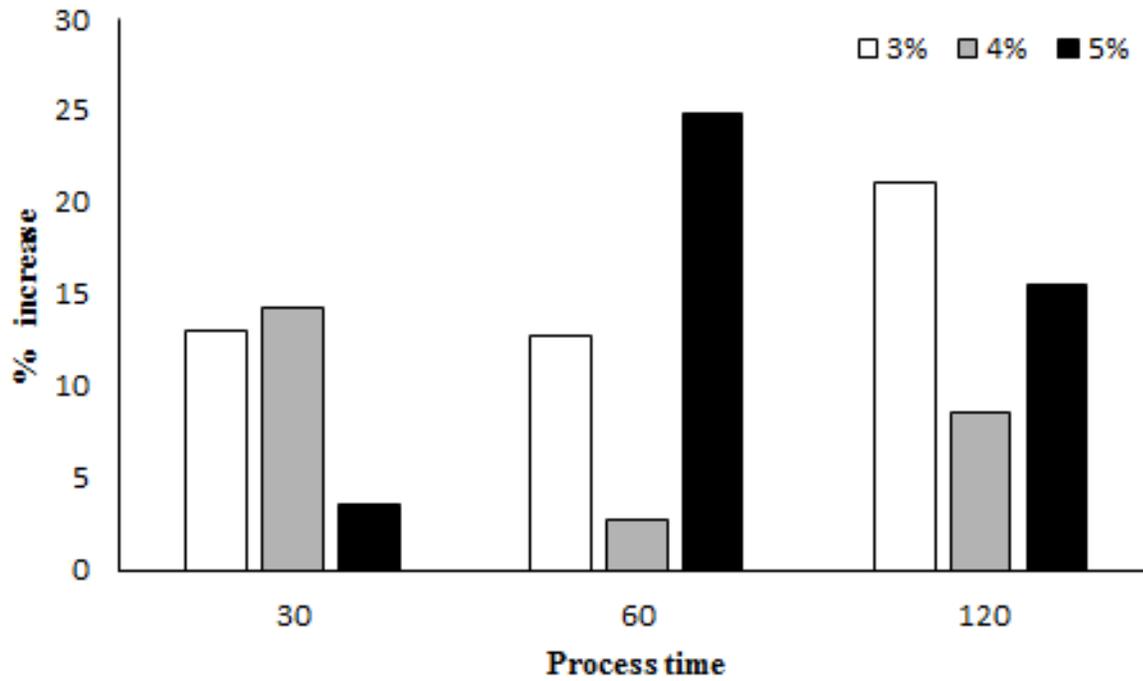
Sang-yod rice bran hydrolysates derived by the co-hydrolysis process:

We discovered that either β -glucan was co-extracted with rice bran protein due to the catalytic activity of protease G6, or protein was co-extracted with β -glucan due to the catalytic activity of amyloglucosidase, which thereby led to an interest in whether the co-hydrolysis of these enzymes may enhance the release of both components. Additionally, there was evidence that co-hydrolysis of amyloglucosidase and protease G6 or A+P process increased both soluble β -glucan and protein contents of the RBHs. The hydrolysate with the highest β -glucan ($4.42\pm 0.18\%$) and protein ($2.16\pm 0.02\%$) content was produced by using an enzyme concentration at 5% for 30 and 120 min respectively.

The results demonstrated in Figure 2A and 2B are the percentage increase of soluble β -glucan and protein contents of the RBHs derived by the co-hydrolysis process relative to those corresponding to the single enzyme hydrolysis process. The process enhanced the release of both components and a massive increase of soluble protein content was noted. The effect of hydrolysis duration on β -glucan content as observed in the single enzyme hydrolysis was dismissed due to the existence of protease G6. This may be accounted for by an increase of accessible sites for amyloglucosidase due to rice bran digestion of Protease G6. Increase of β -glucan content suggests less accumulation of reducing sugar in the hydrolysate [22]. Thus, the results support the presupposition that the destruction of rice bran structure by either enzyme facilitates a release of both β -glucan and protein. In order to verify the advantage of amyloglucosidase activity against protein recovery, the co-enzyme hydrolysis was also arranged into a sequential (A→P) hydrolysis process starting with a pre-digestion of amyloglucosidase before the addition of Protease G6 for the co-hydrolysis. This process protocol was further improved through the deliberation of both β -glucan and protein. The hydrolysates with the highest β -glucan ($4.63\pm 0.05\%$) and protein ($3.37\pm 0.04\%$) content were produced by using the processing of 5A→5P: 30 min and 5A→5P: 60 min respectively.

