Journal of Current Science and Technology, May-August 2021 Vol. 11 No. 2, 324-333

ISSN 2630-0656 (Online)

Cite this article: Wunnakup, T., Monton, C., & Charoenchai, L. (2021, May). Inhibitory activity of protein hydrolysates from rice bran on mushroom tyrosinase. *Journal of Current Science and Technology, 11*(2), 325-334. DOI: 10.14456/jcst.2021.32

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Inhibitory activity of protein hydrolysates from rice bran on mushroom tyrosinase

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Received 7 March 2021;Revised 7 May 2021; Accepted 7 May 2021;
Published online 27 May 2021

Abstract

Rice bran protein hydrolysates (RBPHs) obtained by Alcalase® have attracted attention because of their bioactivity properties (antioxidant, anti-acetylcholinesterase and anti-butyrylcholinesterase). However, there has been no report on their improved inhibition of melanogenesis. This study aimed to investigate the inhibitory tyrosinase activity of RBPH. The defatted Khao Hom Mali RD15 rice bran protein was extracted into water (RBP1), 2% NaCl (RBP2) and 0.1 N NaOH (RBP3) fractions. All protein fractions were hydrolyzed with Alcalase® to produced RBPH1, RBPH2 and RBPH3. The protein content, % yield, degree of hydrolysis (DH) and molecular weight patterns of each fraction were investigated. The RBPH2 and RBPH3 showed high potential inhibition on mushroom tyrosinase activity, the IC_{50} values were determined to be 1.92 mg/ml and 0.46 mg/ml, respectively. The inhibition kinetics showed that RBPH2 was an uncompetitive mechanism, with inhibition constants (K_i and K_{is}) were 6.1 mg/ml and 4.5 mg/ml, respectively. Moreover, the RBPH3 displayed a non-competitive mechanism and K_i and K_{is} were 2.8 mg/ml and 2.5 mg/ml, respectively. Therefore, the RBPHs could be potential candidates for use in the cosmetics and food industries.

Keywords**:** *inhibition; kinetic; mushroom tyrosinase; protein hydrolysates; rice bran protein.*

1. Introduction

The pigment in the skin, hair, and eyes of humans is derived from melanin gained through melanogenesis in melanocytes. The irregularity of melanin biosynthesis causes freckles, melasma, and skin disorders and may increase the risk of skin cancer (Dogra & Sarangal, 2014; Saran, 2004). In melanin biosynthesis, the enzyme that plays a role as a rate-limiting enzyme is tyrosinase (EC 1.14.18.1). Tyrosine is found in mammals, bacteria, plants, and fungi. It catalyzes two activities in melanogenesis: monophenolase activity that hydroxylation L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and diphenolase activity that oxidation L-DOPA to o-dopaquinone (Chai et al., 2018; Gou, 2017). Tyrosinase inhibitors are used for skin-whitening agents and the inhibition of undesired browning in the food industry. Kojic acid, hydroquinone and arbutin are known tyrosinase inhibitors, but they exhibit side effects (Ishikawa, Sasaki, Kawaguchi, Mochizuki, & Nagao, 2006; Kooyers & Westerhof, 2006; Ota et al., 2009).

In recent years, plant-derived protein hydrolysates have gained attention from the nutraceutical and cosmetic industries because of their ability to improve health functions. To obtain protein hydrolysate, either chemical and/or enzymatic methods are used. The modulation of hydrolytic process conditions (type of proteases and time) makes it possible to acquire different bioactive protein hydrolysates. The protein

hydrolysates obtained from oat bran (oatp) by enzymatic hydrolysis with Alcalase®, β-glucan and saccharifying enzyme exhibit antioxidative stress-induced cell injury in skin fibroblasts (Feng et al., 2013). The hydrolyzed wheat protein obtained by enzymatic hydrolysis has shown wound healing and anti-inflammatory activity (Sanguigno et al., 2018). It is safe to use in skin and hair conditioning agents (Burnett et al., 2018). *In vitro* and *in vivo* antioxidant activities have been observed in hemp seed protein hydrolyzed by Neutrase® (Logarušić et al., 2019)

Rice bran is a good source of bioactive hydrolysate protein. Rice bran is a part of the outer layer of a rice grain, which is obtained from the rice-milling process. Interestingly, rice bran contains 10-15% protein, 34-62% carbohydrate and 15-20% oil, in addition to being rich in nutrients and biologically active compounds. Rice bran proteins can be grouped into albumin, globulin, glutelin and prolamin based on their solubility; the values for each protein type depend on rice cultivars (Chanput, Theerakulkait, & Nakai, 2009; Fabian & Ju, 2011). Rice bran protein hydrolysates (RBPHs) have been reported in extraction and hydrolysis with various protease enzymes. The functional peptides from RBPH have been identified and characterized as a good source of pharmacological activities, such as antihypertensive activities, anti-inflammation, and antidiabetic (Boonloh et al., 2015; Jan-On et al., 2020, Phantuwong, Thongraung, & Yupanqui, 2017). In addition, rice protein hydrolysates have high nutrition and hypoallergenic properties (Dupont et al. 2020, Vandenplas, De Greef, Hauser & Paradice Study Group, 2014). However, information about the tyrosinase inhibitory activity of RBP and its fraction Alcalase® hydrolysate remains limited. In this work, RBP was extracted by different methods and RBPHs in all hydrolyzed RBP fractions were separated. Subsequently, the tyrosinase inhibitory activities of RBPs and RBPHs were determined.