The effects of the sequential hydrolysis process on the changes of β -glucan or protein contents of hydrolysates relative to those obtained by using the co-hydrolysis process are presented in Figure 3A and 3B. Even though the process duration of amyloglucosidase activity was the extent by the arrangement we proved that its activity was as high as 80% throughout the experiments. It was discovered that at certain conditions the incorporation of the pre-digestion could increase soluble β -glucan content in addition to the soluble protein content of the hydrolysate. Improvement on digestion of rice bran protein especially by using enzyme concentration at 5% v/w was prominent. Destruction of rice bran matrix by amyloglucosidase activity is thereby likely to facilitate the peptide bond digestion as previously described.

(A)



(B)

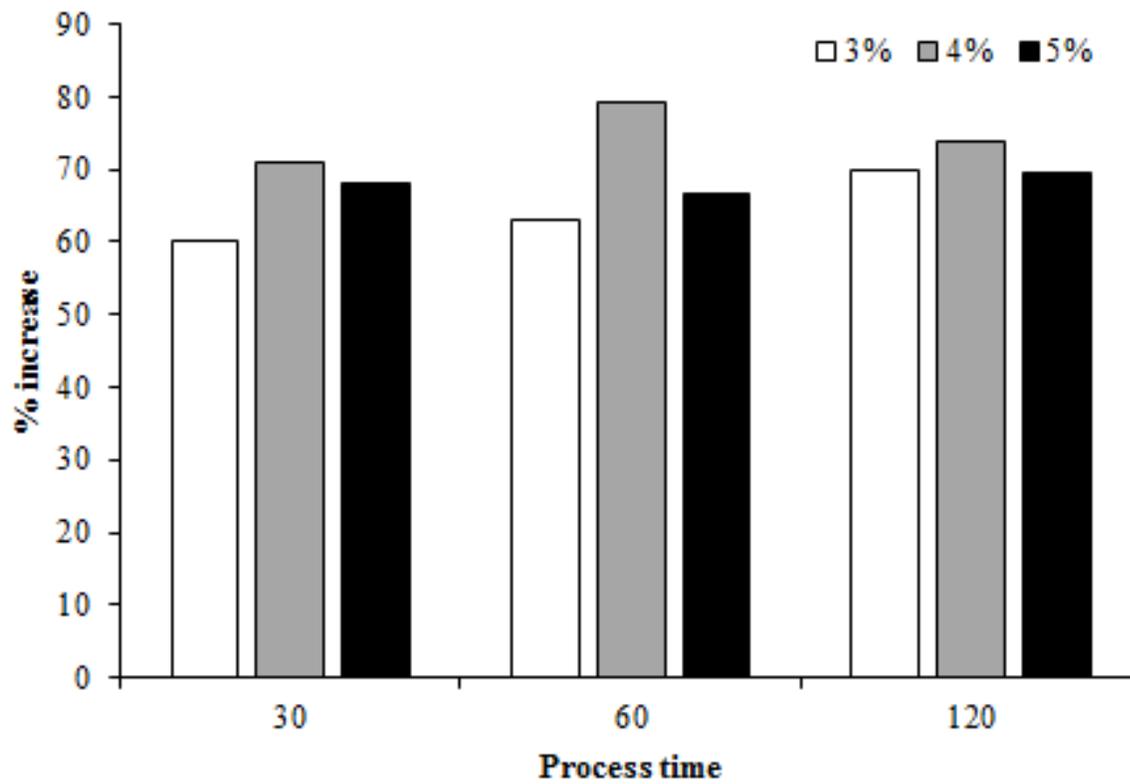
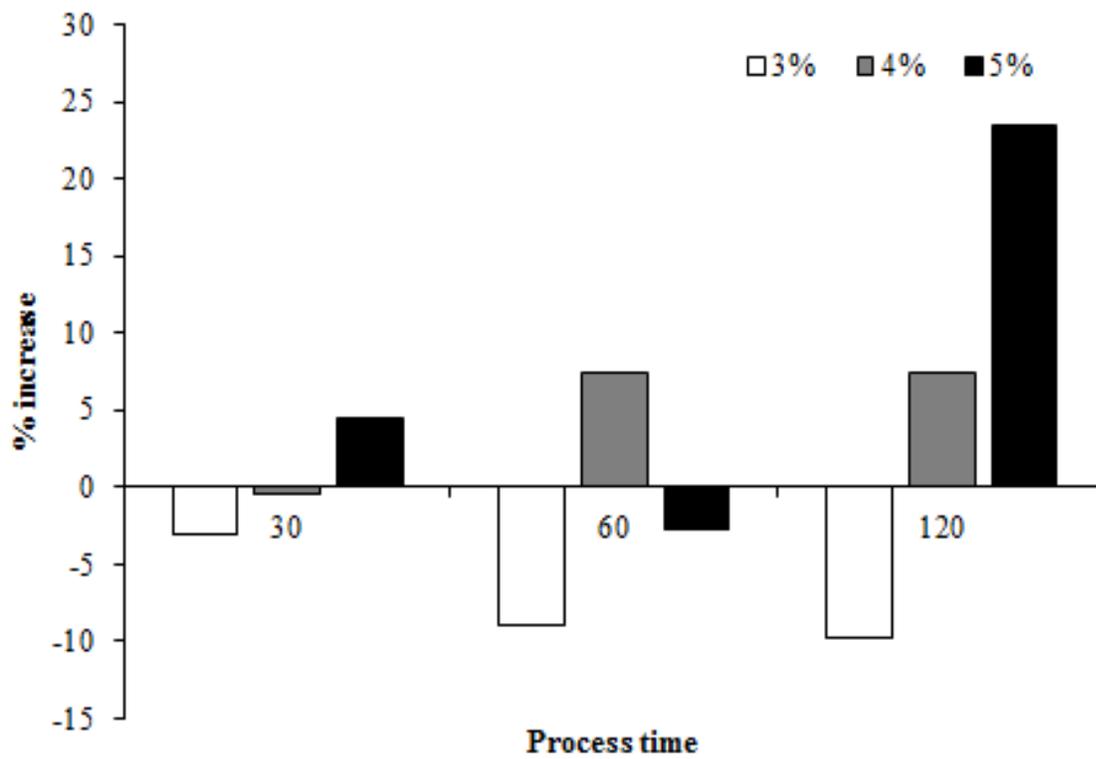