2. Objectives

This study aimed to investigate the potential of mushroom tyrosinase inhibition from rice bran proteins extracted by different extraction conditions as well as their hydrolysates by Alcalase®. The type of inhibition was determined by enzyme kinetic assay.

3. Materials and methods

3.3 Materials

Raw materials of rice bran (Khao Hom Mali RD15) were bought from farmers in Surin Province, Thailand. Tyrosinase from mushroom, 3, 4-Dihydroxy-L-phenylalanine (L-DOPA), β-mercaptoethanol and kojic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alcalase® was purchased from Merck (USA). NaCl and NaOH were purchased from Carlo Erba (Italy). Glycine, Sodium dodecyl sulfate (SDS) and Tris-base were purchased from VWR (USA). Unstained Protein Ladders (1.4-26.6 kD) were purchased from Bio-Rad (USA), while the Blultra pre-stained protein ladder (6.5-270 kD) was purchased from Bio-Helix (Taiwan).

3.2 Rice bran protein hydrolysate (RBPH) preparation

Thai rice bran (Khao Hom Mali RD15) was defatted twice by dispersion in hexane (1:3 w/v), followed by continuous stirring for 1 h. The defatted rice bran (DRB) was air-dried overnight under a hood. The following steps were performed at 4˚C: DRB (200 g) was extracted twice with distilled water (1:5 w/v) by continuous stirring for 1 h and then filtered to obtain the RBP1 fraction. After this step, the residue was extracted twice with 2% NaCl (1:5 w/v) by continuous stirring for 1 h and filtered to recover the RBP2 fraction. Finally, the residue was extracted twice with 0.1 N NaOH (1:5 w/v) by continuous stirring for 1 h and filtered to recover the RBP3 fraction. All fractions were freeze-dried and stored at -20 ˚C. The protein content of extracts was determined by the Bradford method. The bovine serum albumin (BSA) was used as the protein standard.

The RBP1, RBP2, and RBP3 were dissolved in distilled water (1:10 w/v). The suspension was adjusted to pH 8.0 for hydrolysis of Alcalase® (1% E/S). Hydrolysis was performed at 55 ˚C and maintained pH to optimum condition with 0.1 N NaOH. After 3 h, the enzyme was inactivated by heating at 95 ˚C for 10 min. The suspensions were cooled down and centrifuged at 2,000 g for 1 h (4 ˚C) to obtain supernatant for rice bran protein hydrolysates (RBPH1, RBPH2 and RBPH3). All RBPHs were adjusted to pH 7, then freeze-dried and kept at -20 ˚C.

3.2 Determination of the degree of hydrolysis (DH)

After 3 h of protein hydrolysis step, the DH (%) was measured. According to Adler Nissen (1986), the degree of hydrolysis (DH) can be described as the percentage of peptide bonds cleaved to the total number of bonds per unit weight, which can be calculated following the equation below:

$$DH\ (\%) = B \times Nb \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100$$

where B is the base consumption (ml) used to control the pH during enzymatic hydrolysis, Nb is the normality of the base (0.1 N NaOH), and α is the average degree of dissociation of the a α-NH2 groups ($1/\alpha = 1.13$ for hydrolysis of Alcalase®), MP is the mass of protein in g (N) × 5.95, and h_{tot} is the total number of peptide bonds in the protein substrate (8.4 meqv/g rice bran protein).

3.3 Determination of molecular weight of protein by gel electrophoresis

Tricine-SDS-PAGE is used for separate proteins in mass 1-100 kDa. The RBPs and RBPHs (containing 20 µg protein) were mixed with 50 µl of loading buffer: 0.1 M Tris-HCl, pH 6.8, 24% (v/v) glycerol, 8% SDS, 2% β-mercaptoethanol and 0.02% bromophenol blue, and then heated for 5 min in boiling water. The samples (10 µl) were loaded into 4% stacking gel and 16% separating gel. Electrophoresis was conducted using an anode running buffer containing 0.1 M Tris-HCl, pH 8.9, and a cathode running buffer containing 0.1 M Tris, 0.1 M Tricine and 0.1% SDS. SDS-PAGE was performed at 30 mV until samples were transferred into the stacking gel, and then the voltage was changed to 100 mV until the end. For protein visualization, the gel was fixed in fixing solution for 30 min and then immersed in a staining solution for 30 min. The gel was distained overnight in a distaining solution with shaking.