Figure 2: Percentage increase of β -glucan (A) and protein content (B) of the rice bran hydrolysate derived by using the co-hydrolysis process relative to those of the single hydrolysis process.

(A)



(B)

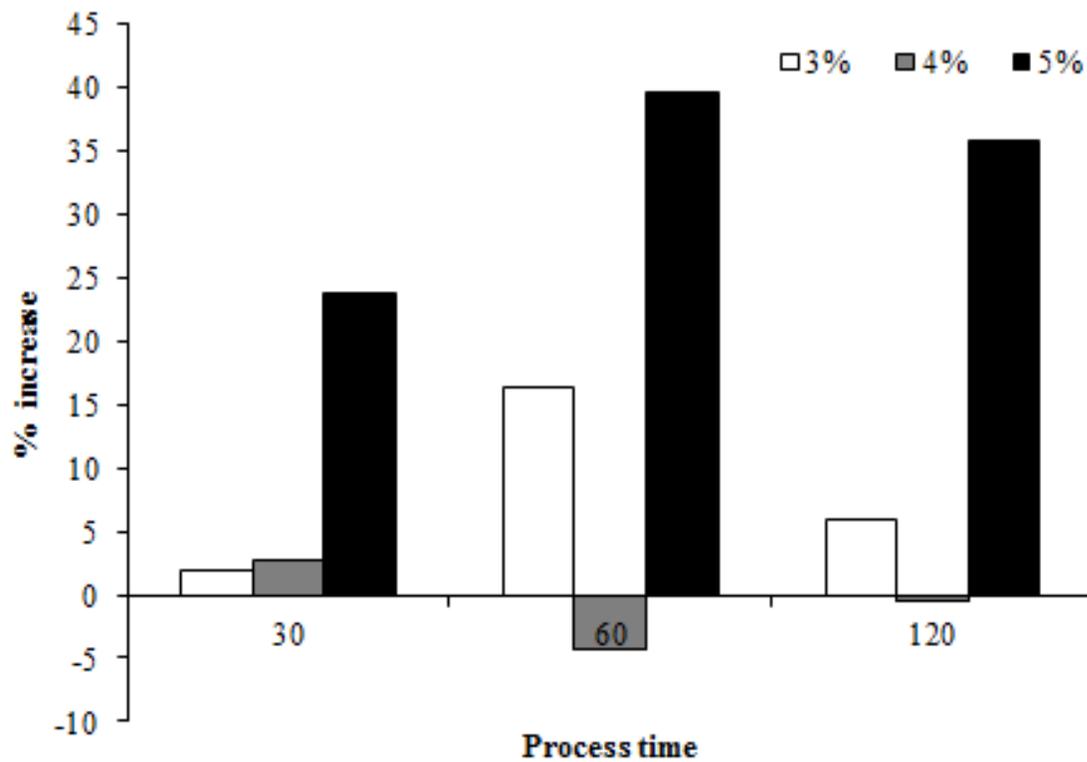


Figure 3: Percentage increase of β -glucan (A) and protein content (B) of the rice bran hydrolysate derived by using the sequential hydrolysis process relative to those of the co-hydrolysis process.

Anti-inflammatory activity and cytotoxicity of RBHs on RAW 264.7 cells:

The RBHs with high protein and/or β -glucan contents prepared by using different processes and conditions were selected and their toxicity evaluated against RAW 264.7 cells. It was discovered that all the selected RBHs at concentrations as high as 2,000 $\mu\text{g/mL}$ exhibited no cytotoxicity against RAW 264.7 cell lines (Figure 4). The results are in accordance with the study reported by Kim and co-workers [24]. Additionally, Thai purple rice bran hydrolysate at 100 $\mu\text{g/mL}$ had been discovered to have no effect on cell death [25].

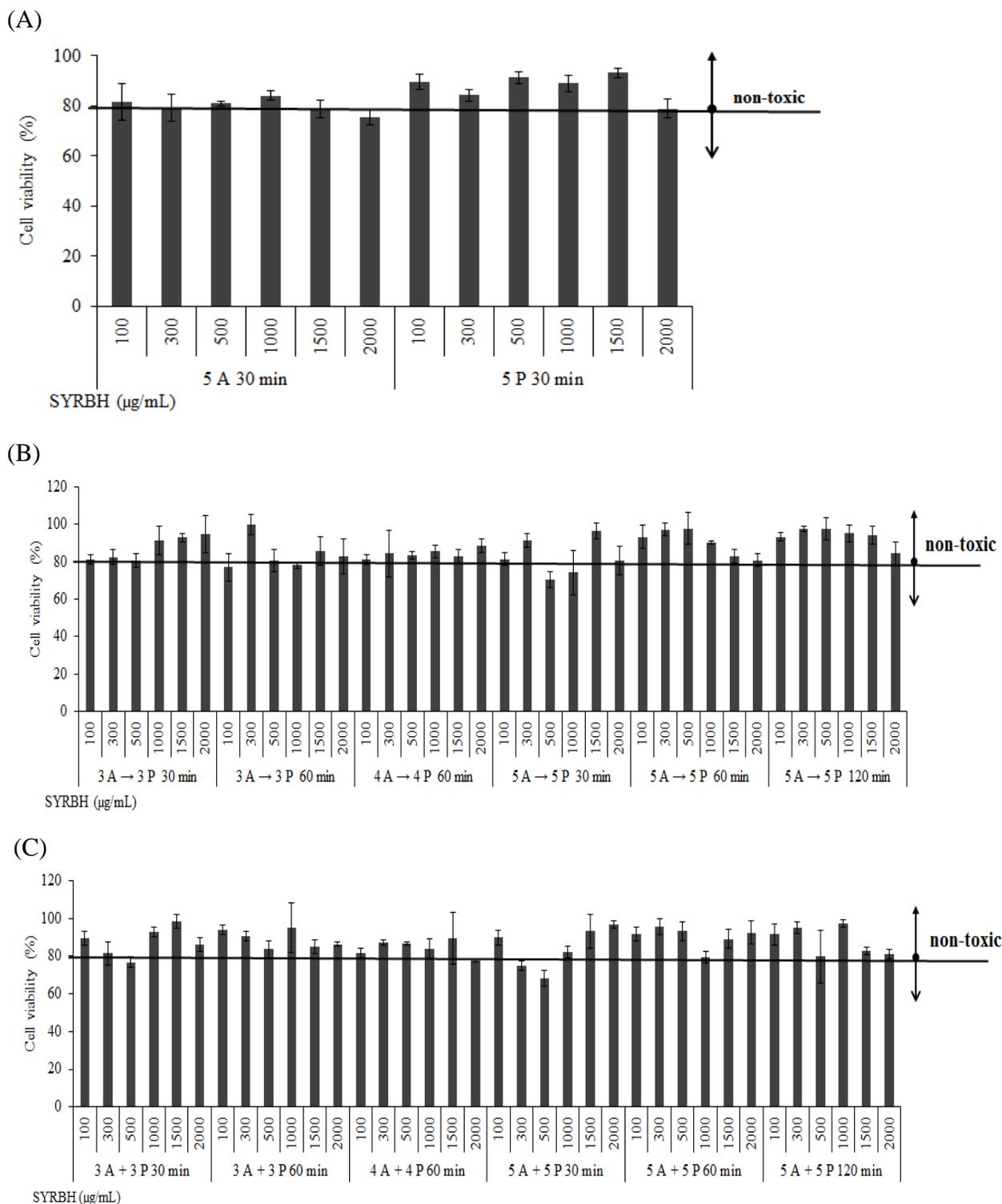


Figure 4. Effect of concentration of the selected RBHs on RAW 264.7 cell viability.

Inhibitory activity of RBHs on nitric oxide production and pro-inflammatory cytokine secretion in LPS-stimulated RAW 264.7 cells:

The selected RBHs at a concentration of 1,500 µg/mL were used to verify their NO inhibitory activity of RAW 264.7 macrophage cells and the results are shown in Table 1. Most of the high-inhibitory activity RBHs were prepared by using the sequential hydrolysis process except that of the 5A+5P-60 min. The result that the activity of the RBH was not related to both protein and β-glucan content was unexpected.

The RBHs containing strong NO inhibitory activity were selected for their inhibitory activity against secretion of pro-inflammatory cytokine. The barley β-glucan was used to compare its activity with those of the RBHs. The result shown in Figure 5 illustrates that TNF-α is a major medium of LPS response followed by IL-6 and IL-1β respectively. Secretion of IL-1β, IL-6, and TNF-α induced by LPS was inhibited by commercial barley β-glucan at 73.52%, 62.94%, and 49.75% respectively. The pro-inflammatory cytokines were inhibited differently by each RBHs. The hydrolysate of 5A→5P: 60 min was the most effective inhibitor. Regardless of the samples, reduction on IL-1β secretion was outstanding with an inhibition range of 45.58-71.96% followed by those of IL-6 and TNF-α which had inhibition range of 35.56-54.55% and 9.37-29.20% respectively.