3.4 Mushroom tyrosinase inhibition assay

Tyrosinase inhibition assay was performed using a slightly modified approach from Kim, Park, Lee, Kim and Lim (2012). In triplicate, the reaction mixture contained 80 µl of sodium phosphate buffer, pH 6.8, 40 µl of RBPs or RBPHs (0.5-4 mg/ml), 40 µl of 12.5 mM L-DOPA and 40 µl of mushroom tyrosinase (125 U/ml). After the addition of the enzyme, the reaction was mixed and incubated at 25 ˚C for 5 min. Subsequently, the dopachrome formation was monitored at 475 nm. Kojic acid (5.68 µg/ml) was used as a positive control. A blank control was a reaction mixture without a sample (RBPs or RBPHs) and tyrosinase. A blank sample was a reaction mixture with sample and without tyrosinase. The percentage inhibition was calculated as follows:

$$\%\ \text{Tyrosinase inhibitory activity} = \frac{(OD_{control} - OD_{blank\ control}) - (OD_{sample} - OD_{blank\ sample})}{(OD_{control} - OD_{blank\ control})} \times 100$$

The kinetics of the catalytic reaction of RBPHs to mushroom tyrosinase was performed using various concentrations of L-DOPA (0.5-5 mM). Inhibitory kinetics of RBPHs (0.1-4 mg/ml) were determined by Lineweaver-Burk plots, and the inhibition constants were measured by second plots of the apparent slope (K_m/V_m) or intercept ($1/V_m$) versus the concentration of the RBPHs.

3.5 Statistical analysis

Data were expressed as mean ± SD, and analyses by one-way ANOVA followed Bonferroni's post hoc test using GraphPad Prism5 (GraphPad Software Inc.). A *p*-value < 0.05 was considered statistically significant.

4. Results

Protein content and yield (%) of RBP1, RBP2, RBP3 and their hydrolysates, together with the degree of hydrolysis (%DH) of the hydrolysate with Alcalase®, are shown in Table 1. The RBP3 showed the highest percentage of protein content (22.07%) and yield (6.85%), followed by RBP1 and RBP2, respectively. After hydrolysis, RBPH1 and RBPH2 showed an increase in protein yield (2.38% and 1.66%), while RBH3 decreased slightly to 5.05%. The hydrolysate of RBPH2 by enzymatic reaction showed the highest DH (%) at 9.54, but the RBH3 resulted in the lowest DH (%) at 3.46.

Table 1 The percentage of yield of RBPs and RBPHs.

Samples	Yield[1] (%)	Protein[2] (%)	DH (%)
RBP1	0.78	7.73 ± 0.34	
RBP2	0.44	2.16 ± 0.31	
RBP3	6.85	22.07 ± 0.05	
RBPH1	2.38	3.52 ± 0.28	7.08
RBPH2	1.66	2.67 ± 0.67	9.54
RBPH3	5.05	5.56 ± 0.42	3.46

[1]The percentage of gram protein/100 gram of defatted rice bran
[2] The percentage of gram protein/100 gram of extract

As shown by the results of tricine-SDS−PAGE in Figure 1, our rice bran protein extract RBP1, RBP2, and RBP3 the molecular weights were 14-95, 6-53 and 6-95 kDa, respectively. After the RBPs were hydrolyzed by Alcalase®, the molecular weight was 6.5 – 37 kDa for RBPH1, 14, 20 and 30 kDa for RBPH2 and less than 6.5 kDa for RBPH3.

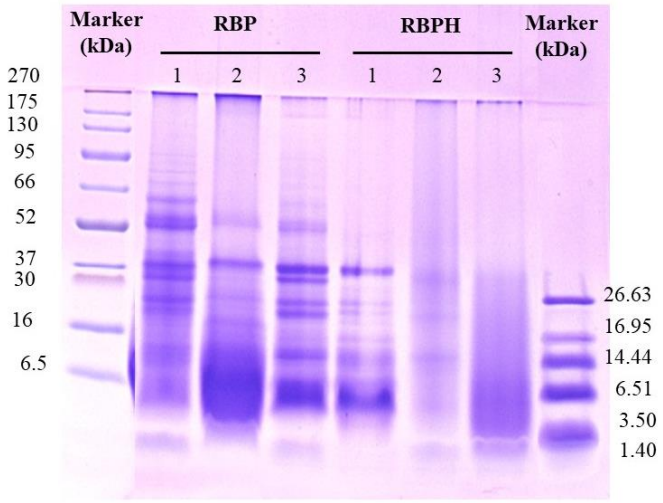

Figure 1 Tricine SDS-PAGE patterns of RBPs and RBPHs hydrolyzed with Alcalase®

L-DOPA was used as a substrate to be investigated against the mushroom tyrosinase diphenolase activity. As shown in Figure 2, all RBPs (RBP1, RBP2 and RBP3) and their hydrolysates (RBPH1, RBPH2 and RBPH3, respectively) were used as inhibitors of the enzymes catalyzing the oxidation of L-DOPA at concentrations of 0.5, 1, 2, and 4 mg/ml. The RBP2 and RBP3 had a significant dose-dependent inhibitory effect on mushroom tyrosinase activity (<50%). Moreover, the hydrolysate RBPH2 and RBPH3 showed higher potent tyrosinase inhibition (2-5-fold) than the protein extract. Also, the RBP1 and RBPH1 showed inhibitory effects on mushroom tyrosinase activity (< 50%), but such inhibitory effect on enzymes was not significantly different. A positive control, kojic acid, showed strong tyrosinase inhibition. In addition, the IC_{50} of RBPH2 and RBPH3 were 1.92 and 0.46 mg/ml, respectively, while the other samples showed IC_{50} higher than 4 mg/ml, as shown in Table 2.

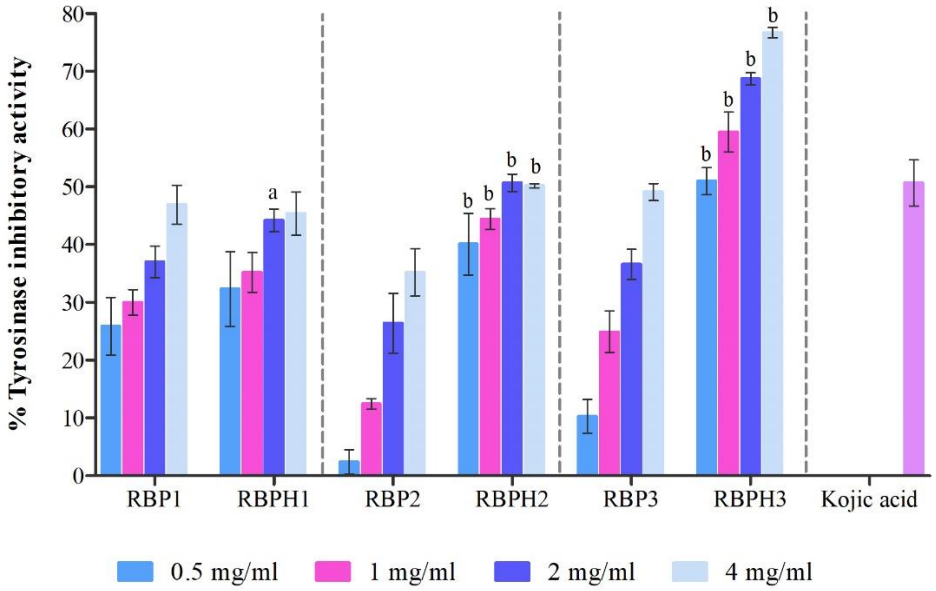

Figure 2 Effect of RBPs and RBPHs on mushroom tyrosinase activity (means ± SD; n = 3). The relative activity of mushroom tyrosinase assayed on L-DOPA oxidation in the presence of different concentrations of each extract (0.5, 1, 2, 4 mg/ml). Kojic acid (5.68 µg/ml) as a positive control was used. a and b indicate $p < 0.05$ and $p < 0.001$ as compare with between group on the same concentration.

Table 2 The inhibitory activity (IC_{50}) of mushroom tyrosinase on RBPs and RBPHs

Samples	IC_{50} (mg/ml)
RBP1	>4
RBP2	>4
RBP3	>4
RBPH1	>4
RBPH2	1.92
RBPH3	0.46

From the results of the inhibitory effect, the inhibition type on the enzyme by RBPH2 and RBPH3 for oxidation of L-DOPA was investigated. Figure 3A shows the double reciprocal Lineweaver-Burk plot, with and without RBPH2. The plot of 1/V versus 1/[S] shows a pattern of parallel straight lines. The values of maximum velocity (V_{max}) and Michael constants (K_m) decreased, but the ratio of K_m/V_m remained unchanged or decreased only slightly. The results suggested that the type of inhibition of RBPH2 on mushroom tyrosinase belonged to the non-competitive type of inhibitor. The inhibition constant for the free enzyme (K_i) was obtained from secondary plots of slopes vs. various concentrations of RBPH2 (Figure 3B) and for the enzyme-substrate (ES) complex (K_{is}), which was measured from the secondary plot of intercept vs. various concentrations of RBPH2 (Figure 3C). The K_i and K_{is} of RBPH2 were 6.1 mg/ml and 4.5 mg/ml, respectively.

The Lineweaver-Burk plot was used to investigate the type of inhibition of RBPH3 on mushroom tyrosinase. Figure 4A shows that five lines with different slopes intersected at the abscissa in the second quadrant. The results showed that V_{max} decreased, and K_m remained unchanged with increasing RBPH3 concentration. These results indicated that RBPH3 was a non-competitive type of inhibitor. The K_i and K_{is} of RBPH2 were 2.8 mg/ml and 2.5 mg/ml, respectively (as shown in Figures 4B and 4C).