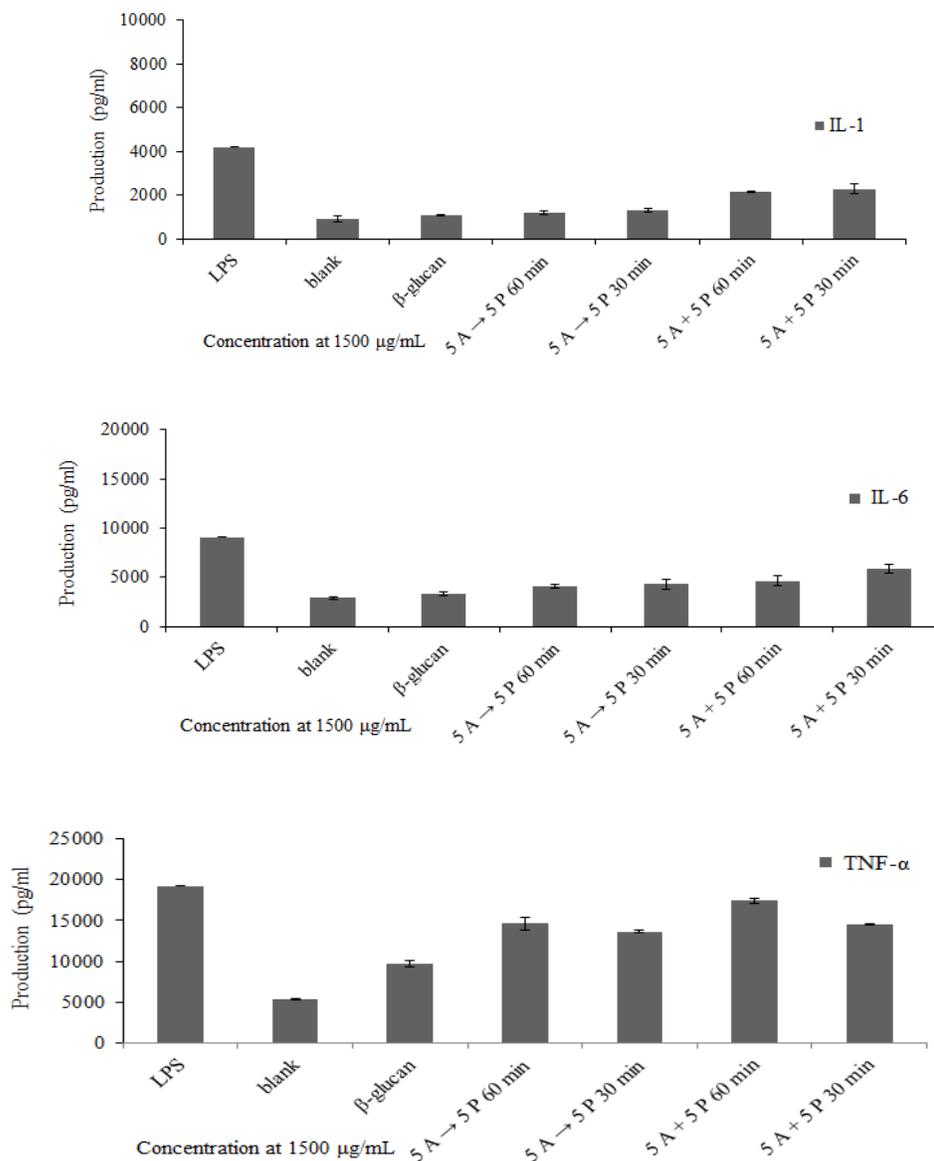
Cereal crude β-glucan extracts and their corresponding commercial forms have been discovered to reduce induction of inflammation-related gene expression kinetics (IL-1β, IL-8, IL-10, and NF-κB) on THP-1 macrophages [26]. Extract of Thai purple rice bran extract demonstrated strong anti-inflammation through the inhibitory effect on nitric oxide production in combined LPS-IFN-γ activated RAW 264.7 murine macrophage cells [25]. Thai colored rice extracts, especially with those with a red color, reduced pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) and MMP-2 expression in LPS-induced HL-60 cells [27]. Moreover, Phangthip and co-worker [28] found that rice-berry bran extract could reduce IL-6 and TNF-α in streptozotocin induced diabetes rats.

Table 1: Effects of the selected RBHs NO inhibition in LPS-stimulated RAW 264.7 cells

Process	Hydrolysate	NO inhibition (%)
Single enzyme hydrolysis	5 A 30 min	29.75 ± 3.13 ^{ef}
	5 P 30 min	24.82 ± 9.06 ^{ef}
Sequential hydrolysis	3 A → 3 P 30 min	23.05 ± 6.51 ^f
	3 A → 3 P 60 min	62.01 ± 8.69 ^{abc}
	4 A → 4 P 60 min	67.31 ± 3.81 ^{ab}
	5 A → 5 P 30 min	58.76 ± 5.49 ^{bcd}
	5 A → 5 P 60 min	71.63 ± 7.63 ^a
	5 A → 5 P 120min	34.49 ± 2.58 ^e
Co-hydrolysis	3 A + 3 P 30 min	28.18 ± 4.28 ^{ef}
	3 A + 3 P 60 min	49.51 ± 2.20 ^d
	4 A + 4 P 60 min	53.36 ± 1.20 ^{cd}
	5 A + 5 P 30 min	49.09 ± 3.36 ^f
	5 A + 5 P 60 min	68.75 ± 5.76 ^{ab}
	5 A + 5 P 120 min	36.07 ± 2.71 ^e

Values are reported as mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$).