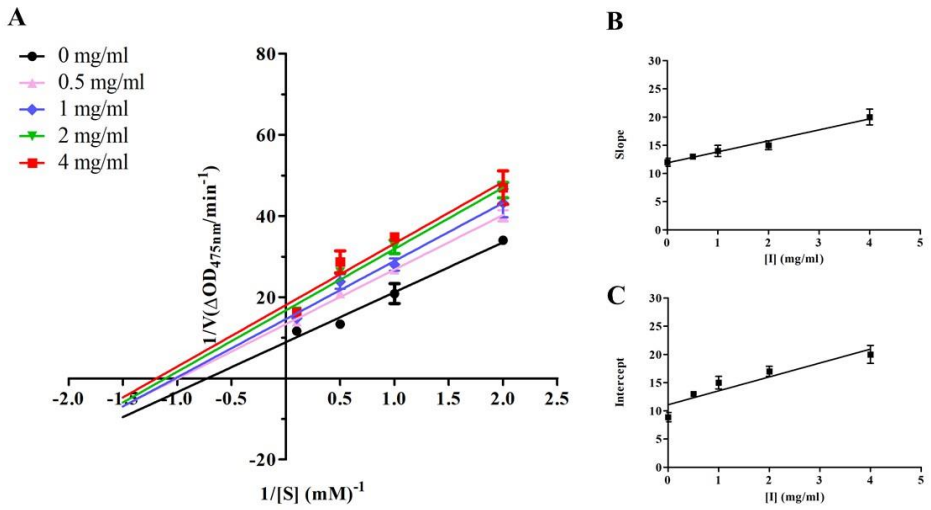

Figure 3 (A) Lineweaver-Burk plots for inhibition of RBPH2 on mushroom tyrosinase for the catalysis of L-DOPA at different concentrations as substrate (0.5, 1.25, 2.5, 5 mM) and different concentrations of RBPH2 (0.5, 1, 2, 4 mg/ml). (B) Secondary plots of slopes vs various concentrations of RBPH2. (C) Secondary plots of intercept vs various concentrations of RBPH2

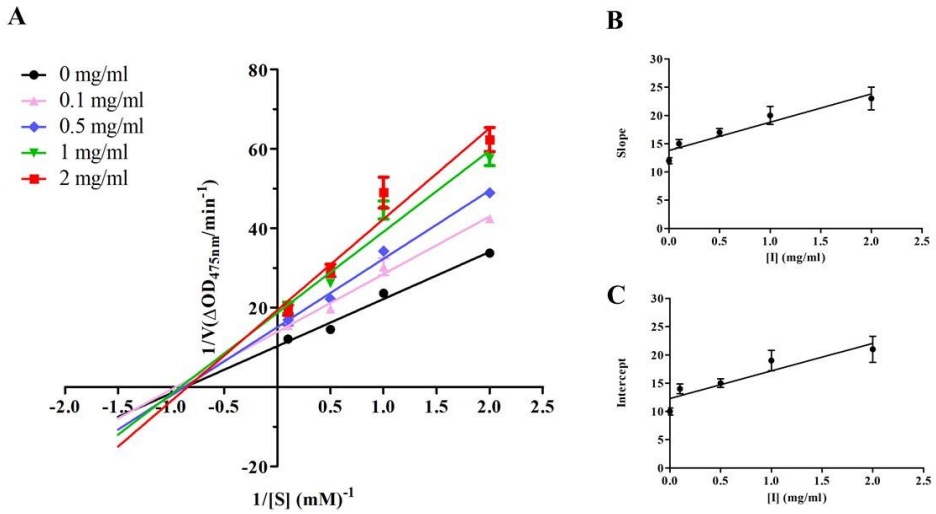

Figure 4 (A) Lineweaver-Burk plots for inhibition of RBPH3 on mushroom tyrosinase for the catalysis of L-DOPA at different concentrations as substrate (0.5, 1.25, 2.5, 5 mM) and different concentrations of RBPH3 (0.1, 0.5, 1, 2 mg/ml). (B) Secondary plots of slopes vs various concentrations of RBPH3. (C) Secondary plots of intercept vs various concentrations of RBPH3.

5. Discussion

More rice proteins were found in rice bran than endosperm (milled rice) (Prakash & Ramaswamy, 1996). Specifically, defatted rice bran showed higher protein content than full-fat rice bran (Jiamyangyuen, Srijesdaruk, & Harper, 2005). The rice bran protein content depends on cultivars and the extraction process. Based on the solubility of each protein, albumin, globulin and glutelin have been extracted by water, salt solution

and alkali solution, respectively (Phongthai, Homthawornchoo, & Rawdkuen, 2017). Similar to this result, protein extraction from *Oryza sativa* L., CV. Khao Dawk Mali 105 and CV. Chainat 1 (1:1 w/w) has reported that protein yielded the highest protein glutelin content (32.6%) followed by albumin (30.9%) and globulin (24.9%). From SDS-PAGE pattern (Figure 1), it was shown that the molecular weight of RBP1 and RBP2 was similar with albumin and globulin in the range of 10-200 kDa and 6.7-36 kDa, respectively. However, RBP3 (glutelin fraction) showed a smear on SDS-PAGE, which might be caused by NaOH. The alkali condition improved protein extraction yield by disrupting the cell wall and changing protein solubility (Kumagai et al., 2006), but also results in denaturation, racemization and lysinoalanine formation (Guan et al., 2017).

Alcalase®, also called subtilisin, is a serine protease. The optimum pH for activity is in the range of pH 8-9. The enzyme has a specificity of peptide bond on the carboxyl side of Glu, Leu, Lys, Met, Phe, Tyr and Trp (Doucet, 2003). According to the increasing of RBPH1 and RBPH2 yield, it might be caused by the Alcalase® producing peptides revealed as amino acids (Lys, Phe, Tyr, and Trp) to react with Bradford reagent (Brunelle et. al., 2017). Similar to many other studies, Alcalase® has efficiency in the hydrolysis of rice bran protein and showed the %DH in the range of 7-14% (Thamnarathip, 2016; Uraipong & Zhao, 2016; Zhao et al., 2012). However, the temperature, pH and NaCl affected the efficiency of Alcalase for hydrolysis (Muyan, Xiumei, Tianxiang, & Chao, 2006; Su, Wang, Kwok, & Lee, 2005). The protein extraction with strong base (NaOH) condition improved the %DH of protein (Dhalleine & Delepierre, 2015). NaOH can extract and hydrolyzed proteins by the breakdown of covalent and non-covalent bonds (Braspaiboon et al., 2020). The proteins were then hydrolyzed by NaOH before enzymatic hydrolysis caused the low % DH value of RBPH3.

Previous studies have demonstrated that the rice bran protein hydrolysate has anti-tyrosinase activity. Ferri et al. (2017) reported that rice protein hydrolysate hydrolyzed by Alcalase® (1- 5 kDa) had high anti-tyrosinase activity. In this work, RBPH3 showed more effective tyrosine inhibition (IC_{50} = 0.46 mg/ml) than rice bran albumin hydrolyzed with papain (IC_{50} = 1.31 mg/ml) (Kubglomsong et al., 2018). The RBPHs showed more inhibitory effect on mushroom tyrosinase than RBPs, suggesting that the protein hydrolysates/peptides could interfere with enzyme activity by changing conformation with Van der Waals and hydrogen bonds (Deng et. al., 2020). The RBPH2 tyrosinase inhibitor was an uncompetitive type. Moreover, the K_{is} value was lower than the K_i value, which indicated that the binding capacity of the inhibitor to the ES complex was better than free enzymes. This result was consistent with the Chia seed peptide that exhibited uncompetitive type with tyrosinase (Aguilar-Toalá & Liceaga, 2020). RBPH3 showed the tyrosinase inhibitor was a non-competitive type. The non-competitive inhibitor is a type of allosteric regulation, with the inhibitor binding to an allosteric site independent of substrate binding. Both RBPH2 and RBPH3 could decrease the turnover rate of enzymes by reduction the Vmax. Our results from kinetic studies revealed the low value of IC_{50} as well as K_i and K_{is}, suggesting that RBPH3 (non-competitive type) showed more potent mushroom tyrosinase inhibitory activity than RBPH2 (uncompetitive type).

6. Conclusion

In the present study, Alcalase® hydrolysis was found to improve the inhibition of tyrosinase activity in rice bran protein fractions. In kinetic studies, the hydrolysate of salt solution extract (RBPH2) showed inhibited tyrosinase in a no-competitive manner. Also, the hydrolysate of alkali extract (RBPH3) exhibited strongly inhibited tyrosinase in a non-competitive manner. These results indicate that the hydrolysis of protease can be an efficient process for the recovery of bioactive compounds from rice bran protein. Thus, RBPHs have the potential for further development in the functional food and cosmetics fields. However, further studies should be carried out to purify and clarify the active protein hydrolysate sequence.

7. Acknowledgements

This research was carried out with financial support of the Research Institute of Rangsit University (Grant No. 1/2650). We would like to express an appreciation to the College of Pharmacy, Rangsit University for providing laboratory facilities.

8. References

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. Elsevier applied science publishers.

Aguilar-Toalá, J. E., & Liceaga, A. M. (2020). Identification of chia seed (*Salvia hispanica* L.) peptides with enzyme inhibition activity towards skin-aging enzymes. *Amino Acids*, *52*(8), 1149-1159. DOI: https://doi.org/10.1007/s00726-020-02879-4.

Burnett, C., Bergfeld, W. F., Belsito, D. V., Hill, R. A., Klaassen, C. D., Liebler, D. C., ... & Heldreth, B. (2018). Safety assessment of hydrolyzed wheat protein and hydrolyzed wheat gluten as used in Cosmetics. *International journal of toxicology*, *37*(1_suppl), 55S-66S. DOI: https://doi.org/10.1177/1091581818776013.

Boonloh, K., Kukongviriyapan, V., Kongyingyoes, B., Kukongviriyapan, U., Thawornchinsombut, S., & Pannangpetch, P. (2015). Rice bran protein hydrolysates improve insulin resistance and decrease pro-inflammatory cytokine gene expression in rats fed a high carbohydrate-high fat diet. *Nutrients*, *7*(8), 6313-6329. DOI: https://doi.org/10.3390/nu7085292.

Braspaiboon, S., Osiriphun, S., Peepathum, P., & Jirarattanarangsri, W. (2020). Comparison of the effectiveness of alkaline and enzymatic extraction and the solubility of proteins extracted from carbohydrate-digested rice. *Heliyon*, *6*(11), e05403. DOI: https://doi.org/10.1016/j.heliyon.2020.e05403.

Brunelle, E., Le, A. M., Huynh, C., Wingfield, K., Halámková, L., Agudelo, J., & Halámek, J. (2017). Coomassie brilliant blue G-250 dye: an application for forensic fingerprint analysis. *Analytical chemistry*, *89*(7), 4314-4319. DOI: https://doi.org/10.1021/acs.analchem.7b00510.