(A)



(B)

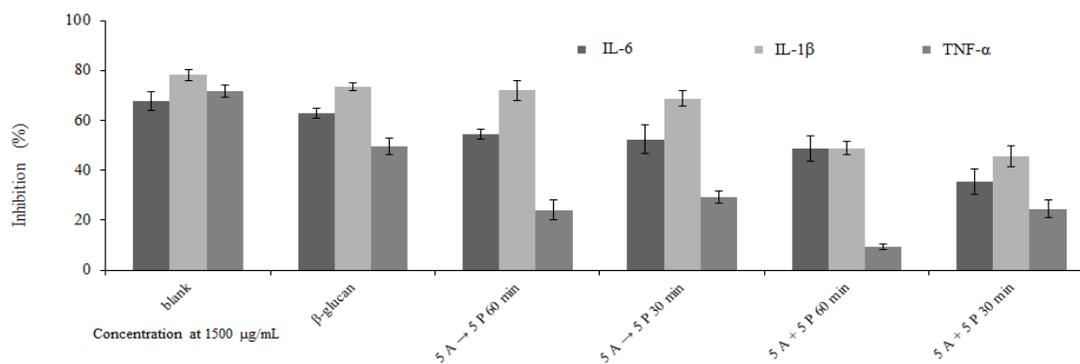


Figure 5. Inhibitory effects of the selected RBHs on IL-6, IL-1β, and TNF-α in LPS-stimulated RAW 264.7 cells. Note: Production of IL-6, IL-1β and TNF-α in LPS-stimulated RAW 264.7 cells (A). Inhibitory of the selected RBHs on the production of IL-6, IL-1β and TNF-α (B).

DISCUSSION

With the results we discovered that the process parameters, including type of enzyme, concentration, process time, and process arrangement determine both β -glucan and the protein of the obtained hydrolysates. Both components could be co-released by either amyloglucosidase or protease. The hydrolysis steps arranged into the sequential hydrolysis (A→P) process was likely to be an optimized process for preparation of Sang-yod rice bran hydrolysate. The hydrolysates revealed high amounts of protein and β -glucan contents, demonstrating an exertion of potent NO inhibition and anti-inflammation cytokines inhibition on RAW 264.7 cells.

CONCLUSION

Digestion of Sang-yod rice bran to deliberate β -glucan and protein was influenced by enzymes and the hydrolysis processes. Pre-digestion with amyloglucosidase followed with co-hydrolysis of amyloglucosidase and protease G6 of the sequential hydrolysis process was the most effective process to release β -glucan and protein from rice bran. The hydrolysate obtained from the process using enzyme concentration at 5% v/w and 60 min hydrolysis duration of each stage had the highest soluble β -glucan and protein content. Moreover, the process provided the hydrolysates with potential anti-inflammatory properties on nitric oxide inhibition and pro-inflammatory cytokines inhibition on RAW 264.7 macrophage cell lines. Consequently, Sang-yod rice bran, a low value by-product, has the potential to be transformed into a functional ingredient for functional foods and nutraceutical products.

Abbreviations: SYRB: Sang-yod rice bran; SYRBHs: Sang-yod rice bran hydrolysates; A: amyloglucosidase; P: protease G6; RBHs: rice bran hydrolysates; MTT: 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide; NO: nitric oxide; TNF- α : tumor necrosis factor-alpha; IL-1 β : interleukin-1-beta; IL-6: interleukin 6; LPS: lipopolysaccharide

Competing interests: The authors have declared that no competing interests exist.

Author's contributions:

Natcha Phantu Wong, Ph.D. candidate, is a major investigator who performed all experiments, in addition to the analysis and writing of this manuscript.

Chakree Thongruang, Ph.D., is an assistant professor of food technology. He provided vision and conceptualization for the research. He also contributed to the study design, writing, and verified the correction of manuscript.

Chutha Takahashi Yupanqui, Ph.D. is a doctor of pharmaceutical sciences of interdisciplinary graduate school of nutraceutical and functional food. She is a research coordinator who provided remarks and suggestions, in addition to verifying the correction of the manuscript.

Financial sponsor: The financial support of the Grant-in-aid for dissertation came from a graduate school, the Prince of Songkla University, Thailand.

Acknowledgements: This work was supported by a graduate school, the Prince of Songkla University. The authors acknowledge Assistant Professor Dr. Chakree Thongruang for his guidance on experimental designs and technical writing.

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