Chai, W. O.-Y., Ou-Yang, C., Huang, Q., Lin, M. Z., Wang, Y. X., Xu, K. L., Huang, W. Y., & Pang, D. D. (2018). Antityrosinase and antioxidant properties of mung bean seed proanthocyanidins: Novel insights into the inhibitory mechanism. *Food chemistry*, *260*, 27-36. DOI: https://doi.org/10.1016/j.foodchem.2018.04.001.

Chanput, W., Theerakulkait, C., & Nakai, S. (2009). Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates. *Journal of Cereal Science*, *49*(3), 422-428. https://doi.org/10.1016/j.jcs.2009.02.001.

Dhalleine, C., & Delepierre, S. (2015). *Method for preparing alkaline hydrolysates of plant proteins*. (U.S. Patent No. 9149063B2). U.S. Patent and Trademark Office. DOI: https://patents.google.com/patent/US9149063B2/en

Deng, Y., Huang, L., Zhang, C., Xie, P., Cheng, J., Wang, X., & Liu, L. (2020). Skin-care functions of peptides prepared from Chinese quince seed protein: Sequences analysis, tyrosinase inhibition and molecular docking study. *Industrial Crops and Products*, 148, 112331. DOI: https://doi.org/10.1016/j.indcrop.2020.112331.

Dogra, S., & Sarangal, R. (2014). Pigmentary disorders: An insight. *Pigment International*, *1*(1), 5. DOI: http://doi.org/10.4103/2349-5847.135429.

Doucet, D., Otter, D. E., Gauthier, S. F., & Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *Journal of Agricultural and Food Chemistry*, *51*(21), 6300-6308. DOI: https://doi.org/10.1021/jf026242v.

Dupont, C., Bocquet, A., Tomé, D., Bernard, M., Campeotto, F., Dumond, P., ... & Kalach, N. (2020). Hydrolyzed Rice Protein-Based Formulas, a Vegetal Alternative in Cow's Milk Allergy. *Nutrients*, *12*(9), 2654. DOI: https://doi.org/10.3390/nu12092654.

Fabian, C., & Ju, Y. H. (2011). A review on rice bran protein: its properties and extraction methods. *Critical reviews in food science and nutrition*, *51*(9), 816-827. DOI: https://doi.org/10.1080/10408398.2010.482678.

Feng, B., Ma, L., Yao, J., Fang, Y., Mei, Y., & Wei, S. (2013). Protective effect of oat bran extracts on human dermal fibroblast injury induced by hydrogen peroxide. *Journal of Zheijang University SCIENCE B*, *14*, 97-105. DOI: https://doi.org/10.1631/jzus.B1200159.

Ferri, M., Graen-Heedfeld, J., Bretz, K., Guillon, F., Michelini, E., Calabretta, M. M., ... & Tassoni, A. (2017). Peptide fractions obtained from rice by-products by means of an environment-friendly process show in vitro health-related bioactivities. *PloS one*, *12*(1), e0170954. DOI: https://doi.org/10.1371/journal.pone.0170954.

Gou, L. L. (2017). The effect of oxaloacetic acid on tyrosinase activity and structure: Integration of inhibition kinetics with docking simulation. *International journal of biological macromolecules*, *260*, 59-66. DOI: https://doi.org/10.1016/j.ijbiomac.2017.03.073.

Guan, J., Takai, R., Toraya, K., Ogawa, T., Muramoto, K., Mohri, S., ... & Cho, S. J. (2017). Effects of alkaline deamidation on the chemical properties of rice bran protein. *Food Science and Technology Research*, *23*(5), 697-704. DOI: https://doi.org/10.3136/fstr.23.697.

Ishikawa, S., Sasaki, Y. F., Kawaguchi, S., Mochizuki, M., & Nagao, M. (2006). Characterization of genotoxicity of kojic acid by mutagenicity in Salmonella and micronucleus induction in rodent liver. *Genes and Environment*, *28*(1), 31-37. DOI: https://doi.org/10.3123/jemsge.28.31.

Jan-On, G., Sangartit, W., Pakdeechote, P., Kukongviriyapan, V., Senaphan, K., Boonla, O., ... & Kukongviriyapan, U. (2020). Antihypertensive effect and safety evaluation of rice bran hydrolysates from Sang-Yod rice. *Plant Foods for Human Nutrition*, *75*(1), 89-95. DOI: https://doi.org/10.1007/s11130-019-00789-9.

Jiamyangyuen, S., Srijesdaruk, V., & Harper, W. J. (2005). Extraction of rice bran protein concentrate and its application in bread. *Extraction*, *27*(1), 55-64.

Kim, J. K., Park, K. T., Lee, H. S., Kim, M., & Lim, Y. H. (2012). Evaluation of the inhibition of mushroom tyrosinase and cellular tyrosinase activities of oxyresveratrol: comparison with mulberroside A. *Journal of enzyme inhibition and medicinal chemistry*, *27*(4), 495-503. DOI: https://doi.org/10.3109/14756366.2011.598866.

Kooyers, T. J., & Westerhof, W. (2006). Toxicology and health risks of hydroquinone in skin lightening formulations. *Journal of the European academy of Dermatology and Venereology*, *20*(7), 777-780. DOI: https://doi.org/10.1111/j.1468-3083.2005.01218.x.

Kubglomsong, S., Theerakulkait, C., Reed, R. L., Yang, L., Maier, C. S., & Stevens, J. F. (2018). Isolation and Identification of Tyrosinase-Inhibitory and Copper-Chelating Peptides from Hydrolyzed Rice-Bran-Derived Albumin. *Journal of agricultural and food chemistry*, *66*(31), 8346-8354. DOI: https://doi.org/10.1021/acs.jafc.8b01849.

Kumagai, T., Kawamura, H., Fuse, T., Watanabe, T., Saito, Y., Masumura, T., ... & Kadowaki, M. (2006). Production of rice protein by alkaline extraction improves its digestibility. *Journal of nutritional science and vitaminology*, *52*(6), 467-472. DOI: https://doi.org/10.3177/jnsv.52.467.

Logarušić, M., Slivac, I., Radošević, K., Bagović, M., Redovniković, I. R., & Srček, V. G. (2019). Hempseed protein hydrolysates' effects on the proliferation and induced oxidative stress in normal and cancer cell lines. *Molecular biology reports*, *46*(6), 6079-6085. DOI: https://doi.org/10.1007/s11033-019-05043-8.

Muyan, C., Xiumei, Z., Tianxiang, G., & Chao, C. (2006). Effects of temperature, pH and NaCl on protease activity in digestive tract of young turbot, Scophthalmus maximus. *Chinese Journal of Oceanology and Limnology*, *24*(3), 300-306. DOI: https://doi.org/10.1007/PL00020776.

Ota, Y., Imai, T., Onose, J. I., Takami, S., Cho, Y. M., Hirose, M., & Nishikawa, A. (2009).

A 55-week chronic toxicity study of dietary administered kojic acid (KA) in male F344 rats. *The Journal of toxicological sciences*, *34*(3), 305-313. DOI: https://doi.org/10.2131/jts.34.305.

Phantuwong, N., Thongraung, C., & Yupanqui, C. T. (2017). Enzymatic hydrolysis on protein and β-glucan content of Sang-yod rice bran hydrolysates and their anti-inflammatory activity on RAW 264.7 cells. *Functional Foods in Health and Disease*, *7*(12), 958-971. DOI: http://doi.org/ 10.31989/ffhd.v7i12.388.

Phongthai, S., Homthawornchoo, W., & Rawdkuen, S. (2017). Preparation, properties and application of rice bran protein: A review. *International Food Research Journal*, *24*(1), 25-34.

Prakash, J., & Ramaswamy, H. S. (1996). Rice bran proteins: properties and food uses. *Critical Reviews in Food Science & Nutrition*, *36*(6), 537-552. DOI: https://doi.org/10.1080/10408399609527738.

Saran, A. S. (2004). Loss of tyrosinase activity confers increased skin tumor susceptibility in mice. *Oncogene*, *23*(23), 4130-4135. DOI: https://doi.org/10.1038/sj.onc.1207565.

Sanguigno, L., Casamassa, A., Funel, N., Minale, M., Riccio, R., Riccio, S., Boscia, F., Brancaccio, P., Pollina, L.E., Anzilotti, S., & Di Renzo, G. (2018). Triticum vulgare extract exerts an anti-inflammatory action in two in vitro models of inflammation in microglial cells. *PLoS One*, *13*(6), e0197493. DOI: https://doi.org/10.1371/journal.pone.0197493.

Su, N. W., Wang, M. L., Kwok, K. F., & Lee, M. H. (2005). Effects of temperature and sodium chloride concentration on the activities of proteases and amylases in soy sauce koji. *Journal of agricultural and food chemistry*, *53*(5), 1521-1525. DOI: https://doi.org/10.1021/jf0486390.

Thamnarathip, P., Jangchud, K., Jangchud, A., Nitisinprasert, S., Tadakittisarn, S., & Vardhanabhuti, B. (2016). Extraction and characterisation of Riceberry bran protein hydrolysate using enzymatic hydrolysis. *International Journal of Food Science & Technology*, *51*(1), 194-202. DOI: https://doi.org/10.1111/ijfs.13008Citations.

Uraipong, C., & Zhao, J. (2016). Rice bran protein hydrolysates exhibit strong in vitro α-amylase, β-glucosidase and ACE-inhibition activities. *Journal of the Science of Food and Agriculture*, *96*(4), 1101-1110. DOI: https://doi.org/10.1002/jsfa.7182.

Vandenplas, Y., De Greef, E., Hauser, B., & Paradice Study Group. (2014). Safety and tolerance of a new extensively hydrolyzed rice protein-based formula in the management of infants with cow's milk protein allergy. *European journal of pediatrics*, *173*(9), 1209-1216. DOI: http://doi.org/ 10.1007/s00431-014-2308-4.

Zhao, Q., Xiong, H., Selomulya, C., Chen, X.D., Zhong, H., Wang, S., Sun, W., & Zhou, Q. (2012). Enzymatic hydrolysis of rice dreg protein: effects of enzyme type on the functional properties and antioxidant activities of recovered proteins. *Food chemistry*, *134*(3), 1360-1367. DOI: https://doi.org/10.1016/j.foodchem.2012.03.033.